DIALYSABLE LEUCOCYTE TRANSFER FACTOR IN MONKEY AND MAN

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ABSTRACT

After review of the literature, this thesis describes the extent of cellular responses to selective antigens *in vivo*, and to antigens and phytohaemagglutinin *in vitro* in immunised and transfer factor-treated rhesus monkeys. Preliminary *in vitro* and fractionation studies of rhesus and human transfer factor preparations are also presented.

In immunised animals an antigen-specific active sensitisation was detected by the skin delayed hypersensitivity (DH), the mixed leucocyte-macrophage migration (LMMI) and the lymphocyte transformation (LT) tests.

In animals treated with rhesus dialysable transfer factor, adoptive transfer of sensitisation, with a specificity related to antigen-sensitivity of the donor(s) (i.e. "Donor-specific") was detected only by the LMMI-test.

The relationship between the various tests was determined. In immunised monkeys, the antigen-stimulated LMMI- and LT-reactivities associated with each other and with the DH-reactivity. In contrast, in transfer factor-treated animals, these reactivities were apparently dissociated. Thus, in transfer factor recipients, dissociation either reflected different sensitivity of the tests or indicated preferential lymphokine production.

Phytohaemagglutinin-stimulated transformation was depressed in immunised animals, whilst it was elevated in transfer factor-treated monkeys probably indicating an "adjuvant-like" activity. Thus the results suggest that transfer factor had specific and non-specific *in vivo* activities in the rhesus monkey model.

The *in vitro* study of human or rhesus monkey dialysable transfer factor in antigen-stimulated transformation tests showed augmenting and suppressive effects with antigen-immune or non-immune transfer factor. The degree of augmentation was related to the degree of antigen-sensitivity of the "recipient" (i.e. "Recipient-specific"). Thus, the *in vitro* activity of rhesus monkey or human transfer factor was non-specific.

Preliminary Sephadex G-25 chromatography of human and rhesus monkey dialysable transfer factor preparations revealed 9-11 fractionable peaks with different absorption ratios, suggesting heterogeneity of composition.

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1. INTRODUCTION: REVIEW OF THE LITERATURE

1.1 Concept of transfer factor as a mediator of the cellular immune response.

1.1.1 Discovery of transfer factor (Table 1).

Landsteiner and Chase (1942) reported that skin delayed hypersensitivity to simple compounds could be transferred from sensitised to unsensitised guinea pigs with live peritoneal exudate cells, but not with heated cells or serum. The transferred sensitivity appeared as early as 2 days after the injection of the lymphoid cells, but waned within a few days. It was, therefore, suggested that the acquired sensitivity in the recipient was due to the surviving, but short-lived, donor lymphoid cells. This was the first demonstration that cell-mediated immunity could be distinguished from the humoral antibody response. Chase (1945) showed that delayed hypersensitivity to tuberculin could similarly be transferred in the guinea pig with peritoneal exudate cells and also with lymphocytes obtained from the spleen and lymph nodes. Such cellular transfer of immunity has since been repeatedly confirmed (Chase, 1946; Cummings et al, 1947; Kirchheimer and Weiser, 1947).

Extending this study to man, Lawrence (1949, 1952) was the first to describe passive or adoptive cellular transfer of both local and systemic cutaneous delayed hypersensitivity to bacterial antigens (tuberculin PPD and streptococcal M antigens) using intact viable blood leucocytes. In contrast to the guinea pig, where transferred delayed hypersensitivity could only be maintained for 3-5 days, it was

reported that, in man, the transferred skin reactivity persisted for 3 months to 2 years. This observation was inconsistent with the view that transferred delayed hypersensitivity lasted only as long as the donor cells remained viable. In 1954 and 1955, Lawrence compared intact and disrupted blood leucocytes for their ability to transfer delayed skin reactivity to tuberculin and streptococcal antigens. Transferred skin reactions seemed to be more intense using the disrupted rather than the intact cells. Disruption of cells was achieved by lysis with distilled water alone and/or freeze-thawing in the presence or absence of deoxyribonuclease (DNase) or ribonuclease (RNase). These methods of disruption did not cause loss of transfer activity. Since DNase and RNase do not affect transfer of delayed hypersensitivity in man, the mechanism of transfer did not appear to depend on highly polymerised DNA or RNA. Nevertheless, it was concluded that since only a small amount of intact or disrupted donor cell mass (0.1-0.4 ml of packed white blood cells) relative to a large recipient cell mass was required to transfer cutaneous sensitivity, the transfer component might be self-replicating.

Lawrence and Pappenheimer (1956) then proceeded to transfer delayed cutaneous hypersensitivity to diphtheria toxin and toxoid, in man, using a washed disrupted extract of white blood cells. It was shown that both donors and recipients possessed little diphtheria antitoxin, the titre being less than 0.001 units/ml of serum. These findings suggested that the transfer extract was not immunogenic, and that the transferred cutaneous delayed hypersensitivity was not a function of humoral antibody. A year later, Lawrence and Pappenheimer (1957), therefore, introduced the term "transfer factor" (TF) to describe the

crude water and DNase-treated human leucocyte extract, which transferred delayed cutaneous hypersensitivity from sensitised to unsensitised individuals.

Rapaport et al (1960) reported successful transfer of delayed cutaneous hypersensitivity to coccidioidin in man with a leucocyte extract, thought to contain transfer factor. This fungal antigen is limited to the West of the United States, but is almost absent in the East with an incidence of only 1.4% in New York (Lawrence, 1974). To test the specificity of transfer factor, the extract was therefore prepared from sensitive donors from California and administered to "naive" recipients from New York. Thirty out of 35 (86%) of the individuals receiving the extract from coccidioidin-positive donors acquired skin test-reactivity to coccidioidin. In contrast, 8 out of 9 (89%) of the control recipients, given extracts prepared from coccidioidin-negative donors, did not acquire such skin test-reactivity. Thus, in these studies coccidioidin sensitivity was transferred de novo and this transfer was donor-specific. The possibility of the elevation of a latent sensitivity to coccidioidin by means of the extract was excluded, since the recipients were not naturally exposed to or skin tested with this antigen before the injection of the extract.

In 1960, Lawrence and colleagues provided evidence that transfer factor-containing leucocyte extract can also specifically transfer sensitivity to human skin transplantation antigens as shown by the accelerated homograft rejection. Transfer factor was prepared from the leucocyte extract of an individual B after repeated exposure to skin grafts from another individual A. Anti-A-sensitive or "allograft-specific"

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transfer factor was then injected into another individual C, who had received a skin graft from the individual A and control grafts from other unrelated individuals (D, E, or F). In such experiments, Lawrence et al (1960) reported that the local injection of an allograft-specific transfer factor, in 4 out of 4 human recipients, transferred homograft-specific hypersensitivity. This was shown in 4 to 5 days by an accelerated homograft rejection ("second-set reaction") of test skin grafts but not control skin grafts. In contrast, non-sensitive transfer factor did not transfer transplantation sensitivity, and could only induce the expected homograft rejection in 10-12 days ("first-set reaction").

Maurer, in 1961, used a non-ubiquitous antigen ("neoantigen") prepared by the ethylene oxide-treatment of human serum, to demonstrate transfer of specific delayed hypersensitivity in man. Freeze-thawed leucocyte extracts or viable whole leucocytes, obtained from sensitive donors, transferred strong delayed cutaneous hypersensitivity to the neoantigen in 7 out of 7 recipients. The transferred skin test-reactivity was still present after 1 year confirming the observation of Rapaport et al (1960) with the coccidioidin antigen. The extracts of the leucocytes were as effective as the viable cells in transferring the antigen sensitivity. This supported the previous findings with the tuberculin and streptococcal antigens (Lawrence, 1954; 1955).

Adoptive transfer of cell-mediated immunity in man was later found to be associated with low molecular weight substances (Lawrence et al, 1963). These substances could be separated, without loss of activity,

from the bulk of the disrupted leucocyte extract by exhaustive dialysis into distilled water and concentrated by freeze-drying. Lawrence et al (1963) named this material "dialysable transfer factor" (TF_D). Traditionally this term was reserved for as yet unidentified factor or factors, rather than the whole crude dialysate, capable of specifically transferring delayed cutaneous hypersensitivity in man (Lawrence, 1969a, b; Lawrence and Al-Askari, 1971). Using the dialysable material, Zuckerman et al (1974) successfully transferred donor-specific delayed hypersensitivity to another neoantigen, called keyhole limpet haemocyanin (KLH). They used dialysable transfer factor prepared from KLH-sensitive donors and, for control experiments, from donors not sensitive to KLH. Ten of 10 recipients of the immune dialysable transfer factor acquired delayed skin test hypersensitivity to KLH. In contrast, none of the 11 recipients of the control dialysable transfer factor converted for KLH. Like the work of Rapaport et al (1960) with coccidioidin, these authors excluded the possibility that the transfer of KLH-sensitivity resulted from a non-specific adjuvant activity upon some precommitted lymphocytes, by not skin testing their recipients with KLH prior to the administration of transfer factor. Hence, table 1 shows some of the properties of dialysable transfer factor as described by Lawrence (1973, 1974).

It has subsequently been suggested that the term dialysable leucocyte extract (DLE) should be used, instead of dialysable transfer factor, to encompass all the other specific and non-specific activities now found in the crude leucocyte extract (Valdimarsson, 1975; Arala-Chaves et al, 1976; Mazaheri et al, 1977). (See section 1.5).

Table 1. Some properties of dialysable transfer factor[a]

Biological[b]	Biochemical[c]	Immunological
Confers upon the recipient specific sensitivity of donor	Soluble, dialysable, lyophilisable, M.Wt. <10,000	Not immunoglobulin Not immunogenic
Acquired sensitivity is systemic Onset early (hours) Duration long (>1 year)	Not protein, albumin, alpha or gamma globulin, Orcinol positive.	Immunologically specific, converts normal lymphocytes <u>in vitro</u> and <u>in vivo</u> to antigen-responsive state.
Capacity for transfer function of donor sensitivity and TF dosage	polypoptide/poly-nucleotide composition. Inactivated at 56°C/30 minutes	Repeated test with antigen may increase intensity and duration of transferred sensitivity: not necessarily its cause
Negative donors incapable of transfer	Resistant to DNase, RNase, lysosomal hydrolases, trypsin	Informational molecule/ depressor/receptor site?
Leucocyte extracts or cell-free supernatants as effective as viable cells.	Not inactivated by distilled water lysis of cells, freeze-thawing (10x), 25°C or 37°C for 6 hours.	

[a]Adapted from Lawrence (1973, 1974).

[b]TF = transfer factor.

[c]DNase = Deoxyribonuclease, RNase = Ribonuclease.

1.1.2 Classification of mediators of cellular immunity (Table 2).

Defence against certain diseases is partly mediated by cellular immunity (World Health Organisation Technical Report Series, 1969). This involves both antigen-specific and non-specific lymphoid cell populations (David, 1973). Antigen apparently reacts with a few specifically sensitised cells, presumably thymus dependent or T-lymphocytes, and results in the recruitment of a large number of non-sensitive cells and the elaboration of a number of soluble mediator substances or "lymphokines" (Dumonde et al, 1969; Lawrence, 1973). These non-antibody substances collectively mediate a variety of in vivo and in vitro biological phenomena, which can be studied to recognise and assess specific cell-mediated immunity. Altogether about 30 lymphokine-activities have apparently been recognised (Waksman and Namba, 1976).

Transfer factor was initially regarded as one such lymphokine, since it was the first cell-free material to be isolated from sensitised cells (Lawrence, 1969a). However, it has been reported to differ from the lymphokines since it can apparently initiate, rather than simply mediate, cellular immunity. Transfer factor is dialysable and therefore has a low molecular weight, (i.e. < 10,000, table 1). In contrast, the lymphokines are non-dialysable and have molecular weights of about 12,000 to 80,000 (Lawrence, 1973, 1974). Table 2, therefore, shows a few examples of biologically active soluble factors relevant to this work. It can be seen that transfer factor apparently requires antigen for expression of cell-mediated immunity, whereas those lymphokines mentioned in the table do not.

Table 2. Some biologically active soluble factors elaborated by sensitised lymphocytes in the expression of cell-mediated immunity

In vivo or in vitro test systems	Name given to soluble factor	Antigen requirement for expression	References
Production of delayed hypersensitivity skin reaction by antigen	Skin reactive factor (SRF : lymphokine)	No	Section 8.1
Inhibition of leucocyte, macrophage or mixed leucocyte-macrophage migration by antigen	Leucocyte and/or macrophage migration inhibition factor (LIF, MIF : lymphokine)	No	Section 8.2
Lymphocyte transformation by antigen or mitogen	Mitogenic or blastogenic factor (MF : lymphokine)	No	Section 8.3
Transfer of delayed hypersensitivity skin reaction, stimulation of lymphocyte transformation and/or migration inhibition factor production.	Transfer factor (TF)	Yes	Section 1

1.2 Clinical studies of human transfer factor (Tables 3-6).

The concept that transfer factor could restore defective cell-mediated immunity, as expressed by the acquisition of delayed-type skin reactions, resulted in its therapeutic use for the treatment of a wide variety of acquired and congenital immunodeficiency states (Lawrence, 1974; Fudenberg et al, 1974). Therapeutic benefit from treatment with transfer factor has been reported for many neoplastic and infectious diseases (Kirkpatrick and Gallin, 1974; Zuckerman, 1975a; Heim et al, 1976; Mazaheri et al, 1977).

1.2.1 Infectious diseases.

1.2.1.1 Fungal infections (Table 3).

A variety of fungal infections have been treated with transfer factor (Table 3). Coccidioidomycosis, usually self-limiting, can become chronic with invasion of the skin and other organs and apparently become resistant to treatment with antifungal agents, such as amphotericin B (Kirkpatrick and Gallin, 1974). One approach for the treatment of disseminated coccidioidomycosis has been the use of transfer factor. Out of 52 treated cases, 33 have been reported to show clinical improvement with recovery of cellular immune reactivity (Graybill et al, 1973; Catanzaro and Spitler, 1976). Catanzaro and Spitler (1976) did not define objectively what they meant by "clinical improvement", but twelve of these improved conditions were directly associated with the beneficial effect of transfer factor, since antifungal agents were, in these cases, not used before or during therapy. Such an association may well be true for 2 other

improved conditions reported by Graybill et al (1973), since these patients were resistant to cure with amphotericin B for up to 5 years previous to treatment with transfer factor. These investigators observed clinical improvement of fungal lesions in the lung and skin of these patients.

Chronic disseminated amphotericin B-resistant histoplasmosis has also been treated with transfer factor with reported clinical improvement for 6 months (Graybill et al, 1976). This patient also had stage III Hodgkin's disease and was, at the same time as transfer factor, being treated with amphotericin B and steroids. The temporary clinical improvement could not, therefore, be attributed to transfer factor only.

Mucocutaneous candidiasis describes a broad variety of disease states and is clinically characterised by fungal lesions on the skin and mucous membrane (Levin et al, 1973). Several patterns of defects in cellular and humoral immunity have been reported among different populations of patients (Valdimarsson et al, 1973). The immune defects include absence of delayed hypersensitivity skin reaction to candida, defective MIF-lymphokine production and candida-induced lymphocyte transformation (Valdimarsson et al, 1973). Some workers have treated 6 patients with candidiasis with transfer factor resulting in clinical improvement and immune conversion to candida antigen (Pabst et al, 1972; Schulkind et al, 1972; Valdimarsson et al, 1972; Moulias et al, 1973; Littman et al, 1976, 1978). Six other patients with chronic mucocutaneous candidiasis were treated with transfer factor by Kirkpatrick et al (1972) and Griscelli (1975).

Table 3. Some clinical applications of transfer factor in fungal and bacterial diseases

Disease[a]	TF source	TF[b] preparation	Immune conversion[c] DH	LIF/MIF	LT	Clinical improvement	References
Fungal							
Coccidioidomycosis	Coccidioidin+	BL,TF_D	3/3	3/3	3/3	2/3	Graybill et al (1973)
	Coccidioidin+	TF_D	32/44	24/28	27/32	31/49	Catanzaro & Spitler (1976)
Histoplasmosis	Histoplasmin+	TF_D	0/1	1/1	0/1	1/1	Graybill et al (1976)
Candidiasis	Candida+	BL	5/6	5/6	1/5	1/6	Kirkpatrick et al (1970, 1972)
	Candida+	TF_D	1/1		1/1	1/1	Pabst et al (1972)
	Candida+	TF_D	1/1	1/1		1/1	Schulkind et al (1972)
	Candida+	TF_D	1/1			1/1	Valdimarsson et al (1972)
	Candida+	TF_D	1/1	1/1	1/1	1/1	Moulias et al (1973)
	Candida+	TF_D				1/1	Griscelli (1975)
	Candida+	TF_D	2/2	2/2	1/1	2/2	Littman et al (1976, 1978)
	Total:		46/60 (77%)	37/42 (88%)	34/44 (77%)	42/66(64%)	
Bacterial							
Tuberculosis	Mantoux+	TF_D	1/1	1/1	1/1	1/1	Whitcomb & Rocklin (1973)
Leprosy	Lepromin+	BL,TF_D	6/9		0/9	? 6/9	Bullock et al (1972)
	Lepromin+	TF_D	2/5			5/5	Hastings et al (1976)
	Total:		9/15 (60%)	1/1	1/10	12/15(80%)	

[a] All persistent and drug resistant.

[b] TF, transfer factor; BL, blood leucocytes; TF_D = dialysable transfer factor.

[c] DH, delayed cutaneous hypersensitivity; LIF/MIF, leucocyte or macrophage migration inhibition factor; LT, lymphocyte transformation with antigen.

Of these, 4 patients developed positive skin hypersensitivity and showed conversion of MIF responsiveness, but only 1 of these recipients developed antigen-induced lymphocyte transformation. One patient, with Nezelof syndrome, did not become skin-test positive or develop reactivity to the in vitro tests (Kirkpatrick et al, 1972). In none of these cases listed in table 3, clinical benefits of transfer factor on patients with candidiasis could totally be related to the beneficial effect of transfer factor, since such an immunotherapy was always undertaken after or during chemotherapy with antifungal agents. Nevertheless, it is believed that transfer factor played an important role, because chemotherapy alone was not adequate for clinical recovery. Drugs were used in an attempt to reduce the antigen load in order to prolong temporary remissions of 220 days previously reported by the use of similar immunotherapy alone (Kirkpatrick et al, 1970).

1.2.1.2 Bacterial infections (Table 3).

Attempts have been made to treat intracellular Mycobacterial infections with transfer factor (Table 3). Whitcomb and Rocklin, in 1973, reported beneficial effects of transfer factor in a case of progressive primary tuberculosis. The patient had failed to respond to 7½ months of chemotherapy, even though the organisms were sensitive to the drugs used. This, they suggest, was due to a defect in the host's immune response and transfer factor was added to the treatment. Recovery of skin test reactivity, accompanied by moderate clearing of the lesions in the lungs, followed the combined drug-transfer factor therapy. Continual chemotherapy and subsequent removal of bacteria-laden spleen

may have sufficiently lowered the antigen load, so that transfer factor could induce the expression of the immunological response, which was absent previous to immunotherapy.

Based on the rationale that there is poor cellular defence against Mycobacterium leprae in patients with lepromatous leprosy, Bullock et al (1972) and Hastings et al (1976) treated 14 patients with transfer factor. Skin test conversions occurred in 8 of these patients. In one study (Bullock et al, 1972), 6 of 9 patients showed slow onset of diffuse erythema and induration within the lepromatous lesions 2 to 6 days after transfer factor-therapy. This was interpreted by these workers as clinical improvement, but it might simply suggest that only some immunological activity was in progress locally. The impact of immunotherapy was, therefore, difficult to assess. Such an assessment was especially difficult, because treatment of some patients was frequently interrupted with chemotherapy which in 3 cases included steroids. On the other hand, the study of Hastings et al (1976) clearly revealed that, after therapy with 6.6 to 7.9 x 10^9 lymphocyte equivalent of transfer factor for a period of 12 weeks, 5 out of 5 patients clinically improved. The beneficial effects of transfer factor were indicated by the observation that the rate of bacterial clearing, during transfer factor therapy, was about 6 times faster than that seen with routine dapsone therapy. Bullock et al (1972) wrote that 40% of patients with arrested leprosy infection may, even after chemotherapy, revert to active disease. They suggested that repeated administration of transfer factor to such patients could reconstitute sufficient cell-mediated immune reactivity to prevent the high rate of relapse.

1.2.1.3 Viral infections (Table 4).

The initial attempts at immunotherapy of viral infections employed viable leucocytes, presumed to contain transfer factor. Thus Kempe (1960) treated a young boy suffering from progressive vaccinia infection with peripheral blood or lymph node leucocytes, and by transplantation of sensitised lymph nodes from vaccinated immune donors. The progression of the lesions was arrested and delayed hypersensitivity to inactivated vaccinia virus was conferred. Similarly O'Connell *et al* (1964) treated an elderly patient suffering from severe progressive vaccinia. Intramuscular and subcutaneous injections of specific immune leucocytes were given on two occasions about 2 months apart. Acquisition of cutaneous delayed hypersensitivity to vaccinia antigen and termination of the infection were reported.

These initial studies were extended to the use of dialysable transfer factor. Drew *et al* (1973) treated a patient with disseminated herpes zoster with dialysable transfer factor prepared from a pool of 27 healthy donors who demonstrated immunity to varicella-zoster *in vitro*. The patient, a 44 year old man with stage IVB Hodgkin's disease and defective cell-mediated immunity was not treated with immunosuppressive drugs or corticosteroids in the three months preceding therapy. Following two subcutaneous injections of dialysable leucocyte extract the vesicular lesions completely healed. Moulias *et al* (1973) treated two children with "giant-cell" measles pneumonia with dialysable transfer factor from immune donors. The patients responded with improvement and regression of the pulmonary lesions. Positive *in vitro* leucocyte migration inhibition responses to measles virus were

Table 4. Some clinical applications of transfer factor in viral diseases

Disease[a]	TF source	TF[b] preparation	Immune conversion[c] DH	LIF/MIF	LT	Clinical improvement	References
Generalised Vaccinia	Vaccinated	BL,LL	1/1			1/1	Kempe (1960)
	Vaccinated	BL	1/1			1/1	O'Connell et al (1964)
Herpes zoster	Herpes$^+$	TF_D			1/1	1/1	Drew et al (1973)
Neonatal herpes	Herpes$^+$	TF_D				1/1	Moulias et al (1973)
Measles pneumonia	Measles$^+$	TF_D		1/1	0/1	1/1	Moulias et al (1973)
SSPE	Measles$^+$	TF_D		5/7	0/2	2/7	Moulias et al (1973)
	Measles$^+$	TF_D	1/1			1/1	Vandvik et al (1973)
CMV retinitis		TF_D	1/1	1/1		1/1	Rytel et al (1975)
CAH	Hep.B$^+$	BL,TF_D				2/2	Kohler et al (1974)
	Hep.A$^+$/B$^+$	TF_D				5/5	Schulman et al (1974, 1976)
	Hep.B$^-$/B$^+$	TF_D				? 4/9	Jain et al (1975, 1977)
	Hep.B$^+$	TF_D	1/1		1/1	0/1	Tong et al (1976)
	Total:		5/5 (100%)	7/9 (78%)	2/5 (40%)	20/31 (65%)	

[a] SSPE, subacute sclerosing panencephalitis; CMV, cytomegalovirus; CAH, chronic active hepatitis.

[b] TF, transfer factor; BL, blood leucocytes; LL, lymph node leucocytes; TF_D, dialysable transfer factor.

[c] DH, delayed cutaneous hypersensitivity; LIF/MIF, leucocyte or macrophage migration inhibition factor; LT, lymphocyte transformation with antigen.

reported after transfer, indicating improved cell-mediated immunity to the virus. Moulias et al (1973) also treated 7 children with sub-acute sclerosing panencephalitis, (SSPE), a degenerative disease associated with measles virus infection. Clinical improvement was seen in 2 out of the 7 children although 5 out of 7 showed conversion of the leucocyte migration inhibition test to measles antigen in vitro. Vandvik et al (1973) treated a 16 year old boy with SSPE with dialysable transfer factor from donors with measles immunity. The patient improved clinically and developed delayed hypersensitivity to various antigens, and a selective reduction in measles antibody titre in the cerebrospinal fluid and serum. Rytel et al (1975) treated a renal transplant patient with cytomegalovirus retinitis with dialysable transfer factor. Inactivation of the natural inflammatory lesions was seen. The patient also acquired delayed skin reaction and in vitro inhibition of leucocyte migration.

Attempts have been made to treat patients with chronic active hepatitis with transfer factor. There was no clinical improvement in a 29 year old patient with chronic active hepatitis who received, over a period of 8 weeks, 17 injections of dialysable transfer factor twice weekly (Tong et al, 1976). Each injection was derived from 5×10^8 leucocytes from a donor who previously had acute hepatitis B, and showed lymphocyte reactivity to hepatitis B surface antigen in vitro. Clinical and biochemical improvement was not observed. However, the transfer of cell-mediated immunity was demonstrated by the appearance of delayed hypersensitivity to mumps and streptokinase-streptodornase, and by in vitro lymphocyte responses to hepatitis B surface antigen. It was suggested that clinical improvement

might have been seen with increased quantity, frequency and duration of treatment. Kohler et al (1974) treated a healthy persistent carrier of hepatitis B surface antigen with lymphocytes from an individual who had recovered from hepatitis B 8 months previously. Within 24 hours of the administration of lymphocytes, there was an increase in the titre of the surface antigen and of serum aspartate transaminase levels, suggesting liver cell damage. It was presumed that the development of cell-mediated immunity to hepatitis B was reflected in the injury of the virus-laden liver cells.

Another patient, a 4 month old infant, who acquired hepatitis B from her mother at birth was given dialysable transfer factor prepared from the mother's leucocytes on two occasions. The recipient promptly developed an increase in the titre of hepatitis B surface antigen and elevation of serum transaminase activity. Subsequently, however, the liver function tests returned to normal and the titre of hepatitis B surface antigen decreased by 95%.

Jain et al (1975) treated 3 patients with dialysates prepared from healthy donors without any history of viral hepatitis ("normal"), and from donors, who had recovered from hepatitis B infection ("specific"). One patient with hepatitis B surface antigen-positive chronic active hepatitis and cirrhosis did not respond to "normal" dialysable transfer factor, but injection of "specific" dialysable transfer factor resulted in a two-fold increase in T lymphocytes as measured by sheep red blood cell rosetting. The second patient, also did not respond to "normal" dialysable transfer factor, but showed a transient increase in transaminase activity. Th. suggested

stimulation of cell-mediated immunity with a resulting hepatic damage. The third patient suffered from antigen-positive active chronic hepatitis complicated by primary liver cancer. This patient was being treated with corticosteroids and did not respond to either "normal" or "specific" dialysable transfer factor. There was no change in the serum titre of hepatitis B surface antigen in any of the patients. Similarly, in another study Jain et al (1977) treated 6 other patients with chronic active hepatitis with "specific" and "non-specific" dialysable transfer factor preparations. No beneficial clinical effects were detected histologically after any of these preparations, but in 3 of 6 patients, the "specific" transfer factor provoked a hepatitic reaction shown by transient elevation of serum transaminase level. In contrast, injection of "non-specific" transfer factor did not result in the elevation of this enzyme, suggesting a "donor-specific" response.

A major criticism of transfer factor therapy has been the lack of controlled clinical trials. Jain et al (1975) were the first to attempt a controlled trial of transfer factor in the treatment of chronic active hepatitis. Schulman et al (1976) initiated a far better small double-blind "controlled" trial. In this study none of the patients had other concomitant complications and were not on chemotherapy. They treated 4 patients with chronic active hepatitis with dialysable transfer factor from pools of donors who had recovered from hepatitis B and A, while 4 others received normal saline. The results indicate that all 4 patients receiving dialysable transfer factor showed biochemical evidence of improvement, with a fall in serum transaminase activity. In 3 of the patients it was considered

that there was also histological improvement, but hepatitis B surface antigen remained in the serum. The patients responding to dialysable transfer factor have remained in remission for periods of 9 months to 1½ years. None of the 4 recipients of normal saline improved. Based on these observations it was considered that dialysable transfer factor may be useful for the management of chronic hepatitis B infection (Schulman et al, 1974; Schulman, 1977 personal communication).

In most of the above clinical studies of transfer factor in infectious diseases, the transfer factor was prepared from donors with strong delayed hypersensitivity skin reaction to the antigens of the infecting organism. During therapy there was, therefore, transfer of donor-specific skin test reactivity and in vitro responses. However, the relationship between specific conversion of cellular immunity and the beneficial clinical effects of transfer factor has not yet been determined.

1.2.2 Neoplastic diseases (Table 5).

The mechanisms by which the host cells inhibit the growth of cancer cells or destroy such cells are not completely understood (Nadler and Moore, 1969). However, the importance of host resistance and immunological surveillance in influencing the growth of tumours has been suspected for many years (Burnet, 1967). Therefore, some investigators have suggested the use of immunocompetent cells or transfer factor in the treatment of neoplastic diseases (Thompson and Mathé, 1972; Ascher et al, 1976).

Table 5. Some clinical applications of transfer factor in neoplastic diseases

Disease	TF source	TF[a] preparation	Immune conversion[b] DH	LIF/MIF	LT	Clinical improvement	References
Alveolar soft-part sarcoma	Contact	TF_D		1/1		?	LoBuglio et al (1973)
Breast cancer	Normal	TF_D	3/5			1/5	Oettgen et al (1974)
Hodgkin's disease	Contacts	TF_D	5/8			?	Khan et al (1975)
Melanoma	Melanoma+	BL	1/2		1/2	2/2	Brandes et al (1971)
	Contacts	TF_D±BCG		4/15	3/19	3/19	Spitler et al (1973, 1976)
Nasopharyngeal carcinoma	IM+	TF_D	1/2			2/2	Goldenberg & Brandes (1972)
Osteogenic sarcoma	Contact	TF_D	1/1			1/1	Levin et al (1972)
Various tumours	Contacts	TF_D				13/35	Vetto et al (1976)
Total:			11/18 (61%)	5/16 (31%)	4/21 (19%)	22/64 (34%)	

[a] BL, blood leucocytes; TF, transfer factor; TF_D dialysable transfer factor; BCG, Bacille Calmette Guérin; IM, infectious mononucleosis.

[b] DH, delayed cutaneous hypersensitivity; LIF/MIF, leucocyte or macrophage migration inhibition factor; LT, lymphocyte transformation.

Table 5 shows that various reports suggest a potential role for transfer factor for treating malignant diseases. Levin et al (1972) have reported temporary restoration of skin test reactivity and arrest of growth of an osteogenic sarcoma in 1 patient. The patient was treated with radiation and multiple injections of dialysable transfer factor from a family member. After 7 months of therapy the patient showed signs of deterioration first shown by decreased skin test reactivity, and 6 weeks later, by tumour growth and lung metastasis. The authors suggested that the temporary clinical improvement may have been due to radiotherapy and/or transfer factor therapy. Goldenberg and Brandes (1972) used transfer factor from donors who had previously recovered from infectious mononucleosis to treat 2 patients with nasopharyngeal carcinoma. In both cases, temporary but definite regression of tumour growth occurred and in one of the patients, a delayed skin hypersensitivity reaction to PPD was restored. In 1973, LoBuglio et al reported that transfer factor prepared from a family contact and administered to a patient with alveolar soft-part sarcoma led to restoration of cellular immunity. The patient's lymphocytes became capable of producing MIF. The tumour neither regressed nor advanced during the 6 months period of therapy. Oettgen et al (1974) treated 5 patients with advanced breast cancer with pooled dialysable transfer factor from healthy donors. Delayed hypersensitivity skin reactions to PPD and/or Streptokinase - Streptodornase was restored in 3 of the patients. Marked partial regression of the tumour occurred in 1 patient and lasted for 6 months. After this period the tumour started to grow again and could no longer be controlled by transfer factor therapy. It cannot be, therefore, suggested that in this

study pooled normal transfer factor offered great promise in the treatment of patients with breast cancer.

Brandes et al (1971) were the first to treat 2 patients with disseminated melanoma with lysed buffy coat cells obtained from the recipients of the tumour graft. One of them developed positive delayed hypersensitivity and lymphocyte transformation responses to tumour-specific antigens. Both patients improved clinically with the melanoma nodules showing an overall regression over the 3 weeks of treatment. Spitler et al (1973) studied effect of transfer factor in 9 patients with such metastatic melanoma. Only 1 such patient, who showed significant conversion of lymphocyte transformation to melanoma antigens, experienced a regression of cancer during treatment. Also, in this patient, MIF lymphokine responses to the same antigen had been positive before and after immunotherapy. New metastases developed after 18 months when transformation and MIF responses were lost. In 1976, Spitler et al treated 10 other patients with disseminated malignant melanoma with transfer factor alone or combined with Bacille Calmette-Guérin (BCG) in 7 cases. Transfer factor was obtained from family contacts or donors who had previously recovered from melanoma. After combined immunotherapy 2 patients improved clinically showing complete regression of all tumour nodules. One had recurrence of disease and died despite continued therapy, but the other patient remained completely free of malignant melanoma for over 3 years. Only 2 patients showed an increase in lymphocyte responses to melanoma antigen, and these were the 2 patients whose lesions regressed after combined immunotherapy. Three patients began to produce MIF after administration of transfer factor and BCG.

Spontaneous regressions can occur in patients with malignant melanoma (Spitler et al, 1976). It is possible that the patients who improved had actually experienced such spontaneous regressions. The clinical benefit had, however, coincided with the administration of combined immunotherapy. Khan et al (1975) treated 8 patients with Hodgkin's disease with transfer factor. Clinical effects were not reported, but cellular immune responses improved in 5 of the patients with restoration of delayed hypersensitivity skin reactions to several antigens.

Vetto et al (1976) treated 35 patients with a variety of advanced recurrent malignant tumours with transfer factor from prolonged close contacts. Types of tumour included melanoma, osteogenic sarcoma, epidermoid carcinoma and others. Within 2 weeks of treatment, 13 (37%) of the patients apparently improved clinically for 1-12 months with over 50% regression of cancer. Eleven (31%) of the patients did not show a benefit from transfer factor. However, 7 of these patients had deficient mitogen-induced in vitro lymphocyte responsiveness, probably due to a blocking factor thought to be present in their serum. The remaining 11 (31%) of the patients treated with transfer factor were not critically evaluated.

Table 5 shows that in the majority of cases, transfer factor donors were healthy contacts of the patients. They were selected on the basis that such contacts might be immune to the aeteological agents of malignant diseases, especially if these agents were viruses (LoBuglio et al, 1973). It has been suggested that

development of *in vitro* responses to tumour antigens may be indicative of clinical benefit in terms of tumour resistance (LoBuglio *et al*, 1973; Spitler *et al*, 1976). The numbers of treated patients are small and, in the majority of cases, the duration of follow-up is too short to allow firm conclusions. Nevertheless, the above observations lead to the following interim conclusions: (a) cell-mediated responses can be restored in patients with neoplastic diseases with transfer factor, and (b) some of the patients can improve with regression of their malignant lesions. These findings do not establish a beneficial therapeutic role for transfer factor in neoplasia, but they do indicate the need for well controlled large scale investigations to determine the efficacy, source and optimum dose of transfer factor in the treatment of malignancies.

1.2.3 Immuno-regulatory diseases (Table 6).

Immuno-regulatory or immunodeficiency diseases can be either congenital or acquired (Roitt, 1977). Levin *et al* (1973) classified congenital or hereditary disorders into: (a) those with defective humoral or B-cell system and intact cellular immunity or T-cell system, (b) those with defective cellular and intact humoral immunity, (c) those with mixed B and T-cell deficiencies, (d) those with variable deficiencies and (e) those with neutrophil dysfunction syndrome. Table 6 shows some of the applications of transfer factor in the treatment of patients with some such congenital defects of immunity.

Wiskott-Aldrich syndrome is an X-linked recessive disorder

Table 6. Some clinical applications of transfer factor in immuno-regulatory diseases

Disease[a]	Defect	Immune conversion[b] DH	Immune conversion[b] LIF/MIF	Immune conversion[b] LT	Clinical improvement	References
Wiskott-Aldrich syndrome	T-cell	7/9	4/8	0/7	6/9	Levin et al (1970, 1973)
		7/12	6/8	0/6	2/11	Spitler et al (1972)
		1/2		2/2	1/2	Ballow et al (1973)
		4/4		1/4	2/4	Griscelli (1975)
Ataxia-telangiectasia	T-cell	4/5			? 0/5	Griscelli (1975)
		3/4	3/4	0/4	1/4	Berkel et al (1977)
SCI		1/1	1/1		2/2	Levin et al (1973)
	Stem cell	1/1			1/1	Arala-Chaves et al (1974)
		0/1			0/2	Griscelli (1975)
CMC	B & T cells	9/10	7/8	4/8	6/11	(See table 3)
Some others	B & T	3/6			2/6	Amman et al (1974)
	T-cell				1/1	Valdimarsson et al (1974)
	B-cell				1/2	Griscelli (1975)
	T-cell	1/1		0/1	1/1	Ballow & Good (1976)
Total:		41/56 (73%)	21/29 (72%)	7/32 (22%)	26/61 (43%)	

[a] SCI, severe combined immunodeficiency; CMC, chronic mucocutaneous candidiasis.

[b] DH, delayed cutaneous hypersensitivity; LIF/MIF, leucocyte or macrophage migration inhibition factor; LT, lymphocyte transformation with antigens.

characterised by thrombocytopenia, eczema, recurrent infections and lack of cellular immunity (Levin et al, 1973). Levin and colleagues, who were the first investigators to use transfer factor therapeutically, in 1970 and 1973 reported the treatment of 9 patients with this syndrome with dialysable transfer factor. Six out of nine patients improved clinically with the clearing of eczema and recurrent infections, some showed skin test conversion and 4 out of 8 produced MIF, but none showed lymphocyte transformation responses. Spitler et al (1972) treated 12 patients with Wiskott-Aldrich syndrome with multiple injections of transfer factor from PPD skin-test positive donors. Seven out of the 12 patients responded with skin-test conversion. Some of these recipients experienced decreased incidence of infections and regression of splenomegaly and clearing of eczema. It was found that response to transfer factor in Wiskott-Aldrich syndrome appears to be correlated with the absence of monocyte IgG receptors. Two other children with this syndrome were treated by Ballow et al (1973) with multiple doses of dialysable leucocyte transfer factor. Although both patients showed positive lymphocyte transformation responses, 1 patient developed strong skin-test reactivity to candida and clinically improved with clearing of eczema and reduction of bleeding. The other recipient showed only delayed cutaneous conversion, but developed autoimmune haemolytic anaemia concomitantly with Coxsackie B5 pneumonitis and subsequently died 7 months later. Griscelli (1975) treated 4 patients with Wiskott-Aldrich syndrome with normal transfer factor. All 4 developed skin test reactivity, while 2 out of 4 patients improved clinically with a reduction in infection and spleen size and clearing of eczema.

Transfer factor can, therefore, have a beneficial effect on some of the patients with Wiskott-Aldrich syndrome. The observed improvement, however, in some tranfer factor-treated patients cannot necessarily be attributed to the beneficial effect of the transfer factor. This disease has a variable course and temporary spontaneous improvement can occur (Spitler et al, 1972).

Ataxia-telangiectasia is an autosomal recessive disease clinically characterised by progressive neurological symptoms and recurrent sinopulmonary infection. Immunologically it involves mainly T-cell deficiency, though some defect in humoral immunity also occurs (Levin et al, 1973). Altogether nine patients suffering from this disease have been treated with transfer factor by Griscelli (1975) and Berkel et al (1977). Among the 5 patients studied by Griscelli in 1975, four showed skin-test conversion, whilst 2 improved clinically. Recently, Berkel et al (1977) observed that delayed hypersensitivity can be restored in 3 out of the 4 patients treated with transfer factor, but clinical improvement in appetite and general well being occurred in only 1 case.

X-linked dual system deficiency or severe combined immunodeficiency generally involves diminished immunoglobulin levels and deficient cellular immune responses with absent skin-test reactivity and low lymphocyte transformation responsiveness to mitogen and antigens (Levin et al, 1973). These authors observed skin-test conversion and production of MIF after transfer factor-therapy of 1 patient with X-linked dual-system immunodeficiency. This patient and another one, with autosomal recessive disease, showed clinical

improvement and were both maintained on transfer factor injections and plasma transfusions. In contrast, Griscelli (1975) did not observe any improvement after transfer factor therapy in 2 patients with autosomal recessive severe combined immunodeficiency syndrome. These apparent inconsistencies in the therapeutic uses of transfer factor are, at least to some extent, due to the heterogeneity of the patients' immunological defects. In 1974, Arala-Chaves et al treated a young patient with unclassified complex immune deficiency disease with 2 injections of dialysable leucocyte transfer factor from healthy donors. Following such a therapy she showed clinical improvement, and developed strong delayed skin reactivity to candida and streptokinase-streptodornase and a moderate and short-lived skin test reactivity to tuberculin PPD. In addition her leucocytes transformed in vitro by induction with phytohaemagglutinin. These manifestations of cellular immunity had been absent prior to treatment with transfer factor.

Chronic mucocutaneous candidiasis and the effect of transfer factor on immunological responses of anergic patients with this disease has been described above (Table 3, section 1.2.1.1).

Some patients with various other immunodef cient disorders have also been treated with transfer factor. Amman et al (1974) treated 6 patients with deficiency in cellular and humoral immunity with dialysable leucocyte transfer factor. Donors had positive skin reactivity to mumps, streptokinase-streptodornase, candida and PPD antigens. One 10 month old patient had sex-linked combined immunodeficiency disease, while two 3½ and 5 year-old patients had

thymic hypoplasia and hyperimmunoglobulinaemia. Of the 3 others, 1 patient had thymic hypoplasia and selective IgA deficiency and severe pulmonary insufficiency, while 1 other suffered from immunodeficiency with thrombocytopenia and eczema, and the last one had thymic hypoplasia, decreased IgG, increased IgM and absent IgA. Following treatment with transfer factor 3 of 6 patients showed improvement in phytohaemagglutinin-induced lymphocyte transformation and conversion of skin-test reactivity. However, there was not any evidence of change in antibody-mediated immunity, but 2 patients showed clinical improvement.

Valdimarsson et al (1974) treated a 3 year old patient with a defective T-lymphocyte function and had severe attacks of extensive skin ulceration and high fever. After injection of dialysable leucocyte transfer factor the patient developed improved phytohaemagglutinin-induced lymphocyte transformation and T-lymphocyte sheep red cell rosetting. The patient also clinically improved with regression of the lesions and remained symptom-free for 9 months. In addition to treatment of patients with Wiskott-Aldrich syndrome, Ataxia-telangiectasia and severe combined immunodeficiency (Table 6), Griscelli (1975) injected transfer factor from healthy donors into 3 normal control subjects and 2 patients with variable hypogammaglobulinaemia. During treatment 2 of 3 control recipients showed skin-test conversion and all 3 responded to various antigens in vitro, while 1 of 2 patients improved clinically. Ballow and Good (1976) treated a 12 year-old patient, who was not capable of producing delayed hypersensitivity skin reactivity to 3 common antigens and suffered from intermittent bronchopulmonary

infections, with transfer factor. After treatment with 4 injections of dialysable leucocyte transfer factor, conversion of skin reactivity occurred to 2 of the 3 antigen markers present in the transfer factor donor. No change occurred in lymphocyte transformation responses, but in the following 4 months there was clinical improvement without any episodes of fever or pneumonia.

From most of the above observations in the clinical application of transfer factor in immunodeficient disorders (table 6) the following conclusions can be drawn: (a) there seems to be a dissociation of skin test reactivity and LIF and/or MIF production from lymphocyte transformation, (b) there is a strong association between the skin test reactivity and LIF and /or MIF production, and (c) there is some association between skin test and cell migration (LIF/MIF) reactivities and clinical improvement.

The clinical use of transfer factor for the treatment of infectious, neoplastic and immuno-regulatory diseases has not been free of investigational problems and reported side effects.

1.2.4 Investigational problems and side effects.

The above accounts of the therapeutic use of transfer factor are largely limited to selected clinical cases. A major criticism of transfer factor therapy has been the lack of properly controlled clinical trials. Since many diseases go through natural cycles of relapse and remission, and patients may be treated with other therapeutic agents, it is often difficult to assess the clinical benefit of treatment with transfer factor.

It has been reported that transfer factor is a safe therapeutic agent. However, reactivation associated with reconstitution of cellular immunity may in itself produce deleterious side-effects. For example Moulias et al (1973) reported that regression of the pulmonary lesions in the children with measles pneumonia was associated with an initial inflammatory response. Drew et al (1973) reported that their patient with Hodgkin's disease and disseminated herpes zoster developed fever and intense erythema around the vesicles following injections of dialysable leucocyte transfer factor. It has also been suggested that the increases in serum transaminase levels in chronic active hepatitis after the administration of dialysable leucocyte transfer factor may reflect liver damage due to cellular immune reactions against the virus-laden liver cells (Kohler et al, 1974).

A further complication was reported by Ballow et al (1973). They reported autoimmune haemolytic anaemia following transfer factor-therapy in patients with Wiskott-Aldrich syndrome. However, they had already observed that there was an unusually high incidence of autoimmune haemolytic anaemia in patients who suffered from Wiskott-Aldrich syndrome and were not treated with transfer factor. This complication may have not, therefore, been due to transfer factor.

Side effects including headache, nausea, arthralgia, malaise and pain at the site of disease lesions have all been observed. (O'Connell et al, 1964; Brandes et al, 1971; Bullock et al, 1972; Graybill et al, 1973).

In contrast to these deleterious effects, Hastings et al (1976) in 180 subcutaneous and intradermal injections of transfer factor did not detect any side effect in his patients. Nor did Oettgen et al (1974) in their patients with breast cancer, or Khan et al (1975) in their's with Hodgkin's disease even when a high dose of transfer factor equivalent to 4.9×10^9 leucocytes was used.

Against such background of mainly uncontrolled clinical applications of transfer factor and only a few reports of possible adverse consequences, it seems prudent to use transfer factor for the therapy of diseases primarily when conventional therapeutic measures are inadequate.

In order to use transfer factor more effectively in the future treatment of various diseases, its in vitro and in vivo biological activities and the specificities of these activities must be determined. The possible relationship between such activities and the therapeutic benefits of transfer factor must be investigated.

1.3 Biological activity of transfer factor preparations in vitro (Tables 7-8).

It is postulated that leucocyte dialysates thought to contain transfer factor can convert non-immune lymphocytes to immune lymphocytes. Thus, it should be possible to culture non-immune lymphocytes with transfer factor and look for the acquisition of the ability to respond to specific antigen. Such specific effects have been reported and were detected by lymphocyte transformation, migration inhibition,

lymphocytotoxicity or other *in vitro* tests. Transfer factor also contains substances which can non-specifically alter *in vitro* lymphocyte responses. Only some of these *in vitro* responses, if any, may be immunologically relevant and reflect non-specific *in vivo* effects.

1.3.1 Lymphocyte transformation (Table 7).

Table 7 shows that, Adler *et al* (1970) and Palmer and Smith (1974) reported that tuberculin-immune human dialysable leucocyte transfer factor could, in transformation cultures, transfer the ability to respond to tuberculin PPD. The "recipient" lymphocytes were from non-immune mice. In contrast, transfer factor obtained from donors not immune to PPD was not effective. Similarly, more recently such *in vitro* transfer of transformation reactivity to leishmania major antigen by leishmania-positive, and not leishmania-negative, transfer factor was reported by Sharma *et al* (1977) using human lymphocyte "recipients" in culture.

Lawrence in 1969 initially added water-dialysed human transfer factor to human lymphocytes *in vitro* in the presence of specific antigen. It was suggested that transfer factor could confer on the "recipient" the antigen sensitivity of the donor, but such activity was irregular. Ascher *et al* (1974) presented evidence that this irregular activity could be overcome by dialysis into tissue culture medium. Such tissue culture medium-dialysed transfer factor (TF_{DM}) was prepared from a donor, who had marked delayed hypersensitivity skin reaction to an antigen and had already transferred this reactivity *in vivo*. On addition of this transfer factor to lymphocyte

Table 7. *In vitro* properties of transfer factor.
I. lymphocyte transformation.

Biological effect	Donor specificity	References
Augmentation of transformation to antigens.	+	Adler et al (1970)
	+	Ascher et al (1974)
	+	Palmer and Smith (1974)
	+	Sharma et al (1977)
	+ & -	Arala-Chaves et al (1976)
	-	Burger et al (1976 a, b)
	-	Hamblin et al (1976 a)
	-	Ascher and Andron (1976)
	-	Salaman (1976)
	-	Littman et al (1977)
	-	This study (Section 6)
Augmentation of transformation to mitogens.	-	Burger et al (1976 b)
	-	Hamblin et al (1976 b)

cultures from a poor responder transformation occurred only in the presence of that antigen. When only such medium-dialysed transfer factor was added to non-immune lymphocytes, significant stimulation or transformation was not observed. Medium-dialysed transfer factor, prepared from skin test-negative individuals, did not cause such antigen-dependent transformation. The stimulation in culture of non-immune lymphocytes occurred only in the presence of those antigens to which the transfer factor donor was sensitive. This reactivity was, therefore, related to the state of the donor's sensitivity (i.e. "donor-specific").

In contrast, there are now reports that similar lymphocyte-stimulation effects have occurred to antigens to which the transfer factor-donor was not sensitive (Hamblin, 1975; Hamblin et al, 1976a; Ascher and Andron, 1976; Salaman, 1976; Littman et al, 1977). This finding led to the concept that such in vitro activity of transfer factor results from the non-specific augmentation of pre-committed cells, rather than the initiation of previously uncommitted lymphocytes (? "recipient-specific"). Similar non-specific activity has been observed in mitogen-induced lymphocyte transformation cultures (Burger et al, 1976 b; Hamblin et al, 1976 b).

A complex set of factors, therefore, govern the in vitro effects of transfer factor on lymphocyte transformation. Sensitivity states of the donor and recipient cells are suggested to be two such contributory factors (Arala-Chaves et al, 1976).

1.3.2 Cell migration inhibition (Table 8).

Table 8 shows that dialysable transfer factor affects migration of guinea pig peritoneal exudate cells and peripheral blood leucocytes alone or mixed with macrophages in the presence of specific antigen.

Salaman (1974) demonstrated that direct migration of normal guinea pig peritoneal exudate cells was inhibited, in the presence of low concentrations of PPD, if dialysable transfer factor from strongly sensitive PPD donors were used. Usually there was not any inhibition in the presence of PPD negative dialysable transfer factor. At higher concentrations of PPD the results were not so consistent. Sephadex column fractions of non-dialysable transfer factor from guinea pigs immunised with ovalbumin or bovine gamma globulin have also been used. Dunnick and Bach (1975) used such fractions and reported a 24 - 28 per cent migration inhibition of guinea pig cells in the presence of specific antigen. In the presence of non-specific antigen migration inhibition was only 5 - 15 per cent.

Read and Zabriskie (1972) employed a direct peripheral blood leucocyte migration test to demonstrate the <u>in vitro</u> effect of transfer factor. Dialysable transfer factor from tuberculin sensitive donors was incubated with non-immune leucocytes. Migration of these cells was inhibited in the presence of PPD. These observations were extended to studies of measles virus-immune dialysable transfer factor in patients with multiple sclerosis, and Epstein-Barr virus-sensitive material in patients with nasopharyngeal carcinoma (Goldenberg and Brandes, 1972; Utermohlen and Zabriskie, 1973).

Table 8. *In vitro* properties of transfer factor.
II. cell migration inhibition and others.

Biological effect	Donor specificity	References
Macrophage migration inhibition	+	Paque *et al* (1973)
	+	Salaman (1974)
	+	Dunnick and Bach (1975)
Leucocyte migration inhibition	+	Goldenberg and Brandes (1972)
	+	Read and Zabriskie (1972)
	+	Utermohlen and Zabriskie (1973)
Mixed leucocyte-macrophage migration inhibition	?	Dabrowska *et al* (1976)
Cytotoxicity	+	Levin (1974)
Chemotaxis	-	Gallin and Kirkpatrick (1974)
T-lymphocyte maturation	-	Mendes *et al* (1975)
	-	Valdimarsson (1975)
	-	Holzman *et al* (1976)

In similar studies Paque et al (1973) used the indirect migration inhibition test. Leucocytes from skin-test negative individuals were incubated with whole cell lysate or non-dialysable transfer factor and specific antigen. Transfer factor donors were strongly positive to histoplasmin and/or coccidioidin. In the presence of any one of these antigens MIF was released and inhibited the migration of guinea pig macrophages. Dialysable transfer factor and the cell lysate or non-dialysable transfer factor, in the absence of antigen or in the presence of non-specific antigen, were ineffective.

The in vitro activity of transfer factor has also been investigated by using a mixed leucocyte-macrophage migration system (Dabrowska et al, 1976). It was indicated that some human dialysable transfer factor preparations, from tuberculin-positive donors, apparently conferred a positive mixed cell migration reactivity to tuberculin PPD upon mixed populations of Mantoux-negative lymphocytes and guinea pig macrophages. However, antigen specificity and reproducibility of this effect were not confirmed.

All these reports suggest that it may be possible to adoptively sensitise previously unsensitised lymphocytes by transfer factor, so that MIF and/or LIF lymphokines are generated to a specific antigen.

1.3.3 Lymphocytotoxicity (Table 8).

Levin (1974) has described an assay system for transfer factor by employing dialysable transfer factor from family contacts of patients with osteogenic sarcoma. Dialysable transfer factor, from

such family contacts, induced increased lymphocytotoxicity to cultured osteogenic sarcoma cells. Transfer factor, prepared from donors whose lymphocytes were not cytotoxic for sarcoma cells or for hypernephroma cells, was not effective.

1.3.4 Leucocyte chemotaxis (Table 8).

Gallin and Kirkpatrick (1974) have found that human dialysable transfer factor was strongly chemotactic for granulocytes, and weakly chemotactic for monocytes *in vitro* and *in vivo* in the monkey skin. Sephadex G-25 fractionation studies revealed that those fractions capable of transferring *in vivo* delayed hypersensitivity skin reactions were also chemotactic both *in vivo* and *in vitro*.

1.3.5 Maturation of lymphocytes (Table 8).

Wybran *et al* (1973) observed that injections of dialysable transfer factor improved cellular immunity in patients with T-lymphocyte deficiency. Such an improvement was measured by a marked increase in circulating T-lymphocytes. This led to the concept that transfer factor contains a component capable of promoting T-cell maturation or mobilisation in the circulation. It is not yet clear whether the number of T-lymphocytes increase following incubation of peripheral blood lymphocytes with transfer factor. Despite this, it has been reported that trypsinised or heat-treated lymphocytes regain their capacity to form rosettes with sheep red blood cells much earlier, if they are incubated with dialysable transfer factor rather than in tissue-culture medium (Mendes *et al*, 1975;

Valdimarsson, 1975). Therefore, these studies suggested that transfer factor contains T-cell receptors or markers. In contrast, the observation that puromycin-treated trypsinised lymphocytes no longer had the ability to reform rosettes with sheep red blood cells, suggested that transfer factor does not contain the receptors (Holzman et al, 1976). The demonstration that dialysable transfer factor preparations stimulate the synthesis of a T-lymphocyte marker, may well account for the beneficial effect of transfer factor observed in patients with T-cell immunodeficiency (Section 1.2.3, table 6).

1.4 Animal models for transfer factor (Tables 9-10).

1.4.1 Previous study of transfer factor in the rhesus monkey (Table 9).

In 1972, Maddison et al demonstrated that both dialysable and non-dialysable transfer factor preparations, from rhesus monkeys infected with Schistosoma mansoni or mycobacteria, or from PPD sensitive humans, were capable of transferring histologically positive delayed hypersensitivity skin reactions to recipient rhesus monkeys. Some specificity was suggested by the fact that monkeys receiving human or monkey tuberculin sensitive transfer factor did not respond to S. mansoni. However, some monkeys receiving transfer factor from "non-sensitised" animals showed positive delayed hypersensitivity to PPD. In some recipients, skin test conversion was associated with in vitro transformation. Maddison (1974) suggested that transfer factor may also be of therapeutic value in infectious diseases in

the rhesus monkey. She observed that immune transfer factor administered together with hyperimmune serum was effective in reducing the worm burden of monkeys infected with Schistosoma mansoni. This implied transfer of immunity to this infection in this animal.

Gallin and Kirkpatrick (1974) demonstrated that human dialysable transfer factor, prepared from donors responsive to mumps, streptokinase-streptodornase and candida antigens, transferred delayed skin reactivity to recipient rhesus monkeys. Reactions were histologically confirmed and reflected the antigen sensitivity of the donors, both in intensity and specificity.

Zanelli and Adler (1975) transferred cutaneous sensitivity from BCG-sensitised rhesus monkeys to rhesus and cynomolgus monkeys. They used viable cells, cell lysates or non-lyophylised dialysable transfer factor. The recipient monkeys converted to skin test positive on histological examination, but their lymphocyte transformation responses to PPD remained negative.

Recently, Dumonde et al (1976) reported transfer of skin test reactivity to keyhole limpet haemocyanin and tuberculin PPD in 1 rhesus monkey given multiple injections of immune transfer factor. These investigators and Scalise et al (1976) have also reported marked transfer of mixed leucocyte-macrophage migration reactivity to keyhole limpet haemocyanin, tuberculin PPD and/or hepatitis B surface antigen by means of immune dialysable rhesus transfer factor.

Table 9. *In vivo* properties of transfer factor in the rhesus monkey

Reference	TF preparation	Assessment of immune transfer
Maddison *et al* (1972)	TF_D/TF_{ND} (rhesus) → rhesus	+ve skin biopsies, +ve LT to Schistosoma mansonii and mycobacteria.
Gallin and Kirkpatrick (1974)	TF_D/TF_{ND} (human) → rhesus	+ve skin biopsies to PPD or to mumps, SKSD, PPD & Candida.
Zanelli and Adler (1975)	Viable cells/cell lysate/TF_D (rhesus) → rhesus & cynomolgus	+ve skin biopsies to PPD, -ve LT to PPD.
Dumonde *et al* (1976)	TF_D (rhesus) → rhesus	+ve skin biopsies to KLH, PPD, +ve LMMI to PPD, KLH, -ve LT.
Scalise *et al* (1976)	TF_D (rhesus) → rhesus	-ve skin biopsies to PPD, KLH, +ve LMMI to PPD, KLH, and HBsAg.
This study (Sections 3-5)	TF_D (rhesus) → rhesus	+ve skin biopsies and LMMI to PPD, KLH & HBsAg, but -ve LT.

TF = transfer factor, TF_D/TF_{ND} = dialysable/non-dialysable transfer factor preparations, PPD = purified protein derivative of tuberculin, SKSD = streptokinase-streptodornase, HSV-1 = herpes simplex virus type 1, DNCB = dinitrochlorobenzene, HBsAg = purified hepatitis B surface antigen, KLH = keyhole limpet haemocyanin, LT = lymphocyte transformation, LMMI = mixed leucocyte-macrophage migration test.

1.4.2 Transfer factor study in other animals (Table 10).

In the guinea pig system, Bloom and Chase (1967) attempted exhaustive studies to transfer delayed hypersensitivity with transfer factor. Guthrie et al (1967) reported the transfer of sensitivity to dinitrofluorobenzene using supernatants, derived from the incubation of sensitised cells with this chemical antigen. These observations were extended by others, some with highly controversial outcome, to a study of transfer of specific delayed hypersensitivity by guinea pig dialysable transfer factor (Burger and Jeter, 1971; Rosenfeld and Dressler, 1974). These findings have proved difficult to repeat in many laboratories.

More recently Clinton and Magoc (1976) have reported that dialysable transfer factor, from animals infected with Leishmania enriettii or actively immunised with Freund's complete adjuvant, transferred specific delayed skin reaction, macrophage migration inhibition and lymphocyte transformation to recipient animals.

Welch et al (1976a, b) reported that guinea pigs, primed with tuberculin PPD or streptokinase-streptodornase, can acquire antigen-specific skin test reactivity. These observations were extended to look for donor and recipient specificity, and the biochemical fractionation of the transfer activity (Vandenbark et al, 1976; Wilson et al, 1976, 1977). Whilst the results are not yet complete, it was suggested that donor specificity may be essential for transfer since transfer factor from antigen-negative or unsensitised donors have proved ineffective.

Table 10. *In vivo* properties of transfer factor in other animals

Reference	TF preparation	Assessment of immune transfer
Guthrie *et al* (1967)	TF_{ND} (guinea pig) → guinea pig	+ve skin test to DNFB
Burger & Jeter (1971), Rosenfeld & Dressler (1974)	TF_D (guinea pig) → guinea pig	+ve skin test to DNCB
Clinton and Magoc (1976)	TF_D (guinea pig) → guinea pig	+ve skin test and MMI to PPD and Leishmania.
Welch *et al* (1976 a, b)	TF_D (human) → guinea pig (primed)	+ve skin tests to PPD and SKSD.
Trepo and Prince (1976)	TF_D (human) → chimp's	+ve skin biopsies & LMT to PPD, HBsAg.
	TF_D/viable leucocytes (chimp's) → chimp's	+ve skin biopsy to HBsAg.
Steele *et al* (1976)	Cell lysates (baboon) → baboon, cebus, marmoset	+ve skin tests (17%) and LT (33%) to monilia, tetanus, mumps, HSV-1, DNCB, PPD.
Shifrine *et al* (1976)	Cell lysate (dogs) → dogs	+ve LT to *Coccidioides immitis*, increased LT to PHA, -ve LT to PPD.
Rifkind *et al* (1976)	TF_D (human) → Mice	+ve skin test, foot pad swelling test to coccidioidin.

TF = transfer factor, TF_D = dialysable transfer factor, TF_{ND} = non-dialysable transfer factor, DNFB = dinitro-fluorobenzene, DNCB = dinitrochlorobenzene, PPD = purified protein derivative of tuberculin, SKSD = streptokinase-streptodornase, HBsAg = purified hepatitis B surface antigen, MMI = macrophage migration inhibition test, LMT = leucocyte migration test, LT = lymphocyte transformation test, PHA = phytohaemagglutinin.

In other animals, Trepo and Prince (1976) observed that transfer factor, from human or chimpanzee donors sensitised to hepatitis B virus and/or PPD, transferred sensitivity to hepatitis B surface antigen (HBsAg) and/or PPD in recipient chimpanzees, which were chronic asymptomatic carriers of HBsAg for over 2 years. Transfer factor used was in the form of dialysable human or chimpanzee transfer factor or viable chimpanzee blood leucocytes. Adoptively transferred delayed hypersensitivity to sensitising antigens was, in the recipient animals, detected by the acquisition of delayed skin test reactivity and inhibition of leucocyte migration test. Specificity of transfer factor was implied by the fact that, when its source was from a person with positive delayed hypersensitivity skin reaction to PPD, the recipient chimpanzee became skin test positive to this antigen. Transfer factor from donors not sensitive to PPD were not effective.

Recently, Steele et al (1976) injected three species of nonhuman primates; baboons, cebus monkeys and marmosets, with dialysable transfer factor from a human donor and non-dialysable transfer factor from baboons. Transfer of sensitivity was evaluated by gross skin testing and lymphocyte transformation. 45% of skin tests and 65% of the lymphocyte transformation tests became positive in recipients injected with human transfer factor. In recipients of baboon transfer factor 17% of skin tests and 33% of transformation tests converted to positive. Using many antigens to which donors were sensitised and not sensitised (monilia, tetanus, herpes simplex type 1, dinitrochlorobenzene and PPD), it was possible to show that recipients never converted to antigens to which transfer factor donors were negative or not sensitised.

In dogs, Shifrine et al (1976) transferred sensitivity to Coccidioides immitis using canine transfer factor in the form of blood leucocyte lysate. This was detected by lymphocyte transformation to antigen. Phytohaemagglutinin stimulation in transfer factor recipients also increased, probably implying transfer of non-specific sensitivity or a host response to injection of transfer factor.

In mice, Rifkind et al (1976) observed that dialysable transfer factor, from skin test and lymphocyte transformation positive human donors, transferred delayed hypersensitivity to coccidioidin antigen in mice. Sensitivity was detected by the skin and foot pad swelling tests. In contrast, transfer factor from non-reactive normal donors was ineffective.

1.5 The problem of specificity.

Immunological specificity of transfer factor has always been a controversial issue (Bloom, 1973; Arala-Chaves and Fudenberg, 1976; Salaman and Valdimarsson, 1976; Mazaheri et al, 1977). Thus, the following sections discuss the various clinical or in vivo and in vitro observations on the specificity of transfer factor.

1.5.1 Clinical observations.

Clinical and immunological observations on the acquisition of delayed type hypersensitivity following the administration of transfer factor have led to the concept, that transfer factor converts non-immune

to immune lymphocytes *in vivo*. Thus, it has been repeatedly reported that previously unresponsive recipients acquire delayed type skin reactions to antigens to which the donor was positive (Lawrence *et al*, 1960; Rapaport *et al*, 1960; Maurer, 1961; Zuckerman *et al*, 1974; Kirkpatrick and Smith, 1976). (See section 1.1.1). However, there are reports that recipients may acquire skin test reactivities which were not demonstrable in the donor (Levin *et al*, 1971; Spitler *et al*, 1972; Griscelli, 1975). It has also been shown that not all positive skin reactions demonstrable in the donor may be transferred to the recipient (Kirkpatrick and Smith, 1976). Other alterations in the cellular-immune responsiveness, which do not apparently relate to donor specificity, have been reported in recipients. These include altered mixed lymphocyte culture responsiveness (Dupont *et al*, 1974), increased responsiveness to phytohaemagglutinin *in vitro* (Arala-Chaves *et al*, 1974; Valdimarsson *et al*, 1974), and increases in the numbers of circulating T-cells as shown by increased sheep red blood cell rosetting (Wybran *et al*, 1973). These observations, in man, have led to the concept that dialysable leucocyte transfer factor, in addition to conferring antigen responsiveness, may also possess non-specific adjuvant like properties. Further support for this hypothesis has been gained from laboratory studies designed to test dialysable leucocyte transfer factor activity *in vivo* in animals and *in vitro*.

1.5.2 *In vivo* and *in vitro* observations.

Much of the scepticism which surrounded the early work on transfer

factor resulted from the inability to demonstrate the transfer phenomenon in animals. Recently, however, studies in nonhuman primates, guinea pigs and other animals (Tables 9-10) have demonstrated that some of the immunological properties of leucocyte extracts, or transfer factor, described in man (Tables 3-6) may be reproduced in animals.

In nonhuman primates successful transfer of delayed hypersensitivity using viable leucocytes, leucocyte extracts, and dialysable transfer factor, has been reported from monkey to monkey and from man to monkey (Table 9: Zanelli and Adler, 1975; table 10: Steele et al, 1976a). Immunological specificity was shown by the observation that recipients never converted to antigens to which the donor did not react in vivo.

In guinea pigs transfer of delayed hypersensitivity has proved extremely difficult (Bloom and Chase, 1967). However, recent studies have shown that guinea pigs, primed with antigen, gave delayed skin reaction following injection of dialysable leucocyte transfer factor (Table 10: Welch et al, 1976a, b). Whilst exposure to antigen is a pre-requisite for the demonstration of subsequent delayed hypersensitivity skin reactions, the role of donor specificity in eliciting such responses has yet to be fully determined.

In vitro tests for transfer factor have also provided evidence for both specific and non-specific activities in dialysable transfer factor preparations (Tables 7-8). Thus, in vivo and in vitro test systems

for transfer factor lend support to the clinical observations that dialysable leucocyte transfer factor contains both specific and non-specific activities. The relative importance of the specific and non-specific activities of transfer factor for the treatment of disease have yet to be delineated. The biochemical properties of dialysable leucocyte transfer factor have been investigated in an attempt to determine which components are responsible for the alterations in cellular immune function *in vivo* and *in vitro*.

1.6 Biochemical properties and fractionation of transfer factor.

Using the transfer of delayed hypersensitivity as the criteria for assessing activity, the properties described in table 1 were reported by Lawrence (1973, 1974).

Fractionation of crude dialysable leucocyte transfer factor has been attempted in order to locate its specific and non-specific components. Sephadex column chromatography has shown the material to be of heterogenous composition, and that it can be separated into a number of fractions (Baram *et al*, 1966; Arala-Chaves *et al*, 1967; Gottlieb *et al*, 1973; Zuckerman *et al*, 1974; Burger *et al*, 1976a; Vandenbark *et al*, 1977). Several publications report that one of the fractions, rich in hypoxanthine, is associated with the ability to transfer delayed skin reactions (O'Dorisio *et al*, 1976; Kirkpatrick *et al*, 1976; Tomar *et al*, 1976). Others have produced evidence for fractions, which stimulate non-specifically weak immune reactivity *in vivo* (Krohn *et al*, 1976a, b; 1977) and augment

lymphocyte transformation *in vitro* (Burger *et al*, 1976a, b; Littman *et al*, 1977). More biochemical studies are required to relate these activities to each other and to the therapeutic efficacy of transfer factor.

1.7 Mode of action of transfer factor.

Various theories have been postulated to explain how transfer factor may initiate and/or promote cell-mediated hypersensitivity.

1.7.1 Informational derepressor theory.

Lawrence (1969a) suggested that transfer factor may be an informational polynucleotide, possibly a short double stranded RNA or a specific gene derepressor. Apparently, previously uncommitted naive lymphocytes are instructed to produce antigen-specific receptors and become specifically sensitised. These lymphocytes subsequently undergo clonal proliferation and donor specific delayed hypersensitivity is thereby induced. In fact, Dumonde and Maini (1971) speculated that, in the presence of a specific antigen, leucocyte dialysates containing transfer factor may induce non-sensitised lymphocytes to produce lymphokines to mediate cell-mediated immunity (Dumonde *et al*, 1969). In the rhesus monkey model, there is evidence that transfer factor may initiate such adoptive sensitisation for selective lymphokine production (Dumonde *et al*, 1976).

1.7.2 Receptor theory.

The easiest explanation would be that dialysable transfer factor carries the donor-specific receptor, but this is thought to be unlikely (Valdimarsson, 1975). Transfer factor may act by stimulating recipient lymphocytes to produce more transfer factor, which then recognises and stimulates a select group of non-immunoglobulin cell surface receptors, called minireceptors. Dialysable transfer factor may react with these receptors thereby initiating an appropriate signal calling for the transfer of cell-mediated immunity (Burnet, 1974).

1.7.3 Superantigen theory.

Another suggestion is that transfer factor may be a macrophage processed highly immunogenic "superantigen". This seems incompatible with the finding that on injection into individuals, it does not apparently lead to antibody synthesis (Lawrence, 1969a). Furthermore, serial transfer of delayed hypersensitivity from individuals A to B to C has been reported and does not support this postulate (Lawrence, 1974; Kirkpatrick and Smith, 1976).

1.7.4 Adjuvant theory.

It has been observed that *in vivo* and *in vitro* recipients of transfer factor are capable of responding to antigens to which donors were skin-test negative (Levin *et al*, 1970, 1971; Spitler *et al*, 1972 ; Griscelli, 1975; Khan *et al*, 1975; Hamblin *et al*, 1976b; Krohn

et al, 1976b; Khan, 1977; Littman et al, 1977). Therefore, an alternative view to the transfer of specific sensitivity is that transfer factor, in some situations, acts as an immunologically non-specific adjuvant and promotes the activity of precommitted lymphocytes. According to this theory the effectiveness of transfer factor depends upon the immune status of the recipient rather than that of the donor.

1.8 Conclusions

1.8.1 The rhesus monkey as a useful experimental model for the study of transfer factor (Table 9).

Some of the investigators mentioned above have reported evidence that, the rhesus monkey (Macaca mulatta) can provide a useful experimental model for the in vivo study of transfer factor (Table 9). Others have reported that immune rhesus transfer factor, in vitro, transferred cellular immune responses to rhesus monkey and human lymphocytes (Baram and Condoulis, 1970). These investigators also later showed that even fractions of antigen-specific rhesus monkey dialysable leucocyte transfer factor, can be assayed by MIF production in vitro (Baram and Condoulis, 1976).

In contrast to man, the rhesus monkey permits relatively free choice of immunogen and a ready monitoring of the cellular immune status of transfer factor donors and recipients. Healthy anergic human volunteers, unlike the rhesus monkey, present ethical, immunological and organisational problems.

Compared with the other nonhuman primates, this species was more readily available during these experiments and was generally more economically purchased and maintained. Unlike the smaller animals, the rhesus monkey, besides providing a larger and more convenient source of transfer factor, provides a larger surface area for a range of skin tests.

In comparison to mice, rats, guinea pigs and rabbits, rhesus monkeys also have the convenience of a much larger volume of blood required for a range of *in vitro* tests.

1.8.2 Background to design of the present experimental work.

In view of the use of dialysable transfer factor for the treatment of various diseases in man (Section 1.2, tables 3-6), and the controversy surrounding its specificity *in vivo* and *in vitro* and mode of action (Sections 1.3-1.5, Section 1.7) the present study was set up. The main aims of this study were (a) to test the rhesus monkey as a useful experimental model for the study of dialysable transfer factor, (b) to extend the limited evidence for the specificity of dialysable transfer factor activity *in vivo* and *in vitro*, (c) to develop satisfactory method or methods of monitoring the *in vivo* activity of transfer factor in the rhesus monkey model, and (d) to make preliminary observations on the fractionation properties of dialysable transfer factor prepared from man or monkey.

Background to the design of parts of the present experimental work has already been described above (Section 1.8.1) and reported by

Dumonde *et al* (1976) and Scalise *et al* (1976). Therefore, the present Thesis reports detailed study of the following:

(a) The extent of delayed skin test hypersensitivity (DH) acquired by both actively immunised donor and dialysable transfer factor-treated recipient rhesus monkeys and its specificity;

(b) the extent of cellular hypersensitivity, as measured by the direct mixed leucocyte-macrophage migration test (LMMI), acquired by both actively immunised and dialysable transfer factor-treated rhesus monkeys and its specificity;

(c) the extent of cellular hypersensitivity, as measured by the whole blood lymphocyte transformation test (LT), acquired by both actively immunised and dialysable transfer factor-treated rhesus monkeys and its specificity;

(d) the *in vitro* activity of dialysable transfer factor preparations, from monkey or man, upon antigen-stimulated human lymphocytes and its specificity, and

(e) the preliminary fractionation properties of rhesus monkey and human dialysable transfer factor preparations, as indicated by their elution through Sephadex G-25 columns.

2. EXPERIMENTAL DESIGN AND METHODS.

In this work the following experiments were carried out:

In vivo study: the experiments consisted of studying the acquisition and specificity of delayed hypersensitivity (DH), mixed leucocyte macrophage migration (LMMI) reactivity and lymphocyte transformation (LT) responses to selective antigens. These tests were done in rhesus monkeys actively immunised with Freund's complete adjuvant (FCA) only, and keyhole limpet haemocyanin or hepatitis B surface antigen in Freund's complete adjuvant (KLH/FCA, HBsAg/FCA). Such tests were also carried out in rhesus monkeys injected with water- or vacuum-dialysed homologous leucocyte transfer factor ($Rh\text{-}TF_{WD}$, $Rh\text{-}TF_{VD}$).

In vitro study: the experiments consisted of studying the activity of both monkey and human dialysable leucocyte transfer factor. Both preparations were made by dialysis into tissue culture medium (monkey: $Rh\text{-}TF_{DM}$, human: $H\text{-}TF_{DM}$). Human leucocyte or purified lymphocyte cultures (LC, PL) were used to detect augmentation of lymphocyte transformation by antigens with dialysable leucocyte transfer factor of monkey and man.

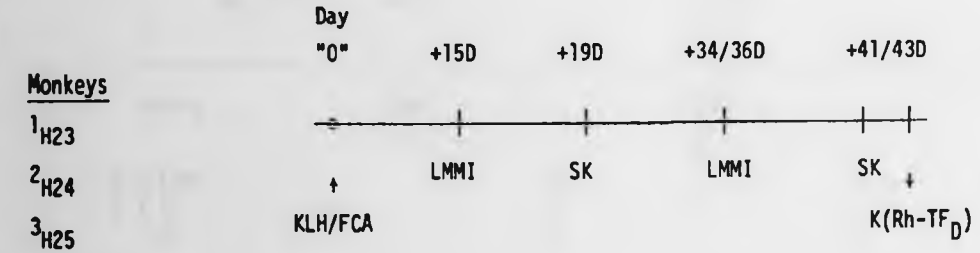
Fractionation of both monkey and human leucocyte dialysates was carried out. Dialysable leucocyte transfer factor was prepared by vacuum-dialysis (monkey: $Rh\text{-}TF_{VD}$, Human: $H\text{-}TF_{VD}$). Fractionation was by column chromatography using Sephadex G-25.

2.1 Protocols for active sensitisation of rhesus monkeys (Figs. 1a-d).

Figure 1a shows that 2 monkeys (H25, H26) were actively immunised with 100 µg KLH in FCA. At the same time two others (H23, H24) were immunised with 1 mg KLH in FCA. The mixed leucocyte-macrophage migration test was carried out, about 2 and 5 weeks after immunisation, with 3-100 µg/ml of sensitising antigens. These antigens were keyhole limpet haemocyanin (KLH), purified protein derivative of tuberculin (PPD) and particulate tubercle bacilli (TB). Skin tests were carried out with 10 µg KLH or PPD about 3 and 6 weeks after active sensitisation. On about the 6th week, after the completion of the second skin test, these animals were killed for the preparation of KLH-immune and FCA-immune Rh-TF_D.

Figure 1b shows immunisation of another 7 animals with FCA with or without KLH. The LMMI and LT tests were carried out 2-3 times before active immunisation. These tests were also done 5 to 42 days after immunisation. The LMMI test was carried out with 3-200 µg/ml antigens and the LT test with 5-100 µg/ml antigens and phytohaemagglutinin. Skin tests were done on two occasions 12-14 and 26-28 days after active sensitisation. These animals' own red blood cells were transfused back. For transfusion, packed red blood cells were made up to the original volume of blood drawn with non-pyrogenic sterile normal saline. These animals were killed 40-42 days after immunisation for preparation of KLH-immune and FCA-immune dialysable rhesus transfer factor.

Fig. 1a. First protocol for active sensitisation of rhesus monkeys with keyhole limpet haemocyanin in Freund's complete adjuvant (KLH/FCA)



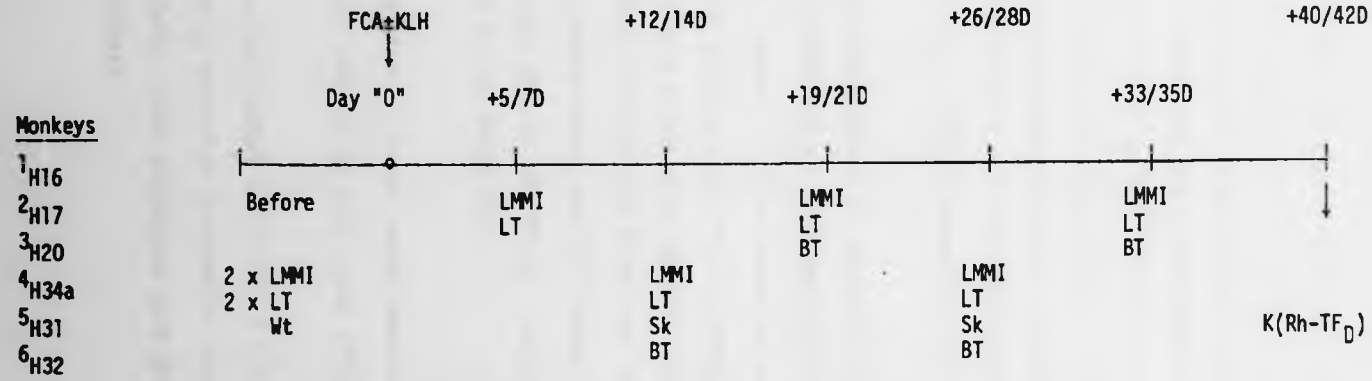
KLH/FCA: contained [1-2] 1 mg KLH in FCA, [3-4] 100 µg KLH in FCA.

LMMI = mixed leucocyte-macrophage migration test: KLH, PPD, TB, at 3-100 µg/ml.

SK = skin test: KLH, PPD at 10 µg.

K = killed and collected blood and lymph nodes/spleen for preparation of KLH- and FCA-immune dialysable leucocyte transfer factor ($Rh\text{-}TF_D$).

Fig. 1b. Second protocol for active sensitisation of rhesus monkeys with keyhole limpet haemocyanin in Freund's complete adjuvant or only this adjuvant (FCA ± KLH).



FCA±KLH: contained $^{1-4}$ 100 µg KLH in FCA or $^{5-7}$ only FCA with 3 mg TB.

$^{1-3}$ LMMI = mixed leucocyte-macrophage migration test: KLH, PPD, TB at 3-200 µg/ml.

$^{1-7}$ LT = lymphocyte transformation test: KLH, PPD, TB, PHA at 5-100 µg/ml.

Wt = weight in kg, Sk = skin test: $^{1-4}$ KLH, $^{1-7}$ PPD at 10 & 30 µg.

BT = blood transfusion.

K = killed and collected blood and lymph nodes/spleen for preparation of KLH- and FCA-immune dialysable leucocyte transfer factor (Rh-TF$_D$).

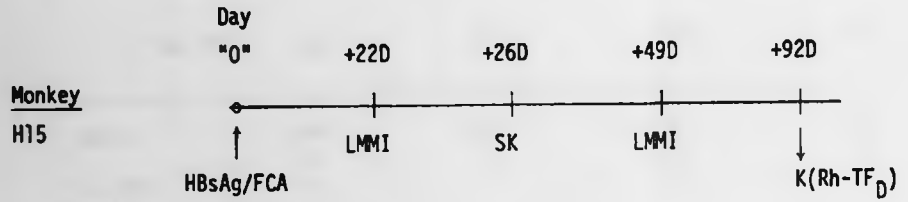
Figure 1c shows that one monkey (H15) was actively immunised with 1 mg purified hepatitis B surface antigen in FCA (HBsAg/FCA). The LMMI test was carried out about 3 and 7 weeks after active sensitisation. Only 1/10 and 1/25 dilutions of a stock solution of purified hepatitis B surface antigen were used in this test. Skin testing was done with 50 µg HBsAg. This was carried out about 4 weeks after active sensitisation. On about the 13th week this animal was exsanguinated and the lymph nodes and spleen were removed for the preparation of HBsAg-immune and FCA-immune Rh-TF_D.

Figure 1d shows immunisation of another 5 rhesus monkeys with HBsAg/FCA. The LMMI and LT tests were carried out 2-3 times before and at various intervals, after immunisation. For the migration test 3-100 µg/ml antigen (KLH, PPD or HBsAg), and for the transformation test 5-100 µg/ml antigen (PPD or HBsAg) were used. All rhesus monkeys were killed 42-51 days after immunisation for preparation of HBsAg-immune and FCA-immune transfer factor. Their mean total weight loss by the end of immunisation experiments in figures 1b and 1d was about 10%.

2.1.1 Selection of rhesus monkey donors of dialysable transfer factor for the *in vivo* study (Table 11).

Table 11 shows that normal unsensitised and actively immunised rhesus monkeys served as donors of dialysable leucocyte transfer factor for the *in vivo* study. Such dialysates were designated normal and immune Rh-TF_D respectively.

Fig. 1c. First protocol for active sensitisation of a rhesus monkey with hepatitis B surface antigen in Freund's complete adjuvant (HBsAg/FCA).



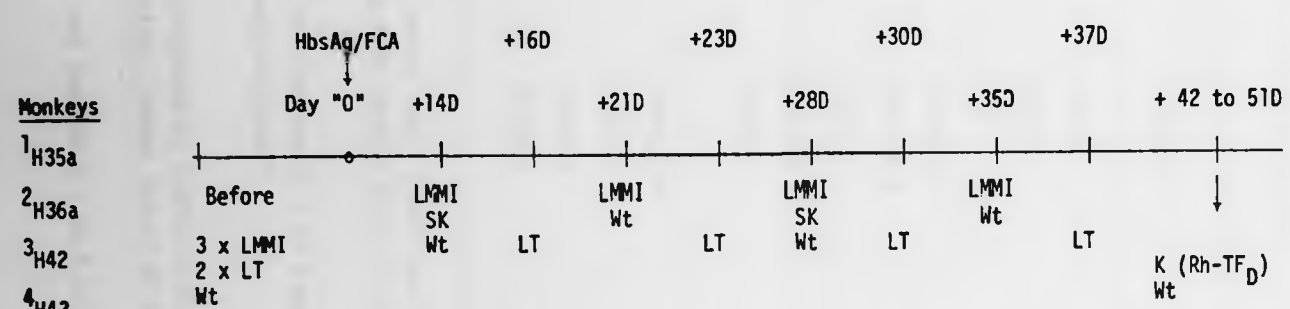
HBsAg/FCA: contained 1 mg HBsAg in FCA

LMMI = mixed leucocyte-macrophage migration test: HBsAg at 1/10, 1/25 dilutions.

SK = skin test: HBsAg at 50 µg.

K = killed and collected blood and lymph nodes/spleen for preparation of HBsAg- and FCA-immune dialysable leucocyte transfer factor (Rh-TF$_D$).

Fig. 1d. Second protocol for active sensitisation of rhesus monkeys with hepatitis B surface antigen in Freund's complete adjuvant (HBsAg/FCA).



HBsAg/FCA: contained 1mg HBsAg in FCA.

3-5 LMMI = mixed leucocyte-macrophage migration test: KLH, PPD, HBsAg at 3-100 µg/ml.

1-2 LT = lymphocyte transformation test: PPD, HBsAg at 5-100 µg/ml.

Wt = weight in kg, SK = skin test: PPD, HBsAg at 10 & 30 µg.

K = killed and collected blood and lymph nodes/spleen for preparation of HBsAg- and FCA-immune dialysable leucocyte transfer factor (Rh-TF_D).

Table 11. Rhesus monkey donors and recipients of dialysable rhesus transfer factor used for the *in vivo* study.

	Rh-TF_D donor[a]		Rh-TF_D recipient[b]	
	Rhesus	Immunity	Rhesus	Dose
(1)	Pool SM	Normal	H35	2 (2.4×10^9)
(2)	H45	Normal	H49	1 (4.5×10^9)
(3)	H32	FCA	H48	1 (5.3×10^9)
(4)	H43	HBsAg/FCA	H37	1 (4.5×10^9)
(5)	H43	HBsAg/FCA	H38	1 (4.5×10^9)
(6)	Pool C	KLH/FCA	H46	1 (4.5×10^9)
(7)	Pool C	KLH/FCA	H47	1 (4.5×10^9)
(8)	Pool C	KLH/FCA	H36	3 (4.5×10^9)
(9)	H15	HBsAg/FCA	H33	1 (4.2×10^9)
(10)	H1-4*	KLH/FCA	H19	1 (8.0×10^9)
(11)	H1-4*	KLH/FCA	H18a	1 (4.0×10^9)
(12)	H23-24*	KLH/FCA	H27	1 (4.0×10^9)

[a]Pool SM, rhesus monkey cells kindly provided by Dr. Shirley Maddison; Pool C, obtained from monkeys H16, 17, 20 and 34a.

[b]Transfer factor in experiments 1 to 8 were "vacuum-dialysed" and in 9 to 12 were "water-dialysed".

*Transfer factor prepared by Professor G. Scalise (Department of Medical Microbiology, London School of Hygiene and Tropical Medicine).

Other details: see section 2.1 to 2.2.1.

Normal Rh-TF_D preparations were obtained from lymphoid cells of 2 healthy rhesus monkeys. Cells for one such preparation were provided by Dr. Shirley Maddison (Division of Parasitology, Centre for Disease Control, Atlanta, Georgia, USA). The other donor was considered normal on the basis that naive monkeys did not generally react to sensitising antigens in vitro.

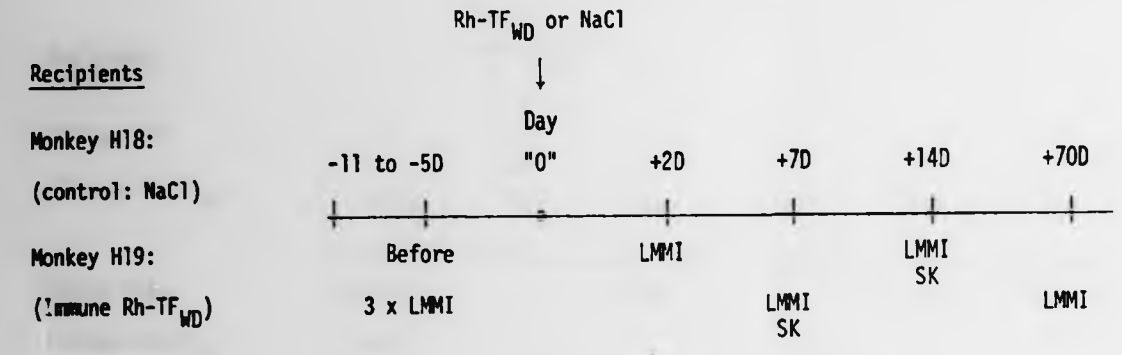
Immune Rh-TF_D was obtained from actively immunised or sensitised rhesus monkeys. 17 male and female monkeys were immunised (3.5 - 8 kg weight) by one set of intramuscular and intradermal injections of Freund's complete adjuvant (FCA) alone or two antigens individually emulsified in FCA. The sensitising antigens used were keyhole limpet haemocyanin (KLH: 100 µg - 1 mg) and hepatitis B surface antigen (HBsAg: 1 mg). FCA always contained 3 mg heat killed tubercle bacilli (TB).

2.2 Protocols for adoptive sensitisation of rhesus monkeys (Figs. 2a-e).

The protocols for adoptive sensitisation of naive rhesus monkeys with water- and vacuum-dialysed rhesus transfer factor were as follows:

Figures 2a-c show that four naive rhesus monkeys received one injection of immune water-dialysed Rh-TF_{WD}. Two others were given

Fig. 2a. First protocol for adoptive sensitisation of a rhesus monkey following an injection of homologous dialysable leucocyte transfer factor immune to keyhole limpet haemocyanin and Freund's complete adjuvant.



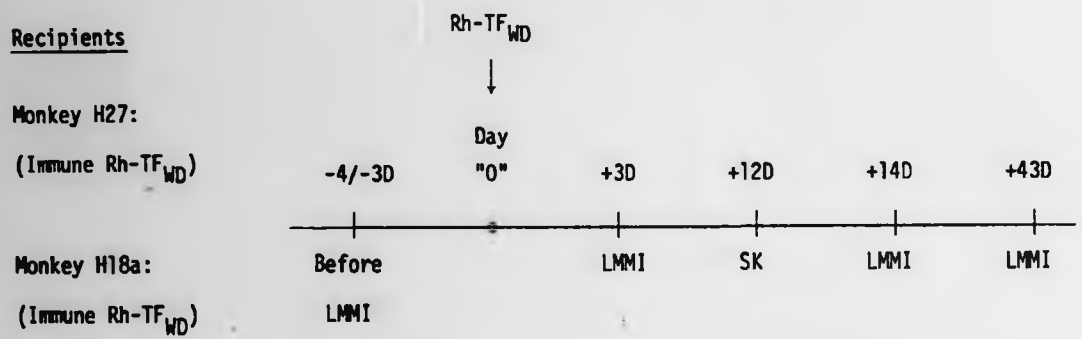
NaCl = sterile normal saline : 20 mls.

Rh-TF$_{WD}$ = water-dialysed rhesus monkey leucocyte transfer factor. Dose : 8×10^9 lymphocytes.

LMMI = mixed leucocyte-macrophage migration test: KLH, PPD, TB, at 3-100 µg/ml.

SK = skin test: KLH, PPD at 30 µg.

Fig. 2b. Second protocol for adoptive sensitisation of rhesus monkeys following an injection of homologous dialysable leucocyte transfer factor immune to keyhole limpet haemocyanin and Freund's complete adjuvant.

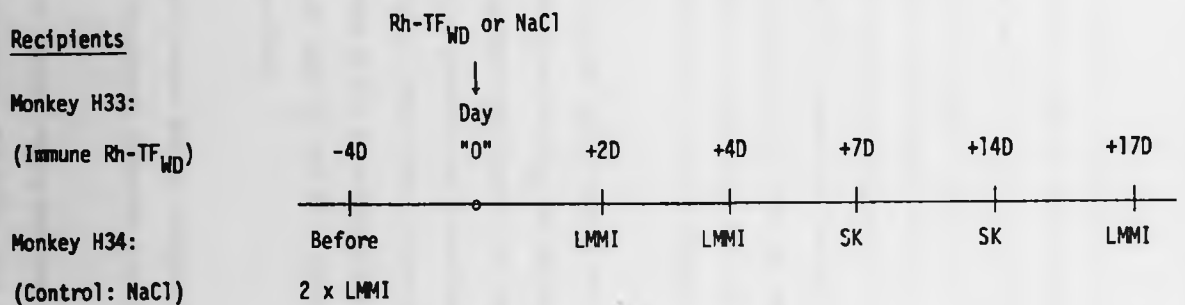


Rh-TF$_{WD}$ = water-dialysed rhesus monkey leucocyte transfer factor.
Dose: 4×10^9 lymphocytes.

LMMI = mixed leucocyte -macrophage migration test: KLH, PPD, TB at 3-100 µg/ml.

SK = skin test: KLH, PPD at 30 µg.

Fig. 2c. First protocol for adoptive sensitisation of a rhesus monkey following an injection of homologous dialysable leucocyte transfer factor immune to hepatitis B surface antigen and Freund's complete adjuvant.



NaCl = sterile normal saline: 30 mls.

Rh-TF$_{WD}$ = water-dialysed rhesus monkey leucocyte transfer factor. Dose: 4.2 x 10^9 lymphocyte equivalent.

LMMI = mixed leucocyte-macrophage migration test: HBsAg at 1/10, 1/25 dilution.

SK = skin test: HBsAg at 50 µg.

only sterile normal saline. These animals were examined only by *in vitro* LMMI test 3-11 days before and 2-70 days after Rh-TF_{WD} injection. Skin tests were carried out 7-14 days after the injection.

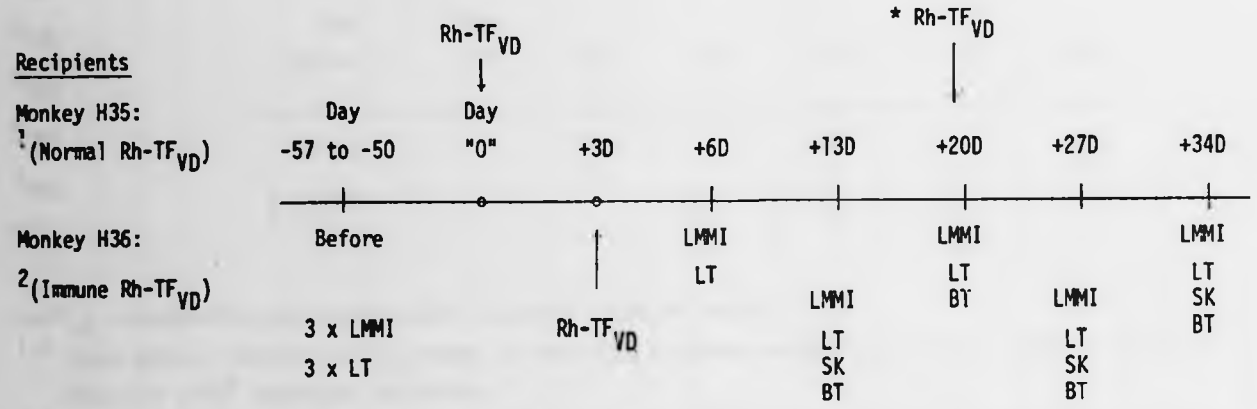
Figure 2d shows that multiple injections of vacuum-dialysed homologous Rh-TF_{VD} was also used in adoptive sensitisation. Both mixed leucocyte-macrophage migration and lymphocyte transformation tests were carried out some days before and 3-34 days after the first injection of normal and immune Rh-TF_{VD}. Skin tests were carried out 13-34 days after this injection. Packed red blood cells from the previous week were suspended in sterile normal saline, stored at $4^{o}C$, and transfused back into the same animal the following week. The final volume of transfusion was the same as the original volume of blood withdrawn the previous occasion.

Figure 2e shows that 6 other naive monkey recipients were given one injection of vacuum-dialysed Rh-TF_{VD} on day zero. Both normal and immune leucocyte dialysates were used. All these animals were examined twice by cell migration and transformation tests 7-28 days before Rh-TF_{VD} injection. After this injection migration tests were carried out between days 2-21, transformation tests between days 2-17, and skin tests on days 14 and 21. Blood transfusion was given on 4 occasions on days 7-21.

2.2.1 Selection of rhesus monkey recipients of homologous dialysable leucocyte transfer factor (Table 11).

Figures 2a-e show that "naive" recipient monkeys were first examined by mixed leucocyte-macrophage migration test and/or lymphocyte

Fig. 2d. The only protocol for adoptive sensitisation of a rhesus monkey with multiple injections of homologous dialysable leucocyte transfer factor.



Rh-TF$_{VD}$ = vacuum-dialysed rhesus monkey leucocyte transfer factor.
[1]Injected on days 0, 3 (2.4 x 10^9 lymphocyte equivalent/dose). *At day 20 NaCl was injected instead of normal Rh-TF$_{VD}$. [2]Injected on days 0, 3, 20. (4.5 x 10^9 lymphocyte equivalent/dose).

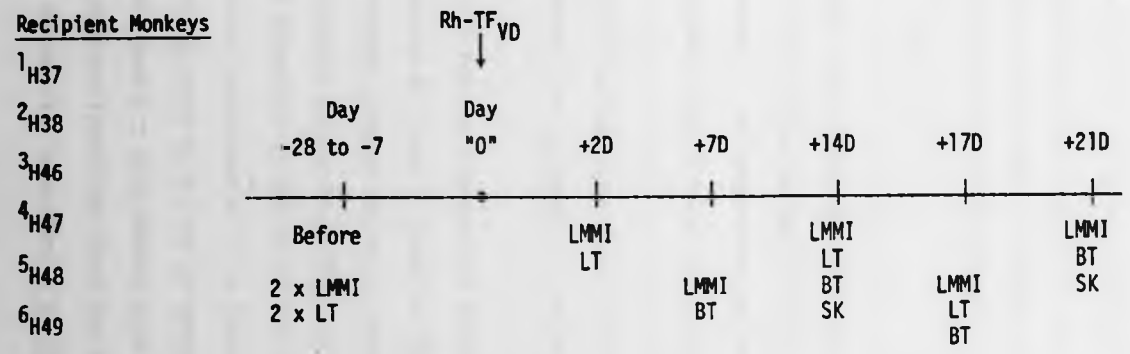
LMMI = mixed leucocyte-macrophage migration test: KLH, PPD, TB at 3-200 µg/ml.

LT = lymphocyte transformation test: KLH, PPD, TB, PHA at 5-100 µg/ml.

SK = skin test: KLH, PPD at 50 µg.

BT = blood transfusion.

Fig. 2e. Last protocol for adoptive sensitisation of rhesus monkeys with one injection of homologous dialysable leucocyte transfer factor.



$Rh\text{-}TF_{VD}$ = vacuum-dialysed rhesus monkey leucocyte transfer factor.

1,2 These animals received $Rh\text{-}TF_{VD}$ immune to hepatitis B surface antigen and Freund's complete adjuvant. Dose: 4.5×10^9 lymphocyte equivalent.

3,4 Received $Rh\text{-}TF_{VD}$ immune to keyhole limpet haemocyanin and FCA. Dose: 4.5×10^9 lymphocyte equivalent.

5 Received $Rh\text{-}TF_{VD}$ immune to only FCA. Dose: 5.3×10^9 lymphocyte equivalent.

6 Received normal $Rh\text{-}TF_{VD}$. Dose: 4.5×10^9 lymphocyte equivalent.

LMMI = mixed leucocyte-macrophage migration test: KLH, PPD, TB, HBsAg at 3-200 µg/ml.

LT = lymphocyte transformation test: KLH, PPD, TB, HBsAg, PHA at 5-100 µg/ml.

BT = blood transfusion, SK = skin test: KLH, PPD, HBsAg at 50 µg.

transformation test. This was to demonstrate absence of cellular hypersensitivity towards KLH, PPD, TB and HBsAg, and to establish baselines. Each dose of lyophilised homologous dialysable leucocyte transfer factor ($Rh\text{-}TF_D$) was made from 4-8 x 10^9 donor lymphocytes. This was dissolved in 20-30 ml volumes of sterile normal saline. It was injected intramuscularly in all four limbs and subcutaneously over the back of individual recipient monkeys. $Rh\text{-}TF_D$ was prepared either by dialysis into water ($Rh\text{-}TF_{WD}$) or by ultrafiltration into vacuum ($Rh\text{-}TF_{VD}$).

Table 11 shows that altogether 10 rhesus monkeys received immune-$Rh\text{-}TF_D$, while 2 received normal-$Rh\text{-}TF_D$. Of the former group four were recipients of the water-dialysed material, whilst all other recipient animals were injected with the vacuum-dialysed material.

2.3 Selection of human donors of dialysable leucocyte transfer factor for *in vitro* and fractionation studies.

Lymphoid cells for the preparation of dialysable transfer factor were supplied by Dr. Anne S. Hamblin (Kennedy Institute of Rheumatology, London). Healthy persons served as donors of normal dialysable leucocyte transfer factor or $H\text{-}TF_D$. Skin testing was carried out by intradermal injection of 100 TU of old tuberculin (Evans Medial Ltd., London), or its equivalent of 2 µg tuberculin PPD. For injection, antigen was dissolved in 0.1 ml of pyrogen-free sterile saline. Normal donors were not skin tested with KLH. Normal human dialysable leucocyte transfer factor was used for both *in vitro* and fractionation studies. The material used for *in vitro* purposes was dialysed into

tissue culture medium ($H\text{-}TF_{DM}$), while that fractionated was vacuum-dialysed ($H\text{-}TF_{VD}$).

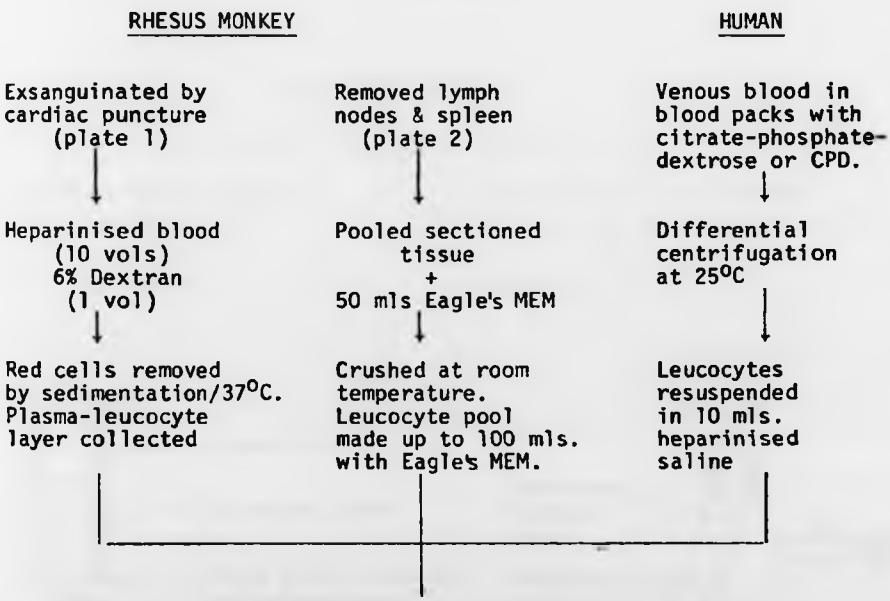
2.4 Selection of human leucocyte or purified lymphocyte culture "recipients" of monkey or human dialysable leucocyte transfer factor.

Leucocytes and purified lymphocytes were obtained from human venous blood. 30-60 mls heparinised blood (25-30 U/ml) were taken from laboratory workers. Majority of the donors had been previously Mantoux tested and were reported to be positive. None of the donors, with the exception of one, were actively sensitised with keyhole limpet haemocyanin. The immunised subject was actively sensitised with 500 µg KLH by one intradermal injection (Dr. R.N. Maini, Kennedy Institute of Rheumatology, London). A positive intradermal skin test with 50 µg KLH was taken as an indication for successful sensitisation.

2.5 Preparation of dialysable leucocyte transfer factor from monkey or man for *in vivo*, *in vitro* and fractionation studies (Fig. 3).

Figure 3 shows that dialysable leucocyte transfer factor for use *in vivo* was prepared only from rhesus monkeys, while that for *in vitro* and fractionation purposes was obtained from both monkey and man. Pooled rhesus monkey lymph node and spleen leucocytes were used for the preparation of leucocyte dialysates for the *in vivo* study. Dialysable leucocyte transfer factor for the *in vitro* study was

Fig. 3. I. Various Methods of Preparing Dialysable Leucocyte Transfer Factor

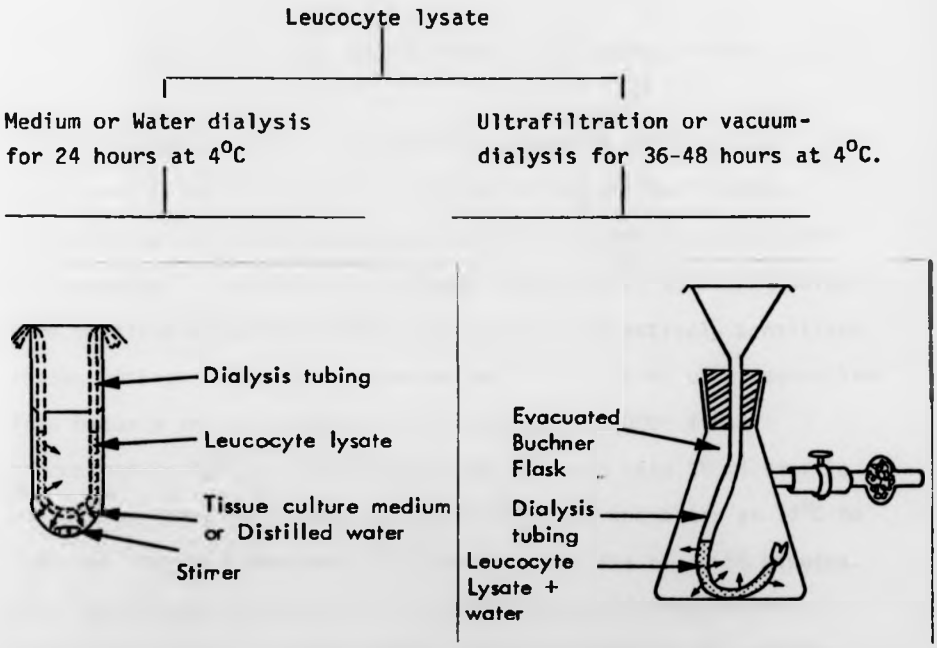


Lymphocytes and polymorphs in the leucocyte suspension were counted. Centrifuged at 200 g for 10-15 minutes at 22-25°C. Leucocyte rich pellet was frozen and stored at -20°C.

1 ml. sterile water and 100 µg DNAse per 10^8 lymphocytes added. Pellet frozen and thawed 10x (-70°C to +37°C) to produce leucocyte lysate. Dialysable leucocyte transfer factor extracted by dialysis for in vivo, in vitro and fractionation studies.

(see Part II).

Fig. 3. II. Various Methods of Preparing Dialysable Leucocyte Transfer Factor



In vitro	In vivo	Fractionation
Medium dialysed rhesus monkey or human transfer factor: (i.e. Rh-TF_{DM} or H-TF_{DM}).	Water or vacuum dialysed rhesus monkey leucocyte transfer factor: (i.e. Rh-TF_{WD} or Rh-TF_{VD}).	Vacuum dialysed rhesus monkey or human leucocyte transfer factor: (i.e. Rh-TF_{VD} or H-TF_{VD}).

For more details see sections 2.5.1 to 2.5.7.

prepared from blood leucocytes of monkey and man. Leucocyte dialysates used for fractionation purposes were from blood leucocytes of man or monkey and from lymph node and spleen leucocytes of rhesus monkey.

2.5.1 Isolation of leucocytes from rhesus monkey blood (Plate 1).

The rhesus monkey donor was anaesthesised by an intramuscular injection of "Ketalar" (stock solution: 50 mg/ml) and the mean concentration of anaesthetic used was 12.5 mg per kilogram weight of the animal. 150-390 mls of blood (mean volume: 45 mls/kg weight) were obtained by cardiac puncture of normal and actively sensitised rhesus monkeys (Plate 1), and mixed with 50 units/ml of preservative-free heparin and 6% dextran (M. wt. 200,000-275,000: final concentration 0.5%). This mixture was taken up into 50 ml sterile plastic syringes and placed vertically into an incubator at 37^{o}C to sediment the red blood cells within the mean time of 40-55 minutes. Wide gauge needles (Yale: 19G 1½: 40/11) were attached to these syringes and the leucocyte-plasma layer was harvested by applying steady, but gentle, pressure on the vertically held syringe plunger. The recovered leucocytes, whose average volume was about 55% of the original volume of blood, were counted for lymphocytes and polymorphs and generally contained > 50% lymphocytes. After counting,the leucocyte layer was divided up into appropriate volumes containing multiples of 10^{8} lymphocytes, placed into sterile plastic universals and centrifuged at 22^{o}C for 15 minutes at 200 g (centrifuge: MSE Mistral 2L). Supernatants were decanted and the cell pellets were quickly frozen in a mixture of dry ice and methanol (-70^{o}C) before being stored at -20^{o}C until required.

Plate 1. Exsanguination of rhesus monkey by cardiac puncture.

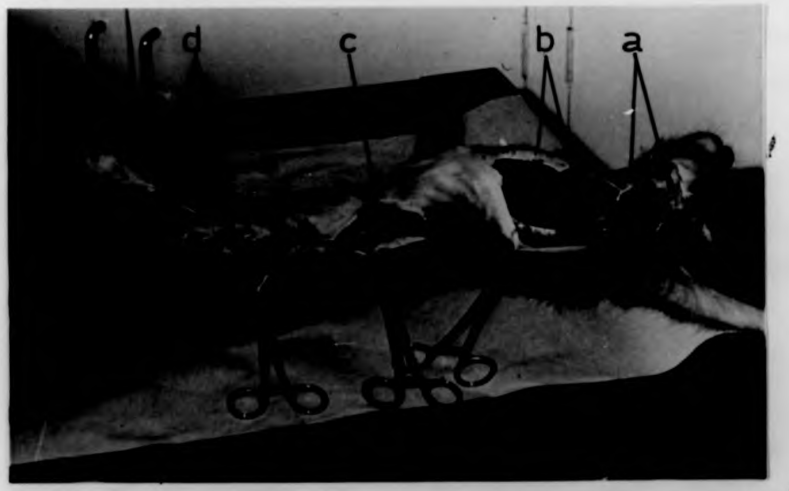


Plate 2. Dissection of rhesus monkey to remove lymph nodes and spleen.
(a) Cervical lymph nodes, (b) thoracic lymph nodes, (c) spleen, (d) inguinal lymph nodes.

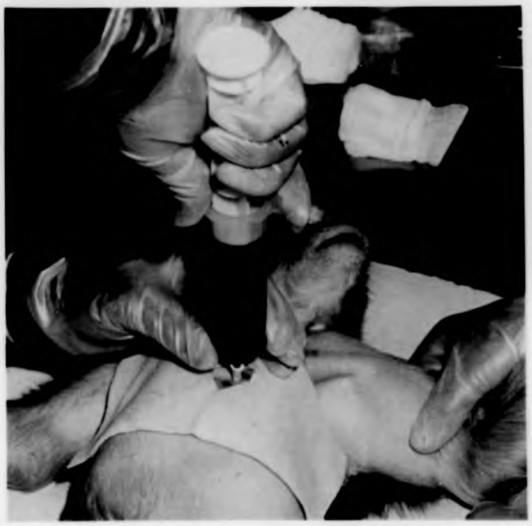


Plate 1. Exsanguination of rhesus monkey by cardiac puncture.

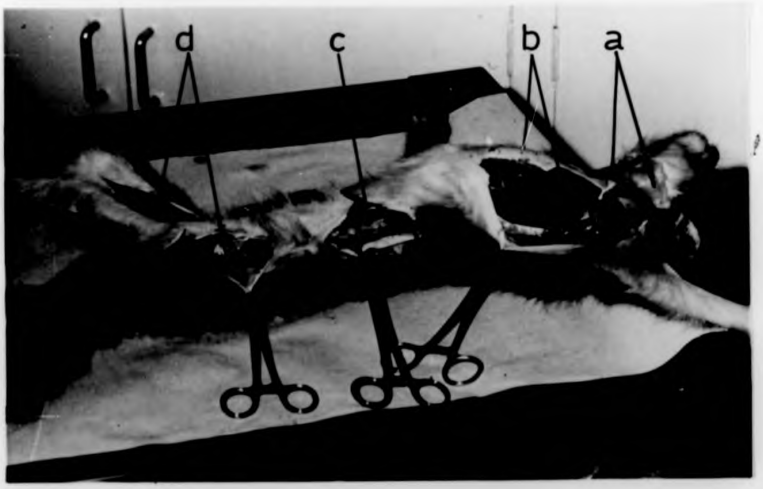


Plate 2. Dissection of rhesus monkey to remove lymph nodes and spleen.
(a) Cervical lymph nodes, (b) thoracic lymph nodes, (c) spleen, (d) inguinal lymph nodes.

2.5.2 Isolation of leucocytes from rhesus monkey lymph-nodes and spleen (Plate 2).

After exsanguination of rhesus monkeys their major lymph nodes and spleen were removed aseptically. Lymoh nodes were obtained from the cervical, thoracic and inguinal regions of normal and actively sensitised donor monkey (Plate 2). These were immediately chopped up into small pieces at room temperature. Lymph-node and spleen pieces were transferred into a sterile bottle containing about 50 ml Eagles minimum essential medium without serum (1 x Eagles MEM). This mixture of lymphoid tissue was crushed through a sterile nickel sieve into a nickel crucible (3.5 cms diameter x 3.5 cms height) under a safety cabinet. The leucocyte pool from the crucible was periodically transferred into another sterile bottle and subsequently volume made up to about 100 ml with Eagles MEM. The lymphoid ceil suspension was mixed gently by hand and counted for lymphocytes and polymorphs and found to contain 97% lymphocytes. As in blood, this leucocyte pool was aliquoted into appropriate volumes containing multiples of 10^8 lymphocytes. Aliquots were centrifuged, supernatants decanted, individual cell pellets quickly frozen and stored at $-20^{o}C$ until required.

2.5.3 Isolation of leucocytes from human blood.

Normal venous blood from healthy human donors was collected into Fenwall double transfer packs (South West Blood Transfusion Centre, St. George's Hospital, Tooting, London). The first one of these bags contained Citrate-Phosphate-Dextrose (CPD). The ratio of

blood to CPD was 7:1 (450 mls: 63 mls). Red and white cells were separated from plasma by differential centrifugation. Red cells were first separated by centrifugation at 3000-4000 g for 20 seconds. The supernatant leucocyte/plasma layer was transferred into the second bag and centrifuged again. This was spun at 2500 g for 15 minutes. Most of the plasma was removed from the leucocyte pellet. These cells were resuspended in the remaining plasma and placed in Sterilin universal containers. This pack was washed out with physiological saline containing 25-30 units/ml heparin. This was also added to the leucocytes in the Sterilin containers. Universals containing cells were centrifuged at 200 g for 10 minutes at 25°C. Supernatant was removed from each leucocyte pellet. The cells from each Universal were pooled and resuspended in 10 mls of physiological saline. A sample was counted for lymphocytes and polymorphs. The leucocyte suspension was divided into appropriate aliquots with known numbers of lymphocytes and polymorphs. Each volume was centrifuged at 200 g for 10 minutes at 25°C. Supernatants were removed from the cell pellets, which were stored at -20°C. The total lymphocyte and polymorph content per 450 mls blood, from different donors, varied considerably. The total lymphocytes varied from 1.5×10^8 to 7×10^8, while polymorphs ranged from 0.13×10^8 to 2.5×10^8 per unit of blood.

2.5.4 Treatment of human and monkey leucocyte pellets to produce lysates.

Each lymphoid cell pellet was thawed at 37°C. It was treated with 1 ml non-pyrogenic sterile distilled water and 100 µg deoxyribonuclease

or DNAse for 10^8 lymphocytes. The DNAse used for the preparation of dialysable leucocyte transfer factor for the in vivo study was crude, while that for in vitro and fractionation studies was pure. The treated cell pellet was freeze-thawed 10 times within 6-8 hours at $-70^{o}C$ to $+37^{o}C$. After such processing a manageable lysed extract of lymphoid cells or a leucocyte lysate was obtained ready for dialysis. All leucocyte lysates were stored at $-20^{o}C$ until required for dialysis.

2.5.5 Water-dialysis of monkey leucocyte lysate for in vivo use (Fig. 3).

Figure 3 above shows that the leucocyte lysate used for the in vivo study was derived from pooled rhesus monkey lymph-node and spleen cells. The method of Lawrence and Al-Askari (1971) was followed with minor modifications. An appropriate length of 8 mm Visking dialysis tube was soaked in sterile distilled water without boiling. The cell lysate was dialysed against sterile distilled water at $4^{o}C$ for 24 hours (50 ml/10^9 lymphocytes). The dialysate or water-dialysed rhesus monkey leucocyte transfer factor ($Rh\text{-}TF_{WD}$) was sterilised by filtration through 0.22 Millipore filter. This was immediately freeze dried and stored at $4^{o}C$ until used.

2.5.6 Medium-dialysis of human and monkey leucocyte lysates for in vitro use (Fig. 3).

Figure 3 above shows that for the in vitro study human and rhesus leucocyte lysates were both obtained from blood. The method of

Hamblin (1975), a modified method of Ascher et al, (1974) was used. The cell lysate from man or monkey was pipetted into 8 mm Visking tube. The dialysis tube had been twice boiled in distilled water to remove heavy metal ions and to sterilise it. This was dialysed against glutamine containing Minimal Essential Medium for suspension culture (1% glutamine in MEMS: 16 ml/10^8 lymphocytes) for 24 hours at 4^oC with constant stirring. Thus 1 ml medium contained the dialysate from 6 x 10^6 lymphocytes. The leucocyte dialysate was sterilised by filtration through 0.22m Millipore filter and was stored at 4^oC until use. Such "medium-dialysed" leucocyte transfer factor from man was designated H-TF_{DM} and that from monkey Rh-TF_{DM}.

2.5.7 Vacuum-dialysis of human and monkey leucocyte lysates for *in vivo* and fractionation studies (Fig. 3).

The method of Burger et al, (1974) with minor modifications was used. A semi-permeable 8 mm Visking tube which was twice boiled in distilled water was connected to a glass funnel ($\sim$ 4.5 cms diameter). After making 3 knots to the bottom of the tube, it was passed through a hole in a silica rubber bung (size: 3.3 cms diameter). This was firmly attached to a 500 ml Buchner type flask. The flask was evacuated until the dialysis tube tautly expended. The leucocyte lysate was transferred into the dialysis tube (approximately 5 x 10^8 lymphocytes/apparatus) and washed with 5 ml non-pyrogenic sterile distilled water. Vacuum-dialysis or ultrafiltration continued for 36-48 hours at 4^oC. More water was periodically added. By the end of the dialysis added water amounted to 4 mls/10^8 lymphocytes. When dialysis was completed the tube was usually dry. The vacuum

was subsequently released, dialysate collected and sterilised by filtration through a 0.22 μ Millipore filter. Such dialysates of man and monkey were designated H-TF_{VD} and Rh-TF_{VD} respectively. Each dose of sterile dialysate used for in vivo or fractionation purposes was freeze-dried twice. Once, because of its large volume, it was freeze-dried in 1 litre round bottomed flasks. For the second time this was taken up into 10 ml non-pyrogenic sterile distilled water, transferred into an ampoule and again freeze-dried. All such preparations were stored at $4^{o}C$ until required.

2.6 Skin tests (Plate 3, table 12).

Skin tests were carried out in normal saline-injected actively sensitised and dialysable leucocyte transfer factor-treated rhesus monkeys. This was to establish presence or absence of delayed hypersensitivity response (DH), an in vivo manifestation of cell-mediated immunity (Maddison, 1973).

Normal rhesus monkeys were skin tested with 0.1 ml saline containing 30 μg PPD or KLH or 50 μg HBsAg (Figs. 2a-b, section 2.3). Actively sensitised animals were skin tested with 10 and/or 30 or 50 μg of the sensitising antigens (Figs. 1a-d, section 2.1). However, to test the in vivo specificity of homologous dialysable transfer factor, recipients of transfer factor were tested with 30 and/or 50 μg of all the antigens (KLH, HBsAg, PPD: figures 2a-e, section 2.3).

The conventional intrapalpebral skin test used in the monkey limits the number of tests in any one animal. Thus, plate 3 shows that skin

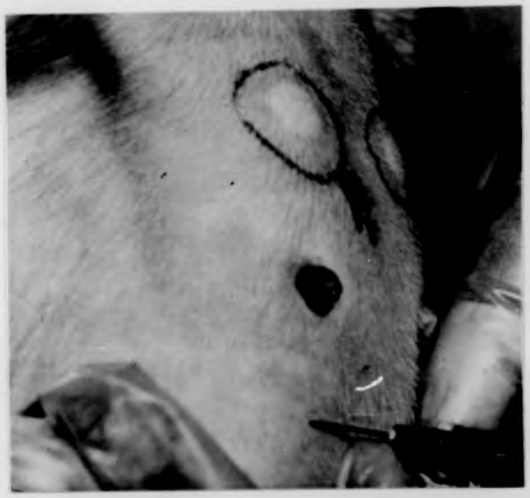


Plate 3. Intradermal skin test of rhesus monkey.

Plate 4. Bleeding of rhesus monkey from its femoral vein.

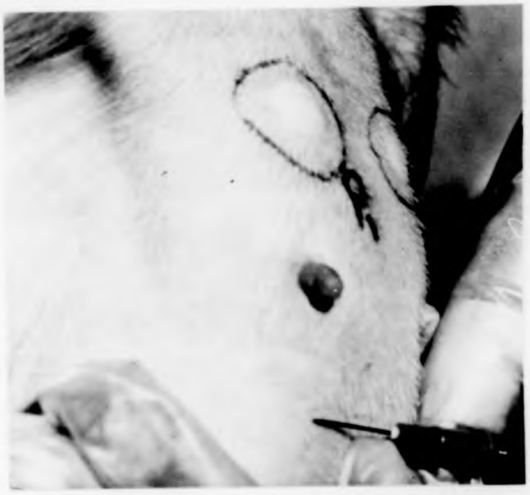


Plate 3. Intradermal skin test of rhesus monkey.

Plate 4. Bleeding of rhesus monkey from its femoral vein.

Table 12. Evaluation of histologically active delayed hypersensitivity in rhesus monkeys.

Reactivity[a] of donor or recipient monkeys	Degree of mononuclear cell exudate		Large mononuclear cells	Maximum[b] total score
	sub-epidermal	deep-dermal		
Active DH	0, 1+, 2+	0, 1+, 2+	0, 1+	1+ to 5+
Baseline	0	0	0	0

[a]DH, delayed cutaneous hypersensitivity.

[b]DH 1+ = very weak, 2+ = weak, 3+ = moderate, 4+ = strong, 5+ = very strong.

For details see section 2.6.

tests were carried out by intradermal injections into the chest area. After gross clinical measurement of the diameter of induration and/or erythema at 4, 24 and 48 hours, the skin test sites were biopsied at 48 hours for the study of lymphocyte infiltration. Skin reactions were considered clinically positive when the diameter of induration and/or erythema was more than 3 mms. This cut-off point was considered reasonable, because none of the skin tests, carried out with saline with or without an antigen in the 2 normal monkeys used, or with saline alone in all the monkeys used, produced a reactivity greater than 3 mms in diameter. Histological examinations were also carried out because of the well recognised lack of consistent appearance of gross delayed hypersensitivity skin reactions in this species (Baram et al, 1971; Maddison et al, 1972). Gross skin reactions with erythema have, however, been described in rhesus monkeys (Mackler et al, 1971).

Table 12 above shows how delayed hypersensitivity reaction was histologically evaluated by the degree of monocytic infiltration. Both sub-epidermal and deep-dermal areas were examined and the degree of exudating monocytes in each area was scored zero to 2+. Thus, so far, total score range was zero to 4+. To this was added a score of zero to 1+ if large monocytes were respectively not prominent or prominent. Thus, a total score of zero to 5+ could be finally obtained. Saline injected sites in all monkeys and all sites in normal monkeys served as the baseline (DH = o).

2.7 *In vitro* cellular hypersensitivity tests.

2.7.1 Mixed leucocyte-macrophage migration or LMMI test (Plates 4-5).

A modified method of mixed cell migration shown previously to be suitable for cellular hypersensitivity testing in man was followed (Rajapakse and Glynn, 1970). Monkey blood leucocytes were mixed with normal guinea-pig macrophages in a ratio of 1:2 respectively before capillary migration. To obtain the macrophages, 1-3 cross-bred albino guinea-pigs of the Hartley strain were injected intra-peritoneally with about 25 mls of sterile warm liquid paraffin. The mean weight of each guinea-pig was about 800 grams. Peritoneal exudate cells were harvested 72-120 hours later with 200 mls of Hank's balanced salt solution. The cell suspension was spun at 175 g for 10 minutes at $4^{o}C$ and the cell pellet was resuspended in 7-10 mls of 10-20% horse serum in Eagle's MEM and washed 3 times. Subsequently it was resuspended in 2-3 mls of the same medium and counted. Peritoneal exudate cells were suspended at 3.0×10^{7} cells/ml and on average consisted of 81% macrophages, 11% lymphocytes and 8% polymorphs. They were kept on melting ice until required.

To obtain normal or immune rhesus leucocytes 10-14 mls of heparinised (50 U/ml) monkey blood were drawn from the femoral vein (Plate 4). Whole blood was mixed with half volumes of 1.5% dextran made up in Hank's solution (final concentration 0.5%). Red cells were largely removed by sedimentation for 30-60 minutes at $37^{o}C$. Leucocytes were centrifuged at 145 g at room temperature and washed three times with 10-20% horse serum in Eagle's MEM. They were suspended at 1.5×10^{7} cells/ml in this medium at room temperature (average > 50% lymphocytes). An equal volume of washed oil-induced guinea-pig

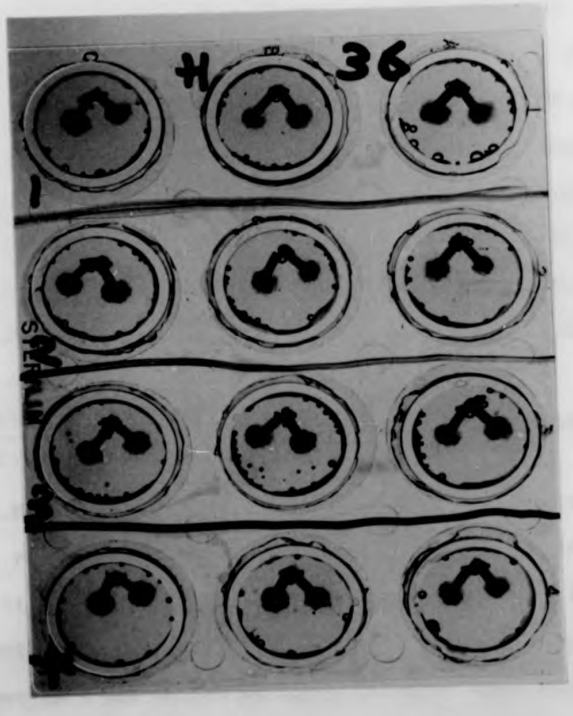


Plate 5. Mixed leucocyte-macrophage migration in a 12-well sterile migration plate.

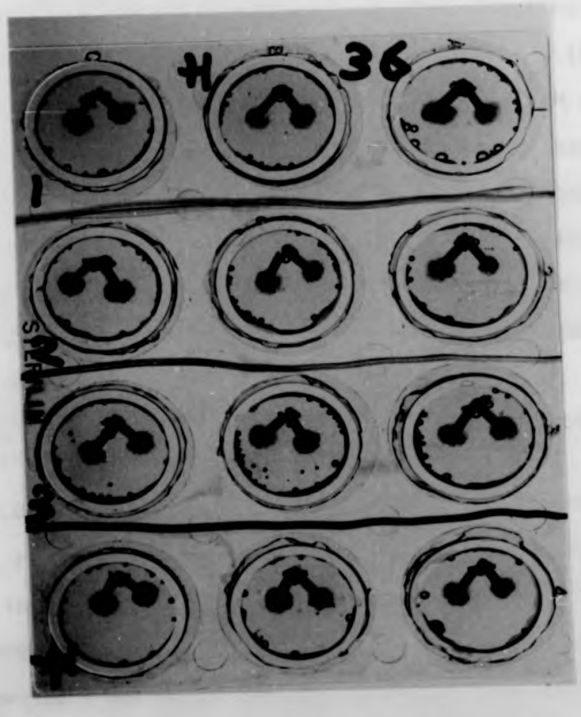


Plate 5. Mixed leucocyte-macrophage migration in a 12-well sterile migration plate.

peritoneal exudate cells at 3 x 10^7 cells/ml was then added. The cell mixtures were kept on ice, taken up into microcapillary tubes, plugged with wax, centrifuged at 145 g in a bench centrifuge for 5 minutes and cut at cell-fluid interphase. The packed cell ends were quickly migrated from microhaematocrit capillary tubes. These were placed in individual wells of a 12-well sterile migrating chamber unit and 3-6 capillaries were used per antigen dilution (Plate 5). Wells were filled with 10-20% horse serum in Eagle's MEM containing 3-200 µg/ml antigen. Antigens were keyhole limpet haemocyanin, purified protein derivative of tuberculin, particulate tubercle bacilli and purified hepatitis B-surface antigen (i.e. KLH, PPD, TB, HBsAg). Migration areas were measured by Planimetry and migration ratios or indices (MI), related to medium alone, were evaluated after 18-20 hours at 37°C in a 5% CO_2: air incubator.

$MI = \frac{\text{Migration with antigen (test)}}{\text{Migration with only medium (control)}}$. A migration index of less than 0.8 was considered significant (MI < 0.8) (Section 8.2.1). The direct, rather than indirect, method of cell migration was used because of its rapidity in setting up a large number of tests. The LMMI test, rather than other cell migration tests, was used in order to develop a new method to assay cellular hypersensitivity in the actively immunised and tranfer factor-treated rhesus monkeys.

2.7.2 Whole blood lymphocyte transformation or LT tests. (Plate 4).

Lymphocyte transformation was measured by using a modification of a whole blood culture technique (Junge *et al*, 1970; Maini *et al*, 1974). 0.1 ml quantities of heparinised monkey whole blood was added to 0.9 ml of Eagle's MEM containing an antigen or a mitogen. Venous

blood was obtained as before (Plate 4). The antigens were KLH, PPD, TB, HBsAg; while the mitogen was reagent grade phytohaemagglutinin (PHA). The concentration range was 5-100 µg/ml. Cultures were set up in triplicate for each concentration. Tubes were maintained for 6 days at 37°C in 5% CO_2: air. On the 5th day they were pulsed with 1µCi ^{3}H-thymidine in 0.1 ml Eagle's MEM (specific activity 15-19 Ci/mM). All cultures were harvested 18 hours later for ß-scintillation counting. In harvesting of cultures red cells were lysed with 3% acetic acid, once washed with phosphate buffered saline and protein precipitated with cold 5% trichloro-acetic acid. After 1-2 hours of storage at 4°C, each culture precipitate was filtered through a 2.5 cm fibreglass filter. Tubes were once again washed with cold 5% trichloro-acetic acid and precipitates dried with a Methanol wash. Filters were dried at 60°C "hot oven" for 30-60 minutes. Each one was transferred into a clean dry disposable glass or plastic vial containing 5 mls scintillation fluid. Vials were counted for ß-particles in a Tracerlab Spectro Matic Scintillation counter.

LT results were expressed as stimulation ratios or indices (SI) in the presence of antigen or PHA in relation to medium in the absence of stimulant. These were corrected for background:

$$SI = \frac{\text{counts per minute with stimulant (test)}}{\text{counts per minute in medium alone (control)}}$$

A stimulation index of greater than 2.0 (SI > 2) was considered positive (Section 8.3.1). The <u>in vitro</u> lymphocyte transformation test has been suggested to be more sensitive than skin tests for detecting tuberculin

hypersensitivity in the rhesus monkey (Baram et al, 1971). Mackler et al (1971, 1972) have demonstrated transformation responses to ascaris, trinitrophenyl-keyhole limpet haemocyanin and phytohaemagglutinin in rhesus monkeys. Thus, to extend the studies above, a simple in vitro lymphocyte transformation test was used here to: (a) monitor cellular hypersensitivity in the rhesus monkey, and (b) to determine its relationship to skin tests in the actively immunised or transfer factor-treated animals.

2.8 Lymphocyte transformation tests used to detect the in vitro activity of dialysable leucocyte transfer factor from monkey or man.

2.8.1 Leucocyte culture "recipients" (LC) of dialysable leucocyte transfer factor.

Leucocytes were separated, from human venous blood mixed with 25-30 units/ml heparin and 0.5% dextran, by sedimentation at 37°C (mean lymphocyte content 33%). The leucocyte-plasma layer was centrifuged at 25°C for 10 minutes at 200 g. The supernatant plasma was clarified of platelets by centrifugation for 15-30 minutes again at 25°C at 200 g. The leucocyte pellet was suspended in tissue culture medium (minimum essential medium for suspension cultures: MEM-S). This contained 30% homologous platelet-free plasma at a concentration of 1×10^6 lymphocytes/ml. Test cultures contained 1 ml (1×10^6 lymphocytes) of leucocyte suspension made up to 2 ml with MEMS alone or MEMS containing medium-dialysed leucocyte transfer factor (0.25, 0.5, 1.0 ml). The final plasma

concentration was 15%. Both human and monkey leucocyte dialysates were used (H-TF_{DM} and Rh-TF_{DM}). Augmentation activity of both H-TF_{DM} and Rh-TF_{DM} was tested on transformation by purified protein derivative of tuberculin and keyhole limpet haemocyanin. Tuberculin was added at 2 µg/tube and haemocyanin at 10-100 µg/tube. Each concentration was made up in 0.1 ml MEMS. Culture tubes, at least in triplicate tests, were incubated at 37°C in an atmosphere of 5% CO_2 in air. On the 6th day 0.1 ml (i.e. 1µCi) ^{3}H-thymidine with a specific activity of 5 Ci/mM was added to each tube. Leucocytes were harvested for β-counting after 24 hours by the method of Wolstencroft and Dumonde, (1970).

2.8.2 Purified lymphocyte "recipients" (PL) of dialysable leucocyte transfer factor.

Lymphocytes were isolated in 2 steps (73-95% pure). In the first step leucocytes, from 500 mls blood, were separated by sedimentation with 50 mls dextran (10:1). The leucocyte/plasma supernatant was centrifuged as before and the leucocyte pellet resuspended in 16 mls homologous plasma. In the second step, the leucocyte suspension was carefully layered onto 8 mls of freshly made mixture of Ficoll/ Triosil (i.e. 2 vols. leucocytes: 1 vol. Ficoll/Triosil). The gradient was centrifuged at 25°C for 30 minutes at about 260 g. The white purified lymphocyte layer was transferred to a fresh tube and washed 3 times with 20 mls MEMS. Purified lymphocytes were counted and made up to a concentration of 1 x 10^6 lymphocytes/ml as above for <u>in vitro</u> experiments.

2.9 Fractionation of monkey and human dialysable leucocyte transfer factor preparations.

Only vacuum-dialysed leucocyte transfer factor was fractionated. Leucocyte dialysate was prepared from 5-6 x 10^8 lymphocytes of normal monkey or man. Fractionation was by Sephadex G-25 column chromatography.

2.9.1 Sephadex G-25 column chromatography.

Freeze-dried dialysable leucocyte transfer factor was taken up into above 0.5 - 2 mls of distilled deionised water. This was filtered through a 0.22m Millipore. 50 µl was removed and stored at -20°C, so that extinction readings of the crude or unfractionated material could later be determined at 260 nm and 280 nm wavelengths (E_{260} and E_{280}). The remainder was loaded onto a Sephadex G-25 column. One-two columns were used (1.6 cms x 90 cms each). When two columns were used, they were joined in series. The columns were eluted with a volatile buffer of 0.02M ammonium bicarbonate/acetic acid, pH 7.4. Flow rate was adjusted to deliver 10 mls eluent per hour using a peristaltic pump. About five to fifteen minutes aliquots were collected by the automatic fraction collector at 4°C (~ 0.9 to 2.7 mls). Extinction readings of each aliquot were taken at 260 nm and 280 nm and the fractionation profile or graph of extinction against aliquot number was plotted. The appropriate aliquots, making up each peak, were pooled to give each fraction of dialysable leucocyte transfer factor. The extinction of each fraction at 260 nm and 280 nm was now determined and each freeze-

dried in round bottomed flasks. Each new dialysable leucocyte transfer factor fraction was then taken up into 5 ml of volatile buffer at room temperature and sterilised by filtration through 0.22 μ Millipore filter. This was divided into five 1 ml aliquots and again freeze-dried in ampoules and stored at -20°C.

2.10 Properties of test antigens and phytohaemagglutinin (Plate 6).

2.10.1 Keyhole limpet haemocyanin (KLH).

This is a strong, non-ubiquitous ("neoantigen"), protein antigen isolated from the haemolymph of giant keyhole limpets (Megathura crenulata) found in the Pacific Ocean (Weigle, 1964). KLH molecules can readily associate in acid pH (6.5, tris) and dissociate in alkali pH (> 7.5, tris: Fay, 1975: Personal communications). It has been used as a sensitising antigen in immunisation studies of man or monkey (Curtis et al, 1970; Mackler et al, 1971). It has also been employed for the in vivo and in vitro studies of transfer factor (Baram and Condoulis, 1970; Zuckerman et al, 1974; Burger et al, 1976a).

Thus, KLH was used in this work to test the specificity of the acquired responses in actively immunised and transfer factor-treated rhesus monkeys, and in the in vitro study of transfer factor because of (a) its rarity in nature, and (b) past use in the study of transfer factor (see appendix for the preparation of the stock solution).

2.10.2 Purified protein derivative of tuberculin (PPD).

This is a common soluble protein antigen isolated from cultures of human tubercle bacilli. It was obtained in neutralised freeze-dried form (PPD 292). This was used in the *in vivo* and *in vitro* studies of transfer factor. Its effects were compared with those of particulate tubercle bacilli in the mixed-cell migration and transformation tests in the *in vivo* study (see appendix for the preparation of the stock solution).

2.10.3 Tubercle bacilli (TB).

This antigen consisted of particulate, heat-killed, freeze-dried human mycobacteria (TB 292). It was used in parallel with PPD to determine if it had a different effect in the mixed-cell migration and lymphocyte transformation tests used in the *in vivo* study (see appendix for the preparation of the stock solution).

2.10.4 Hepatitis B surface antigen (HBsAg: Plate 6).

Infection with hepatitis B virus is intimately associated with the appearance in the human serum of the Dane particle containing a 42 nm particle, called hepatitis B core antigen. The surface of the core antigen is covered by the hepatitis B surface antigen displaying complex reactivities (HBsAg: plate 6). The group-specific antigen has been named "a" and there are at least four phenotypes: adw, adr, and ayr. There may be other determinants. The surface

Plate 6. Electron micrograph of hepatitis B surface antigen purified on caesium chloride gradient by ultracentrifugation (Total magnification: 126,000 X).

antigen reactivity is shared by small spherical 18-22 nm particles, the tubular forms and the outer shell of the 42 nm Dane particles (Zuckerman, 1975b). In this study HBsAg/ad was used because of its clinical importance in man. Cellular immune responses to HBsAg were followed in actively sensitised and transfer factor-treated rhesus monkeys (see appendix for the preparation of the purified stock solution of HBsAg).

2.10.5 Phytohaemagglutinin (PHA).

Ling, in 1968, described PHA as a plant agglutinin or lectin, prepared from a bean called Phaseolus vulgaris. It contains both protein and carbohydrate, is water-soluble and has a molecular weight of about 118,000. It agglutinates red cells of many species, and is mitogenic and can, therefore, stimulate lymphocytes to enter blastogenisis in vitro. Thus, PHA was used in the present study to determine (a) the normal lymphocyte transformation response to PHA in virgin rhesus monkeys, and (b) the effects of active immunisation or transfer factor upon the PHA-response in this species (see appendix for the preparation of the stock solution).

3. RESULTS OF THE DELAYED HYPERSENSITIVITY (DH) SKIN TESTS

Skin tests in normal, actively sensitised and transfer factor-treated monkeys were examined clinically and histologically as described above (Section 2.6).

3.1 Normal and actively sensitised monkeys (Figs. 4-6, plates 7-10, tables 13-14).

3.1.1 Clinical DH-responses to KLH, PPD, or HBsAg (Figs. 4a-c, plate 7).

Two normal control monkeys, injected with 20-30 mls normal saline, were skin tested twice with 30-50 µg KLH, PPD or HBsAg (Section 2.2: Figs. 2a, 2c, monkeys H18, H34). The first skin test was carried out on day 7 and the second on day 14. None of the animals showed clinically positive DH-responses to any of the antigens, since induration and/or erythema were not detected at 24-48 hours in any of the 12 tests carried out (Diameter < 3mms).

In contrast, when monkeys were actively immunised with FCA, with or without KLH or HBsAg (Section 2.1: figs. 1a-d), clinically positive skin-test reactivity to 10-30 µg KLH, PPD or HBsAg was detected. These responses were measured in some representative animals for 10 µg antigen in 37 out of 40 tests (93%) (Figs. 4a-c). Maximum reactivity, after the first or the second skin test, developed at 24 or 48 hours. There was also evidence of immediate hypersensitivity response, since in those animals examined some had developed measurable induration and/or skin colour change at 4 hours (Erythematous or yellow). This was detected in 13 out of 15 tests (89%) after the first and the

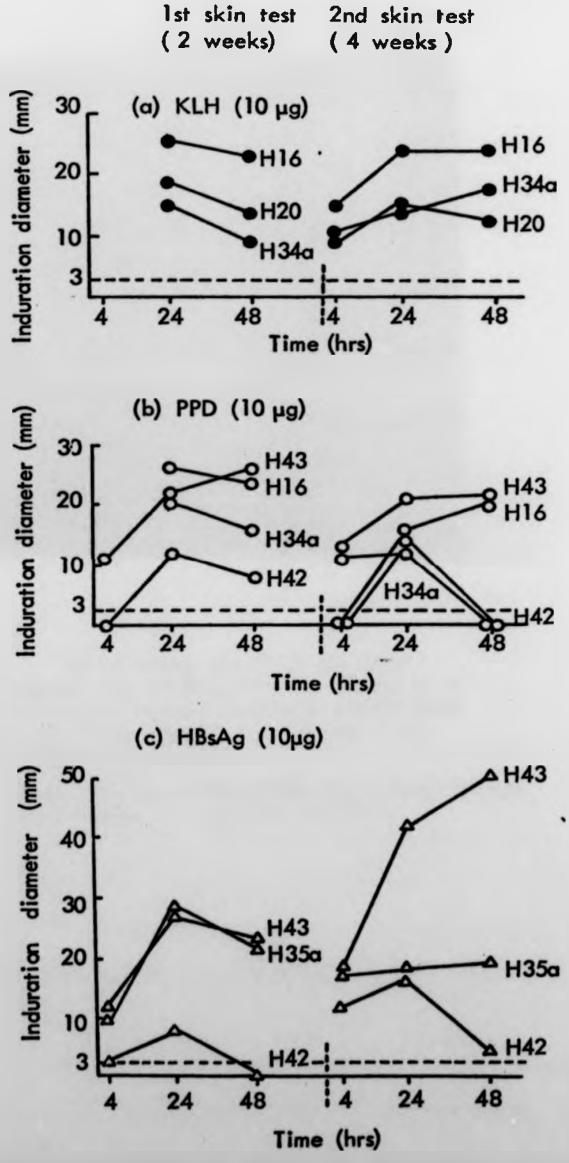


Fig. 4. Induration diameters in skin test reactivity at 4 to 48 hours against (a) KLH, (b) PPD, (c) HBsAg in representative rhesus monkeys actively sensitised with (a) 100 µg KLH in FCA, (b) FCA + KLH or HBsAg, and (c) 1 mg HBsAg in FCA.

Saline control sites and normal animals were negative, i.e. induration diameter < 3mms. For protocols see section 2.1, figures 1b, d. Monkeys are coded from H16-H43.

Plate 7. Clinically positive 48 hour DH-responses to 10-30µg PPD or HBsAg in a representative monkey actively sensitised with 1 mg HBsAg in FCA containing 3 mg killed tubercle bacilli.

P_1 = 10µg PPD, P_3 = 30µg PPD, H_1 = 10µg HBsAg, H_3 = 30µg HBsAg, C = saline control.

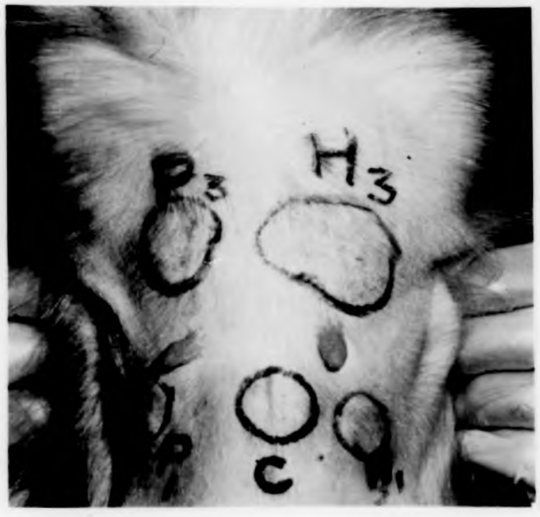


Plate 7. Clinically positive 48 hour DH-responses to 10-30μg PPD or HBsAg in a representative monkey actively sensitised with 1 mg HBsAg in FCA containing 3 mg killed tubercle bacilli.

P_1 = 10μg PPD, P_3 = 30μg PPD, H_1 = 10μg HBsAg, H_3 = 30μg HBsAg, C = saline control.

second skin test. The general pattern of the clinically positive DH-responses to all the sensitising antigens was similar (Figs. 4a cf 4b cf 4c). Such 4-hour observations were not carried out after the first skin test in animals actively sensitised with KLH in FCA (Fig. 4a). One animal, immunised with HBsAg in FCA, showed an exceptionally large induration diameter at 24 or 48 hours after the second skin test with 10 μg HBsAg (Fig. 4c: monkey H43).

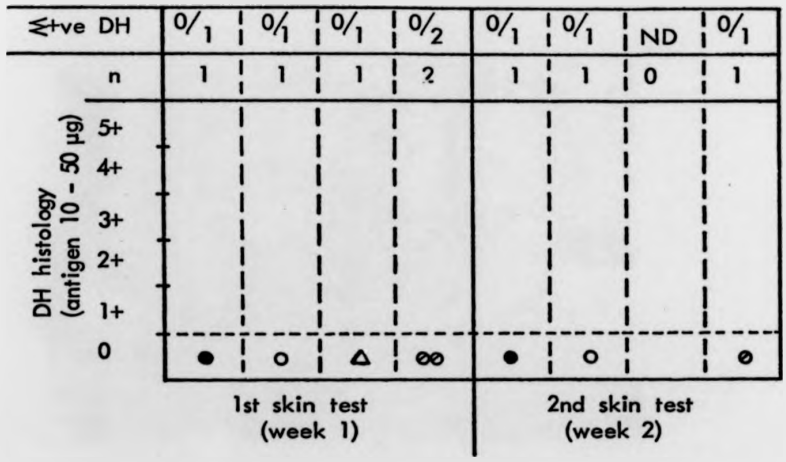
An example of an immunised monkey with clinically positive DH-responses to 10-30 μg PPD and HBsAg is shown in plate 7. Higher skin test concentration of these antigens produced larger induration and erythema than the lower concentration.

3.1.2 Histological DH-responses to KLH, PPD or HBsAg (Figs. 5-6, plates 8-10).

Figure 5a demonstrates that a normal monkey, injected with only saline, was skin tested with 10-30 μg KLH and PPD, and another one skin tested with 50 μg HBsAg. They showed no histological evidence of DH-response. The 48 hour histological examination of such baseline DH-responses to an antigen or only saline always revealed little infiltration of dermal layers with cells (Plates 8-9).

Compared with the normal monkeys above, all the 17 rhesus monkeys actively immunised with FCA, with or without KLH or HBsAg, showed histologically positive DH-responses. Such responses to KLH, PPD and HBsAg antigens were all well marked after the first skin test by 1 to 4 weeks, and were still present after the second skin test

(a) Normal monkeys



(b) Actively sensitised monkeys

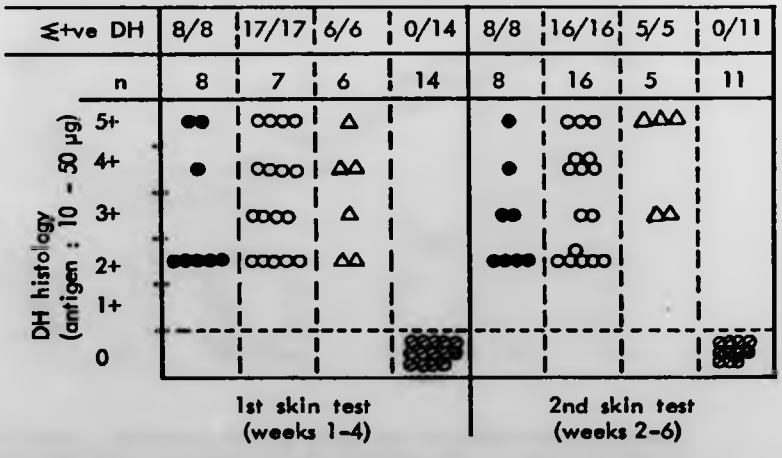
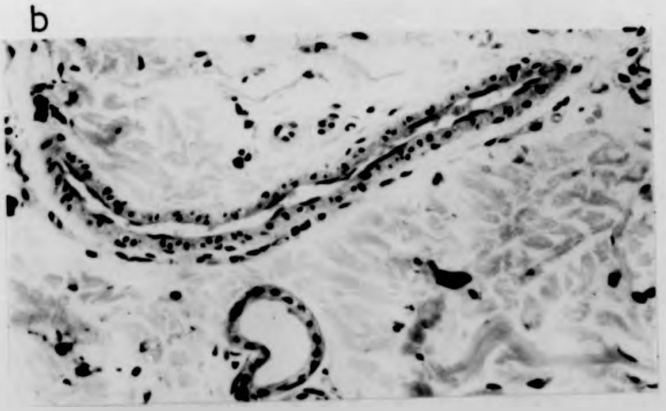


Fig. 5. Histological results of maximum DH-responses to 10-50 µg antigen in (a) normal saline-treated, and (b) actively sensitised rhesus monkeys (FCA ± KLH or HBsAg).

n, number of monkeys; (●) KLH; (○) PPD; (△) HBsAg; (⊘) Saline.

a

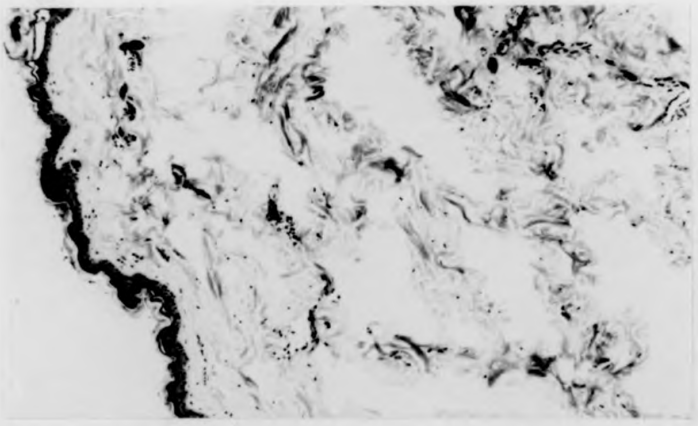
b



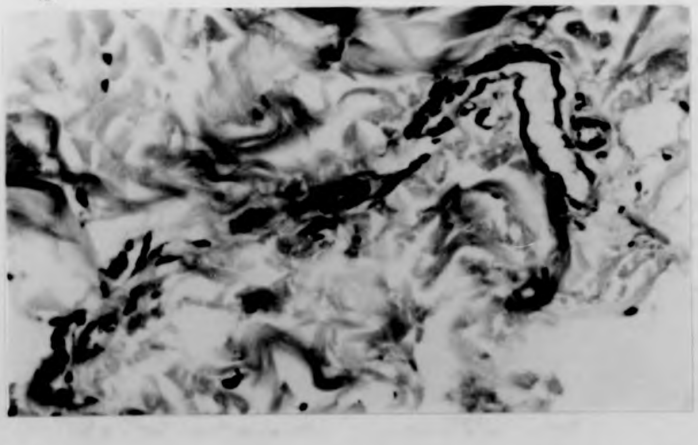
Plates 8a-b. Histologically negative baseline skin delayed hypersensitivity response to an antigen in a representative normal rhesus monkey (DH=o)

(a) Low power : 72x, (b) high power : 288x

a



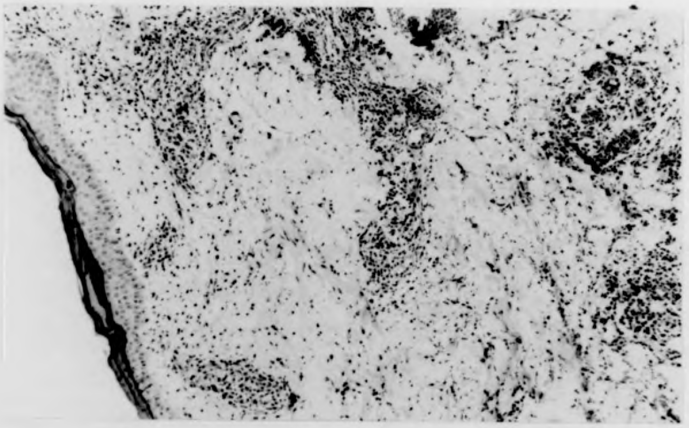
b



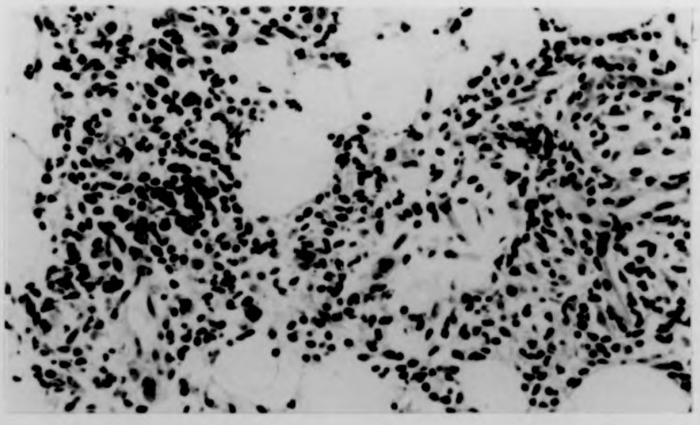
Plates 9a-b. Histologically negative baseline skin delayed hypersensitivity response to saline alone in a representative rhesus monkey (DH = 0)

(a) Low power : 72x, (b) high power : 288x

a



b



Plates 10a-b. Histologically positive skin delayed hypersensitivity response to an antigen in an actively sensitised representative rhesus monkey (DH > 3+)

(a) Low power : 72x, (b) high power : 288x

by 2 to 6 weeks (Fig. 5: DH = 2+ to 5+). Positive DH-responses were histologically characterised by widespread infiltration of all dermal layers with lymphocytes, macrophages and polymorphs. The latter were mainly prominent in the more intense reactions (Plates 10a-b: DH > 3+).

In the actively immunised animals, skin tests carried out with 30 µg PPD or HBsAg produced a more marked mean histological reactivity than those with 10 µg PPD or HBsAg (Fig. 6). Therefore, as for the clinical observations (Plate 7), the intensity of the acquired DH-responses depended upon the dose of PPD or HBsAg used for the skin test.

3.1.3 Comparison of DH-conversion rates to KLH, PPD or HBsAg in normal and actively sensitised monkeys (Table 13).

Table 13 shows that, none of the tests in the normal control animals became histologically positive for KLH, PPD or HBsAg, whilst all the tests in the actively immunised animals became positive. When sum of all the tests with these antigens were taken together, the acquired DH-reactivity was highly significant in the immunised animals ($P < 0.001$).

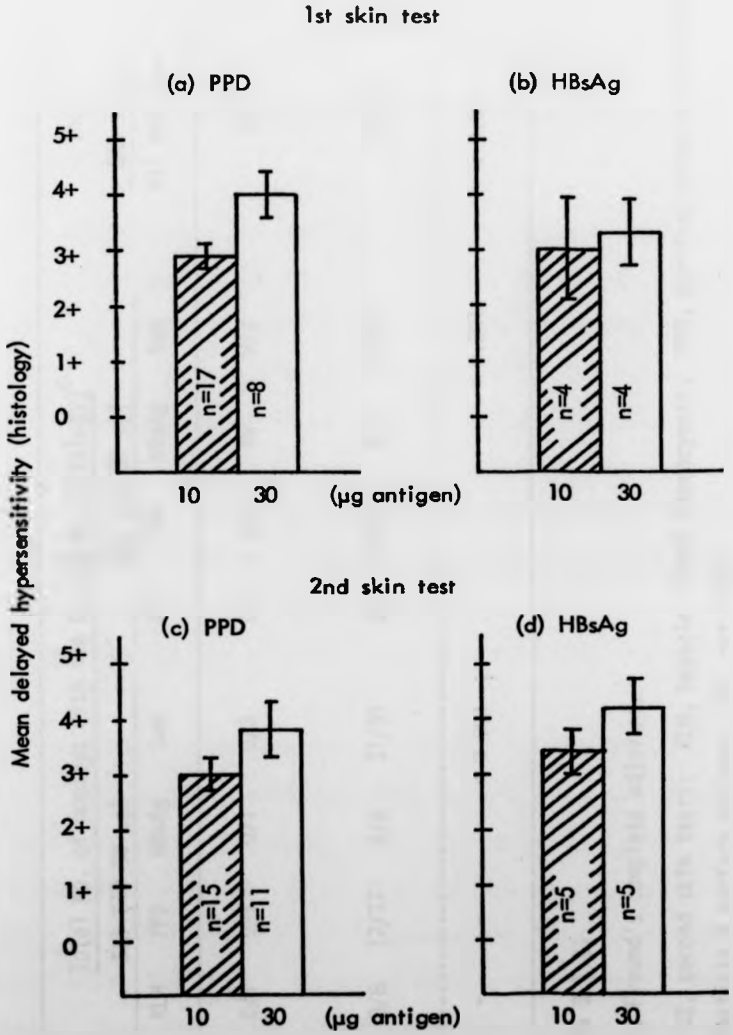


Fig. 6. Mean intensity of histologically positive DH-responses to different doses (10-30 µg) of PPD or HBsAg in actively sensitised rhesus monkeys (FCA ± HBsAg).

n, number of monkeys; I mean ± SEM.

Table 13. Comparison of DH-conversion rates to KLH, PPD and HBsAg in normal and actively immunised rhesus monkeys.[a]

Immunisation[b] with	Total no. of monkeys with +ve DH (48 hr histology)[c]								Total SK1 + SK2
	SK1 (10-50 µg)				SK2 (10-30 µg)				
	KLH	PPD	HBsAg	Sum	KLH	PPD	HBsAg	Sum	All antigens
Nothing/NaCl (Control)	0/1	0/1	0/1	0/3	0/1	0/1	ND	0/2	0/5
FCA ± antigen	8/8	17/17	6/6	31/31	8/8	16/16	5/5	29/29	60/60
P for χ^2	-	-	-	< 0.001	-	-	-	< 0.001	< 0.001

[a] From figures 5a-b (Section 3.1.2).

[b] NaCl, normal saline; FCA, Freund's complete adjuvant.

[c] SK1, first skin tests; SK2, second skin tests; KLH, keyhole limpet haemocyanin; PPD, purified protein derivative of tuberculin; HBsAg, hepatitis B surface antigen; ND, not done.

3.2 Transfer factor-treated monkeys (Figs. 7-8, plates 11-13, tables 14-16).

3.2.1 Clinical DH-responses to KLH, PPD or HBsAg (Plate 11, tables 14-15).

Four control animals were treated with a single injection of dialysable rhesus transfer factor (Table 14). None of the recipients showed clinical DH-responses to 50 µg KLH, PPD or HBsAg after the first skin test (Induration diameter < 3 mms). However, after the second skin-test 1 animal became positive to 50 µg KLH (Monkey H38; induration diameter 5.5 mms). Similarly, 9 virgin rhesus monkeys treated with a single injection of immune dialysable rhesus transfer factor, equivalent to 4 to 8 x 10^9 lymphocytes, did not demonstrate skin test-reactivity to 30 or 50 µg KLH, PPD or HBsAg up to 21 days after the injection in any of the 44 tests carried out.

Table 15 shows that, in another control animal treated with 2 injections of normal dialysable transfer factor, there was no clinical DH-response to KLH, or PPD (Monkey H35). In contrast, in one animal (Monkey H36), given 3 injections of KLH- and FCA-immune transfer factor, each equivalent to 4.5 x 10^9 lymphocytes, skin test reactivity was detected after the second and third skin test on days 27 and 34 respectively. Such reactivity to PPD or HBsAg, was not detected after the first injection of transfer factor.

Thus, altogether clinically positive DH-responses were seen in only 1 out of 23 (4%) skin tests in 5 animals treated with control dialysable transfer factor (ie. normal or unrelated to test antigen), and in 4 out of 53 tests (8%) in 10 test animals treated with immune dialysable transfer factor. Clinically negative results in transfer factor-treated animals were characterised by the absence of any induration or

Table 14. The extent of clinical DH-responses in 4 monkeys following one injection of control vacuum-dialysed rhesus transfer factor ($Rh\text{-}TF_{VD}$).

Recipient monkey and dose of $Rh\text{-}TF_{VD}$	*SKIN TEST 14 days Induration diameter (mms) KLH 50 µg	PPD 50 µg	HBsAg 50 µg	NaCl 0.1 ml	21 days Induration diameter (mms) KLH 50 µg	PPD 50 µg	HBsAg 50 µg	NaCl 0.1 ml
H37: HBsAg/FCA 1(4.5×10^9)	0			0	0			0
H38: HBsAg/FCA 1(4.5×10^9)	0			< 3	5.5			0
H48: FCA 1(5.3×10^9)	0		0	0	0		0	0
H49: Normal 1(4.5×10^9)	0	0	0	0	0	0	0	0

* Average diameter of 24 hour induration: > 3 mms, positive (Section 2.6).

Table 15. Acquisition of delayed hypersensitivity following multiple injections of vacuum-dialysed rhesus monkey dialysable leucocyte transfer factor ($Rh\text{-}TF_{VD}$).

Skin test antigen and dose		+Mean diameter of 24 hr-induration (mm) at different times after first $Rh\text{-}TF_{VD}$ injection. 13 days	27 days	34 days
Monkey H35*				
(Normal $Rh\text{-}TF_{VD}$)				
KLH		0	0	0
PPD	50 µg	0	0	0
HBsAg		ND	ND	ND
NaCl	(0.1 ml)	0	0	0
Monkey H36*				
(KLH/FCA $Rh\text{-}TF_{VD}$)				
KLH		0	7	10
PPD	50 µg	0	12	13
HBsAg		ND	ND	ND
NaCl	(0.1 ml)	0	0	0

*Monkeys skin tested about 2, 4 and 5 weeks after first injection of $Rh\text{-}TF_{VD}$. Normal $Rh\text{-}TF_{VD}$ {total ≡ 2 (2.4×10^9) lymphocytes} injected at days 0 and 3 followed with saline on day 20. Immune $Rh\text{-}TF_{VD}$ injected on day 0, 3 and 20 {total ≡ 3(4.5×10^9) lymphocytes}.

+Diameter > 3 mms considered positive (Section 2.6); ND, not done.

Also see appropriate protocol (Section 2.2.1, fig. 2d).

Plate 11. Clinically negative 48 hour delayed hypersensitivity responses to antigens in a representative rhesus monkey given one injection of homologous dialysable transfer factor.

H = 50µg HBsAg, K = 50µg KLH,
P = 50µg PPD, S = saline control.

Plate 11. Clinically negative 48 hour delayed hypersensitivity responses to antigens in a representative rhesus monkey given one injection of homologous dialysable transfer factor.

H = 50μg HBsAg, K = 50μg KLH,
P = 50μg PPD, S = saline control.

erythema (Plate 11). This was similar to the appearance of the skin on the chest of a normal monkey, but was in direct contrast to the highly reactive DH-response seen in the actively immunised animals (Plate 7). The clinically positive DH-response seen in 1 recipient animal was not unfortunately photographed (Table 15: monkey H36).

3.2.2 Histological DH-responses to KLH, PPD or HBsAg (Fig. 7, plates 12-13).

Figure 7a shows that 2 to 5 animals, depending on the antigen, were treated with 4.2 to 5.3 x 10^9 lymphocyte-equivalent of control dialysable rhesus transfer factor. After the first skin test, none reacted to KLH, or HBsAg (DH = 0), but 1 showed reactivity to PPD (DH = 2+). After the second skin test, 2 animals acquired positive DH-responses to KLH and 1 to HBsAg. The animal which showed no DH-reactivity to PPD after the first skin test, also remained non-reactive to PPD after the second skin test. The one animal with positive DH-response to PPD after the first skin test, was not unfortunately biopsied after the second skin test for histological examination.

Figure 7b shows that 3 to 9 animals injected with immune dialysable rhesus transfer factor were skin tested with various antigens. Animals received a single injection of transfer factor, equivalent to 4-8 x 10^9 lymphocytes, or 3 injections on days 1, 3 and 20, each equivalent to 4.5 x 10^9 lymphocytes (See section 2.2.1: table 11). After the first skin test, 1 animal became positive for KLH (DH = 2+), 5 animals for PPD (DH = 2+ to 3+) and 1 for HBsAg (DH = 2+).

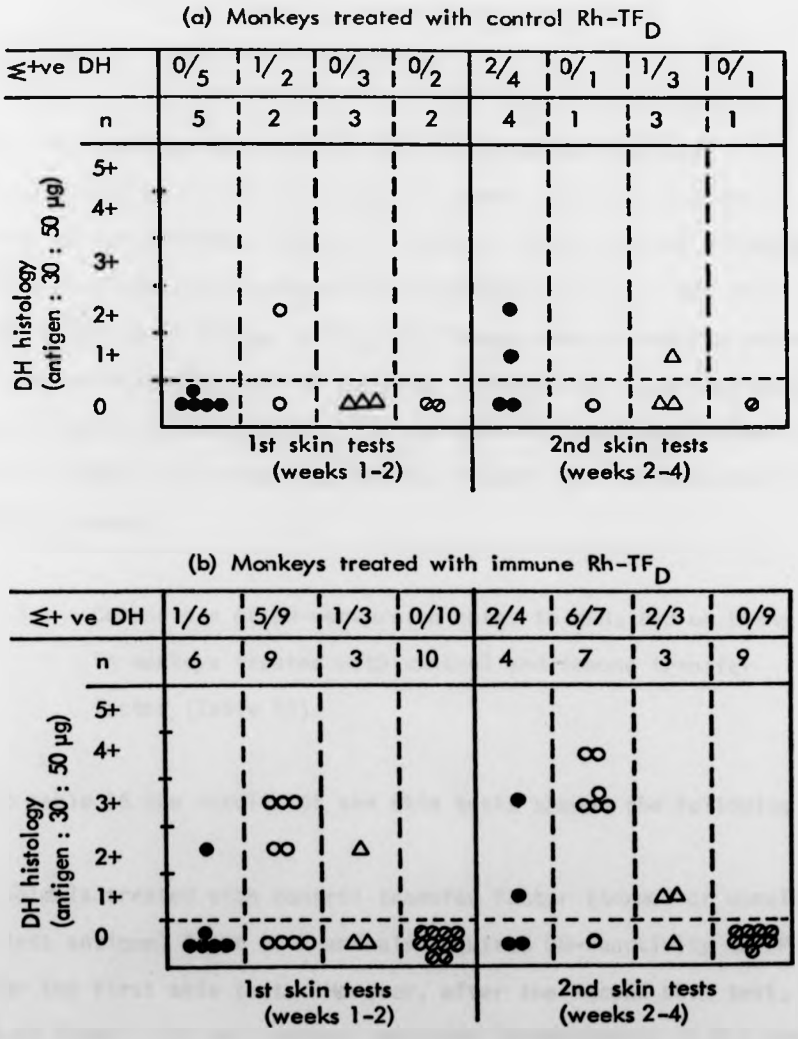


Fig. 7. Histological results of maximum DH-responses to 30-50 µg antigen in rhesus monkeys treated with (a) control or (b) immune homologous dialysable transfer factor (Rh-TF$_D$).

n, number of monkeys; (●) KLH; (o) PPD; (Δ) HBsAg; (⊘) saline; control Rh-TF$_D$ = normal or unrelated to test antigen; immune Rh-TF$_D$ = immune to test antigen.

After the second skin test, 2 animals had converted for KLH, 7 for PPD and 2 for HBsAg (DH = 1+ to 4+).

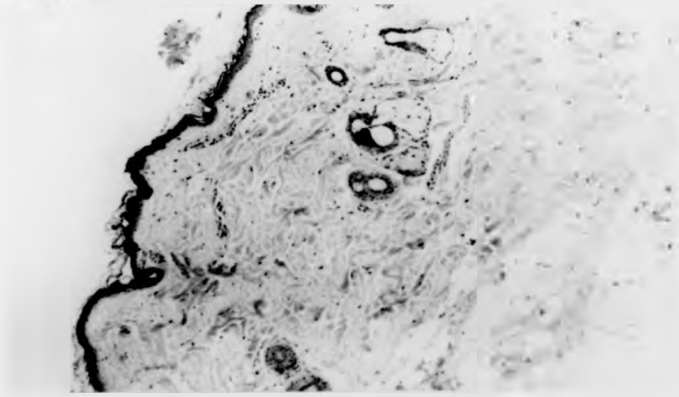
Negative histology in transfer factor-treated monkeys was characterised by the infiltration of dermal layers with minimum number of cells (Plates 12a-b). This was similar to the histology of the baseline skin test-reactivity (Plates 8-9). On the other hand, positive histology in transfer factor-treated animals showed various perivascular foci of cellular infiltration in one or more dermal layers (Plates 13a-b). In the actively immunised animals, all the dermal layers were relatively densely infiltrated with cells (Plates 10a-b).

3.2.3 Comparison of DH-conversion rates to KLH, PPD or HBsAg in monkeys treated with control and immune transfer factor (Table 16).

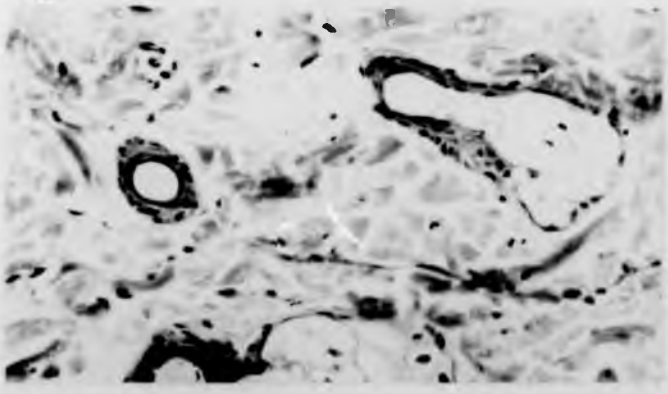
From table 16 the results of the skin tests showed the following:

In animals treated with control transfer factor (normal or unrelated to test antigen) 1 out of 2 animals acquired DH-reactivity to PPD after the first skin test. However, after the second skin test, 2 out of 4 and 1 out of 3 animals acquired DH-reactivity to KLH and HBsAg respectively. In animals treated with the immune transfer factor (related to test antigen), after the first skin test 1 out of 6 animals acquired DH-reactivity to KLH, 5 out of 9 to PPD and 1 out of 3 to HBsAg. After the second skin test, 2 out of 4 animals showed DH-reactivity to KLH, 6 out of 7 to PPD and 2 out of 3 to HBsAg.

a



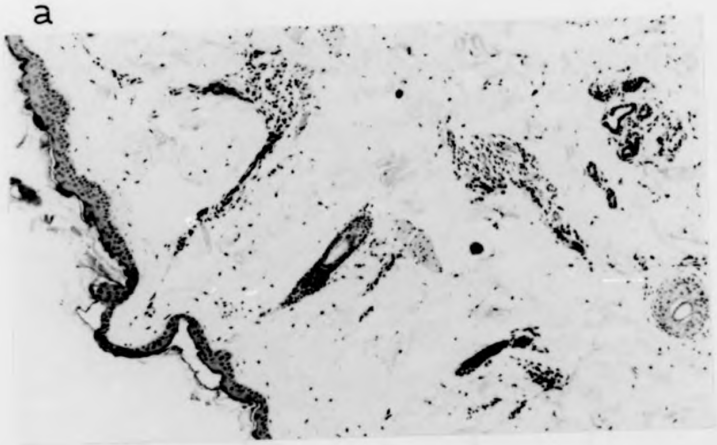
b



Plates 12a-b. Histologically negative skin delayed hypersensitivity response to an antigen in a representative rhesus monkey injected with homologous dialysable transfer factor (DH = 0).

(a) Low power : 72x (b) high power : 288x

a



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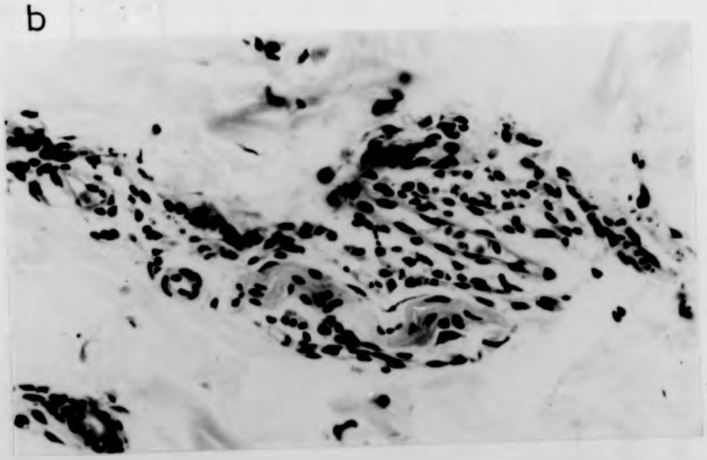

Plates 13a-b. Histologically positive skin delayed hypersensitivity response to an antigen in a representative rhesus monkey injected with homologous dialysable transfer factor (DH < 3+).

(a) Low power : 72x, (b) high power : 288x

Table 16. Comparison of DH-conversion rates to KLH, PPD and HBsAg in monkeys treated with control and immune dialysable rhesus transfer factor.[a]

Rh-TF_D[b] Status	Total no. of monkeys with +ve DH (48 hr histology)[c]								Total
	SK1 (30-50 µg)				SK2 (10-30 µg)				SK1 + SK2
	KLH	PPD	HBsAg	Sum	KLH	PPD	HBsAg	Sum	All antigens
Control	0/5	1/2	0/3	1/10 (10%)	2/4	0/1*	1/3	3/8 (38%)	4/18 (22%)
Immune	1/6	5/9	1/3	7/18 (39%)	2/4	6/7	2/3	10/14 (71%)	17/32 (53%)
P for χ^2	-	-	-	> 0.05	-	-	-	> 0.05	> 0.05

[a] From figures 7a-b (Section 3.2.2).

[b] Rh-TF_D, dialysable rhesus transfer factor.

[c] SK1, first skin tests; SK2, second skin tests; KLH, keyhole limpet haemocyanin; PPD, purified protein derivative of tuberculin; HBsAg, hepatitis B surface antigen.

* Histology of the recipient with positive DH-response in the first skin test was not available at the time of the second skin test.

The sum of the first and/or the second skin tests, with all the antigens taken together, revealed that the number of skin tests showing DH-reactivity increased after the second skin test. This occurred in animals treated with control or immune transfer factor. Comparison of the cumulative results showed no significant difference between the control and immune transfer factor preparations in their histological conversion of skin test reactivity to the antigens used ($P > 0.05$).

3.2.4 Relationship of the dose and/or methods of dialysis of transfer factor to the acquired DH-reactivity to KLH, PPD or HBsAg (Fig. 8).

Figure 8 shows the mean DH-responses acquired after the first skin test carried out 1-2 weeks after injection(s) of vacuum-dialysed or water-dialysed KLH-immune, FCA-immune or HBsAg-immune rhesus transfer factor. The total doses of KLH-immune or FCA-immune transfer factor, given before 20 days, was equivalent to 4 to 9 x 10^9 lymphocytes (Section 2.2.1: table 11).

Figure 8a shows the mean histological DH-responses in animals treated with the vacuum-dialysed KLH-immune transfer factor. The 2 monkeys treated with the low dose ($\equiv$ 4.5 x 10^9 lymphocytes) did not acquire any DH-reactivity to 50 µg KLH. In contrast, when a higher dose ($\equiv$ 9 x 10^9 lymphocytes) was given to another animal some DH-response was detected (Mean DH = 2+). Unlike the vacuum-dialysed KLH-immune transfer factor, none of the animals treated with the water-dialysed

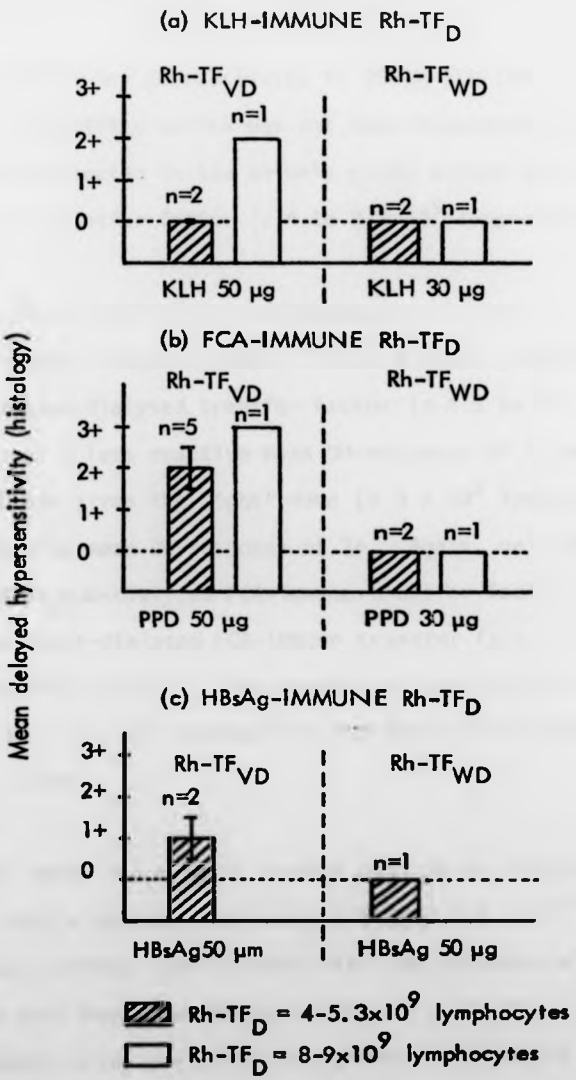

Fig. 8. Effect of the dose and/or different methods of dialysis of immune transfer factor upon the mean DH-responses to (a) KLH, (b) PPD, and (c) HBsAg in recipient monkeys.

Rh-TF$_D$, rhesus dialysable transfer factor; Rh-TF$_{VD}$, Vacuum-dialysed rhesus transfer factor; Rh-TF$_{WD}$, Water-dialysed rhesus transfer factor; n, number of monkeys; I, mean response ± SEM.

transfer factor developed any DH-reactivity to 30 µg KLH (Mean DH = 0). The absence of such reactivity to KLH was not dose-dependent, since DH-reactivity was not detected in the animals given either the lower or the higher dose of transfer factor (≡ 4 to 8 x 10^9 lymphocytes).

Figure 8b shows the mean histological DH-responses in animals injected with FCA-immune transfer factor. Those animals given the lower dose of the vacuum-dialysed transfer factor (≡ 4.5 to 5.3 x 10^9 lymphocytes), acquired a less reactive mean DH-response of 2+ to 50 µg PPD, whilst those given the higher dose (≡ 9 x 10^9 lymphocytes), acquired a more reactive mean DH-response of 3+. Again, unlike the 6 recipients of the vacuum-dialysed FCA-immune transfer factor, the 3 recipients of the water-dialysed FCA-immune transfer factor did not respond to 30 µg PPD (DH = 0). The absence of such reactivity to PPD, as for KLH above, was not dependent on the dose of the water-dialysed transfer factor.

Similarly figure 8c shows the animals treated with HBsAg-immune transfer factor. Only a low dose was used (≡ 4.2 to 4.5 x 10^9 lymphocytes). When 2 animals were treated with the vacuum-dialysed transfer factor, a very weak mean DH-reactivity of 1+ to 50 µg HBsAg was obtained. However, when one animal was given a comparable dose of water-dialysed transfer factor, it did not react to 50 µg HBsAg (DH = 0).

Thus, from figure 8 the following were concluded: (a) that the acquisition of DH-reactivity to KLH, PPD or HBsAg resulted from the injection of only the vacuum-dialysed and not the water-dialysed

transfer factor, and (b) that the degree of such acquired DH-responses to KLH and PPD was dependent on the dose of transfer factor.

4. RESULTS OF THE MIXED LEUCOCYTE-MACROPHAGE MIGRATION (LMMI) TESTS.

LMMI tests were carried out in normal, actively sensitised and transfer factor-treated rhesus monkeys. The migration indices were calculated as described previously (Section 2.7.1), and a migration index (MI) of less than 0.8 was arbitrarily considered positive (Plates 14a-b). The reason for this cut-off point will be discussed later (Section 8.2.1).

4.1 Normal and actively sensitised monkeys (Figs. 9-16, plates 14a-b, tables 17-18).

4.1.1 Maximum LMMI-responses to KLH, PPD, TB and HBsAg (Figs. 9-10).

The LMMI test was carried out in naive rhesus monkeys in order to determine the normal baseline responses to 3-200 µg/ml KLH, PPD, TB and HBsAg (Figs. 9a-d). The mean migration indices in these monkeys were above 0.8 (MI > 0.8) for all the concentrations of all the antigens, though some individual animals showed non-specific reactivity to KLH, PPD, TB and HBsAg at one or more concentration. Such reactivity was most prevalent for KLH (Fig. 9a).

In comparison with the virgin monkeys above, when animals were actively immunised with FCA, with or without KLH or HBsAg, the results shown in figures 10a-d were obtained. FCA always contained 3 mgs particulate tubercle bacilli. Depending on the concentration

a

b

Plates 14a-b. Mixed leucocyte-macrophage migration from capillary tubes in an actively or adoptively sensitised rhesus monkey (MI < 0.8)

(a) Area of fan in the presence of antigen (test).

(b) Area of fan in the absence of antigen (control).

MI, migration index = (a) ÷ (b).

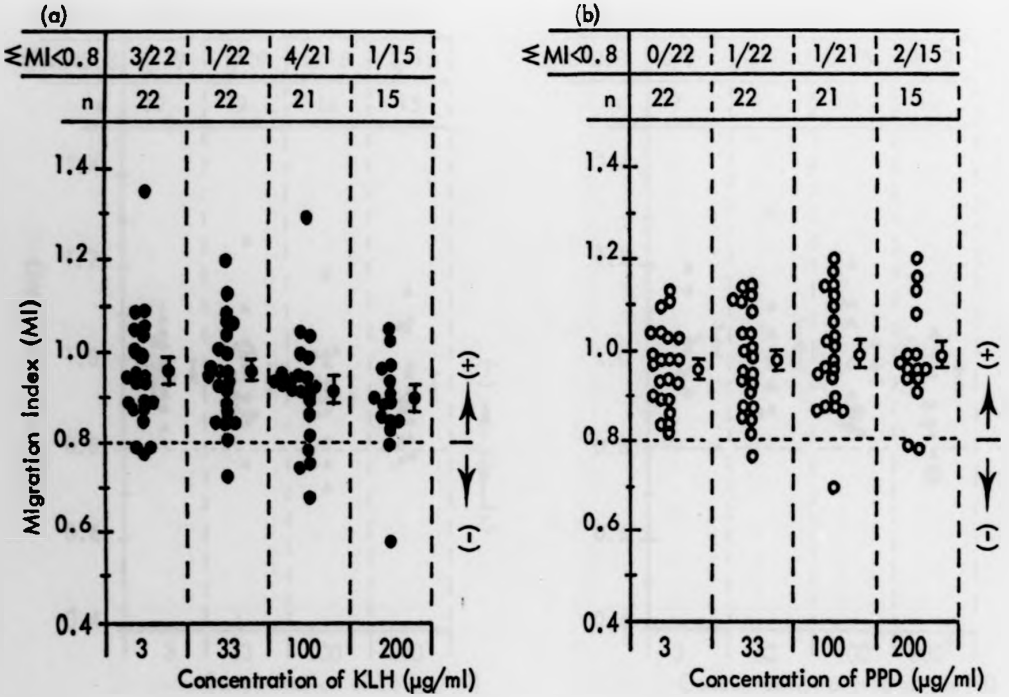

Fig. 9. Maximum baseline LMMI dose-response to (a) KLH or (b) PPD in normal rhesus monkeys.

n, number of monkeys; ⊥ ⊥, mean MI ± SEM.

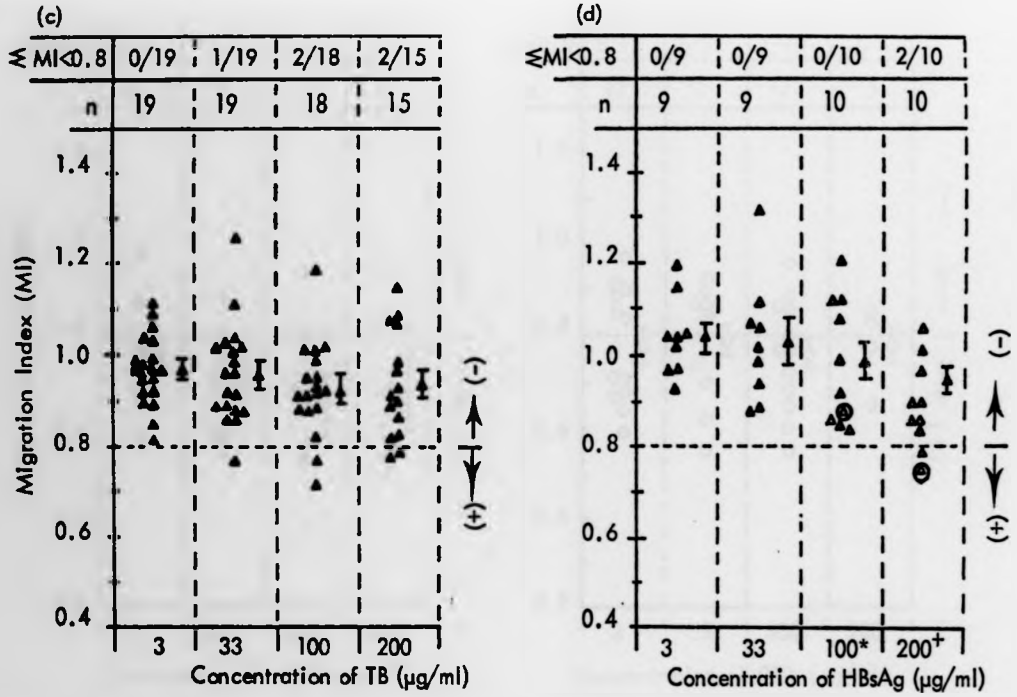

Fig. 9. Maximum baseline LMMI dose-response to (c) TB or (d) HBsAg in normal rhesus monkeys.

n, number of monkeys; ⊥ ⊥, mean MI ± SEM; * ⊙ 1/25, + ⊙ 1/10 dilutions of HBsAg.

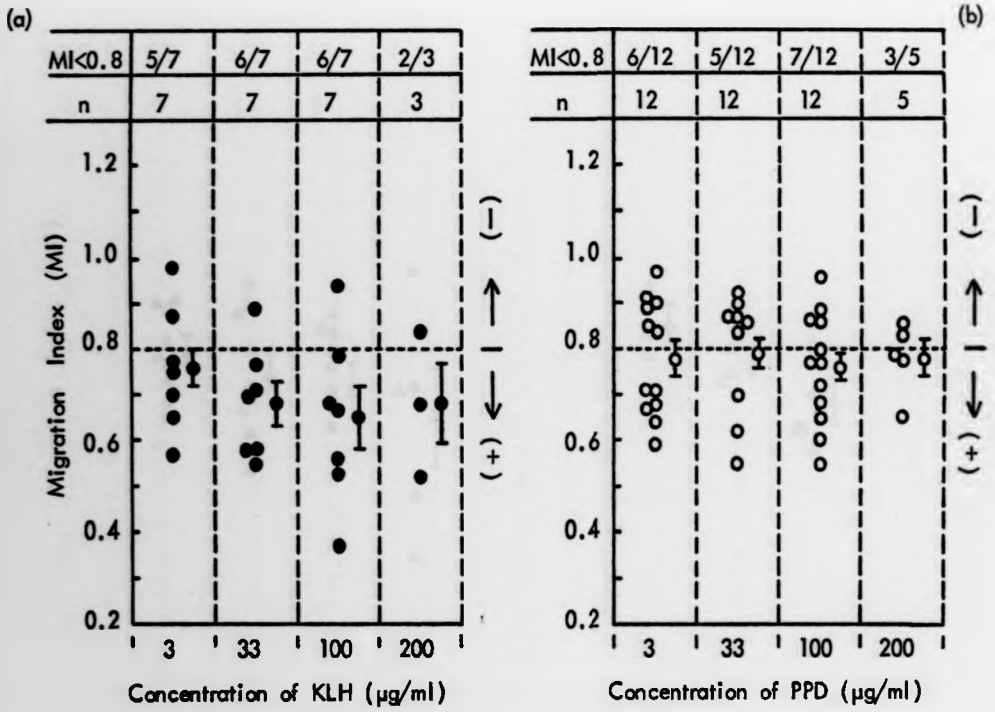

Fig. 10. Maximum LMMI dose-response to (a) KLH or (b) PPD in rhesus monkeys actively sensitised with FCA with or without KLH or HBsAg.

n, number of monkeys; ▮ ▯ mean MI ± SEM.

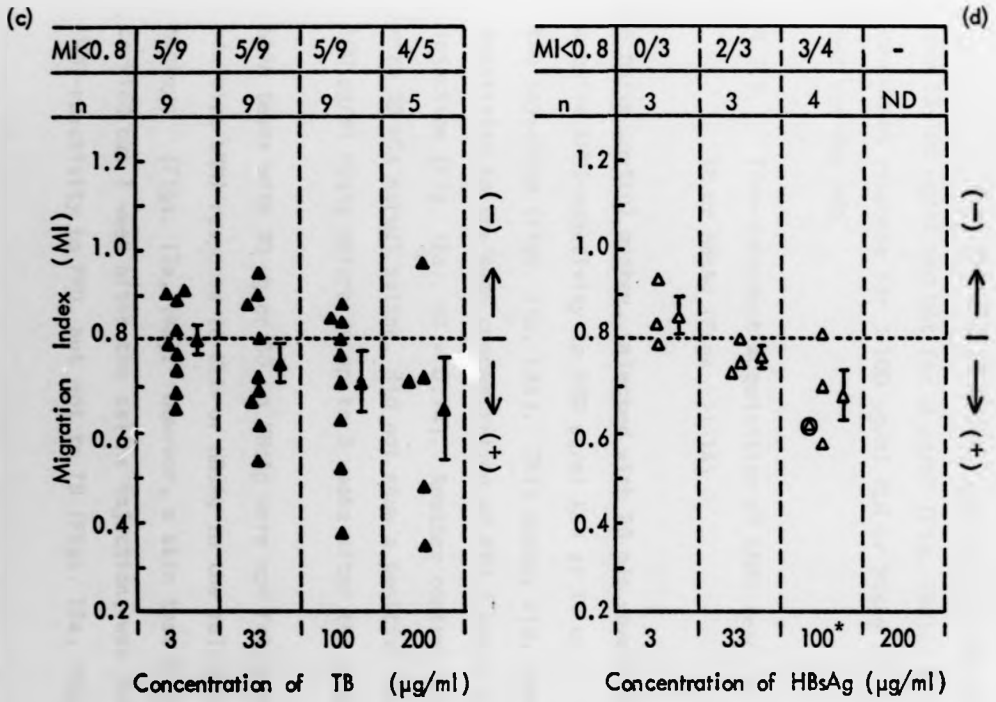

Fig. 10. Maximum LMMI dose-response to (c) TB or (d) HBsAg in rhesus monkeys actively sensitised with FCA with or without KLH or HBsAg.

n, number of monkeys; I mean MI ± SEM; ND, not done; * ⊙ 1/25 dilution of HBsAg.

of each antigen, LMMI-reactivity (MI < 0.8) to KLH was detected in 2 to 6 animals (Fig. 10a), to PPD in 3 to 7 animals (Fig. 10b), to TB in 4 to 5 animals (Fig. 10c) and to HBsAg in 2 to 3 animals (Fig. 10d). Mean migration indices were always equal to or below 0.8 for 3-200 µg/ml KLH, PPD and TB (Figs. 10a-c), but for HBsAg this was so for 33-100 µg/ml and not for 3 µg/ml (Fig. 10d). There was a dose-dependent response for 3-100 µg/ml KLH or HBsAg and for 3-200 µg/ml TB, but not for PPD.

4.1.2 Time-dependent acquisition of LMMI-reactivity to KLH, PPD TB or HBsAg (Figs. 11-14).

A naive control monkey, injected with 20 mls normal saline, did not acquire LMMI-reactivity to 100 µg/ml KLH or TB up to 10 weeks after the injection (Figs. 11a, 13a). This monkey did, however, acquire reactivity to the same concentration of PPD 2 weeks after the saline injection (Fig. 12a: MI = 0.74). Another control monkey, injected with 30 mls normal saline, did not show a positive response to 100 µg/ml HBsAg before or up to 3 weeks after the injection (Fig. 14a).

Skin tests with 30 µg or 50 µg HBsAg were not followed with a positive LMMI-response to KLH or HBsAg in the saline-injected control monkeys (Figs. 11a, 14a). However, a skin test with 30 µg PPD, carried out 1 week after the saline injection, was followed by LMMI-reactivity to PPD, but not to TB (Figs. 12a, 13a).

Unlike the saline-injected virgin monkeys, animals immunised with FCA, with or without an antigen (KLH or HBsAg), acquired LMMI-reactivity towards KLH, TB or HBsAg 2 to 5 weeks after the immunisation

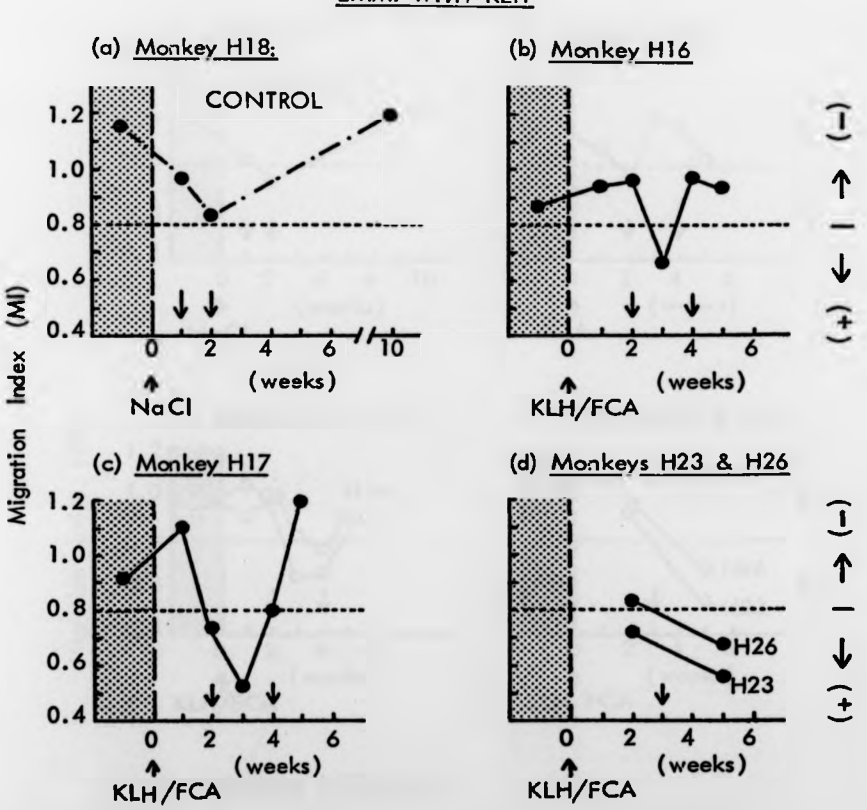

Fig. 11. Time course of LMMI-responses to 100 µg/ml KLH before and after (a) injection of a control monkey with saline, and (b-d) immunisation of 4 monkeys with KLH in FCA.

NaCl, sterile normal saline; ↓ skin test with KLH; shaded area, any time before saline injection or immunisation; immunised H16, H17, H26 with 100 µg KLH in FCA; H23 with 1 mg KLH in FCA; (•) 100 µg/ml KLH.

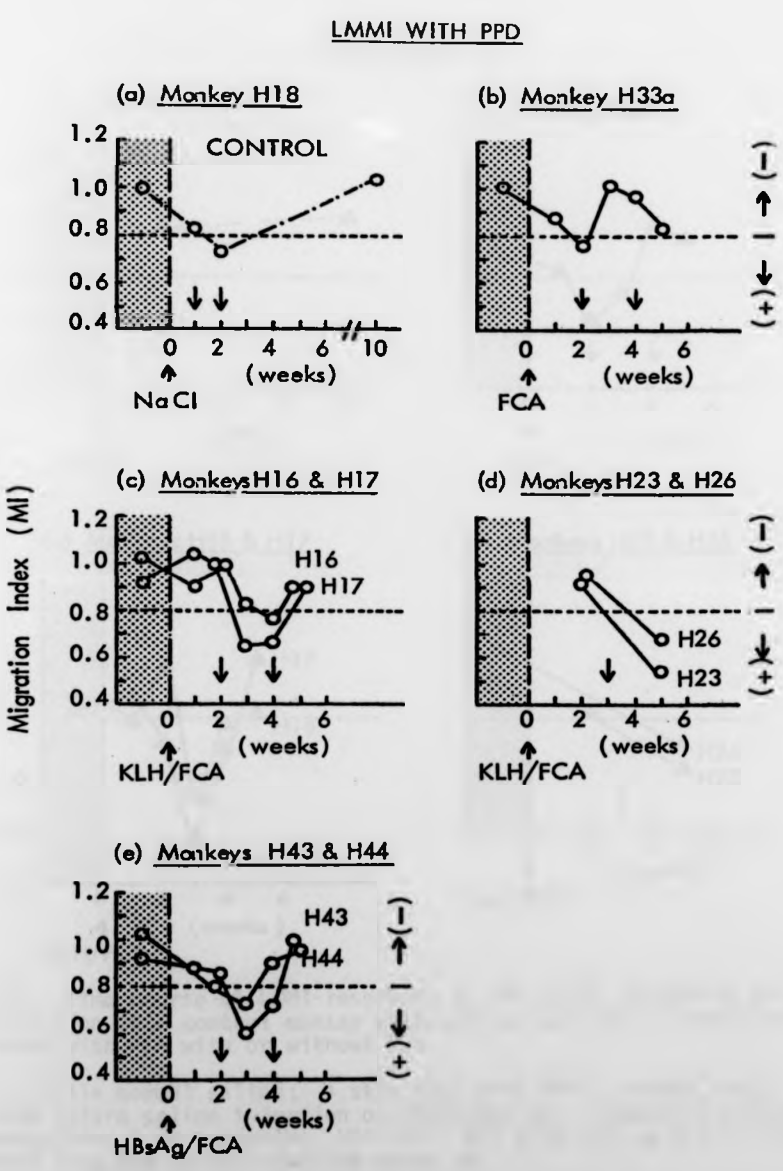

Fig. 12. Time course of LMMI-responses to 100 µg/ml PPD before and after (a) injection of a control monkey with saline, and (b-e) immunisation of 7 monkeys with FCA with or without KLH or HBsAg.

NaCl, sterile normal saline; ↓ skin test with PPD; shaded area, any time before saline injection or immunisation; immunised H33a with FCA containing 3 mg tubercle, H16, H17, H26 with 100 µg KLH in FCA, H23 with 1 mg KLH, and H43, H44 with 1 mg HBsAg in FCA; (o) 100 µg/ml PPD.

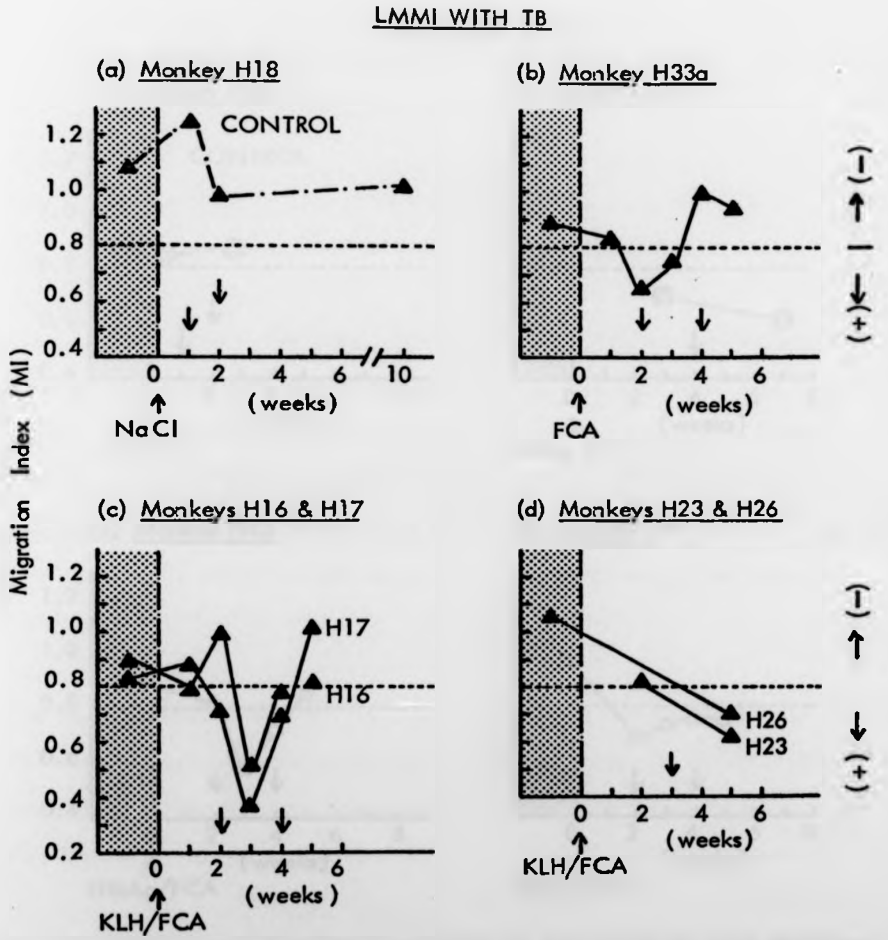

Fig. 13. Time course of LMMI-responses to 100 µg/ml TB before and after (a) injection of a control monkey with saline, and (b-d) immunisation of 5 monkeys with FCA with or without KLH.

NaCl, sterile normal saline; ↓ skin test with PPD; shaded area, any time before saline injection or immunisation; immunised H33a with FCA containing 3 mg tubercle; H16, H17, H26 with 100 µg KLH in FCA; H23 with 1 mg KLH in FCA; (▲) 100 µg/ml TB.

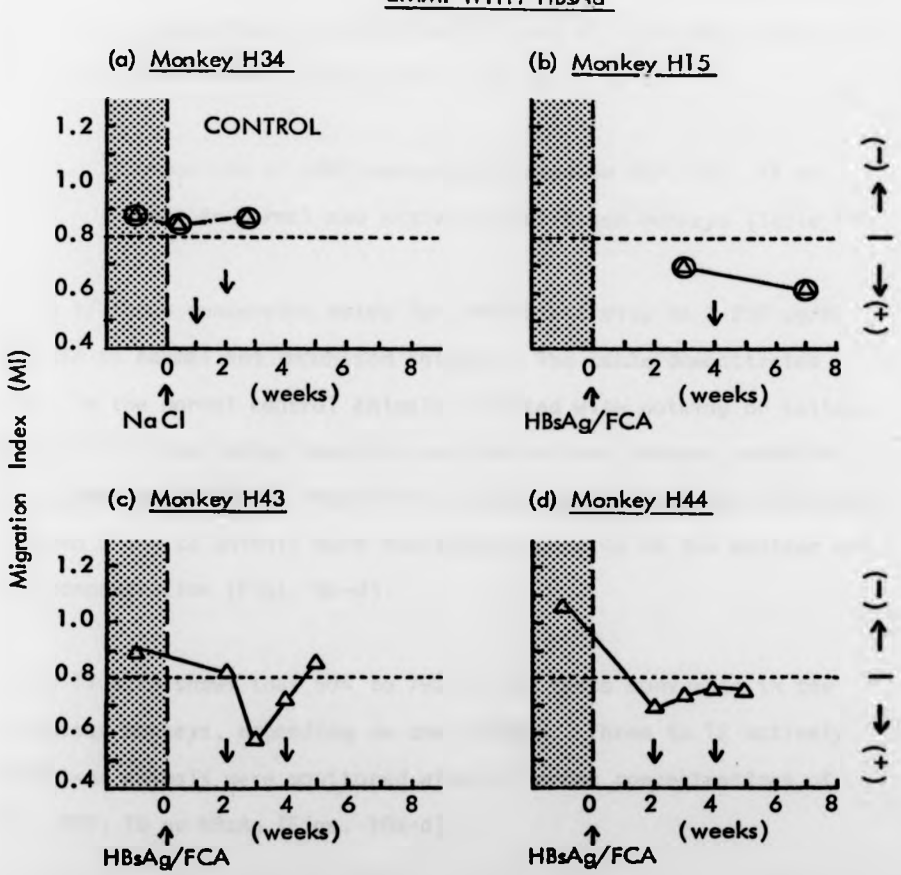

Fig. 14. Time course of LMMI-responses to 100 µg/ml or 1/25 dilution of HBsAg before and after (a) injection of a control monkey with saline, and (b-d) immunisation of 3 monkeys with HBsAg in FCA.

NaCl, sterile normal saline; ↓ skin test with HBsAg; shaded area, any time before saline injection or immunisation; immunised H15, H43, H44 with 1 mg HBsAg in FCA; (●) 1/25 dilution HBsAg; (Δ) 100 µg/ml HBsAg.

(Figs. 11b-d, 13b-d, 14b-d). Such reactivity also developed towards PPD 2 to 5 weeks after the immunisation, but this was not unlike the saline-injected monkey (Figs. 12a cf 12b-e).

4.1.3 Comparison of LMMI-conversion rates to KLH, PPD, TB or HBsAg in normal and actively sensitised monkeys (Table 17).

Table 17 shows conversion rates for LMMI-reactivity to 3-200 µg/ml antigen in normal and immunised animals. The table demonstrates that, in the normal control animals injected with nothing or saline, 5% to 11% of the tests, depending on the antigen, became positive. Thus, some non-specific reactivity to the antigens used was detected. Between 9 and 22 animals were monitored depending on the antigen and its concentration (Figs. 9a-d).

Table 17 also shows that 50% to 79% of the tests converted in the immunised monkeys, depending on the antigen. Three to 12 actively immunised animals were monitored with different concentrations of KLH, PPD, TB or HBsAg (Figs. 10a-d).

Comparison of the above results revealed that the likelihood for the acquisition of LMMI-reactivity by the immunised monkey was highly significant for all these antigens, but more so for KLH, PPD and TB ($P < 0.001$), than for HBsAg ($P < 0.005$).

4.1.4 Relationship of the immunising dose of KLH and the acquired LMMI-reactivity (Fig. 15).

Table 17. Comparison of LMMI-conversion rates to KLH, PPD, TB or HBsAg in normal and actively immunised monkeys.[a]

Immunisation[b] with	Total no. of +ve LMMI (MI < 0.8) tests[c] 3-200 µg/ml KLH	PPD	TB	HBsAg	Sum
Nothing/NaCl (control)	9/80 (11%)	4/80 (5%)	5/71 (7%)	2/38 (5%)	20/269 (7%)
FCA ± KLH or HBsAg	19/24 (79%)	21/41 (51%)	19/32 (59%)	5/10 (50%)	64/107 (60%)
P for χ^2	< 0.001	< 0.001	< 0.001	< 0.005	< 0.001

[a]From figures 9-10 (Section 4.1.1).

[b]NaCl, normal saline; FCA, Freund's complete adjuvant.

[c]LMMI, mixed leucocyte-macrophage migration test done at any time in normal controls and at 2 to 7 weeks after active immunisation. KLH, keyhole limpet haemocyanin; PPD, purified protein derivative of tuberculin; TB, particulate tubercle bacilli; HBsAg, purified hepatitis B surface antigen; MI, migration index.

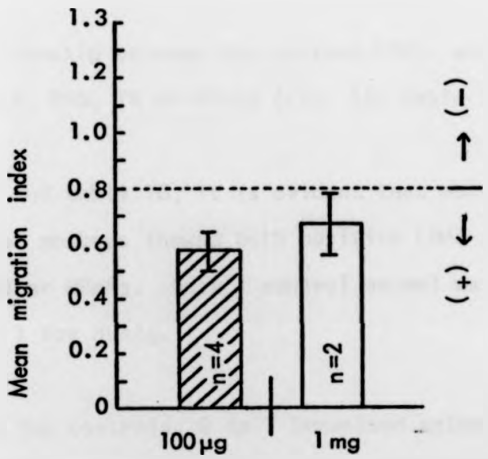

Fig. 15. The effect of different concentrations of KLH in FCA upon the LMMI-responses in actively sensitised monkeys.

n, number of monkeys; I, SEM.

Figure 15 shows that the amount of KLH in FCA affected the intensity of the acquired reactivity to 100 µg/ml KLH in the immunised monkeys. The 2 animals immunised with 100 µg KLH in FCA acquired a stronger mean LMMI-reactivity (Mean MI = 0.57), than the 4 animals immunised with 1 mg KLH in FCA (Mean MI = 0.67).

4.1.5 Relationship between the maximum LMMI- and DH-responses to KLH, PPD, TB or HBsAg (Fig. 16, table 18).

From figure 16 and table 18, it is evident that none of the saline-injected control monkeys showed both positive LMMI- and DH-responses to KLH, PPD, TB or HBsAg. Only 1 control animal was used for KLH, PPD and TB and 1 for HBsAg.

In contrast to the controls, 2 to 7 immunised animals, depending on the antigen, acquired both positive LMMI- and DH-responses to KLH, PPD, TB or HBsAg. Three to 12 immunised animals were monitored with the LMMI and skin tests at the same time. Thus, the table shows that in the actively immunised animals, when all antigens were considered, there was 66% and 65% association between the positive LMMI-tests and the positive DH-responses after the first or the second skin tests respectively. Such an association was significantly different from the controls after the first skin test ($P < 0.05$), but not after the second skin test ($P > 0.1$).

4.2 Transfer factor-treated monkeys (Figs. 17-28, tables 19-20).

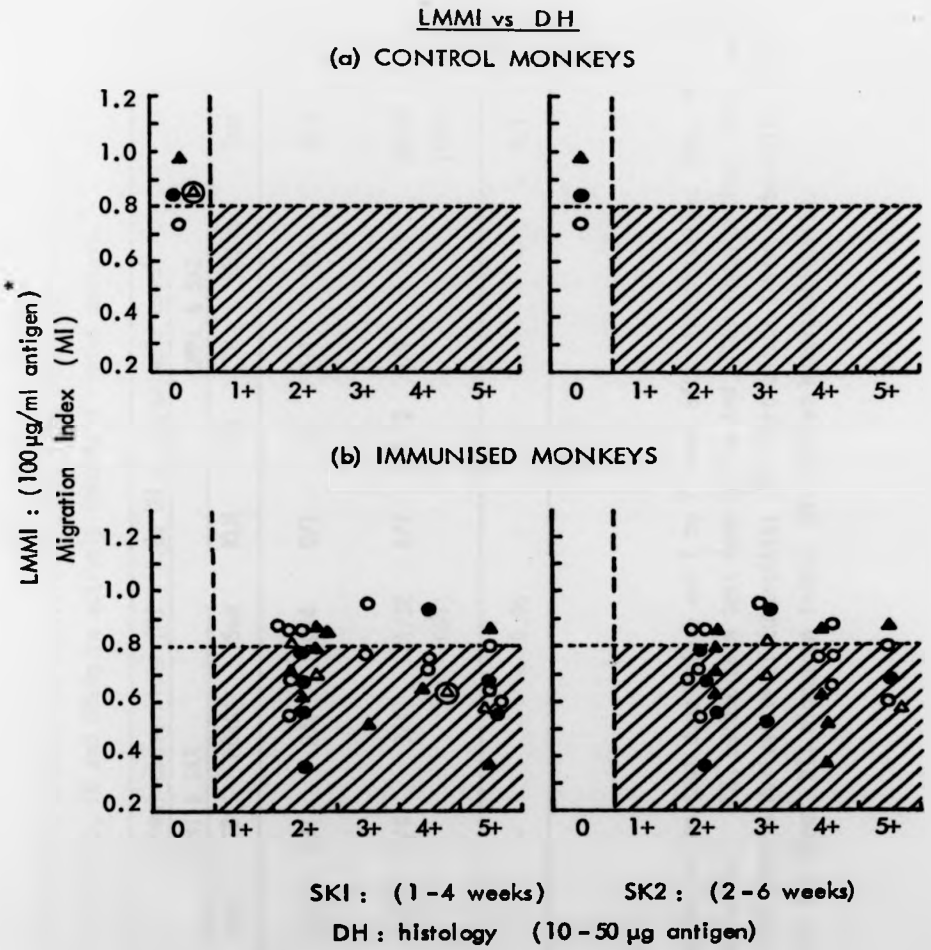

Fig. 16. Relationship between the maximum LMMI- and DH-responses to KLH, PPD, TB and HBsAg in (a) normal control, and (b) actively immunised monkeys.

SK1, first skin test; SK2, second skin test; (●) KLH; (○) PPD; (▲) TB; *(HBsAg : △ 100 µg/ml, ◎ 1/25 dilution); ▨ positive LMMI and DH.

Table 18. Relationship between the acquisition of maximum LMMI- and DH-responses to KLH, PPD, TB and HBsAg in actively immunised rhesus monkeys.[a]

Immunisation[b] with	Animal conversions (MI < 0.8, DH ≥ 1+)/animals used[c]									
	LMMI & SK1					LMMI & SK2				
	KLH	PPD	TB	HBsAg	Sum	KLH	PPD	TB	HBsAg	Sum
Nothing (control)	0/1	0/1	0/1	0/1	0/4	0/1	0/1	0/1	ND	0/3
FCA ± KLH or HBsAg	6/7	7/12	5/9	3/4	21/32 (66%)	6/7	7/12	5/9	2/3	20/31 (65%)
P for x^2	-	-	-	-	< 0.05	-	-	-	-	> 0.1

[a] From figure 16.

[b] FCA, Freund's complete adjuvant.

[c] LMMI, mixed leucocyte-macrophage migration test done before and 2 to 7 weeks after immunisation; SK1, first skin test done before and after immunisation; SK2, second skin test done before and after immunisation; KLH, keyhole limpet haemocyanin; PPD, purified protein derivative of tuberculin; TB, particulate tubercle bacilli; HBsAg, hepatitis B surface antigen; ND, not done; MI, migration index; DH, delayed hypersensitivity.

4.2.1 Maximum LMMI-responses to KLH, PPD, TB and HBsAg (Figs. 17-18).

Figures 17a-d demonstrate that 2 to 4 naive monkeys depending on the antigen, received control dialysable transfer factor. None developed LMMI-reactivity to 3 to 33 µg/ml KLH, or to 3 to 200 µg/ml PPD or TB, or to 3 to 100 µg/ml HBsAg. However, reactivity was acquired by 1 to 3 animals for 100-200 µg/ml KLH and by 2 animals for 200 µg/ml HBsAg (Figs. 17a, d). Therefore, some non-specific reactivity was detected to KLH and HBsAg, but not to PPD or TB. Mean migration indices revealed absence of reactivity (MI > 0.8) towards 3-100 µg/ml KLH, PPD, TB and HBsAg, but they showed a dose response for KLH or HBsAg. However, dose-response for PPD or TB was irregular.

In contrast to the results of the controls above, figures 18a-d show the results of the animals treated with the immune dialysable rhesus transfer factor. Depending on the concentration of the test antigen, 2 to 6 recipients of KLH-immune transfer factor converted when tested with 3-200 µg/ml KLH (Fig. 18a: MI < 0.8). Similarly, 3 to 5 converted for PPD, 4 for TB and 1 to 3 for HBsAg (Figs. 18b-d). Mean migration indices showed irregular dose-response for KLH, PPD or TB, but a good dose-response for HBsAg. Unlike most of the controls (Figs. 17a-d), here mean indices revealed reactivity (MI < 0.8) for 3-200 µg/ml KLH (Fig. 18a), for 3, 100 and 200 µg/ml PPD (Fig. 18b), for 33-100 µg/ml TB (Fig. 18c), and for 33-200 µg/ml HBsAg (Fig. 18d). These reactivities were stronger for KLH or HBsAg, than for PPD or TB.

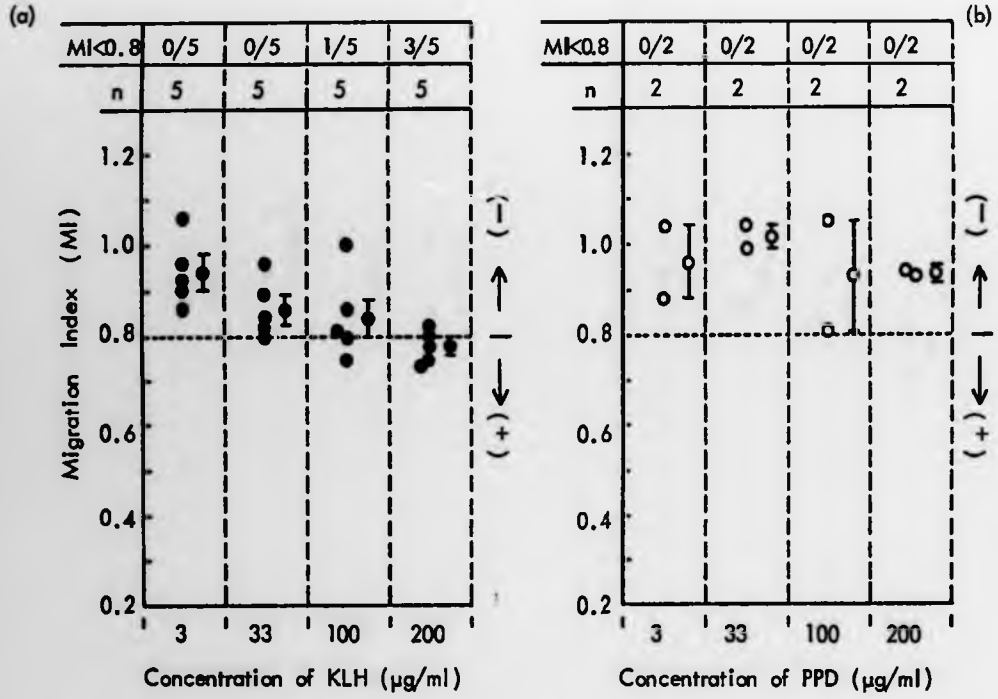

Fig. 17. Maximum LMMI dose-response to (a) KLH, or (b) PPD in rhesus monkeys treated with control dialysable rhesus transfer factor.

n, number of monkeys; $\bar{\bar{I}}$, mean MI $\pm$ SEM.

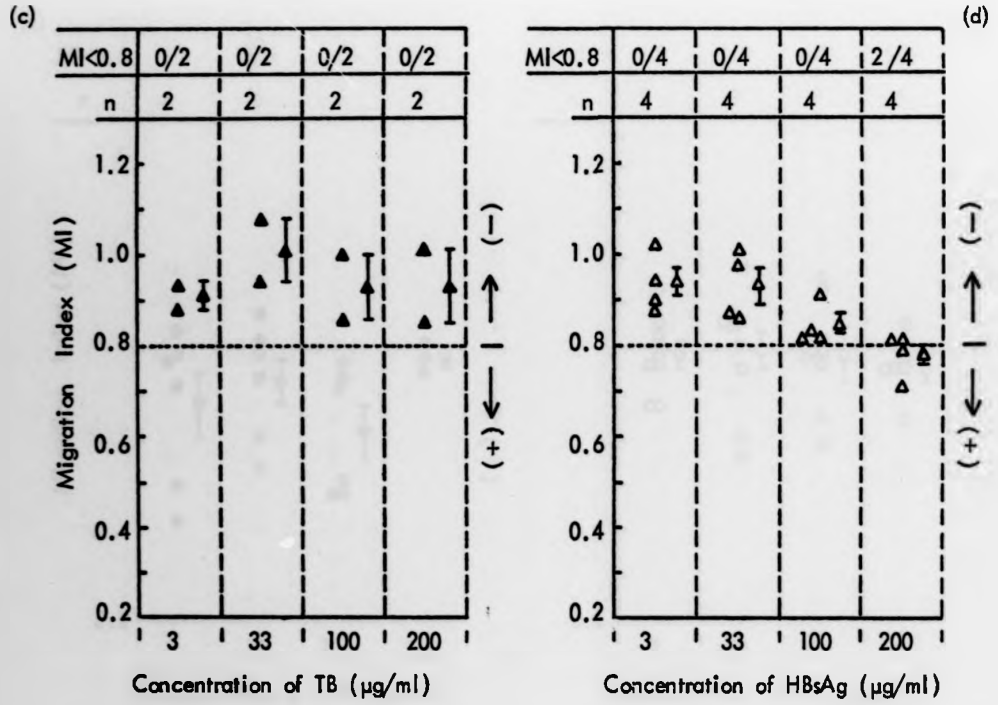

Fig. 17. Maximum LMMI dose-response to (c) TB, or (d) HBsAg in rhesus monkeys treated with control dialysable rhesus transfer factor.

n, number of monkeys; I, mean MI ± SEM.

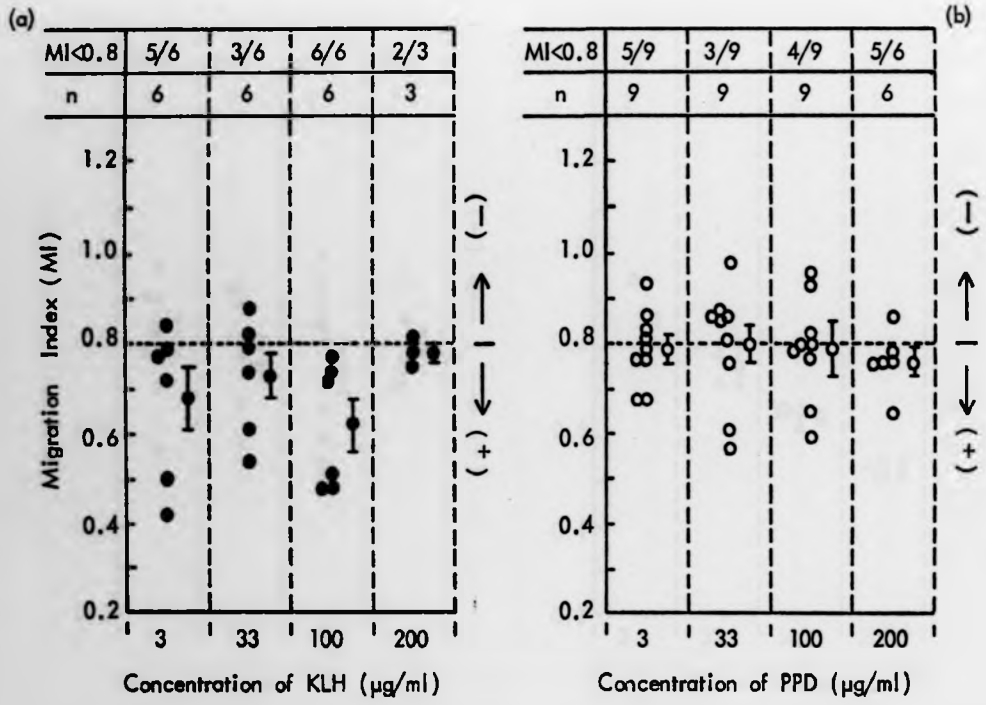

Fig. 18. Maximum LMMI dose-response to (a) KLH or (b) PPD in rhesus monkeys treated with immune dialysable rhesus transfer factor.

n, number of monkeys; $\bar{\bar{x}}$, mean MI ± SEM.

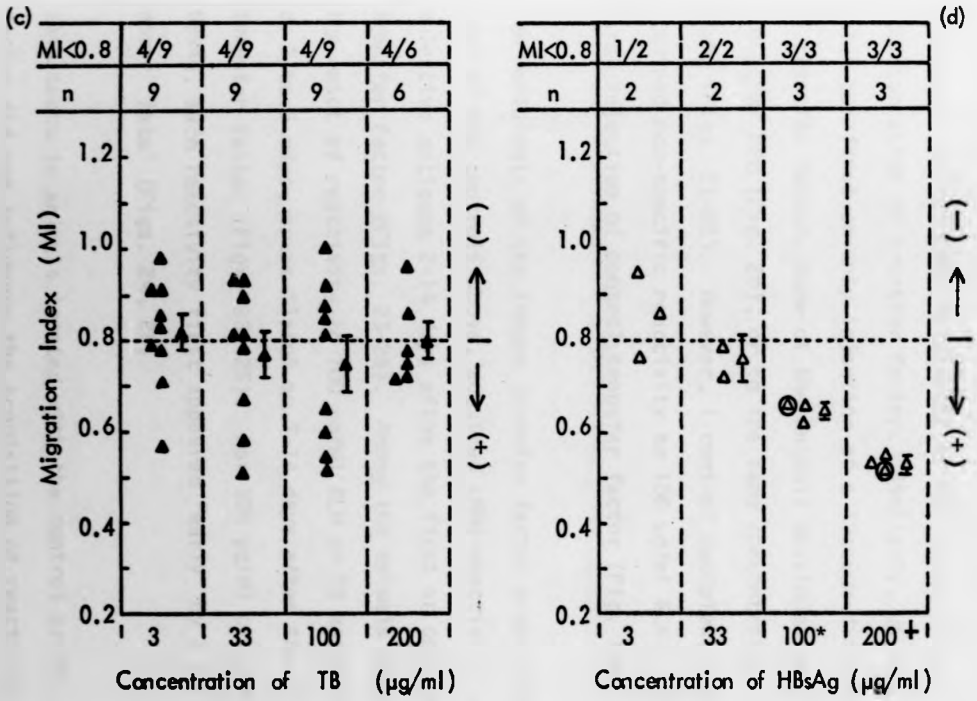

Fig. 18. Maximum LMMI dose-response to (c) TB or (d) HBsAg in rhesus monkeys treated with immune dialysable rhesus transfer factor.

n, number of monkeys; ⊥, mean MI ± SEM; ⊙, *1/25 and +1/10 dilutions of HBsAg.

4.2.2 Time-dependent acquisition of LMMI-reactivity to KLH, PPD, TB and HBsAg (Figs. 19-26).

From figures 19 to 22 it can be seen that none of the control recipients showed LMMI-reactivity to 100 µg/ml antigen before the administration of transfer factor. Similarly, up to 21 to 34 days after the first or only injection of a "vacuum-dialysed" preparation of transfer factor, none of the animals developed reactivity to 100 µg/ml PPD (Fig. 20), or to the same concentration of TB or HBsAg (Figs. 21-22). However, 1 control recipient unexpectedly acquired non-specific reactivity to 100 µg/ml KLH 6 days after the first injection of control transfer factor (Fig. 19a).

The recipients of the immune transfer factor preparations, unlike most of the controls above, acquired LMMI-reactivity towards selective antigens 2-14 days after the first or only injection of transfer factor (Figs. 23-26). Among the animals which converted, the onset of reactivity to 100 µg/ml KLH or TB appeared as"early" as 2 to 6 days, or as "late" as 7-14 days after the injection(s) of transfer factor (Figs. 23, 25). For 100 µg/ml or 1/25 dilution of HBsAg, such reactivity first appeared "early" by 2 to 6 days, but never "late" (Figs. 24, 26).

Skin tests in animals treated with the control or the immune transfer factor did not influence the acquisition of reactivity (Figs. 19-26). This was because the acquired reactions towards all the antigens always appeared before the first skin test.

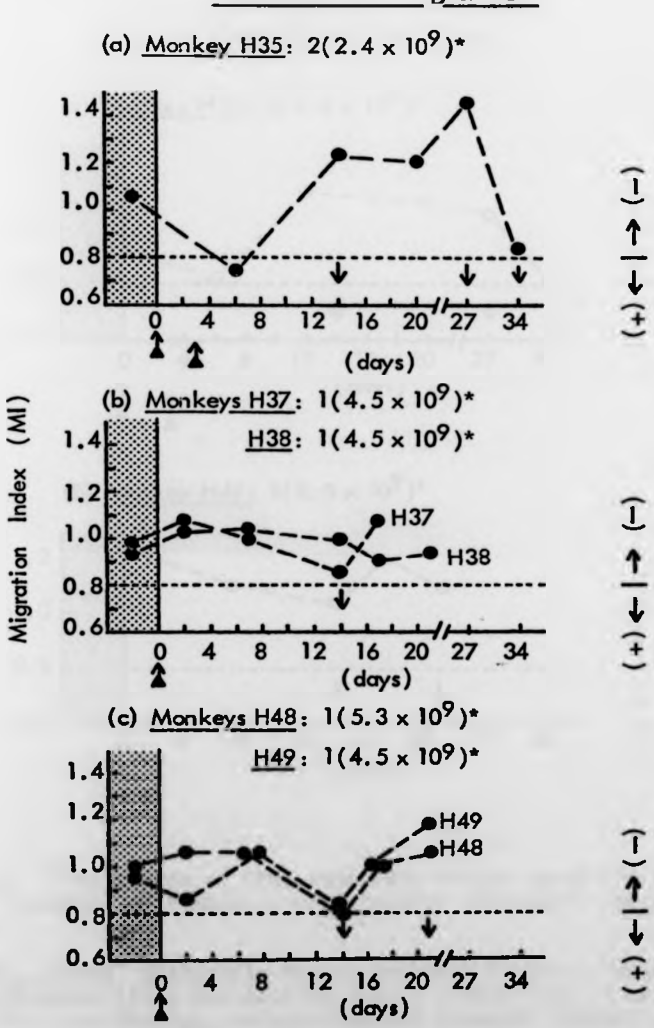

Fig. 19. Time course of LMMI-responses to 100 µg/ml KLH before and after treatment of 5 monkeys with control dialysable rhesus transfer factor.

↑ Rh-TF$_D$, normal or unrelated dialysable rhesus transfer factor; ▲ * dose of Rh-TF$_D$ in lymphocytes (Also see section 2.2.1: table 11); + skin test with KLH; --- Rh-TF$_{VD}$, vacuum-dialysed rhesus transfer factor; shaded area, any time before Rh-TF$_D$.

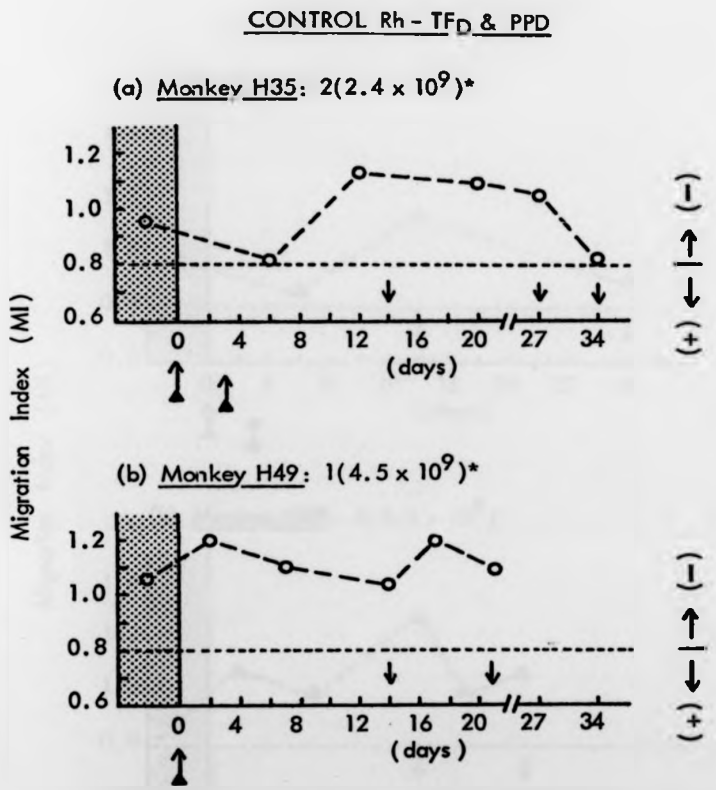

Fig. 20. Time course of LMMI-responses to 100 µg/ml PPD before and after treatment of 2 monkeys with control dialysable rhesus transfer factor.

↑ Rh-TF$_D$, normal dialysable rhesus transfer factor; *dose of Rh-TF$_D$ in lymphocytes (Also see section 2.2.1: table 11); + skin test with PPD; --- Rh-TF$_{VD}$, vacuum-dialysed transfer factor; shaded area, any time before Rh-TF$_D$.

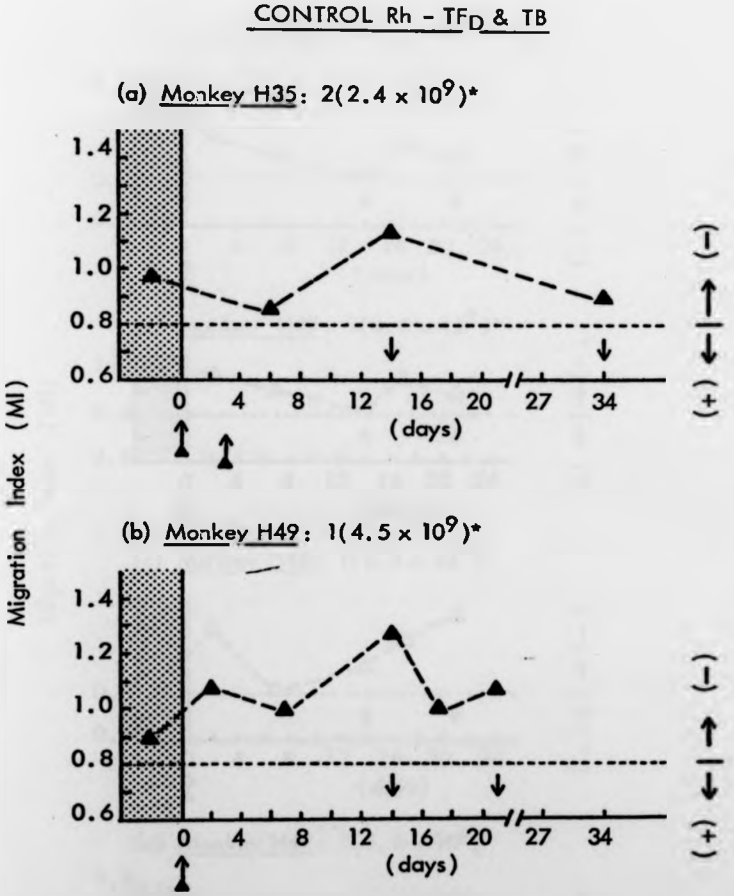

Fig. 21. Time course of LMMI-responses to 100 µg/ml TB before and after treatment of 2 monkeys with control dialysable rhesus transfer factor.

↑ Rh-TF$_D$, normal dialysable rhesus transfer factor; * dose of Rh-TF$_D$ in lymphocytes (Also see section 2.2.1: table 11); + skin test with PPD; --- Rh-TF$_{VD}$, vacuum-dialysed transfer factor; shaded area, any time before Rh-TF$_D$.

CONTROL Rh - TF_D & HBsAg

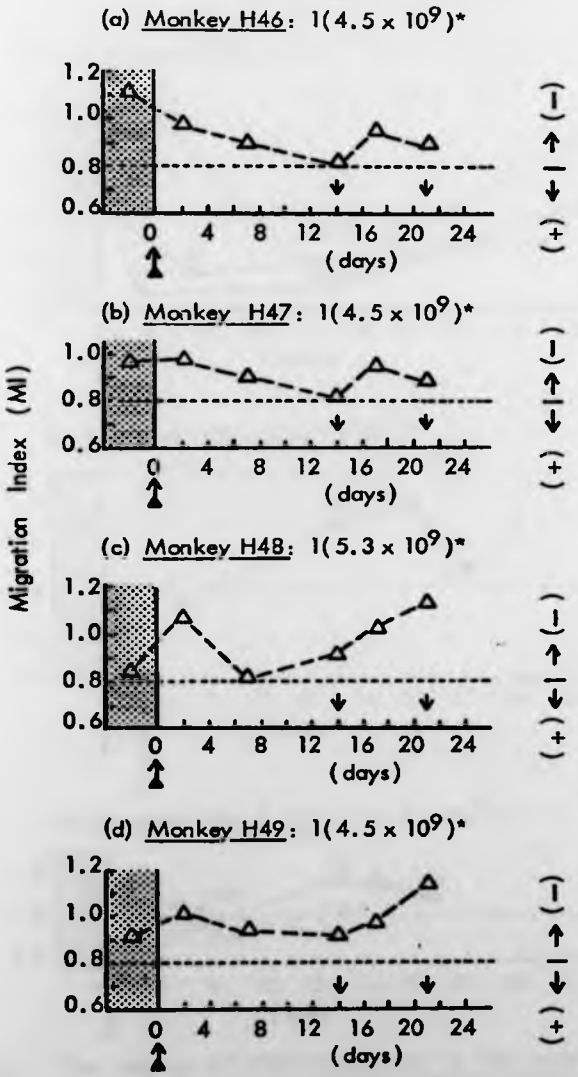

Fig. 22. Time course of LMMI-responses to 100 µg/ml HBsAg before and after treatment of 4 monkeys with control dialysable rhesus transfer factor.

↑Rh-TF_D, normal or unrelated dialysable rhesus transfer factor; * dose of Rh-TF_D in lymphocytes (Also see section 2.2.1: table 11); ↓ skin test with HBsAg; --- Rh-TF_{VD}, vacuum dialysed transfer factor; shaded area, any time before Rh-TF_D.

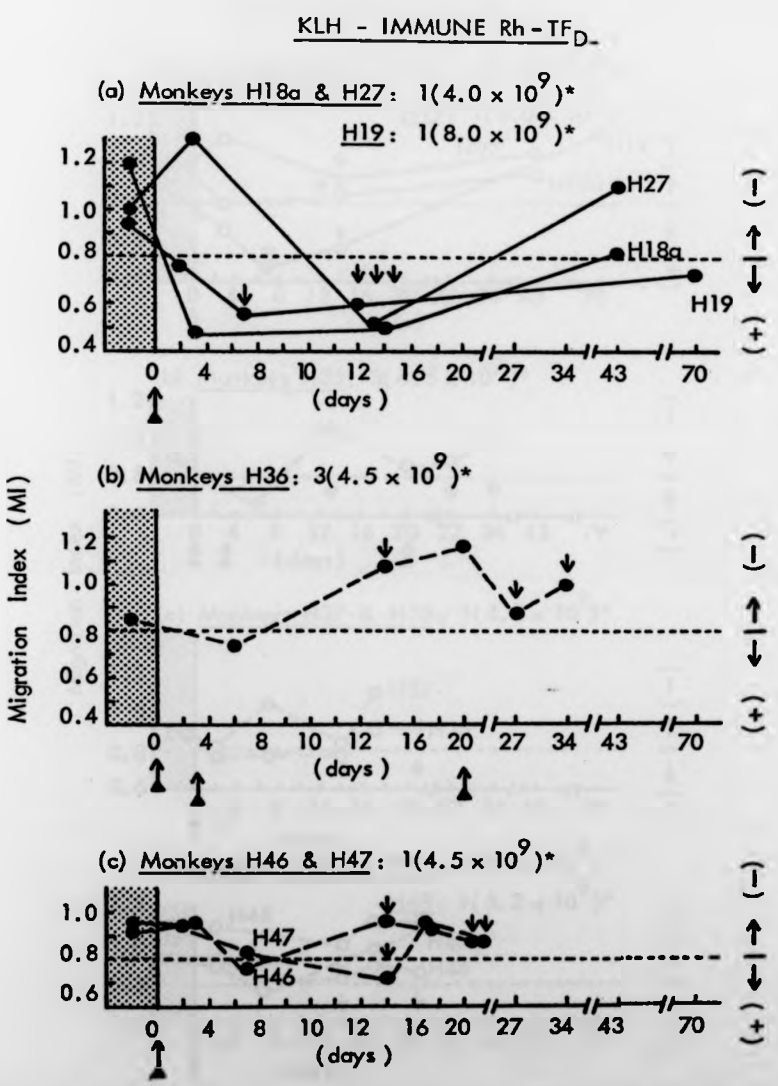

Fig. 23. Time course of LMMI-responses to 100 µg/ml KLH before and after treatment of 6 monkeys with KLH-immune dialysable rhesus transfer factor.

↑ Rh-TF$_D$, dialysable rhesus tranfer factor; * dose of Rh-TF$_D$ in lymphocytes (Also see section 2.2.1: table 11); + skin test with KLH; --- Rh-TF$_{VD}$, vacuum-dialysed transfer factor; —— Rh-TF$_{WD}$, water-dialysed transfer factor; shaded area, any time before Rh-TF$_D$.

FCA - IMMUNE Rh-TF_D

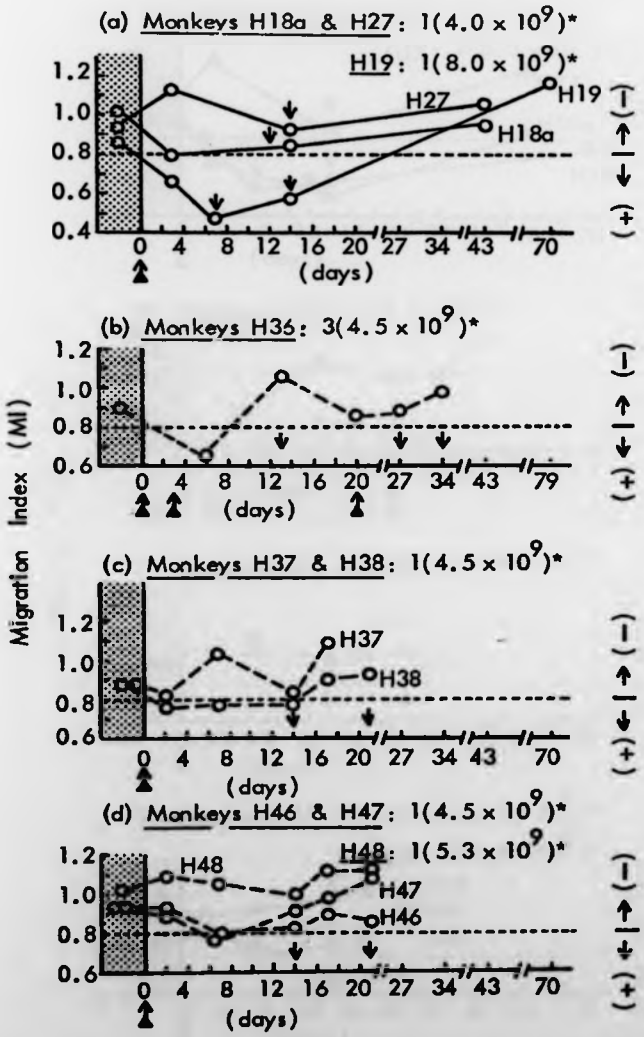

Fig. 24. Time course of LMMI-responses to 100 µg/ml PPD before and after treatment of 9 monkeys with FCA-immune dialysable rhesus transfer factor.

↑ Rh-TF_D, dialysable rhesus transfer factor; *dose of Rh-TF_D in lymphocytes (Also see section 2.2.1: table 11); ↓ skin test with PPD; --- Rh-TF_{VD}, vacuum-dialysed tranfer factor; —— Rh-TF_{WD}, water-dialysed transfer factor; shaded area, any time before Rh-TF_D.

FCA - IMMUNE Rh-TF_D-

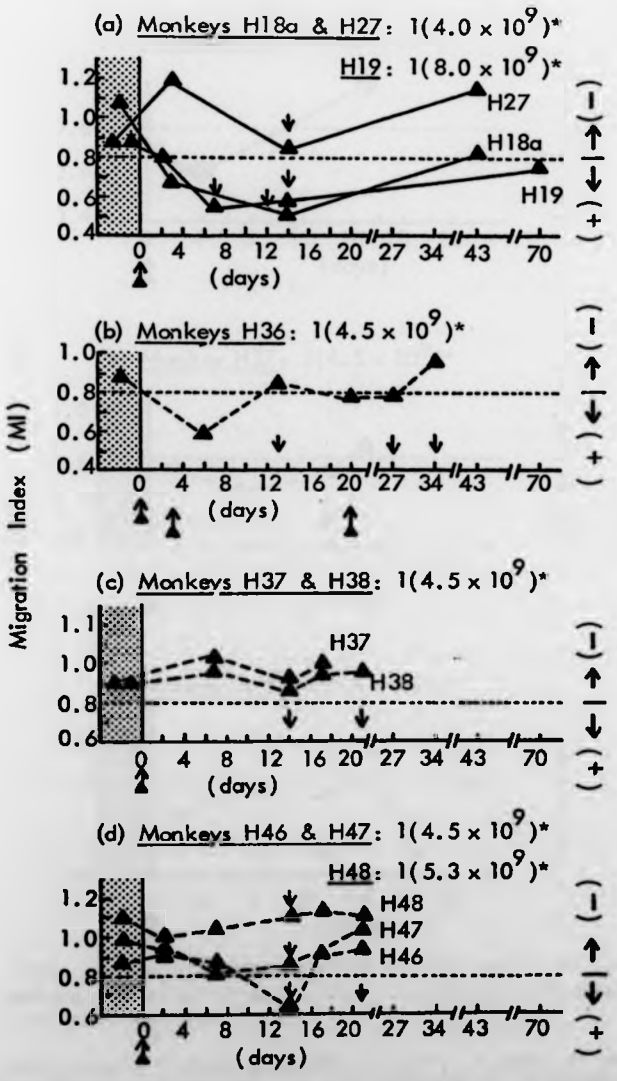

Fig. 25. Time course of LMMI-responses to 100 µg/ml TB before and after treatment of 9 monkeys with FCA-immune dialysable rhesus transfer factor.

↑ Rh-TF_D, dialysable rhesus transfer factor; * dose of Rh-TF_D in lymphocytes (Also see section 2.2.1: table 11); ↓ skin test with PPD; --- Rh-TF_{VD}, vacuum-dialysed transfer factor; —— Rh-TF_{WD}, water-dialysed transfer factor.

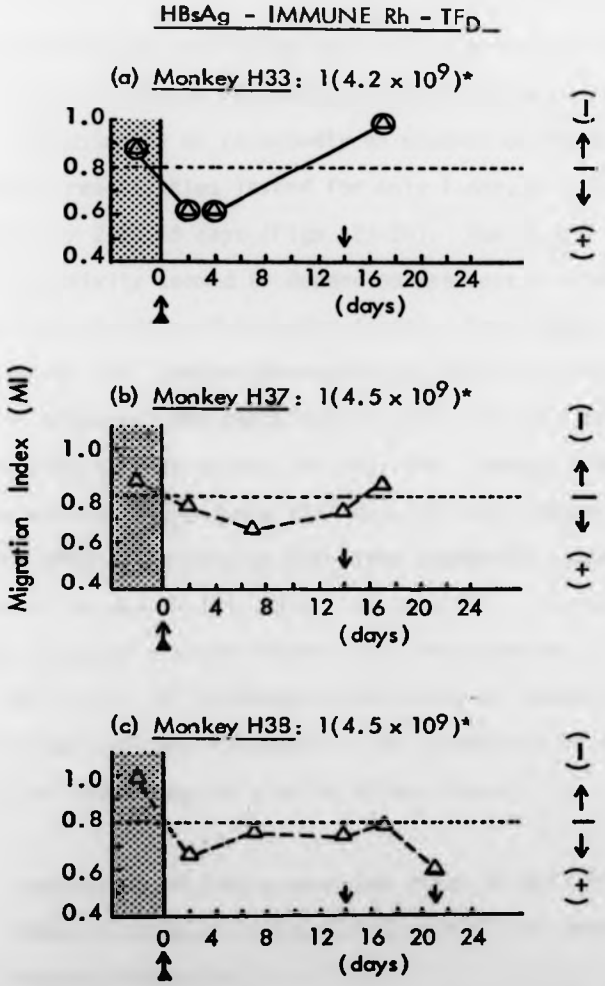

Fig. 26. Time course of LMMI-response to 100 µg/ml or 1/25 dilution of HBsAg before and after treatment of 3 monkeys with HBsAg-immune dialysable rhesus transfer factor.

↑ Rh-TF$_D$, dialysable rhesus transfer factor; * dose of Rh-TF$_D$ in lymphocytes (Also see section 2.2.1: table 11); + skin test with HBsAg; --- Rh-TF$_{VD}$, vacuum-dialysed transfer factor; —— Rh-TF$_{WD}$, water dialysed transfer factor; shaded area, any time before Rh-TF$_D$; ⓐ, 1/25 dilution HBsAg; Δ, 100 µg/ml HBsAg.

In various recipients of the immune transfer factor, some of the acquired reactions to various antigens were detected either on one occasion ("Transient") or repeatedly on several occasions ("Lasting"). The transient reactivities lasted for only 1 day, but the lasting ones lasted for 2 to 68 days (Figs. 23-26). The length of this period of reactivity seemed to depend on the test antigen, the method of dialysis or the dose of transfer factor. For example, after the injection of 4×10^9 lymphocyte-equivalent of water-dialysed transfer factor, the acquired LMMI-reactivity to 100 µg/ml KLH appeared by day 3 and persisted through to day 14 (Fig. 23a: monkey H18a). When another animal was given twice this dose of water-dialysed transfer factor, the LMMI-reactivity to KLH first appeared by day 2 and was still present by day 70 (Fig. 23a: monkey H19). In comparison to such water-dialysed transfer factor, the injection of a total dose of 4.5×10^9 or 9×10^9 lymphocyte-equivalent of vacuum-dialysed transfer factor was only followed by the appearance of a transient reactivity to KLH on day 6, 7 or 14 (Figs. 23b-c).

4.2.3 Comparison of LMMI-conversion rates to KLH, PPD, TB or HBsAg in monkeys treated with control and immune transfer factor (Table 19).

The data in table 19 show the acquired maximum LMMI-reactivity to 3-200 µg/ml antigen in animals treated with control (normal or unrelated to test antigen) or immune transfer factor (related to test antigen).

The table reveals that in animals treated with control transfer factor, 13% or 20% of the tests became positive for HBsAg or KLH

Table 19. Comparison of LMMI-conversion rates to KLH, PPD, TB and HBsAg in monkeys treated with control and immune dialysable homologous transfer factor.[a]

$Rh\text{-}TF_D$[b] Status	Total no. of +ve LMMI (MI < 0.8) tests[c] 3-200 µg/ml KLH	PPD	TB	HBsAg	Sum
Control	4/20 (20%)	0/8	0/8	2/16 (13%)	6/52 (12%)
Immune	16/21 (76%)	17/33 (52%)	16/33 (49%)	9/10 (90%)	58/97 (60%)
P for χ^2	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

[a]From figures 17-18 (Section 4.2.1).

[b]$Rh\text{-}TF_D$, dialysable rhesus transfer factor.

[c]LMMI, mixed leucocyte-macrophage migration test done at any time before or after the first or only injection of $Rh\text{-}TF_D$. KLH, keyhole limpet haemocyanin; PPD, purified protein derivative of tuberculin; TB, particulate tubercle bacilli; HBsAg, hepatitis B surface antigen; MI, migration index.

respectively, but none became positive for PPD or TB. Thus, non-specific reactivity was only detected for HBsAg or KLH. A total of 2 to 5 control animals were monitored, depending on the antigen (Figs. 17a-d).

In contrast to the controls, 49% to 90% of the tests, depending on the antigen, converted when monkeys were treated with the immune transfer factor preparations. Between 2 and 9 recipients were monitored with different concentrations of the various antigens (Figs. 18a-d).

A comparison of these results showed that, the difference between the acquisition of LMMI-reactivity, in control and immune transfer factor-treated animals was highly significant for KLH, PPD, TB and HBsAg, when antigens were taken individually or altogether (Table 19: $P < 0.001$).

4.2.4 Relationship between the different doses and/or methods of dialysis of transfer factor and the intensity of maximum LMMI-responses to KLH, PPD and TB (Fig. 27).

From Fig. 27 it can be seen that dose per dose, monkeys treated with the water-dialysed transfer factor, rather than the vacuum-dialysed transfer factor, produced a stronger mean LMMI-response to KLH or TB (Figs. 27a, c). However, for PPD, this was so only for the higher dose of transfer factor, and not the lower dose (Fig. 27).

4.2.5 Relationship between the maximum LMMI- and DH-responses to KLH, PPD, TB or HBsAg (Fig. 28, table 20).

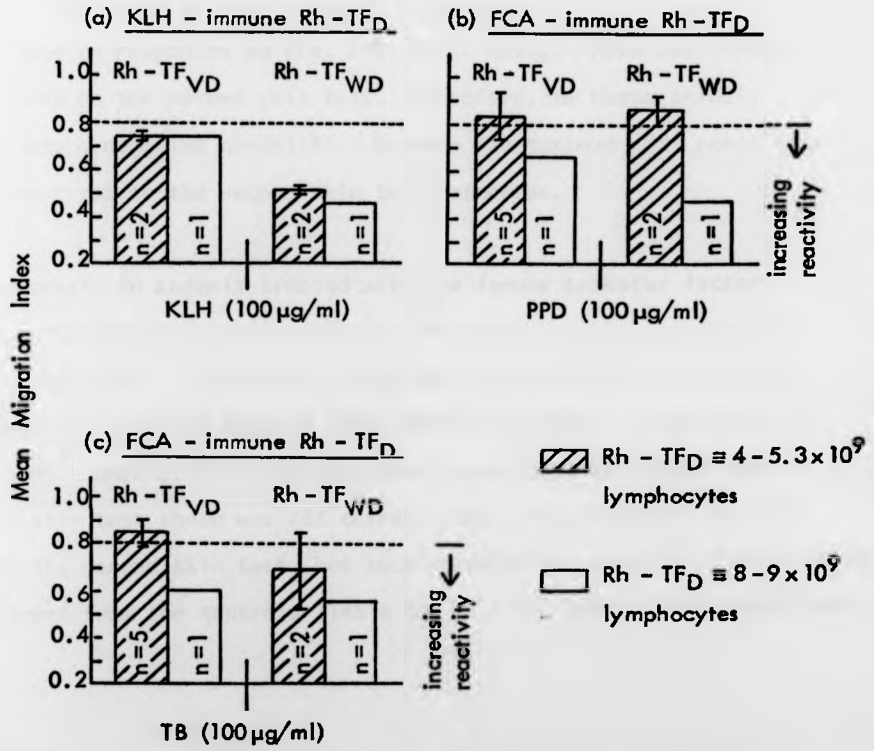

Fig. 27. Relationship between the different doses and/or methods of dialysis of immune transfer factor and the intensity of maximum LMMI-responses to (a) KLH, (b) PPD, and (c) TB.

n, number of monkeys; $\overline{\mathrm{I}}$, mean ± SEM; Rh-TF$_D$, dialysable rhesus transfer factor; Rh-TF$_{VD}$, vacuum dialysed transfer factor; Rh-TF$_{WD}$, water-dialysed transfer factor.

From figure 28 and table 20 it is evident that none of the monkeys injected with the control transfer factor showed both positive LMMI- and DH-responses to KLH, PPD, TB or HBsAg. This was so after the first or the second skin test. Therefore, in these animals there was a negative correlation between the maximum LMMI reactivity and the first or the second skin test responses.

In contrast, in animals treated with the immune transfer factor, some animals converted for both the LMMI test and the first or the second skin test. Therefore, there was some positive correlation between the acquired maximum LMMI- and DH-responses to the various antigens. When all the antigens were taken together, after the first skin test there was 26% correlation, which increased to 43% after the second skin test, but such correlations were not significantly different from the controls (Table 20: $P > 0.1$ and > 0.05 respectively).

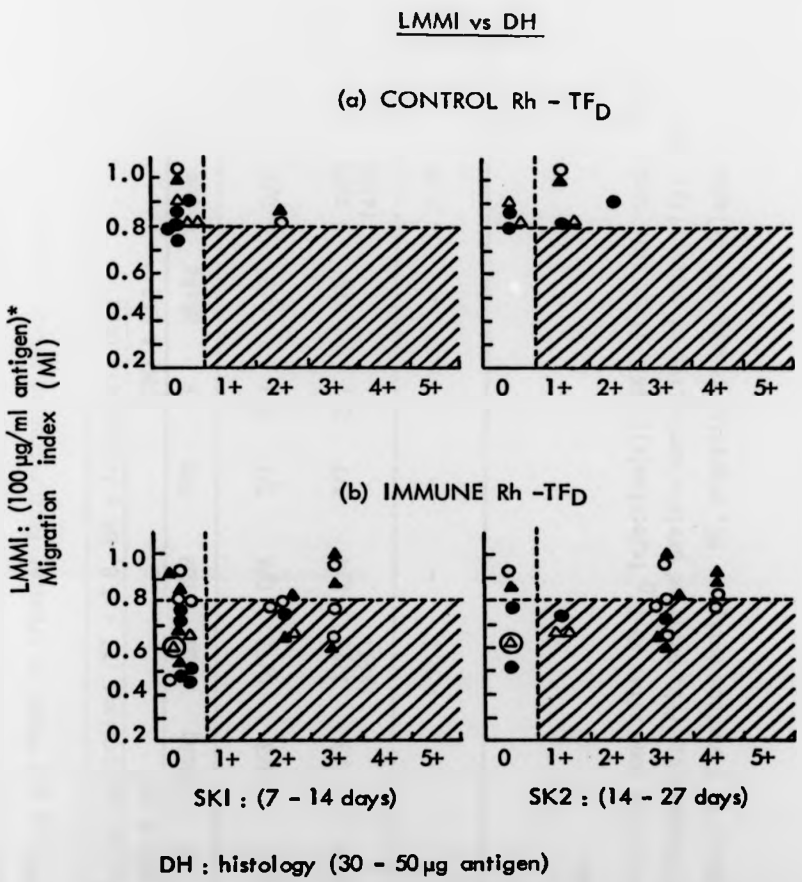

Fig. 28. Relationship between the maximum LMMI- and DH-responses to KLH, PPD, TB and HBsAg in monkeys treated with (a) control or (b) immune rhesus dialysable transfer factor.

SK1, first skin test; SK2, second skin test; • KLH; o PPD; ▲ TB; * (Δ 100 µg/ml HBsAg; Ⓐ 1/25 dilution of HBsAg); ▨ positive LMMI and DH; Rh-TF$_D$, rhesus dialysable transfer factor.

Table 20. Relationship between the acquisition of maximum LMMI- and DH-responses to KLH, PPD, TB and HBsAg in transfer factor-treated rhesus monkeys.[a]

Rh-TF_D[b] Status	Animal conversions (MI < 0.8, DH ≥ 1+)/animals used[c]									
	LMMI & SK1					LMMI & SK2				
	KLH	PPD	TB	HBsAg	Sum	KLH	PPD	TB	HBsAg	Sum
Control	0/5	0/2	0/2	0/3	0/12	0/4	0/1	0/1	0/3	0/9
Immune	1/6	3/9	2/9	1/3	7/27 (26%)	2/4	3/7	2/7	2/3	9/21 (43%)
P for x^2	-	-	-	-	> 0.1	-	-	-	-	> 0.05

[a] From figures 28a-b.

[b] Rh-TF_D, dialysable rhesus transfer factor.

[c] LMMI, mixed leucocyte-macrophage migration test done after Rh-TF_D injection(s); SK1, first skin test; SK2, second skin test; KLH, keyhole limpet haemocyanin; PPD, purified protein derivative of tuberculin; TB, particulate tubercle bacilli; HBsAg, hepatitis B surface antigen; MI, migration index; DH, delayed hypersensitivity.

5. RESULTS OF THE LYMPHOCYTE TRANSFORMATION (LT) TESTS

LT tests were carried out in normal, actively sensitised and transfer factor-treated rhesus monkeys and the stimulation indices were calculated as described before (Section 2.7.2). A stimulation index (SI) of more than 2.0 was arbitrarily considered positive. Reasons for this cut-off point will be discussed later (Section 8.3.1).

5.1. Normal and actively sensitised monkeys (Figs. 29-39, Tables 21-23).

5.1.1 Maximum LT-responses to KLH, PPD, TB and HBsAg (Figs. 29-30).

To determine the normal range of baseline responses to 1-100 µg/ml KLH, PPD, TB and HBsAg. LT tests were carried out in virgin rhesus monkeys (Figs. 29a-d). Non-specific reactivity was not detected for TB at any concentration (Fig. 29c: SI $<$ 2), but some such reactivity was detected for the other antigens (SI $>$ 2). Two to 5 virgin animals converted for 5-100 µg/ml KLH (Fig. 29a), 1 animal for 1-5 µg/ml PPD (Fig. 29b), and 1 to 2 for 50-100 µg/ml HBsAg (Fig. 29d). Despite these non-specific reactivities in individual monkeys, mean values of the stimulation indices revealed absence of LT-reactivity to all the antigens at 1-100 µg/ml concentration range (Figs. 29a-d: mean SI $<$ 2).

In comparison with the virgin monkeys above, the actively immunised animals showed positive LT-responses. Figure 30 demonstrates that

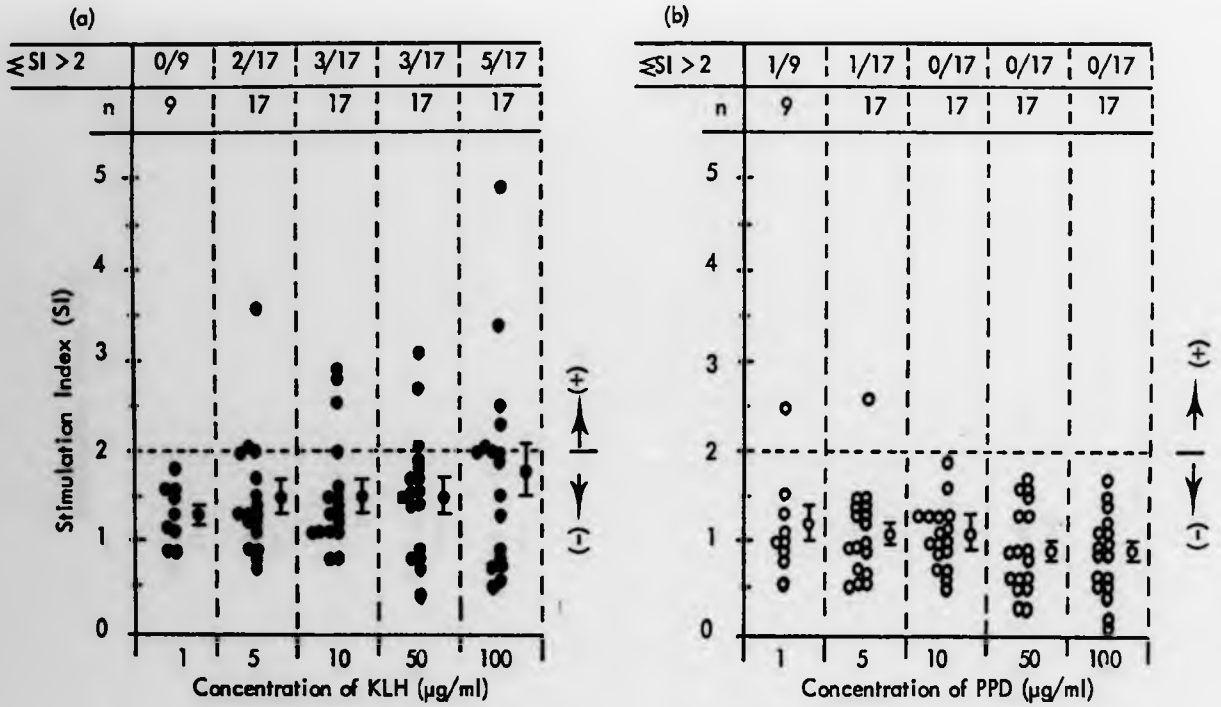

Fig. 29. Maximum baseline LT dose-response to (a) KLH or (b) PPD in normal rhesus monkeys. n, number of monkeys; $\bar{x}$, mean SI ± SEM.

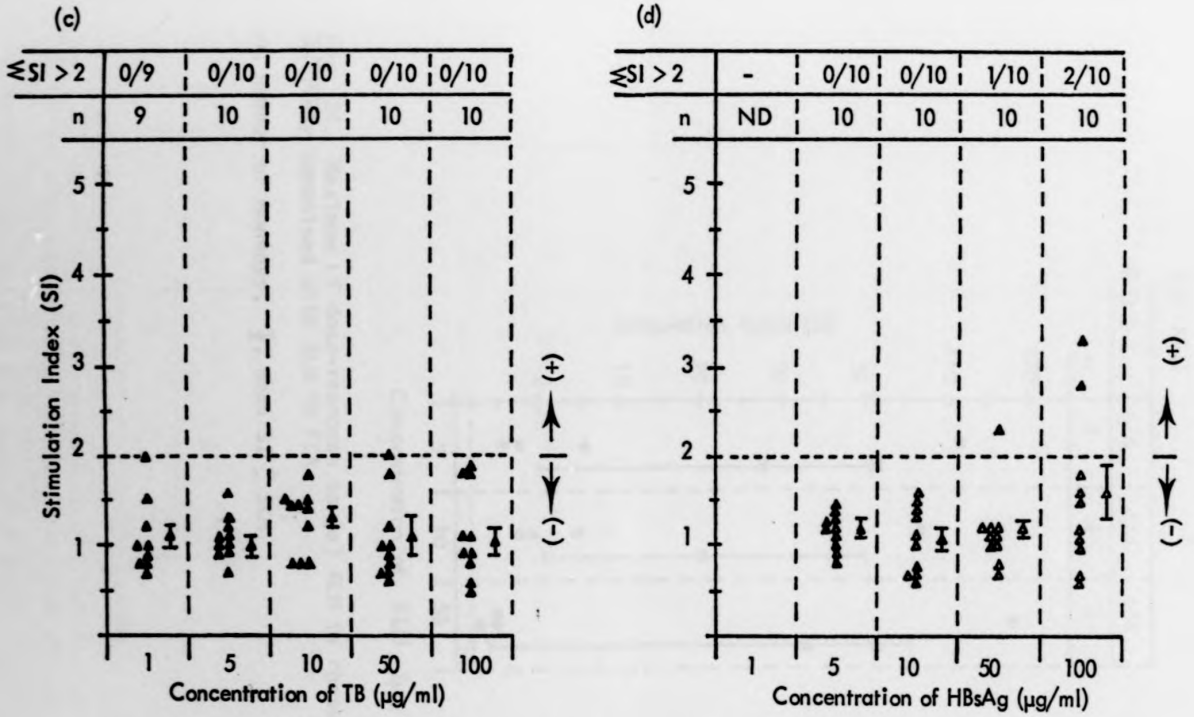

Fig. 29. Maximum baseline LT dose-response to (c) TB or (d) HBsAg in normal rhesus monkeys. n, number of monkeys; ⊥ ⊥, mean SI ± SEM; ND, not done.

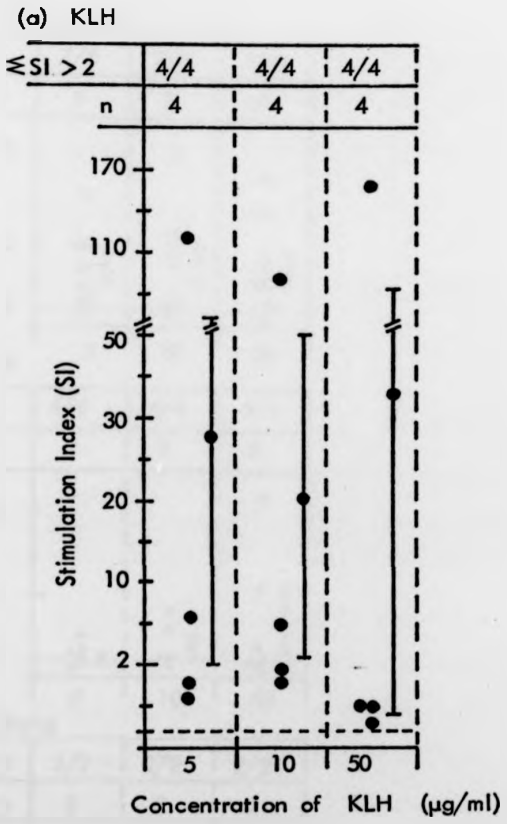

Fig. 30. Maximum LT dose-response to (a) KLH in rhesus monkeys actively immunised with KLH in FCA.

n, number of monkeys; ⧱, mean SI ± SEM.

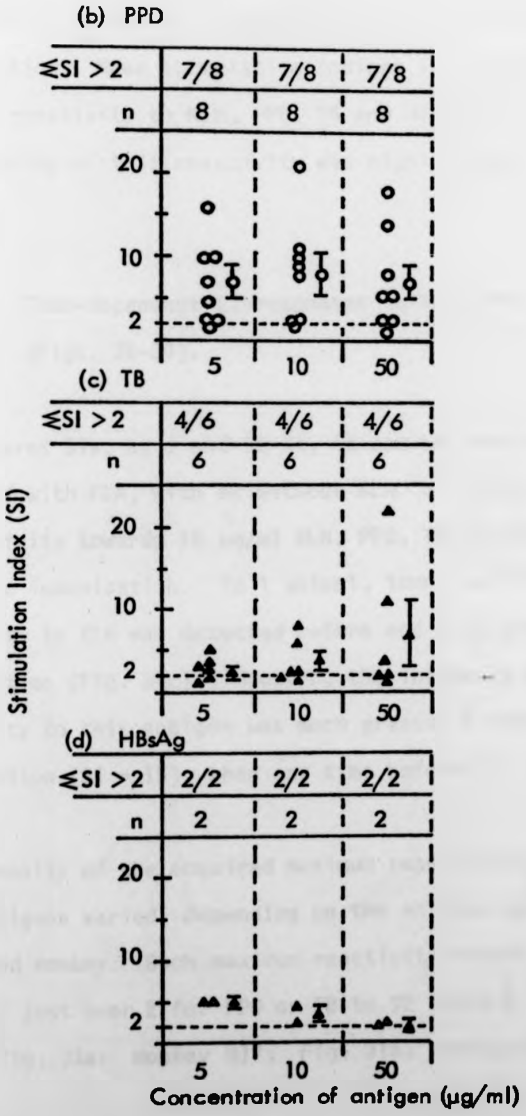

Fig. 30. Maximum LT dose-response to (b) PPD, (c) TB or (d) HBsAg in rhesus monkeys actively immunised with FCA with or without HBsAg.

n, number of monkeys; ⊻ ⊺ ⊼, mean SI ± SEM.

1 to 7 animals converted, depending on the antigen and its concentration. Mean stimulation indices for 5-50 µg/ml antigen revealed reactivity to KLH, PPD, TB and HBsAg (Mean SI > 2), and the intensity of this reactivity was highly marked for KLH (Mean SI > 30).

5.1.2 Time-dependent LT-responses to KLH, PPD, TB and HBsAg (Figs. 31-34).

From figures 31a, b, d and 32-34, it can be seen that animals immunised with FCA, with or without KLH or HBsAg, began to acquire LT-reactivity towards 10 µg/ml KLH, PPD, TB or HBsAg 1 to 4 weeks after the immunisation. In 1 animal, immunised with KLH in FCA, reactivity to KLH was detected before and 2 to 5 weeks after the immunisation (Fig. 31c). However, the intensity of maximum reactivity to this antigen was much greater 4 weeks after the immunisation (SI = 15), than any time before (SI = 3).

The intensity of the acquired maximum reactivity towards 10 µg/ml test antigens varied, depending on the antigen and the particular immunised monkey. Such maximum reactivity ranged from a stimulation index of just over 2 for PPD or TB to 92 for KLH (Fig. 32d: monkey H36a; Fig. 33a: monkey H17; Fig. 31a: monkey H16).

5.1.3 Comparison of LT-conversion rates to KLH, PPD, TB or HBsAg in normal and actively sensitised monkeys (Table 21).

The data in table 21 shows conversion rates for LT-reactivity to 5-50 µg/ml antigen in normal and actively immunised monkeys.

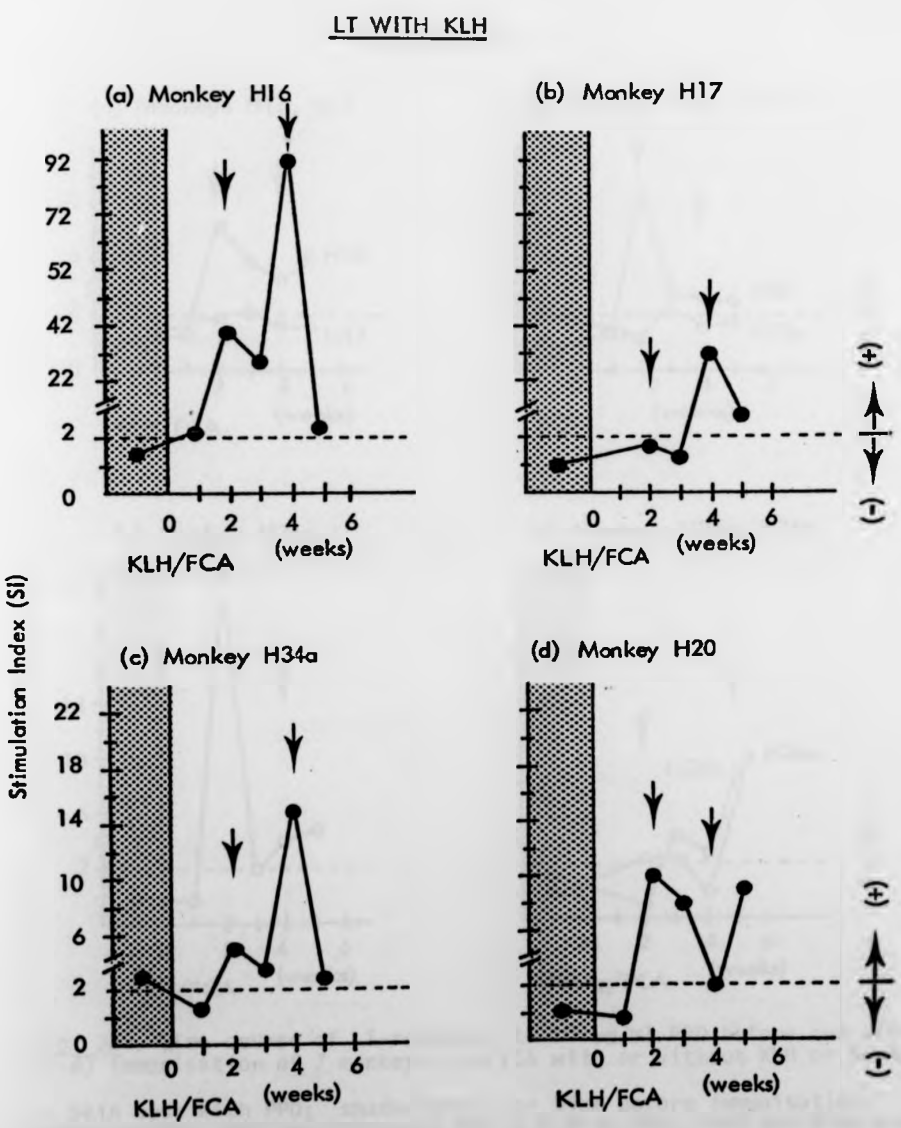

Fig. 31. Time course of LT-responses to 10 µg/ml KLH before and after (a-d) immunisation of 4 monkeys with 100 µg KLH in FCA.

+ skin test with KLH; shaded area. any time before immunisation; (•) 10 µg/ml KLH.

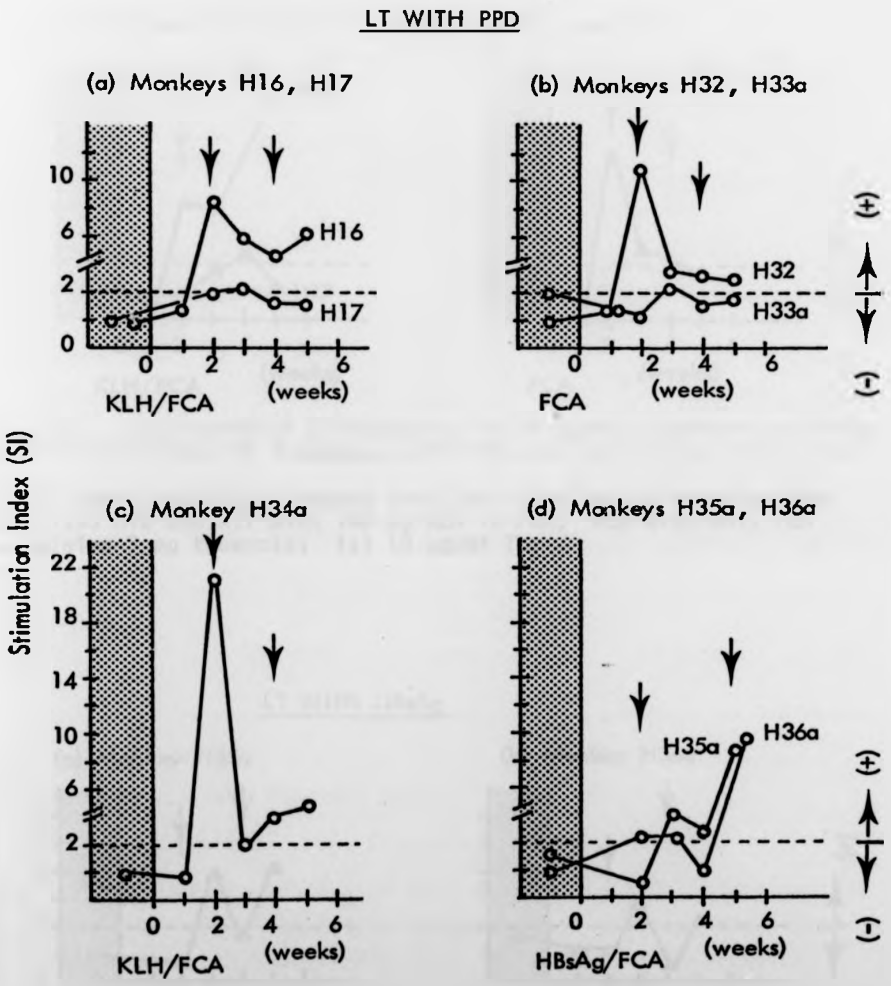

Fig. 32. Time course of LT-responses to 10 µg/ml PPD before and after (a-d) immunisation of 7 monkeys with FCA with or without KLH or HBsAg.

+, Skin test with PPD; shaded area, any time before immunisation; immunised H16, H17 and H34a with 100 µg KLH in FCA; H32 and H33a with FCA containing 3 mg tubercle; H35a and H36a with 1 mg HBsAg in FCA; (o) 10 µg/ml PPD.

LT WITH TB

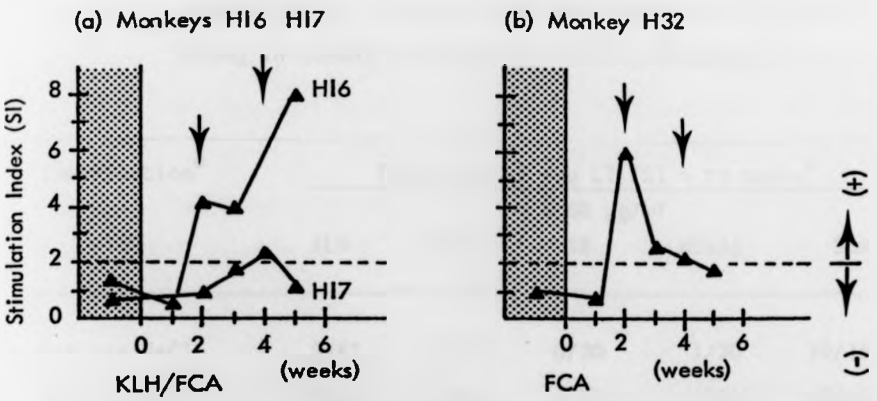

Fig. 33. Time course of LT-responses to 10 µg/ml TB before and after (a-b) immunisation of 3 monkeys with FCA with or without 100 µg KLH.

+, Skin test with PPD; shaded area, any time before immunisation; immunised H16 and H17 with 100 µg KLH in FCA; H36 with only FCA containing 3 mg tubercle; (▲) 10 µg/ml TB.

LT WITH HBsAg

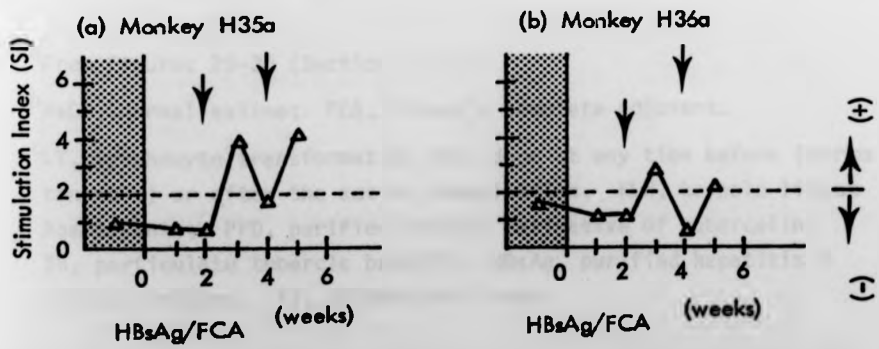

Fig. 34. Time course of LT-responses to 10 µg/ml HBsAg before and after (a-b) immunisation of 2 monkeys with 1 mg HBsAg in FCA.

+, Skin test with HBsAg; shaded area, any time before immunisation; (Δ) 10 µg/ml HBsAg.

Table 21. Comparison of LT-conversion rates to KLH, PPD, TB or HBsAg in normal and actively immunised monkeys.[a]

Immunisation[b] with	Total no. of +ve LT (SI > 2) tests[c] 5-50 µg/ml KLH	PPD	TB	HBsAg	Sum
Nothing/NaCl (control)	8/51 (16%)	1/51 (2%)	0/30 (0%)	1/30 (0%)	10/162 (6%)
FCA ± KLH or HBsAg	12/12 (100%)	21/24 (88%)	12/18 (67%)	5/6 (83%)	50/60 (83%)
P for χ^2	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01

[a]From figures 29-30 (Section 5.1.1).

[b]NaCl, normal saline; FCA, Freund's complete adjuvant.

[c]LT, lymphocyte transformation test done at any time before (normal controls) or after the active immunisation. KLH, keyhole limpet haemocyanin; PPD, purified protein derivative of tuberculin; TB, particulate tubercle bacilli; HBsAg, purified hepatitis B surface antigen; SI, stimulation index.

Depending on the antigen, none to 16% of the tests became reactive in the normal control monkeys, compared with 67% to 100% conversion for tests in the immunised animals. Therefore, although some non-specific reactivity appeared for KLH and PPD, the difference in the acquisition of reactivity to antigen between the normal and immunised animals was highly significant when all the antigens were taken separately or altogether ($P < 0.01$).

5.1.4 Relationship between the maximum LT- and DH-responses to KLH, PPD, TB or HBsAg (Fig. 35, table 22).

After the first or the second skin test, depending on the antigen, 2 to 7 actively immunised animals acquired both positive LT- and DH-responses (Fig. 35, table 22). When all the antigens were taken together, the rate of such double-conversion was 85%, regardless of the number of skin tests. Therefore, there was an association between the lymphocyte transformation and the delayed hypersensitivity tests in the actively immunised animals. Such data were not available for naive animals.

5.1.5 Relationship between the maximum LT- and LMMI-responses to KLH, PPD or TB (Fig. 36, table 23).

From the figure and the table it can be seen that 2 to 3 out of 3 to 4 immunised animals converted when both LT and LMMI tests were carried out with KLH, PPD or TB. In the few animals, where these tests were performed at the same time and when all the test antigens were considered altogether, the double-conversion rate was 73%.

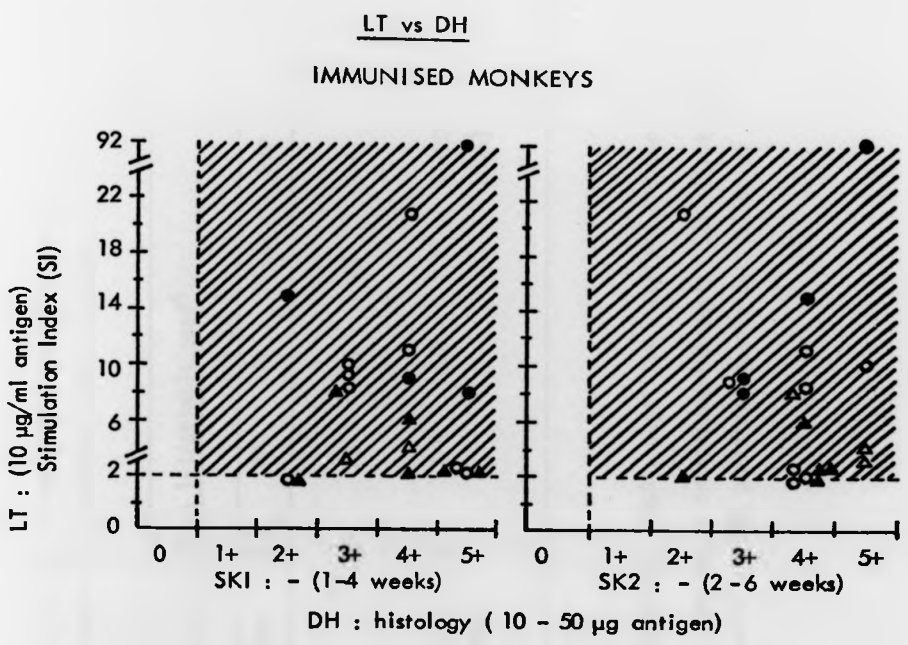

Fig. 35. Relationship between the maximum LT- and DH-responses to KLH, PPD, TB and HBsAg in actively immunised monkeys.

SK1, first skin test; SK2, second skin test; • KLH; o PPD; ▲ TB; Δ HBsAg; ▨ positive LT and DH.

Table 22. Relationship between the acquisition of maximum LT and DH-responses to KLH, PPD, TB and HBsAg in actively immunised rhesus monkeys.[a]

Immunisation[b] with	Animal conversions (SI > 2, DH ≥ 1+)/animals used[c]									
	LT & SK1					LT & SK 2				
	KLH	PPD	TB	HBsAg	Sum	KLH	PPD	TB	HBsAg	Sum
Nothing	ND	ND	ND	ND	-	ND	ND	ND	ND	-
FCA ± KLH or HBsAg	4/4	7/8	4/6	2/2	17/20 (85%)	4/4	7/8	4/6	2/2	17/20 (85%)

[a] From figure 35.

[b] FCA, Freund's complete adjuvant; KLH, keyhole limpet haemocyanin; HBsAg, hepatitis B surface antigen.

[c] SI, stimulation index; DH, delayed hypersensitivity; LT, lymphocyte transformation test; SK1, first skin test; SK2, second skin test; TB, particulate tubercle bacilli; PPD, purified protein derivative of tuberculin; ND, not done.

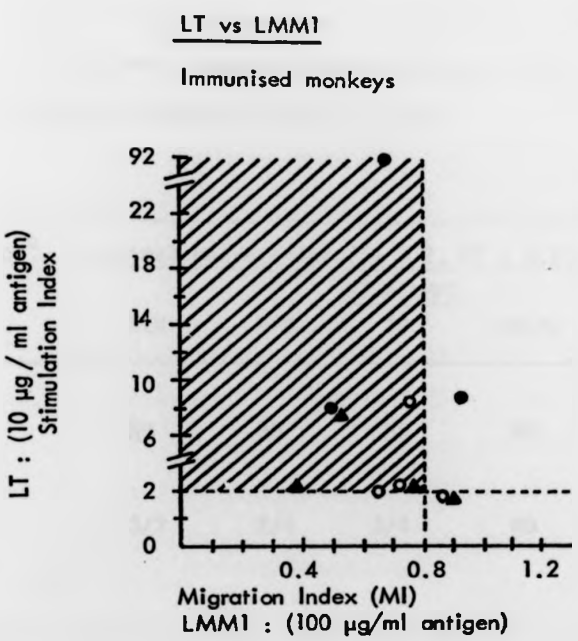

Fig. 36. Relationship between the maximum LT- and LMMI-responses to KLH, PPD and TB in actively immunised monkeys.

▨ positive LT and LMMI; • KLH; o PPD; ▲ TB.

Table 23. Relationship between the acquisition of maximum LT and LMMI-responses to KLH, PPD and TB in actively immunised rhesus monkeys.[a]

Immunisation[b] with	Animal conversions (SI > 2, MI < 0.8)/animals used[c]				
	LT & LMMI				
	KLH	PPD	TB	HBsAg	Sum
Nothing	ND	ND	ND	ND	-
FCA ± KLH or HBsAg	3/3	2/4	3/4	ND	8/11 (73%)

[a] From figure 36.

[b] FCA, Freund's complete adjuvant; KLH, keyhole limpet haemocyanin; HBsAg, hepatitis B surface antigen.

[c] SI, stimulation index; MI, migration index; LT, lymphocyte transformation test; LMMI, mixed leucocyte-macrophage migration test; PPD, purified protein derivative of tuberculin; TB, particulate tubercle bacilli; ND, not done.

Thus, an association existed between the transformation and the migration tests in the actively immunised monkeys. Again such data were not available for naive monkeys.

5.1.6 Baseline LT dose-response to PHA in normal rhesus monkeys (Fig. 37).

When 6 to 15 normal monkeys were monitored by the LT test using 5-300 µg PHA, the dose-response shown in figure 37 was obtained. Except for 5 µg/ml PHA, all the monkeys showed reactivity to 10-300 µg/ml PHA, with the maximum mean reactivity detected for 100 µg/ml PHA (Mean SI = 428). The normal baseline response in these animals for 100 µg/ml PHA ranged from a stimulation index of about 70 to 4250.

5.1.7 Maximum LT dose-response to PHA in actively immunised rhesus monkeys (Fig. 38).

The figure demonstrates that, in monkeys actively immunised with FCA, with or without KLH or HBsAg, there was a good PHA dose-response. Like the normal animals above, positive reactivity was not detected for all the monkeys when LT-tests were carried out with 5 µg/ml PHA, but was detected for all when a concentration of 25 or 100 µg/ml PHA was used. For 100 µg/ml PHA, maximum mean stimulation index was about 93 and, therefore, fell within the normal range of reactivity found in the unsensitised monkeys (Section 5.1.6: mean SI = 70 to 4250).

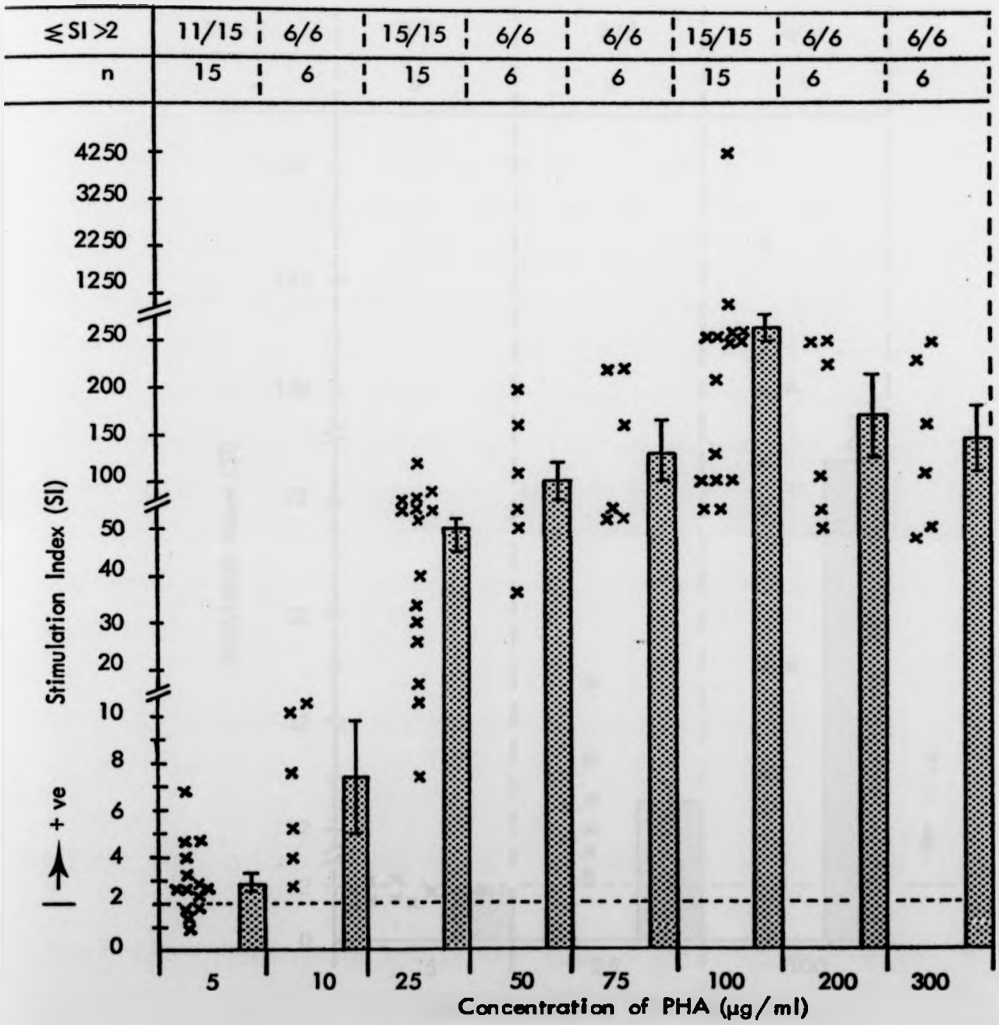

Fig. 37. Maximum baseline LT dose-response to 5-300 µg/ml PHA in 15 normal rhesus monkeys.

n, number of monkeys; vertical bars represent mean SI ± SEM; (×) stimulation index of individual animals to 5-300 µg/ml PHA.

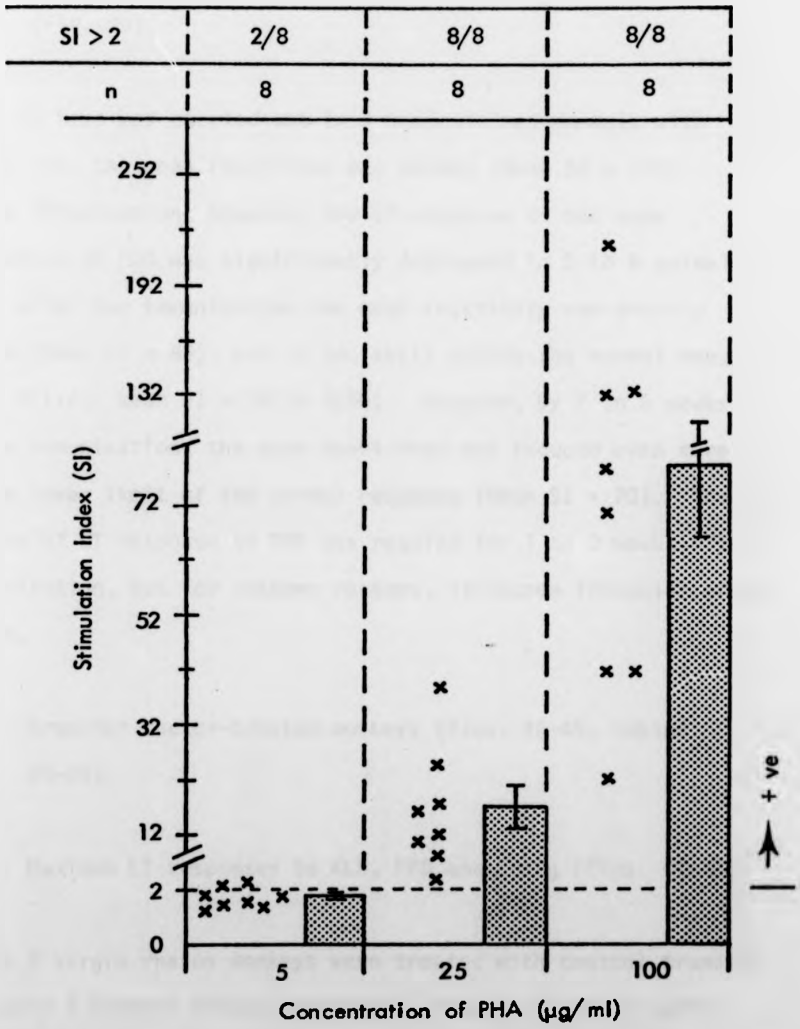

Fig. 38. Maximum LT dose-response to 5-100 µg/ml PHA in 8 rhesus monkeys actively immunised with FCA with or without KLH or HBsAg.

n, number of monkeys; vertical bars represent mean SI ± SEM; (×) stimulation index of individual animals to 5-100 µg/ml PHA.

5.1.8 Depression of LT-response to PHA after active immunisation (Fig. 39).

When the LT test was carried out in 7 unsensitised monkeys with 100 µg/ml PHA, the mean reactivity was normal (Mean SI = 173). After the immunisation, however, the LT-response to the same concentration of PHA was significantly depressed in 5 to 8 animals. One week after the immunisation the mean reactivity was greatly depressed (Mean SI = 83), but it was still within the normal range (Section 5.1.6: mean SI = 70 to 4250). However, by 2 to 5 weeks after the immunisation, the mean reactivity was reduced even more to below the lower limit of the normal response (Mean SI = 70). The depression of LT response to PHA was regular for 1 to 3 weeks after the immunisation, but for unknown reasons, it became irregular after this time.

5.2 Transfer factor-treated monkeys (Figs. 40-45, tables 24-26).

5.2.1 Maximum LT-responses to KLH, PPD and HBsAg (Figs. 40-41).

When 2 to 5 virgin rhesus monkeys were treated with control transfer factor, only 2 animals showed positive LT-reactivity to 10 µg/ml KLH, but none showed reactivity to 5 or 50 µg/ml KLH or to 5-50 µg/ml PPD or HBsAg. Such tests were not carried out with TB (Fig. 40).

In contrast, when 2 to 6 virgin monkeys were given immune transfer factor, depending on the concentration of the test antigen, 1 to 2

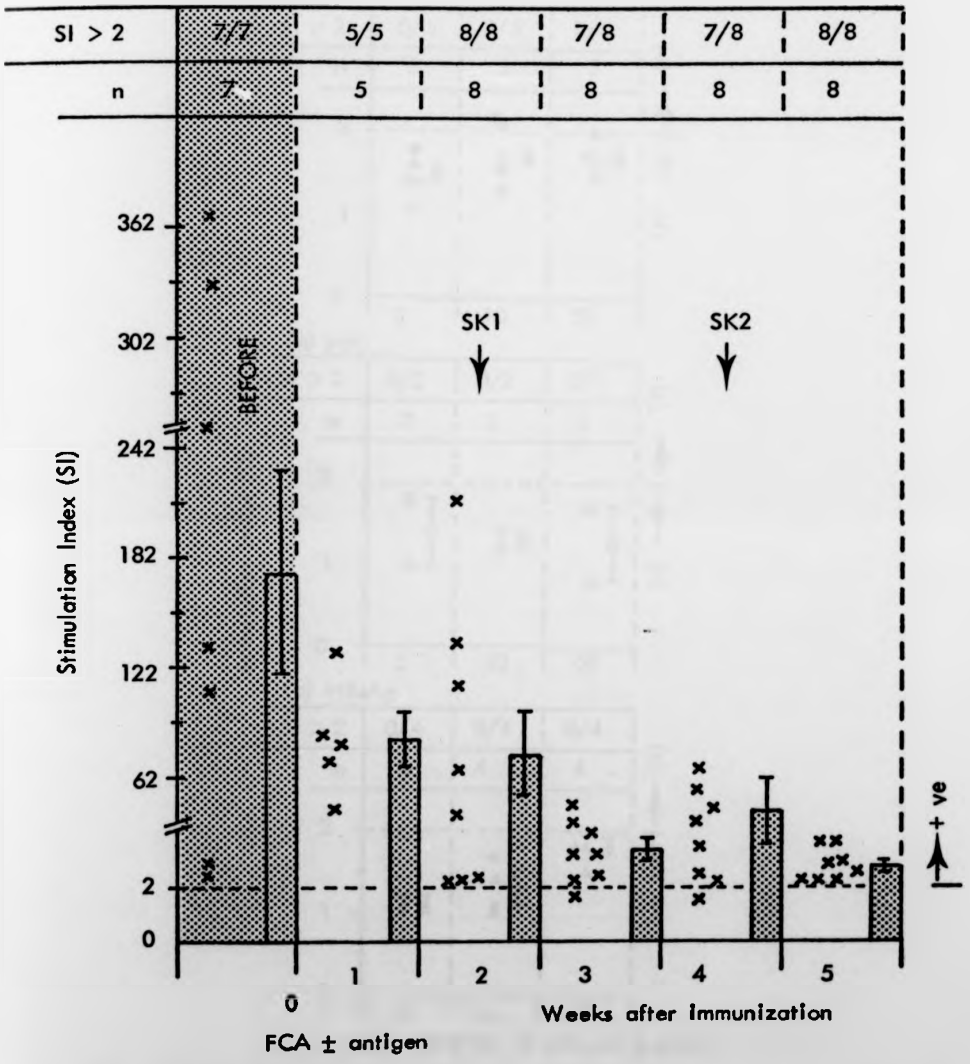

Fig. 39. LT-response to 100 µg/ml PHA in 8 rhesus monkeys before and after active immunisation with FCA with or without KLH or HBsAg.

n, number of monkeys; vertical bars represent mean SI ± SEM; SK1, SK2 first and second skin tests with an antigen; shaded area, any time before immunisation; (×) stimulation index of individual animals to 100 µg/ml PHA.

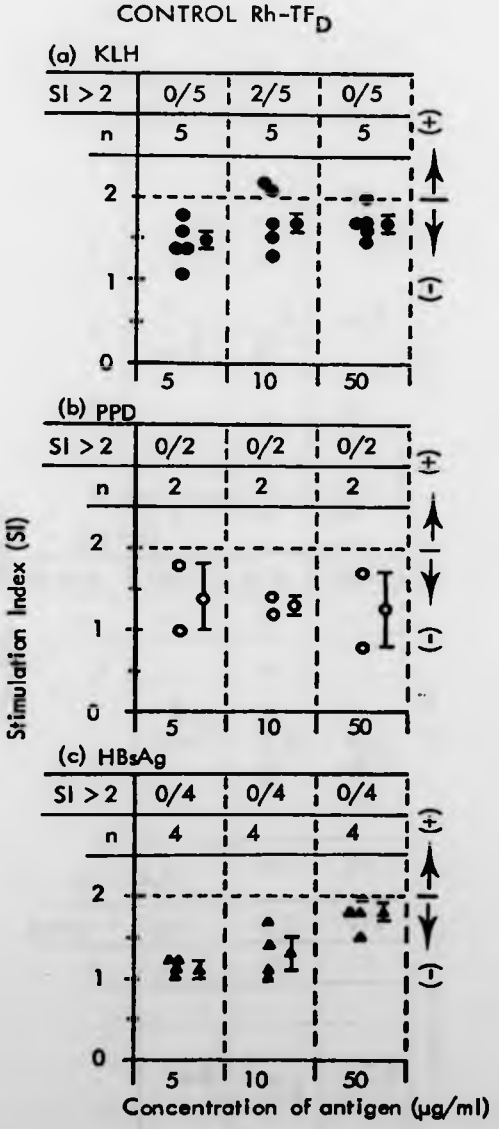

Fig. 40. Maximum LT dose-response to (a) KLH, (b) PPD, or (c) HBsAg in rhesus monkeys treated with control dialysable rhesus transfer factor.

n, number of monkeys; $\bar{x}$ $\bar{x}$ $\bar{x}$, mean SI ± SEM; Rh-TF$_D$, rhesus dialysable transfer factor.

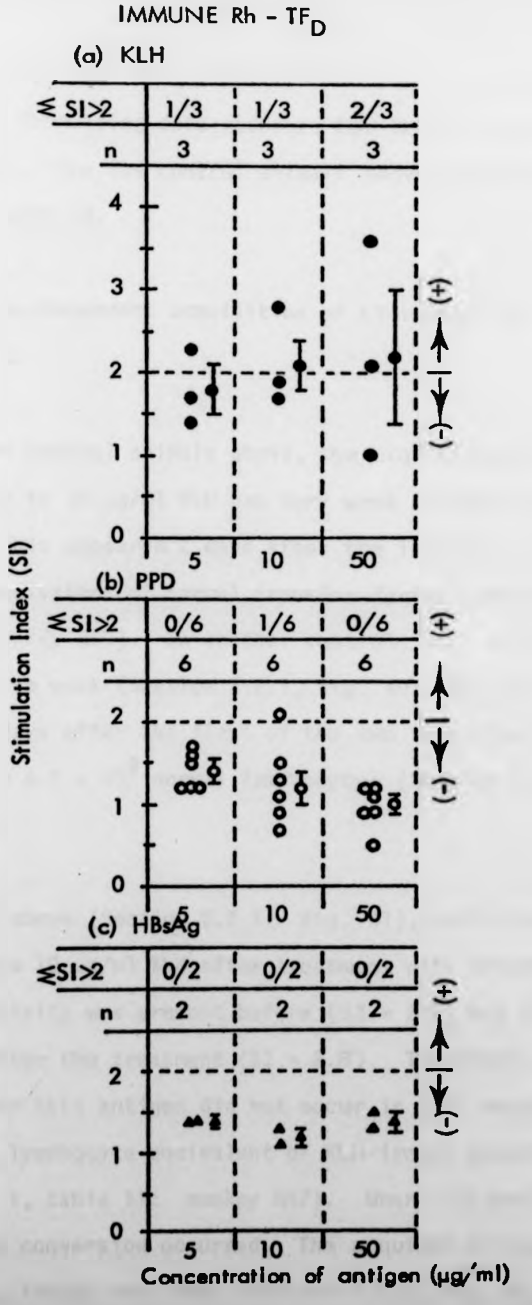

Fig. 41. Maximum LT dose-response to (a) KLH, (b) PPD, or (c) HBsAg in rhesus monkeys treated with immune dialysable rhesus transfer factor.

n, number of monkeys; $\bar{x}$ $\bar{\bar{x}}$ $\bar{\bar{\bar{x}}}$, mean SI ± SEM; Rh-TF$_D$, rhesus dialysable transfer factor.

animals converted for KLH, 1 for PPD and none for HBsAg (Fig. 41). There was an increasing dose-response for KLH, but not for PPD or HBsAg. Again, like the control animals above, tests were not carried out with TB.

5.2.2 Time-dependent acquisition of LT-reactivity to KLH or PPD.

In one of the control animals above, the acquisition of non-specific LT-reactivity to 10 µg/ml KLH was very weak (Section 5.2.1, fig. 40: SI = 2.2). This appeared 2 days after the injection of 4.5×10^9 lymphocyte-equivalent of normal transfer factor (Section 2.2.1, table 11: monkey H49). In another control, such acquired reactivity to KLH was also weak (Section 5.2.1, fig. 40: SI = 2.1), but it appeared 34 days after the first of the two injections together obtained from 4.2×10^9 normal lymphocytes (Section 2.2.1, table 11: monkey H35).

The 1 animal above (Section 5.2.1: fig. 41), which showed positive LT-response to 10 µg/ml KLH after treatment with immune transfer factor, reactivity was present before (SI = 2.6) and 2 days (SI = 2.4) or 14 days after the treatment (SI = 2.8). Therefore, true conversion for this antigen did not occur in this monkey recipient of 4.5×10^9 lymphocyte-equivalent of KLH-immune transfer factor (Section 2.2.1, table 11: monkey H47). Where PPD was concerned, however, true conversion occurred. The acquired LT-reactivity to 10 µg/ml PPD, though very weak (Section 5.2.1, fig. 41: SI = 2.1), appeared 27 days after the first of the 3 injections of immune

transfer factor, each prepared from 4.5×10^9 lymphocytes, (Section 2.2.1, table 11: monkey H36).

5.2.3 Comparison of LT-conversion rates to KLH, PPD or HBsAg in monkeys treated with control and immune transfer factor (Table 24).

The table shows that when all the tests for 5-50 µg/ml were considered, in 5 animals treated with control (normal or unrelated to test antigen) transfer factor, 2 out of 15 tests converted to KLH, but none to PPD or HBsAg. In 6 animals treated with immune transfer factor (related to test antigen), 4 out of 9 tests converted to KLH and 1 out of 18 to PPD, but none to HBsAg. When sum of all the tests with each or all the antigens were considered, the difference between the cumulative rates of conversion in the animals treated with control and immune transfer factor preparations was not significant ($P > 0.2 < 0.6$).

5.2.4 Relationship between the maximum LT- and DH-responses to KLH, PPD or HBsAg (Fig. 42, table 25).

The figure and the table demonstrate that, after the first skin test, none of the recipients of the control or immune transfer factor showed double conversion of LT- and DH-responses to KLH, PPD or HBsAg. After the second skin test, only 1 animal showed such double conversion for KLH, but none for PPD or HBsAg. Therefore, unlike the actively immunised monkeys (Section 5.1.4, fig. 35, table 22), there was no positive correlation between the lymphocyte transformation and delayed hypersensitivity skin tests in transfer factor-treated animals.

Table 24. Comparison of LT-conversion rates to KLH, PPD and HBsAg in monkeys treated with control and dialysable homologous transfer factor.[a]

$Rh\text{-}TF_D$[b] Status	Total no. of +ve LT (SI > 2) tests[c] 5-50 µg/ml KLH	PPD	TB	HBsAg	Sum
Control	2/15 (13%)	0/6	ND	0/12	2/37 (5%)
Immune	4/9 (44%)	1/18 (6%)	ND	0/6	5/33 (15%)
P for χ^2	> 0.2 < 0.3	> 0.5 < 0.6			> 0.3 < 0.4

[a] From figures 40-41 (Section 5.2.1).

[b] $Rh\text{-}TF_D$, dialysable rhesus transfer factor.

[c] LT, lymphocyte transformation test done any time before or 2 to 34 days after transfer factor.
KLH, keyhole limpet haemocyanin; PPD, purified protein derivative of tuberculin; TB, particulate tubercle bacilli; HBsAg, hepatitis B surface antigen; SI, stimulation index; ND, not done.

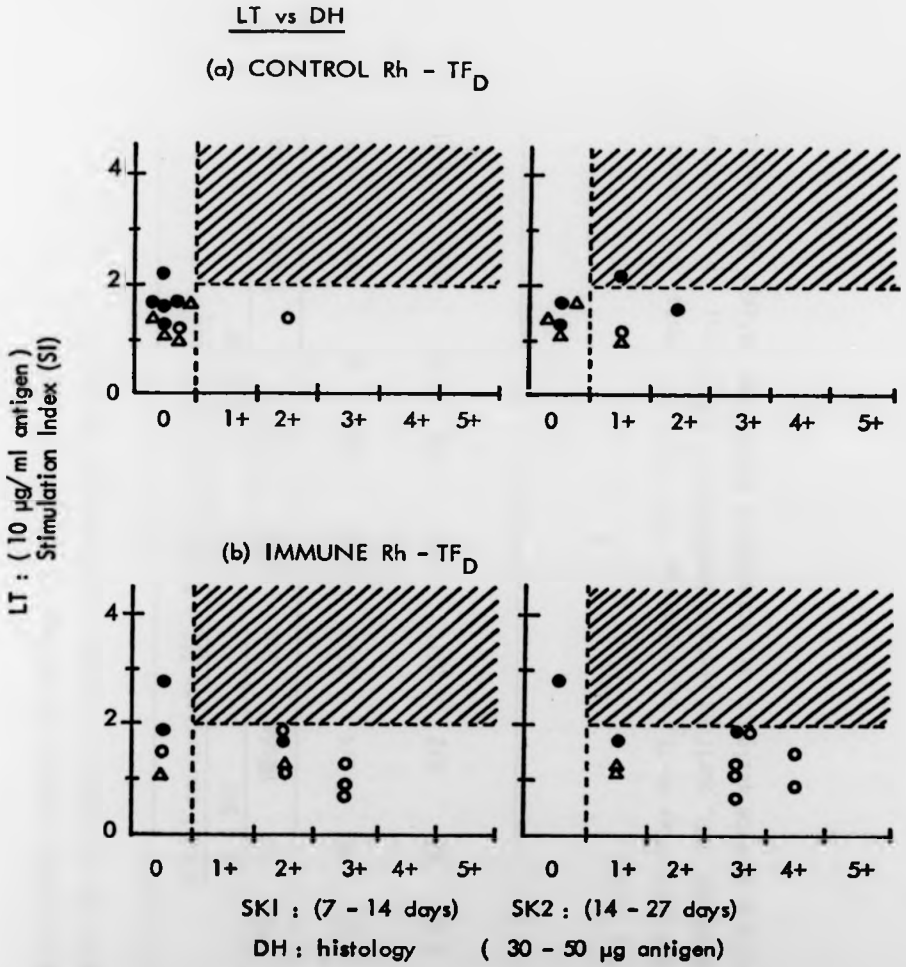

Fig. 42. Relationship between the maximum LT- and DH-responses to KLH, PPD or HBsAg in (a) control and (b) immune transfer factor-treated monkeys.

LT, lymphocyte transformation test; DH, delayed hypersensitivity; Rh-TF$_D$, dialysable rhesus transfer factor; SK1, first skin test; SK2, second skin test; • KLH: ○ PPD: Δ HBsAg; ▨ positive LT and DH.

Table 25. Relationship between the maximum LT and DH-responses to KLH, PPD and HBsAg in transfer factor-treated monkeys.[a]

Rh-TF_D[b] Status	Animal conversions (SI > 2, DH ⩾ 1+)/animals used[c]									
	LT & SK1					LT & SK2				
	KLH	PPD	TB	HBsAg	Sum	KLH	PPD	TB	HBsAg	Sum
Control	0/5	0/2	ND	0/4	0/11	1/4	0/1	ND	0/4	1/9
Immune	0/3	0/6	ND	0/2	0/11	0/3	0/6	ND	0/2	0/11

[a] From figures 42a-b.

[b] Rh-TF_D, dialysable rhesus transfer factor.

[c] LT, lymphocyte transformation test done after Rh-TF_D injection(s); SK1, first skin test; SK2, second skin test; KLH, keyhole limpet haemocyanin; PPD, purified protein derivative of tuberculin; TB, particulate tubercle bacilli; HBsAg, hepatitis B surface antigen; SI, stimulation index; DH, delayed hypersensitivity; ND, not done.

5.2.5 Relationship between the maximum LT and LMMI-responses to KLH, PPD or HBsAg (Fig. 43, table 26).

It is evident from figure 43 and table 26 that, none of the animals treated with control transfer factor converted for KLH, PPD or HBsAg when monitored with the lymphocyte transformation and the migration tests. When recipients of the immune transfer factor preparations were monitored, only 1 animal showed the double conversion of response to KLH, but none to PPD or HBsAg. Therefore, in contrast to the immunised monkeys (Section 5.1.5, fig. 36, table 23), there was a negative correlation between the transformation and migration tests in the transfer factor-treated monkeys.

5.2.6 Maximum LT dose-response to PHA (Fig. 44).

The figure reveals that, in 6 transfer factor-treated rhesus monkeys, there was a good dose-response to 5-100 µg/ml PHA, and the positive reactivity to each concentration was detected in all the recipient monkeys. The pattern of the dose-response for these animals was similar to that of the actively immunised animals, but the mean reactivity per concentration of PHA was greater in the transfer factor-treated animals than in the actively immunised ones (Figs. 44 cf. 38).

5.2.7 Elevation of LT-response to PHA (Fig. 45).

When the LT-tests were carried out with 100 µg/ml PHA, any time

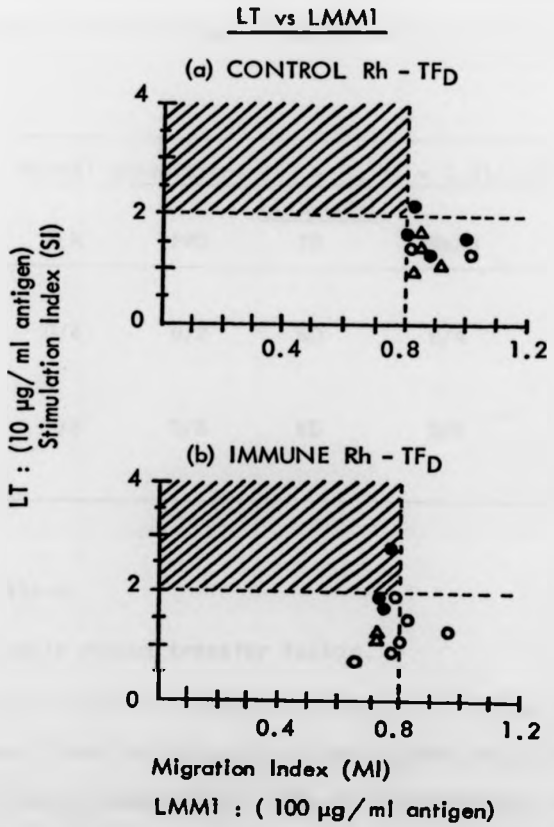

Fig. 43. Relationship between the maximum LT- and LMMI- responses to KLH, PPD or HBsAg in (a) control and (b) immune transfer factor-treated monkeys.

LT, lymphocyte transformation; LMMI, mixed leucocyte-macrophage migration; Rh-TFD, dialysable rhesus transfer factor; • KLH: ○ PPD: △ HBsAg; ▨ positive LT and LMMI.

Table 26. Relationship between the maximum LT and LMMI-responses to KLH, PPD and HBsAg in transfer factor-treated monkeys.[a]

Rh-TF_D[b] Status	Animal conversions (SI > 2, MI < 0.8)/animals used[c]				
	LT & LMMI				
	KLH	PPD	TB	HBsAg	Sum
Control	0/4	0/2	ND	0/4	0/10
Immune	1/3	0/6	ND	0/2	1/11

[a] From figures 43a-b.

[b] Rh-TF_D, dialysable rhesus transfer factor.

[c] SI, stimulation index; MI, migration index; LT, lymphocyte transformation; LMMI, mixed leucocyte-macrophage migration; KLH, keyhole limpet haemocyanin; PPD, purified protein derivative of tuberculin; TB, particulate tubercle bacilli; HBsAg, hepatitis B surface antigen; ND, not done.

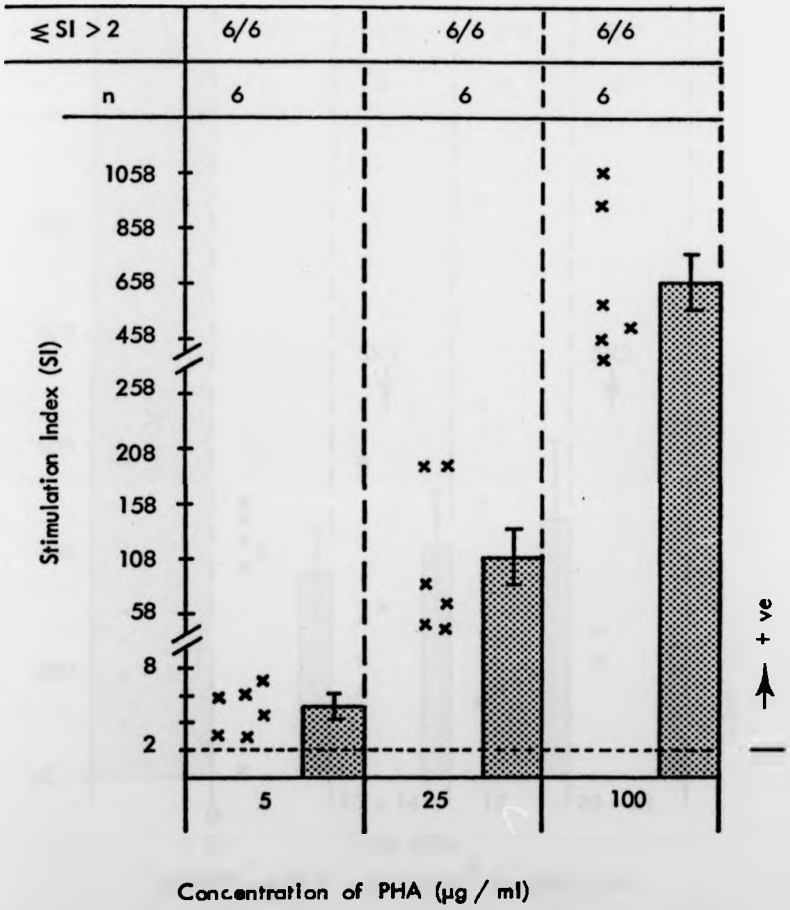

Fig. 44. Maximum LT dose-response to 5-100 µg/ml PHA in 6 rhesus monkeys treated with dialysable rhesus transfer factor.

n, number of monkeys; vertical bars represent mean SI ± SEM; (×) stimulation index of individual animals to 5-100 µg/ml PHA.

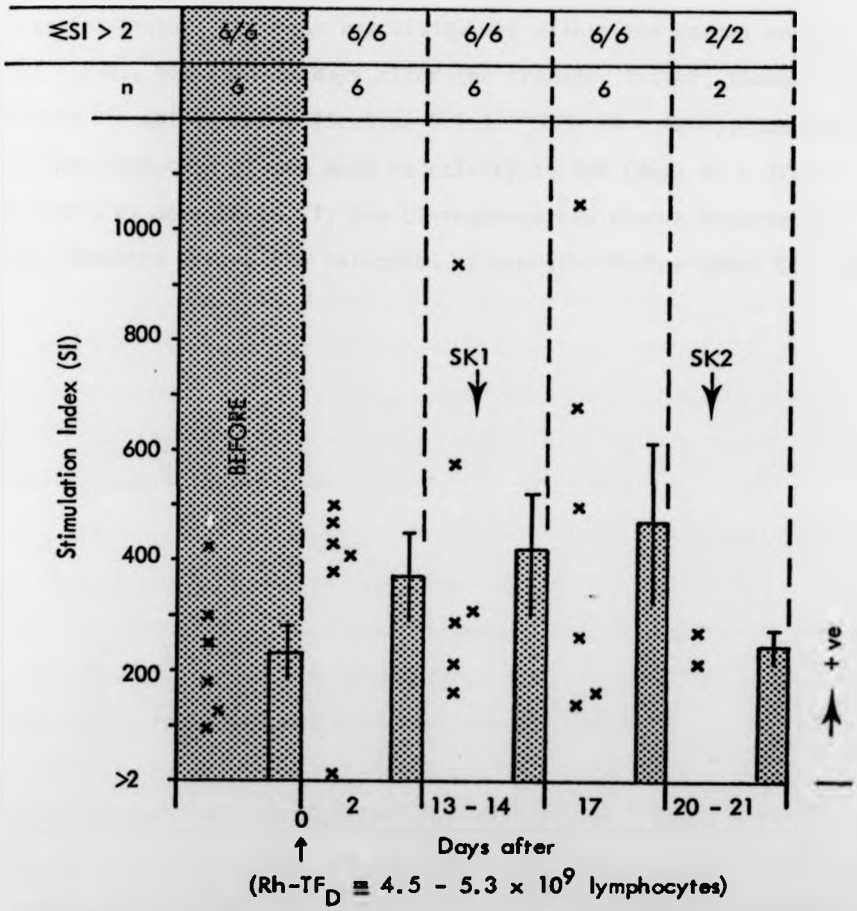

Fig. 45. LT-response to 100 µg/ml PHA in 6 rhesus monkeys before and after one injection of dialysable rhesus transfer factor.

n, number of monkeys; vertical bars represent mean SI ± SEM; SK1, SK2, first and second skin tests done with KLH, PPD or HBsAg; shaded area, any time before rhesus dialysable transfer factor (Rh-TF$_D$); (×) stimulation index of individual animals to 100 µg/ml PHA.

before and 2 to 21 days after the administration of a single injection of transfer factor, the results in figure 45 were obtained. Before transfer factor, the mean reactivity was within the normal range (SI = 230), but 2 to 17 days after the transfer factor, though still within the normal range (Section 5.1.6: SI > 70 < 4250), there was uniform elevation of the mean reactivity to PHA (Mean SI > 370). However, by days 20 to 21, the LT-response had almost returned to that detected before the injection of transfer factor (Mean SI = 240).

6. RESULTS OF THE IN VITRO STUDY OF DIALYSABLE LEUCOCYTE TRANSFER FACTOR FROM MONKEY OR MAN.

An attempt has been made to determine whether transfer factor *in vitro* can confer and/or augment lymphocyte transformation responses to the antigens KLH and PPD in recipient cultures.

A "neoantigen" KLH (Section 2.10.1) was included in the study, since it is unlikely that the human donors of the "recipient" lymphocytes *in vitro* would have come into contact with this antigen. Thus, in experiments where an attempt was made to transfer KLH-sensitivity *in vitro* with dialysable transfer factor, such "unsensitised" cells were considered ideal "recipients". In contrast, tuberculin PPD was used, because donors of the "recipient" cells would most likely be Mantoux-positive due to the United Kingdom's vaccination programme with BCG. Therefore, to demonstrate the augmenting activity of dialysable transfer factor *in vitro*, such "sensitised" cells were used as the recipients of transfer factor in culture.

The effects of "medium-dialysed" transfer factor (TF_{DM}), prepared from man or rhesus monkey as described before (H-TF_{DM} or Rh-TF_{DM}: section 2.5.6 & fig. 3), were studied on cultured human leucocytes or purified lymphocytes (LC or PL: section 2.8). These "recipient" cells were obtained from individuals who had been immunised or not immunised with KLH and were mainly Mantoux-positive (Section 2.4).

The concentrations of the antigens used to monitor the *in vitro* activity of transfer factor were 10, 33 or 100 µg KLH and 2 µg PPD

per culture. The augmenting effect of transfer factor on lymphocyte transformation, demonstrated by increased incorporation of tritiated thymidine by the "recipient" human lymphocytes in vitro, was calculated according to the method of Hamblin (1975: Fig. 46).

6.1 Augmentation of lymphocyte transformation to KLH with rhesus or human transfer factor (Figs. 46-48, table 27).

Figures 47-48 show that, in 4 experiments, KLH-positive or KLH-negative rhesus transfer factor (obtained from monkeys immunised with KLH in FCA or only FCA respectively) was added to leucocyte cultures in the presence of 10 µg KLH. The "recipient" leucocytes in culture were obtained from individuals who were actively immunised or not immunised with KLH.

Figure 47 shows that there was no evidence for transfer of KLH-sensitivity by KLH-positive rhesus transfer factor, to unsensitised "recipient" human leucocytes in vitro. There was no significant difference in the mean disintegrations per minute for cultures incubated with 0.25 to 1.0 ml of KLH-positive or KLH-negative rhesus transfer factor alone or in the presence of 10 µg KLH.

In contrast, figure 48 shows the same KLH-positive or KLH-negative rhesus transfer factor added to cultured "recipient" leucocytes, obtained from an individual immunised with KLH. Both KLH-positive and KLH-negative preparations of transfer factor caused augmentation of lymphocyte transformation to 10 µg KLH. Thus, such augmenting activity was not related to the sensitivity of the transfer factor

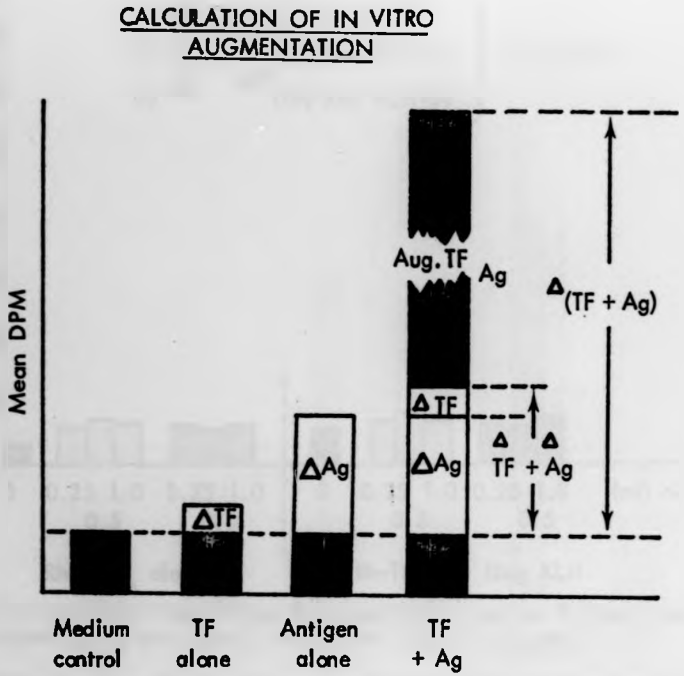

Fig. 46. Calculation of the augmenting effect of a single volume of medium-dialysed transfer factor in lymphocyte transformation (Hamblin, 1975).

Mean DPM of at least triplicate cultures is represented by the bars. Augmentation to an antigen or Aug. TF_{Ag} = Δ (TF + Ag) minus Δ TF +ΔAg . TF = transfer factor, Ag = antigen, DPM = disintegration per minute.

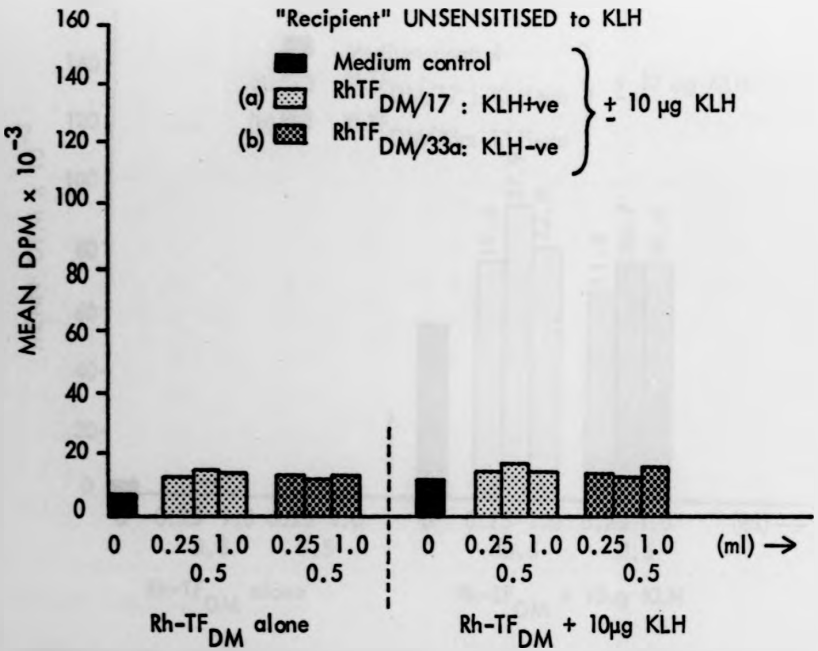

Fig. 47. Absence of in vitro transfer of KLH-sensitivity by (a) KLH-positive, or (b) KLH-negative rhesus transfer factor to unsensitised "recipient" human leucocytes.

$Rh\text{-}TF_{DM}$, rhesus monkey medium-dialysed transfer factor; DPM, disintegration per minute.

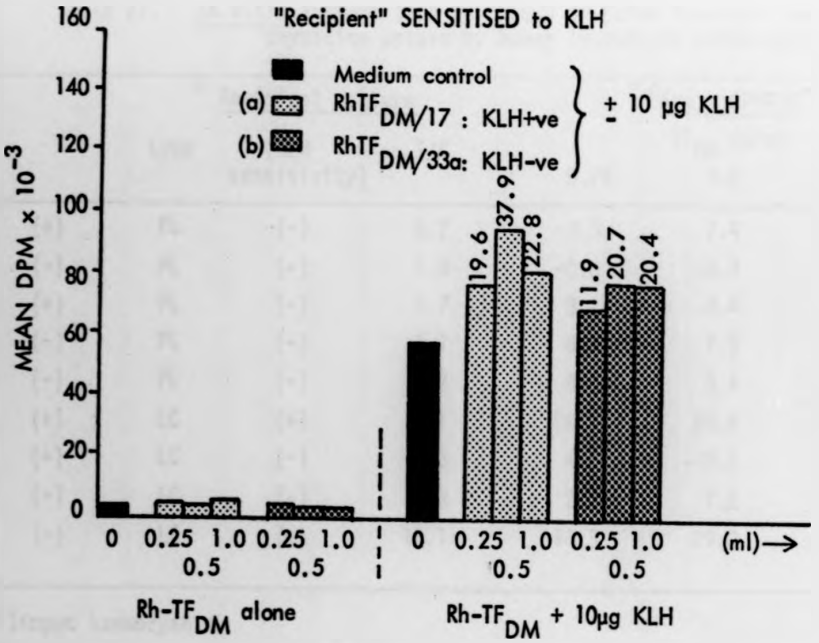

Fig. 48. Presence of augmentation of lymphocyte transformation to KLH by (a) KLH-positive, and (b) KLH-negative rhesus transfer factor.

$Rh\text{-}TF_{DM}$, rhesus monkey medium-dialysed transfer factor; DPM, disintegration per minute; numbers on the bars represent augmentation in DPM x 10^{-3}.

Table 27. In vitro augmentation by rhesus or human transfer factor of tritiated thymidine uptake by human lymphocyte stimulation with KLH.[a]

[b] TF_{DM} donor (KLH sensitivity)		[c] Recipient culture type	(KLH sensitivity)	T/C	[d] Aug.: DPMX10^{-3} TF_{DM} (mls) 0.25	0.5	1.0	KLH conc. (µg/ml)
1. Rh-$TF_{DM/16}$	(+)	PL	(-)	2.2	3.3	7.4	-0.7	10
2. H-$TF_{DM/2}$	(-)	PL	(-)	1.0	-0.8	-0.4	2.9	10
3. Rh-$TF_{DM/16}$	(+)	PL	(-)	1.7	8.1	2.4	1.0	33
4. Rh-$TF_{DM/32}$	(-)	PL	(-)	1.7	6.3	7.9	-5.1	33
5. H-$TF_{DM/1}$	(-)	PL	(-)	1.7	4.4	5.4	-3.8	33
6. Rh-$TF_{DM/17}$	(+)	LC	(+)	13.1	26.7	29.2	10.9	100
7. Rh-$TF_{DM/17}$	(+)	LC	(-)	4.3	4.6	2.5	2.9	100
8. Rh-$TF_{DM/33a}$	(-)	LC	(-)	4.3	2.7	7.2	-6.7	100
9. Rh-$TF_{DM/33a}$	(-)	LC	(+)	13.1	18.5	29.0	0.1	100

[a] KLH, keyhole limpet haemocyanin.

[b] TF_{DM}, medium-dialysed transfer factor; Rh-TF_{DM}, medium-dialysed rhesus transfer factor; H-TF_{DM}, medium-dialysed human transfer factor.

[c] T/C = counts per minute with antigen (test) ÷ counts per minute without antigen (control); PL, purified lymphocyte culture; LC, leucocyte culture.

[d] Augmentation in disintegrations per minute calculated as in figure 46.

donor to KLH. The optimum augmentation was observed with 0.5 ml of either preparation of transfer factor.

Table 27 shows the results of 9 other experiments with 3 batches of rhesus transfer factor ($Rh\text{-}TF_{DM/16}$, $Rh\text{-}TF_{DM/32}$, $Rh\text{-}TF_{DM/17}$) and 2 batches of human transfer factor ($H\text{-}TF_{DM/2}$, $H\text{-}TF_{DM/1}$) used at 0.25, 0.5 and 1 ml volumes. As in figure 48, augmentation of lymphocyte transformation to 10-100 µg KLH was caused by all batches of the rhesus or human transfer factor with at least one of the volumes of transfer factor used. However, depending on the KLH-sensitivity of the donor or the in vitro "recipient" of transfer factor, all batches of rhesus or human transfer factor also caused suppression of lymphocyte transformation to 10, 33 or 100 µg KLH. Such suppressive activity was caused by 0.25, 0.5 or 1.0 ml of the human transfer factor, but only by 1.0 ml of the rhesus transfer factor (Table 27: experiments 1, 2, 4, 5, 8).

The augmenting or suppressive effects were not donor-specific, since KLH-positive or KLH-negative rhesus transfer factor and KLH-negative human transfer factor preparations augmented or suppressed tritiated thymidine uptake by KLH-stimulated lymphocytes in vitro.

6.2 Augmentation of lymphocyte transformation to PPD with rhesus or human transfer factor (Fig. 49, table 28).

Figure 49 shows the results of 3 experiments with 2 batches of rhesus transfer factor and 1 batch of human transfer factor. Each transfer factor preparation was added, at different volumes (0.25 to 1.0 ml),

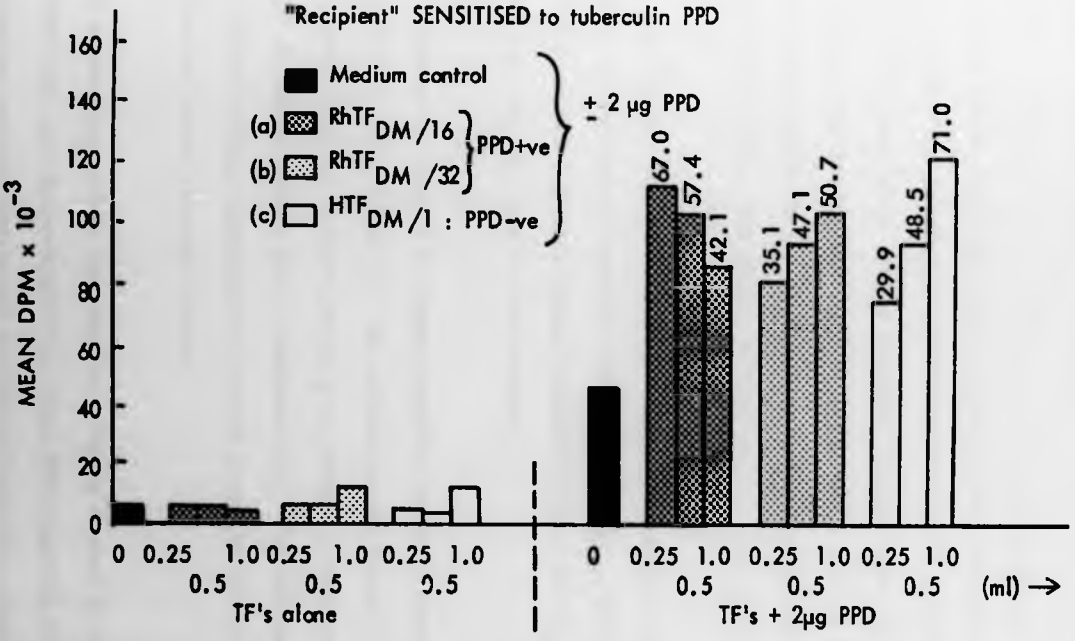

Fig. 49. Comparative augmentation of in vitro responses of sensitised human lymphocytes by (a-b) PPD-positive rhesus, and (c) PPD-negative normal human transfer factor preparations.

Rh-TF_{DM}, rhesus monkey medium-dialysed transfer factor; H-TF_{DM}, human medium-dialysed transfer factor; DPM, disintegration per minute; numbers on bars represent augmentation in DPM x 10^{-3}.

to purified "recipient" human lymphocytes *in vitro* in the presence or absence of 2 µg PPD. The 2 rhesus donors of transfer factor were actively immunised with Freund's complete adjuvant with or without an antigen (Rh-TF_{DM} : PPD +ve), whilst the human donor was Mantoux-negative (H-TF_{DM} : PPD -ve). Both preparations of PPD-positive rhesus transfer factor and the one preparation of PPD-negative human transfer factor, like the KLH-system above (Section 6.1), augmented lymphocyte transformation to 2 µg PPD *in vitro*. In 1 experiment with rhesus transfer factor, the volume producing maximum augmentation was 0.25 ml (Fig. 49a), whilst in the other 2 experiments with rhesus or human transfer factor the optimum volume was 1.0 ml (Figs. 49b-c).

Table 28 shows the results of 5 other experiments with 2 batches of PPD-positive rhesus transfer factor (Rh-$TF_{DM/16}$, Rh-$TF_{DM/34}$) and 2 batches of PPD-negative human transfer factor (H-$TF_{DM/1}$, H-$TF_{DM/3}$) used at 0.25, 0.5 and 1.0 ml volumes.

All batches of rhesus or human transfer factor showed only an augmenting activity at all the volumes used, except for one batch of human transfer factor, which also showed a suppressive effect at 0.25 ml volume (Table 28: experiment 5). The optimum volume producing maximum augmentation varied with the various batches of rhesus or human transfer factor.

The augmenting and/or suppressive effects caused by the human transfer factor were not donor-specific, since PPD-negative transfer factor augmented and/or suppressed tritiated thymidine uptake by PPD-

Table 28. *In vitro* augmentation by rhesus or human transfer factor of tritiated thymidine uptake by human lymphocyte stimulation with PPD.[a]

[b] TF_{DM} donor (PPD sensitivity)		[c] Recipient culture type	(Mantoux)	T/C	[d] Aug.: DPMX10^{-3} TF_{DM} (mls) 0.25	0.5	1.0	PPD conc. (µg/mls)
1. Rh-$TF_{DM/16}$	(+)	PL	?	21.6	97.9	88.7	87.8	2
2. Rh-$TF_{DM/34}$	(+)	LC	+	59.8	98.1	135.2	95.8	2
3. H-$TF_{DM/1}$	(-)	LC	+	59.8	9.9	10.4	12.8	2
4. H-$TF_{DM/1}$	(-)	PL	?	7.6	33.9	62.9	86.0	2
5. H-$TF_{DM/3}$	(-)	LC	+	35.1	-8.8	29.6	17.3	2

[a] PPD, purified protein derivative of tuberculin.

[b] TF_{DM}, medium-dialysed transfer factor; Rh-TF_{DM}, medium-dialysed rhesus transfer factor; H-TF_{DM}, medium-dialysed human transfer factor.

[c] T/C = counts per minute with antigen (test) ÷ counts per minute without antigen (control); PL, purified lymphocyte culture; LC, leucocyte culture.

[d] Augmentation in disintegrations per minute calculated as in figure 46.

stimulated lymphocytes *in vitro*. It was not possible to determine the donor-specificity of the augmenting activity of the rhesus transfer factor, because PPD-negative transfer factor was not used in any experiment.

6.3 Correlation of *in vitro* augmentation by rhesus or human transfer factor and antigen sensitivity of the recipients (Figs. 50a-d).

Figure 50 shows correlation of the extent of *in vitro* augmentation by 0.25 to 1.0 ml transfer factor and the extent of sensitivity of the "recipient" human lymphocyte or leucocyte cultures to 10-100 µg KLH or 2 µg PPD.

Figures 50a and 50c show that, when the rhesus transfer factor was used, there was a linear relationship between the increasing degree of *in vitro* augmentation and the increasing degree of the "recipient" culture-sensitivity to KLH or PPD. This correlation was higher and more significant for KLH (Fig. 50a: $r = +0.7$ $P < 0.001$) than for PPD (Fig. 50c: $r = +0.6$ $P < 0.05$). Similarly, figure 50b shows that, when the human transfer factor was used, the degree of augmentation also seemed to increase with the increasing degree of the "recipient" culture-sensitivity to KLH, but the number of tests was too few for this relationship to be statistically significant ($n = 3$, $r = +0.6$ $P > 0.1$). In contrast, figure 50d shows that, the degree of augmentation by the human transfer factor decreased significantly as the degree of the "recipient" culture-sensitivity to PPD increased ($r = -0.7$, $P < 0.05$).

RECIPIENT SPECIFICITY

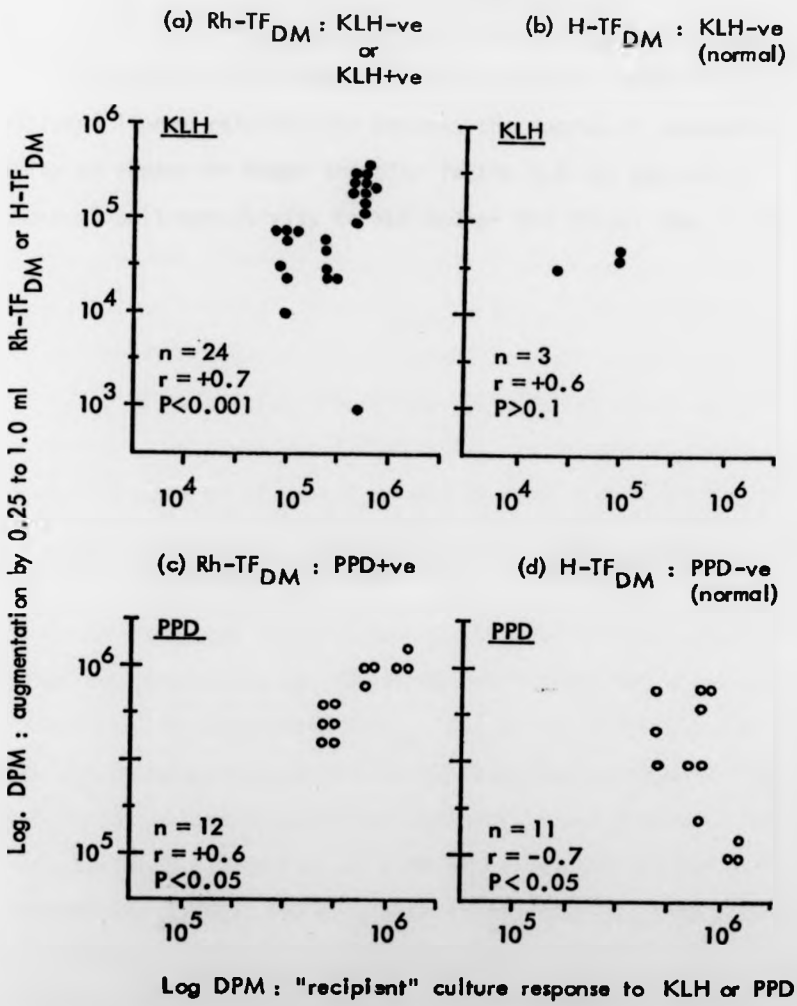

Fig. 50. Correlation of *in vitro* augmentation by human or rhesus transfer factor and recipient sensitivity to (a-b) KLH, or (c-d) PPD.

From figures 48-49 and tables 27-28. Rh-TF$_{DM}$ or H-TF$_{DM}$, medium-dialysed rhesus or human transfer factor; DPM, disintegration per minute; n, number of tests with different volumes of transfer factor; r, sample correlation coefficient; P, level of significance.

Therefore, the *in vitro* augmenting activity of rhesus or human transfer factor was "recipient-specific", because there was a significant linear relationship between the degree of augmenting activity by rhesus or human transfer factor and the degree of "recipient" cell-sensitivity to KLH and/or PPD (Figs. 50a, c, d).

7. RESULTS OF THE PRELIMINARY BIOCHEMICAL ANALYSIS OF DIALYSABLE LEUCOCYTE TRANSFER FACTOR FROM MAN OR MONKEY

7.1 Fractionation of transfer factor on Sephadex G-25.

Crude transfer factor was prepared by "vacuum-dialysis" of human peripheral blood leucocytes ($H\text{-}TF_{VD}$), rhesus monkey peripheral blood leucocytes ($Rh\text{-}TF_{VD/BI}$) or pooled lymph-node and spleen cells ($Rh\text{-}TF_{VD/LSI}$) (See section 2.5.7, fig. 3). Each preparation was fractionated on column(s) of Sephadex G-25 as described before (Section 2.9), and an attempt was made to compare some of the fractionation properties of the above transfer factor preparations.

The extinction of each fraction was measured at 260 nm, predominantly to detect derivatives of nucleic acids, and at 280 nm, chiefly to detect derivatives of proteins (E_{260}, E_{280}). In addition, the elution volume (V_e) of each fraction in the gel was measured and the ratio of the elution volume to total bed volume (V_t) was determined (V_e/V_t). To characterise the behaviour of each fraction, without reference to the size of the column, the partition coefficient (K_{av}) between the liquid phase and the gel phase was calculated. This was calculated according to the method of Krohn et al (1976b, 1977), by the formula $K_{av} = (V_e - V_o) \div (V_t - V_o)$ or $(V_e - V_o) \div V_i$, where V_o represented the void or dead volume, V_t the total volume of the gel bed, V_i the inner volume. Thus, compounds adsorbing to the gel have K_{av} values of greater than 1.0, whilst non-adsorbing substances have K_{av} values of less than 1.0.

7.1.1 Normal transfer factor from human or rhesus monkey blood or rhesus lymph-nodes and spleen (Figs. 53-56, table 29).

Crude dialysable transfer factor, prepared from normal human blood leucocytes, containing 6 x 10^8 lymphocytes, was dissolved in 0.5 ml volatile buffer and fractionated on one column of 1.6 x 90 cms of Sephadex G-25 (Kindly carried out by Mr. Brian Ellis, Immunology Division, Kennedy Institute of Rheumatology, Hammersmith, London) (Section 2.9.1). These leucocytes were contaminated with red blood cells and, on average, contained about 17% polymorphs (Section 2.5.3). Similarly, transfer factor, prepared from 5 x 10^8 lymphocytes of normal rhesus monkey blood or pooled lymph-node and spleen leucocytes, was dissolved in 2 mls volatile buffer and fractionated on 2 serially connected columns of 1.6 x 90 cms each (Section 2.9.1). The rhesus leucocytes were contaminated with red blood cells and, on average, contained less than 50% or 3% polymorphs respectively (Sections 2.5.1 & 2.5.2). Such fractionation of human and rhesus monkey transfer factor preparations led to the following results.

Figures 51-53 show that, depending on the species and lymphocyte source of transfer factor, fractionation resulted in 9 to 11 distinguishable elution peaks. The appropriate tubes were recombined to give 9 to 11 fractions, with various elution volumes. Figure 51 shows that, for the human transfer factor ($H\text{-}TF_{VD}$), there were 9 fractions, coded A - H, with fraction B being divided into B1 and B2. Similarly, figure 52 demonstrates that, for the rhesus transfer factor prepared from blood ($Rh\text{-}TF_{VD/BI}$), there were 10 fractions, designated BI-BX. Figure 53 shows that fractionation of the rhesus

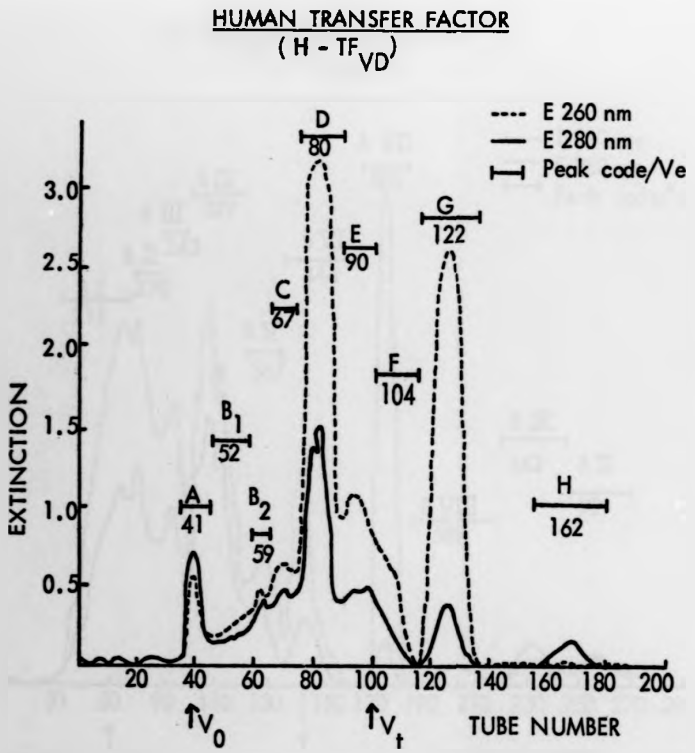

Fig. 51. Sephadex G-25 fractionation of dialysable transfer factor from normal human blood (H-TF$_{VD}$).

A single column of 1.6 x 90 cms was used and 0.9 ml aliquots collected (Section 2.9.1). V_o = void volume (35 mls); V_t = total bed volume (95 mls); V_e = elution volume (41 - 162 mls).

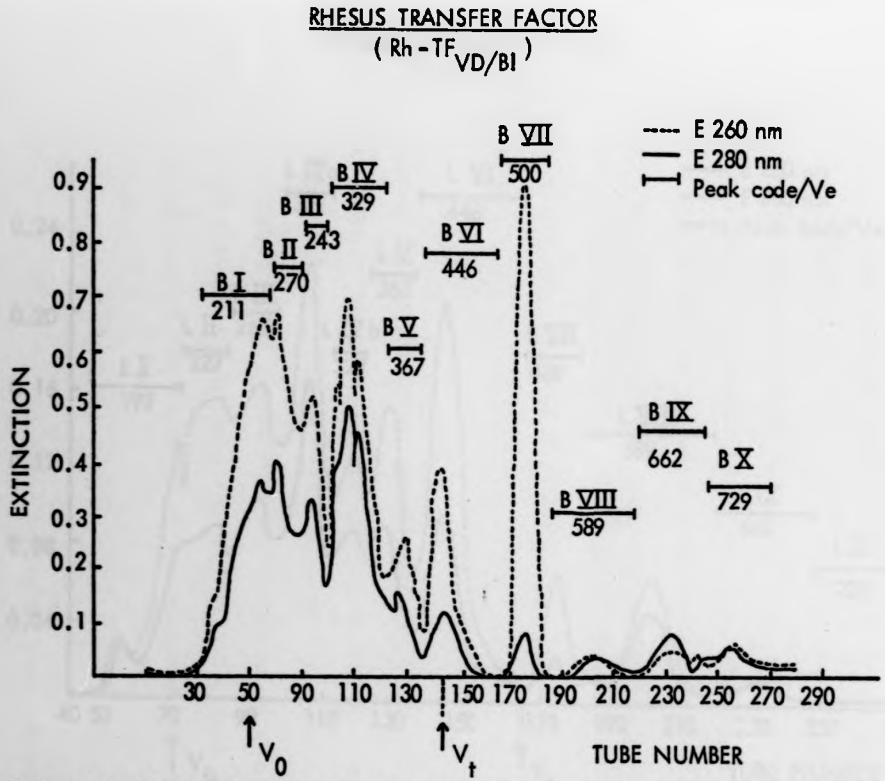

Fig. 52. Sephadex G-25 fractionation of dialysable transfer factor from normal rhesus monkey blood (Rh-TFVD/BI : monkey H41).

Two serially connected columns of 1.6 x 90 cms each were used and 2.7 mls aliquots collected. V_o = void volume (190 mls); V_t = total bed volume (390 mls); V_e = elution volume (211-729 mls).

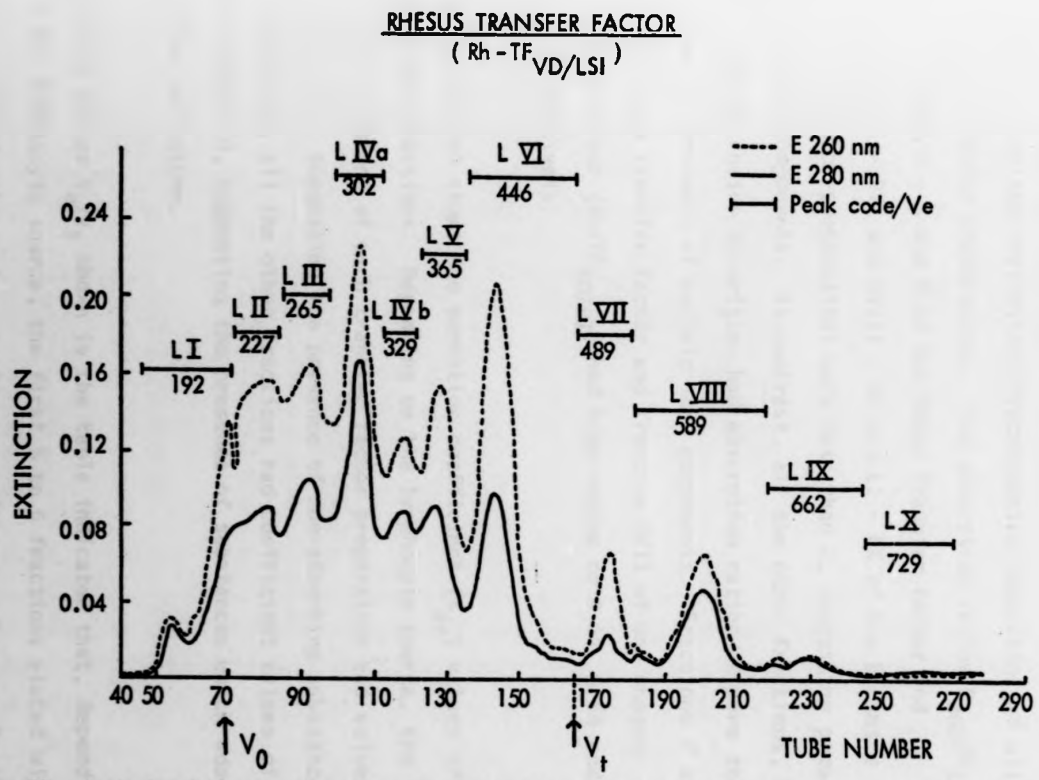

Fig. 53. Sephadex G-25 fractionation of dialysable transfer factor from pooled lymph node and spleen cells of normal rhesus monkey (Rh-TF$_{VD/LSI}$: monkey H41).

Two serially connected columns of 1.6 x 90 cms each were used and 2.7 mls aliquots collected. V_o = void volume (190 mls); V_t = total bed volume (390 mls), V_e = elution volume (192-729 mls).

transfer factor, derived from pooled lymph-nodes and spleen ($Rh\text{-}TF_{VD/LSI}$), resulted in 11 fractions, coded LI - LX, with fraction LIV being divided into LIVa and LIVb.

Table 29 shows the preliminary fractionation properties of all the 3 transfer factor preparations. The absorption ratios E_{260}/E_{280} of fractions A - C and H of the human transfer factor and fractions BI - BVI and BVIII - BX and LI - LX of the 2 rhesus transfer factor preparations were less than 2, suggesting presence of protein components. In contrast, all the other fractions, be it human or rhesus in origin, had absorption ratios of more than 2, suggesting presence of nucleic acid components. Fractions F and G of the human transfer factor and fraction BVII of one rhesus transfer factor ($Rh\text{-}TF_{VD/BI}$) had high ratios of 4.14, 6.54 and 4.39 respectively.

The table also shows the partition coefficient (K_{av}) values of the different fractions. Depending on the lymphocyte source, the first 5 to 6 fractions of each transfer factor preparation had values of 1.0 or less, suggesting the presence of non-adsorbing substances. In contrast, all the other fractions had coefficient values of more than 1.0, suggesting the presence of substances which adsorbed to the gel column.

Elution ratios V_e/V_t shown in the table indicated that, depending on the lymphocyte source, the first 5 to 6 fractions eluted within the total bed volume since ratios were either equal to or less than 1.0. This was not so for the rest of the fractions ($V_e/V_t > 1$).

Table 29. Summary of data for Sephadex G-25 fractionation of dialysable leucocyte transfer factor preparations from man and monkey.

Fraction	*H-TF_{VD} E_{260}/E_{280}	K_{av}	V_e/V_t	Fraction	*Rh-$TF_{VD/BI}$ E_{260}/E_{280}	K_{av}	V_e/V_t	Fraction	*Rh-$TF_{VD/LSI}$ E_{260}/E_{280}	K_{av}	V_e/V_t
A	0.88	0.10	0.46	BI	1.47	0.11	0.54	LI	1.29	0.01	0.49
B1	1.09	0.28	0.58	BII	1.40	0.27	0.62	LII	1.48	0.19	0.58
B2	1.20	0.40	0.66	BIII	1.27	0.28	0.69	LIII	1.40	0.38	0.68
C	1.19	0.53	0.74	-	-	-	-	LIVa	1.33	0.56	0.77
D	2.08	0.75	0.89	BIV	1.17	0.70	0.84	LIVb	1.24	0.70	0.84
E	2.00	0.92	1.00	BV	1.42	0.89	0.94	LV	1.60	0.88	0.94
F	4.14	1.15	1.16	BVI	1.64	1.28	1.14	LVI	1.75	1.28	1.14
G	6.54	1.45	1.28	BVII	4.39	1.55	1.28	LVII	1.58	1.50	1.25
-	-	-	-	BVIII	1.11	2.00	1.51	LVIII	1.13	2.00	1.51
-	-	-	-	BIX	0.82	2.36	1.70	LIX	0.78	2.36	1.70
H	0.60	2.12	1.80	BX	0.90	2.70	1.87	LX	0.85	2.70	1.87

* Dialysable leucocyte transfer factor preparations were obtained by vacuum-dialysis from normal donors: human blood = H-TF_{VD}, rhesus blood = Rh-$TF_{VD/BI}$, pooled rhesus lymph-node and spleen cells = Rh-$TF_{VD/LSI}$.

Absorption ratio: E_{260}/E_{280} = Extinction at 260 nm ÷ Extinction at 280 nm.

Partition coefficient: $K_{av} = (V_e - V_o) \div (V_t - V_o)$. Elution ratio = $V_e \div V_t$.

Abbreviations: V_e = elution volume (ml), V_o = void volume (ml), V_t = total bed volume (ml).

Thus, this may suggest the presence of substances with different molecular weights.

Figures 54a-b show absorption spectra of the crude rhesus monkey transfer factor, prepared either from blood or mixed lymph-node and spleen cells. Both preparations revealed peak absorption at 250-260 nm, suggesting presence of substances derived from nucleic acids. Concentration of these components was more marked in the transfer factor preparations from the rhesus blood, than that from the rhesus lymph-node-spleen cells. Such an analysis was not carried out for the human transfer factor preparation.

The above results of preliminary biochemical analysis of normal dialysable transfer factor preparations from man and monkey, as indicated by Sephadex G-25 gel filtration, therefore, revealed some species similarity of their elution properties.

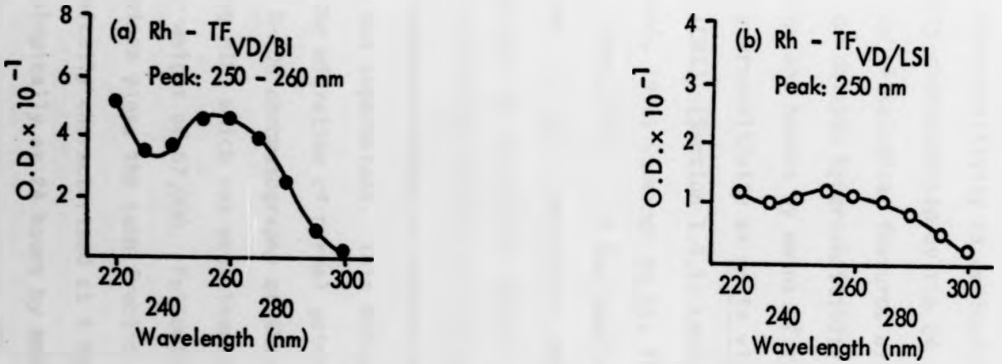

Fig. 54. Optical density scanning of crude preparation of dialysable transfer factor derived from (a) normal rhesus monkey blood ($Rh\text{-}TF_{VD/BI}$) or (b) pooled lymph node-spleen, ($Rh\text{-}TF_{VD/LSI}$).

O.D., optical density.

8. DISCUSSION

8.1 Delayed hypersensitivity and the skin reactive factor (SRF): the DH-skin test as the *in vivo* indicator of cell-mediated immunity.

Delayed hypersensitivity is defined here as that *in vivo* sensitivity characterised by the 24 to 48 hour clinical and the 48 hour histological features already described (Section 2.6). Transfer of delayed hypersensitivity from sensitised to non-sensitised guinea pigs and humans by means of lymphoid cells established delayed hypersensitivity as an *in vivo* manifestion of cell-mediated immunity (CMI) (Section 1.1.1: Landsteiner and Chase, 1942; Chase, 1945, 1946; Cummings *et al*, 1947; Kirshheimer and Weiser, 1947; Lawrence, 1949, 1952). It has been suggested that soluble substances produced by activated lymphocytes mediate delayed hypersensitivity skin reactions to an antigen (Bennett and Bloom, 1968). They showed that PPD stimulated cultures of lymphocytes, from guinea pigs with delayed hypersensitivity to tuberculin, produce a soluble substance MIF into the supernatant. This MIF-containing supernatant could inhibit the migration of normal guinea pig peritoneal exudate cells. Sephadex G-100 chromatography of the supernatant produced a fraction containing MIF, which was proteinaceous with an estimated average molecular weight of 67,000. Following an intradermal injection into normal guinea pigs, the same fraction produced skin reactions which were clinically characterised at 4 hours by erythema and induration, and histologically at 24 hours by monocytic infiltration. Antigen-cell interaction, therefore, produces SRF as well as MIF (Dumonde *et al*, 1969; Pick *et al*, 1969). This SRF lymphokine may mediate delayed cutaneous hypersensitivity (Dumonde, 1970; Dumonde and Maini, 1971). Based on the clinical and histological criteria of delayed hypersensitivity

(Section 2.6), this thesis reports on the extent of such a reaction in the actively immunised and dialysable transfer factor-treated monkeys (Section 3). The conversion rates of the clinically and/or histologically acquired delayed hypersensitivity to KLH, PPD or HBsAg, the suitability of the chest as the skin test area, and the specificity of the reactions in the immunised and transfer factor-treated monkeys are discussed below.

8.1.1 Acquisition and antigen-specificity of DH-skin test reactivity in immunised monkeys: active sensitisation.

Clinically and/or histologically positive 24-48 hour delayed hypersensitivity to various antigens have previously been described in immune rhesus monkeys (Phillips et al, 1970; Baram et al, 1971; Mackler et al, 1971; Maddison et al, 1972; Zanelli and Adler, 1975; Dumonde et al, 1976). In the representative immunised animals here, clinically positive 24-48 hour delayed hypersensitivity was seen in the majority of skin tests. The conversion rates for delayed hypersensitivity were 14 out of 16 tests (88%) with 10 µg PPD in 4 animals, 11 out of 12 tests (92%) with 10 µg HBsAg in 3 animals and 12 out of 12 tests (100%) with 10 µg KLH in 3 animals (Section 3.1.1: figs. 4a-c). The mean clinical conversion rate with all these antigens was 93%, a rate comparable to that of 82% observed for PPD by Baram et al (1971) in 22 BCG-immunised animals. However, these conversion rates are in contrast to that (0%) observed by others who were not able to clinically demonstrate delayed hypersensitivity to Schistosoma antigen or PPD in any of the 8-29 monkeys either infected with the parasite or various mycobacteria or immunised with adult Schistosoma worm extract in

FCA (Maddison et al, 1971, 1972). It therefore seems that clinical evaluation of skin tests for delayed hypersensitivity, in this species, can produce inconsistent results. As a result, like others, (Zanelli and Adler, 1975) clinical observations for delayed hypersensitivity were routinely followed with 48 hour histological examination of biopsies (Section 2.6).

Histologically positive 48 hour delayed hypersensitivity skin test reactions to KLH, PPD or HBsAg were observed. The reactions occurred after the first or the second skin test at 1 to 4 and 2 to 6 weeks respectively in all the 60 tests carried out in 17 actively immunised monkeys (Section 3.1.2: fig. 5b; section 3.1.3: table 13). Similar conversion rates were observed by others for sensitising antigens in the few rhesus monkeys immunised with FCA containing trinitrophenyl-KLH (Mackler et al, 1971) and immunised with BCG (Zanelli and Adler, 1975). The difficulties in interpreting the appearance of clinically positive skin test reactions at 48 hours, in some animals with PPD or HBsAg (Section 3, fig. 4b: monkeys H34a, H42 after second skin test with PPD; fig. 4c: monkey H42 after the first skin test with HBsAg) were overcome by histological examination of the skin biopsy. The lack of clinically positive reactions in some tests was probably related to the low levels of histologically positive reactions detected (DH : 1+ to 3+). This view is consistent with a report by Baram et al (1971), who have also described difficulties in skin testing and the lack of consistent appearances of clinically positive delayed hypersensitivity skin test reactions for low levels of delayed hypersensitivity in sensitised rhesus monkeys. These difficulties may have been associated with the site of the skin test (Chase, 1976).

Intradermal skin tests in the rhesus monkey have been carried out on the eyelid (Baram et al, 1971; Maddison et al, 1971) and on the chest or the back (Maddison et al, 1972). In these experiments, intradermal skin tests were carried out on the chest area (Section 2.6, plate 3). Chase (1976) has said that, in screening African-imported monkeys the chest is not proven to be a good test area for delayed hypersensitivity. In this study, the majority of the tests in the immunised animals showed reactivity. Clinically the cumulative 24 to 48 delayed hypersensitivity reaction was detected in 38 out of 40 tests (95%) (Section 3.1.1: figs. 4a-c), whilst histologically the cumulative 48 hour biopsies revealed active delayed hypersensitivity in 60 out of 60 tests (100%) (Section 3.1.2 : fig. 5b). Such high clinical and histological reactions indicate that the chest area is a suitable area for skin testing the immunised animals according to the protocols used (Section 2.1: figs. 1a-d). This was supported by the evidence for good dose-responses to 2 of 3 skin test antigens. Histologically, 30µg PPD or HBsAg produced a more marked mean delayed hypersensitivity response than 10µg PPD or HBsAg (Section 3.1.2: fig. 6). Unlike the intrapalpebral skin tests, recommended by Chase (1976) and carried out by some workers in this species (Baram et al, 1971; Maddison et al, 1971), the chest area has some advantages not shared by the eyelids.The chest does not limit the number of skin tests to one or two antigens, or to a few dilutions, or only to clinical examinations. It provides a relatively large area which can be used for many skin tests and can easily be biopsied for histological studies.

Two comparisons may be considered for assessing the antigen-specificity of delayed hypersensitivity reactions in the immunised animals. The reactions can either be compared with those produced by the same antigens in other naive control animals (Baram et al, 1971) or with those produced with unrelated antigens in the same animals (Maddison et al, 1972). To determine antigen-specificity of the acquired delayed hypersensitivity to KLH,PPD and HBsAg in the immunised animals reported here, skin tests with the same antigens were carried out in 2 saline-injected normal rhesus monkeys as controls. Comparison of the results showed a highly significant difference between the control (naive) and immunised animals in their conversion of the skin test from negative to positive delayed hypersensitivity after the first or the second skin test taken either separately or together (Section 3.1.3: table 13, $P<0.001$). In agreement with this report, similar antigen-specific acquisition of delayed hypersensitivity responses have been reported by Baram et al (1971) in rhesus monkeys immunised with BCG or infected with mycobacterium tuberculosis, and by Maddison et al (1972) in animals infected with Schistosoma mansoni or mycobacteria. However, as in this study, Baram et al, did not carry out skin tests in the immunised animals with an unrelated (non-specific antigen), but compared their results with skin tests done in naive control animals.

Thus, immunisation of rhesus monkeys with FCA, with or without KLH or HBsAg, seemed to induce specifically active sensitisation for delayed hypersensitivity skin test to an antigen. Such sensitisation could qualitatively and quantitatively be monitored with intradermal skin tests on the chest area. These findings led

to the use of such skin tests to monitor delayed hypersensitivity in rhesus monkeys treated with homologous dialysable transfer factor.

8.1.2 Acquisition and specificity of DH-skin test reactivity in transfer factor-treated monkeys.

Transfer factor often fails to transfer delayed hypersensitivity responses clinically in rhesus monkeys (Maddison et al, 1972; Zanelli and Adler, 1975). In support of this observation, there were low cumulative conversion rates for clinical observation of skin tests. Only 1 out of 23 (4%) skin tests converted in animals treated with control dialysable transfer factor, ie. transfer factor normal or unrelated to test antigen (Section 3.2.1: table 14, monkey H38; KLH: induration diameter = 5.5mms), and 4 out of 53 (8%) skin tests converted in recipients treated with immune dialysable transfer factor i.e. transfer factor immune to test antigen (Section 3.2.1: table 15, monkey H36, KLH: induration diameter = 7-10mms, PPD; induration diameter = 12-13mms). In contrast to these conversion rates, those rates reported by Steele et al (1976) for clinically positive delayed hypersensitivity skin tests were lower for control dialysable transfer factor (unrelated to test antigen) and higher for immune dialysable transfer factor. In 3 other nonhuman primate species as recipients, these investigators observed cumulative conversion rates of zero out of 17 (0%) and 19 out of 42 (45%) with control (unrelated to test antigen) and immune human dialysable transfer factor preparations respectively. A higher cumulative clinical conversion rate of 32 out of 42 tests (80%), for delayed hypersensitivity to Eimeria bovis and PPD has recently been reported by Klesius

and Fudenberg (1977) in 17 cattle recipients of unfractionated and alcohol-precipitated immune homologous dialysable transfer factor. Thus, the source of lymphocytes and the procedure for preparation of the transfer factor and the species sensitivity of the recipients are possible factors influencing the differences in the clinical conversion rates after administration of dialysable transfer factor. In addition, skin testing of recipients before treatment with dialysable transfer factor (Steele et al, 1976; Klesius and Fudenberg, 1977) may have played a role in the higher clinical rates of conversion of negative to positive delayed hypersensitivity.

Histologically some transfer of delayed cutaneous hypersensitivity responses occurred in the course of the study in the recipients of dialysable transfer factor (Section 3.2.3: table 16). In all the 12 recipients, the cumulative histological conversion rate of 4 out of 18 (22%) skin tests occurred with the control transfer factor (normal or unrelated to test antigen). In contrast, a higher cumulative conversion rate of 17 out of 32 (53%) skin tests occurred with the immune transfer factor. These cumulative rates are comparable to those found by others with 4 out of 23 test conversions (17%) with control homologous dialysable transfer factor and with 8 out of 12 test conversions (67%) with immune dialysable rhesus transfer factor in 3 rhesus recipients (Maddison et al, 1972). However, these results are contrary to those for three rhesus monkeys used by Zanelli and Adler (1975). These workers reported no conversion in 8 tests with control (unrelated to test antigen) dialysable rhesus transfer factor, but obtained a higher conversion

rate of 13 out of 15 tests (87%) with the immune dialysable transfer factor. Similarly, Gallin and Kirkpatrick (1974), after administration of dialysable human transfer factor into 3 rhesus monkey recipients, observed no conversion in 8 tests after the control unrelated dialysable transfer factor, but 5 out of 7 test conversions (71%) after the immune dialysable transfer factor. Therefore, higher rates of conversion were indicated histologically rather than clinically (see above). The delayed hypersensitivity results obtained here by histological examination of skin biopsies were considered more reliable and used to question the possible effect of skin testing the recipients before the administration of dialysable transfer factor.

It has been reported that, guinea pigs treated with dialysable human transfer factor, only acquire delayed hypersensitivity skin test reactivity when they have been pre-exposed or 'primed' to subsensitising doses of antigen (Welch et al, 1976a,b). In the experiments reported here none of the recipient rhesus monkeys were previously skin tested with HBsAg. One recipient was skin tested with KLH, but it did not convert to this antigen. In contrast, all the recipients were previously Mantoux tested by the suppliers. Thus, the majority of the recipients were not primed with HBsAg or KLH, but all were with tuberculin. After immune transfer factor administration, 2 out of 9 first skin tests converted to KLH and HBsAg, and 5 out of 9 to PPD (Section 3.2.3: table 16). Thus, there seems to be some indication that priming might increase the rate of the first skin test conversion after the administration of rhesus dialysable transfer factor.

This is perhaps supported by the observation that the cumulative rate of conversion to antigen increased after control or immune transfer factor at the time of the second skin test (Section 3.2.3: table 16). The use of delayed hypersensitivity skin test, in either primed or unprimed recipients, has not unequivocally established the biological specificity of the *in vivo* activity of transfer factor (Section 1.5). Despite this, however, the skin test was used here to investigate the problem of specificity. The *in vivo* specificity of the rhesus dialysable transfer factor throughout this thesis is discussed in terms of antigen-sensitivity of the donor ("Donor-specific"). Donor-specific acquisition of reactivity is defined as that sensitivity transferred to an antigen to which the donor is sensitised. On the basis of this definition, a comparison of the cumulative results of control and test recipients indicated that, the acquisition of delayed hypersensitivity after the first and the second skin test together, in transfer factor-treated monkeys, seemed to be non-specific, i.e. not related to antigen-sensitivity of the donor(s) (Section 3.2.3, table 16: $P>0.05$). This non-specificity is supported by similar findings of others in man (Section 1.5.1 : Levin *et al*, 1971; Spitler *et al*, 1972; Griscelli, 1975) (Khan *et al*, 1975; Krohn *et al*, 1976a; Khan, 1977) and the rhesus monkey (Section 1.4.1: Maddison *et al*, 1972). Rhesus dialysable transfer factor, like the human material, may therefore contain components which augment non-specifically delayed hypersensitivity skin tests. The apparent non-specificity of rhesus dialysable transfer factor seen here can be interpreted in two ways. Firstly, that repeated skin testing alone may result in the conversion of the skin test from negative to positive

(Baram et al, 1966; Maini et al, 1976). Secondly, that repeated skin testing may bring out the dormant nonspecific reactivity induced by dialysable transfer factor (Khan, 1977). The nonspecific activity was found here to PPD after the first skin test, whilst against KLH and HBsAg only after the second skin test (Section 3.2.3: table 16). This difference between PPD and the other antigens may be explained as follows. Firstly, the normal control dialysable transfer factor was prepared from cell pellets made from a monkey recently imported and skin tested three times with old tuberculin and was therefore primed with this antigen. Secondly all recipient animals were also probably primed with tuberculin, since they are routinely screened for tuberculosis by a Mantoux test prior to laboratory use. Thus, in this work, the delayed skin test reactivity to PPD in the recipients does not seem to be an appropriate test for the determination of the specificity of the in vivo activity of the rhesus dialysable transfer factor. For the other antigens (KLH, HBsAg), the nonspecific effects were not detected after the first skin test, but were after the second. This may be because repeated skin testing may have resulted in the induction of delayed hypersensitivity and/or dialysable transfer factor had nonspecific in vivo activity. The former was unlikely in this study because the two normal saline-injected monkeys were skin tested twice with KLH or HBsAg and showed no histological evidence of delayed hypersensitivity (Section 3.1.3: table 13). Thus the rhesus dialysable transfer factor may have had nonspecific activity towards these antigens. However, the number of DH-skin tests with control transfer factor are too few for meaningful detailed study of the in vivo specificity of the thesus dialysable transfer factor with such selective antigens.

Therefore, DH-skin test on the chest of the rhesus monkeys seemed to be an appropriate *in vivo* test of CMI for detecting antigen-specific active sensitisation of animals with FCA, with or without an antigen. It does not appear to be an appropriate test for monitoring CMI in transfer factor-treated monkeys, or for determining the *in vivo* specificity of transfer factor in this species.

8.2. Cellular hypersensitivity and the macrophage or leucocyte inhibition factor (MIF, LIF): the LMMI-test as an *in vitro* indicator of cell-mediated immunity.

Cellular hypersensitivity is indicated here by significant migration inhibition of indicator cells *in vitro*. Several *in vitro* cell migration tests have been described to measure cellular hypersensitivity to antigens in man and animals. These are the direct and indirect macrophage or leucocyte migration tests and the mixed cell migration test (LMMI).

In 1932, Rich and Lewis reported that tuberculin PPD specifically inhibited the *in vitro* migration of macrophages from explants of spleen and lymph nodes taken from guinea pigs with delayed hypersensitivity skin reaction to PPD, but PPD had no effect on explants from normal animals. Thus, they lay the foundation for the development of the *in vitro* cell migration assay system of cellular hypersensitivity as a parameter of delayed hypersensitivity reaction *in vivo*. In 1962, George and Vaughan introduced the technique of inhibition of migration of guinea pig peritoneal

exudate cells (> 70% macrophages) from capillary tubes. This correlated with delayed hypersensitivity to PPD and egg albumin in guinea-pigs immunised with BCG or egg albumin in FCA. These studies were soon confirmed and extended by others in the guinea-pig system (David et al, 1964a, b; Bloom and Bennett, 1966, 1968; David, 1966, 1968). It was found that only as few as 10 - 20% of the peritoneal exudate cells need be specifically sensitive to inhibit the migration of the whole population of peritoneal exudate cells (David et al, 1964b). Bloom and Bennett (1966) and David (1966) found that the macrophages, and not the lymphocytes, constitute the migrating indicator cells of the peritoneal exudate cells, but the sensitive lymphocytes were necessary for inhibition to occur. The sensitive lymphocytes, during incubation with specific antigen, elaborated a soluble, non-dialysable and heat-stable substance which specifically inhibited the in vitro migration of normal guinea-pig peritoneal exudate cells, presumably the macrophages, and reflected the state of delayed hypersensitivity in vivo (Bloom and Bennett, 1966; David, 1966, 1968). This mediator was given the name migration inhibition factor i.e. MIF (Bloom and Bennett, 1966). MIF mediates the inhibition of migration of the indicator cells either alone or by interaction with antigen (Bennett and Bloom, 1967), and is synthesised by the sensitive lymphocytes upon contact with the specific antigen (Bloom and Bennett, 1968; Dumonde et al, 1969).

Thor and Dray (1968) used the direct capillary macrophage migration test for studies in man and found that the specific

inhibition of macrophages, obtained from lymph nodes of sensitised human subjects, correlated with delayed hypersensitivity reaction to PPD or histoplasmin. However, the relative inaccessibility of human macrophages made it difficult to apply such direct macrophage capillary tube technique to man. Thus, in an attempt to develop a more useful clinical test, Thor et al, (1968) developed an indirect cell migration system and first showed MIF in man, as opposed to guinea pig. Human peripheral blood lymphocytes, obtained from sensitised subject and incubated for 72 hours with PPD or histoplasmin or coccidioiodin, produced MIF in the supernatant. This specifically inhibited the migration of normal guinea pig macrophages. They suggested that this factor in man was analagous to that in the guinea pig, and since the human MIF inhibited guinea pig macrophages, it lacked species specificity. Such an in vitro detection of cellular hypersensitivity has been confirmed by others in man with PPD, streptokinase-streptodornase and candida antigens (Rocklin et al, 1970a, b). This indirect MIF assay has proved to be slow for clinical tests and led to the development of a direct in vitro assay system.

Some investigators have shown that human buffy coat cells can also serve as indicator cells. In 1968, Soborg demonstrated that the 24-hour migration of human peripheral leucocytes, obtained from a human subject after primary immunisation with killed Brucella bacteria, was inhibited in the presence of the

specific antigen. He suggested this direct and rapid test as a useful *in vitro* test of specific delayed hypersensitivity in man. These findings were later confirmed and extended by Bendixen and Soborg (1969) with PPD, Brucella or "organ-specific" antigens, by Curtis and Hersh (1973) with KLH, by Maini *et al* (1973) with particulate TB, and by others with HBsAg (Yeung-Laiwah *et al*, 1973; Reed *et al*, 1974; Lee *et al*, 1975). Clausen (1971) developed a leucocyte migration test in agarose and demonstrated a correlation between inhibition of migration and delayed hypersensitivity skin reaction to PPD. He showed such agarose plate cultures to be more sensitive than the capillary tube technique for the PPD system, and to have the advantage of using less human leucocytes and less antigen than the capillary leucocyte migration method. The migration indicator cells in the leucocyte capillary technique have been identified as both mononuclear and polymorphonuclear leucocytes (Bendixen and Soborg, 1969; Rosenberg and David, 1970; Clausen, 1971; Read and Zabriskie, 1972).

Like the capillary macrophage migration test, it was suggested that a mediator, similar to or the same as MIF, may also cause the *in vitro* inhibition of the leucocyte migration (Bendixen and Soborg, 1969). Recently, Rocklin (1974) purified the products of activated human lymphocytes by column chromatography. He showed that both the *in vitro* macrophage and leucocyte migration inhibition systems correlate with the *in vivo* delayed hypersensitivity to tuberculin PPD, streptokinase-streptodornase and candida, but are assay systems for two functionally and physically different lymphokines. One is MIF (M. wt. 23,000) and inhibits the

migration of guinea pig macrophages or human monocytes, but not the human polymorphs. The other is the leucocyte inhibition factor or LIF (M. wt. 60,000), which inhibits the migration of human polymorphs, but not that of guinea pig macrophages or human monocytes.

There have been reports of difficulties in relating inhibition of leucocyte migration from capillaries or in agarose to delayed hypersensitivity in vivo (Kaltreider et al, 1969). In some cases, inhibition of leucocyte migration may be adversely affected by abnormalities of the indicator cells, which may even be absent or insufficiently sensitive (Marsman et al, 1972). Thus, another cell migration technique has been developed in an attempt to overcome some of these problems.

In 1970, Rajapakse and Glynn introduced an in vitro method as an assay of cellular hypersensitivity to PPD in man. They migrated, from capillaries, normal guinea pig peritoneal exudate cells (macrophages) mixed with purified human peripheral blood lymphocytes obtained from sensitive or normal subjects. They suggested that the guinea pig macrophages were the indicator cells and found, that only as few as 3-15% of sensitive human lymphocytes were required to cause significant inhibition of migration of the indicator cells. A close correlation was reported between the mixed cell migration inhibition and delayed hypersensitivity skin test reaction to PPD. Lack of inhibition

of migration of normal peritoneal exudate cells alone or mixed with 3-15% normal or sensitive human lymphocytes, in the absence of PPD (controls), showed that mixing of such cells from two different species did not spontaneously cause inhibition of migration. The usefulness of such mixed lymphocyte-macrophage migration inhibition test, as an index of antigen-specific cellular hypersensitivity and, as an *in vitro* correlate of antigen-specific delayed hypersensitivity skin test, was later confirmed and extended by others in man with tuberculin PPD, candida and streptokinase-streptodornase antigens (Marsman, *et al*, 1972; Marsman and Van der Hart, 1974).

A mixed leucocyte-macrophage migration test (LMMI) was used for this study, which was adapted to the rhesus monkey leucocytes (not pure lymphocytes), and extended to four antigens, KLH, PPD, TB and HBsAg. Such a test was considered to have the following advantages over other migration tests.

Sabioncello *et al*, (1976) compared the direct and indirect macrophage migration inhibition with direct and indirect leucocyte migration inhibition in guinea pigs. It was found that in animals with delayed hypersensitivity skin test positive reactions to tuberculin PPD, all such systems behave alike and reflected the delayed hypersensitivity status of the test animals. Thus, they suggested that both macrophages and peripheral leucocytes seemed to be equally valuable for such *in vitro* cell migration tests of cellular hypersensitivity. It therefore seems advantageous to

combine both the macrophage migration and leucocyte migration tests, in the form of mixed leucocyte-macrophage migration test, to detect cellular hypersensitivity in vitro as an index of delayed hypersensitivity in vivo. The inhibition of the mixed leucocyte-macrophage migration probably indicates production of MIF and LIF. Thus, such a mixed cell migration test may be a more effective indicator of cellular hypersensitivity than either macrophage migration or leucocyte migration test alone. The other advantages of the LMMI test are as follows. Compared with the direct macrophage migration test, the LMMI test does not use monkey peritoneal exudate cells (macrophages). This could only be obtained by killing an expensive animal and would limit the number of tests to one. Compared with the direct capillary leucocyte migration test, the LMMI test does not need a large volume of blood to carry out many tests with multiple dilutions of different antigens. Compared with indirect MIF or LIF assay, the LMMI test is quick and relatively simple to set up.

In these experiments the LMMI test was sequentially carried out in normal, actively immunised and transfer factor-treated rhesus monkeys (Sections 2.1 and 2.2), and used to monitor cellular hypersensitivity. The LMMI results obtained are shown above (Section 4), and the conversion rates for acquisition of inhibition of migration and its specificity to KLH, PPD, TB and HBsAg are discussed below on the basis of the criteria used to indicate significant cellular hypersensitivity LMMI-reactivity (Sections 8.2.1-8.2.3).

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8.2.1 Criteria for LMMI-reactivity to antigen (Tables 30-31).

In experiments carried out for this work a migration index of less than 0.8 (MI < 0.8: section 2.7.1) was used throughout as a criteria for positive LMMI-response or presence of cellular hypersensitivity. This index as a point of significance was selected for two main reasons. Firstly, a line drawn at 80% migration (MI = 0.8) clearly separated the majority of the actively sensitised monkeys from the normal control animals for all dilutions of KLH, PPD, TB and HBsAg (3-200 µg/ml: section 4.1.1: figs. 9 cf 10). Secondly, using similar or other cell migration techniques in man or guinea pigs, other investigators have also considered a MI < 0.8 as indicative of acquisition of cellular hypersensitivity to various antigens (George and Vaughan, 1962; David et al, 1964a, b; Soborg, 1968; Rocklin et al, 1970a; Marsman et al, 1972; Marsman and Van der Hart, 1974; Ramsey et al, 1976). Using this cut-off point the potentially toxic effects of the antigens on the migration test was excluded in order to validate the acquisition of cellular hypersensitivity LMMI-reactivity.

It has been reported that in similar tests with mixed human lymphocyte and normal guinea pig peritoneal exudate cells, the latter are the migrating indicator cells (Rajapakse and Glynn, 1970). In order to exclude the possible non-specific influence of direct antigen toxicity on the migration of these cells, normal guinea pig peritoneal exudate cells (≡ Average > 80% macrophages)

were migrated from capillaries in the presence or absence of 3-200μg/ml KLH, PPD, TB or HBsAg. Table 30 clearly shows almost total absence of non-specific toxic reactivity to the antigens used. Only 2 out of 64 tests (3%) showed toxic effects (200μg/ml KLH and HBsAg: MI<0.8). In the mixed leucocyte-macrophage migration tests carried out here, peripheral rhesus polymorphs and/or monocytes as well as the guinea pig macrophages could have been the indicator cells. To exclude the possible non-specific influence of direct toxicity of the antigens on the migration of the mixed indicator cells, normal rhesus leucocytes mixed with normal guinea pig peritoneal exudate cells were migrated from capillaries in the proportions and numbers described previously (Section 2.7.1). Table 31 shows that, in a total of 269 tests carried out with all the antigens at all dilutions, only 20 tests (7%) converted indicating minimal direct antigen toxicity on the mixed cells. Furthermore it indicated that significant inhibition (MI<0.8) was not due to the mixing of the normal cells from the two species. Thus, in the LMMI tests carried out in the actively immunised rhesus monkeys (Section 4.1.1: fig. 10a-d), the acquisition of apparent cellular hypersensitivity migration responses to various concentrations of the different antigens used was not due to the direct toxicity of the antigens on the normal guinea pig peritoneal exudate cells alone (Table 30), or mixed with the rhesus leucocytes (Table 31).

8.2.2 Acquisition and antigen-specificity of LMMI-reactivity in immunised monkeys: active sensitisation (Table 32).

Table 30 Migration indices (MI) of 18-20 hour capillary migration of normal guinea-pig peritoneal exudate cells (PEC) alone in the presence of antigens KLH, PPD, TB and HBsAg.[a]

Test antigens (µg/ml)		MI : $4x10^7$ macrophages/ml[b] (1)	(2)	(3)	(4)	Mean MI[c] ± SEM
KLH:	3	0.96	1.25	1.10	1.23	1.14 ± 0.01
	33	1.12	1.19	1.01	0.99	1.08 ± 0.01
	100	1.03	0.96	0.80	0.95	0.94 ± 0.00
	200	0.97	0.92	0.64	0.83	0.84 ± 0.01
PPD:	3	1.17	1.19	1.23	1.18	1.19 ± 0.00
	33	1.15	1.14	1.03	1.22	1.14 ± 0.00
	100	1.09	1.09	1.10	1.30	1.11 ± 0.01
	200	0.99	0.87	1.10	1.14	1.03 ± 0.01
TB:	3	1.21	1.17	1.20	0.95	1.13 ± 0.01
	33	1.07	1.18	1.14	1.13	1.13 ± 0.01
	100	1.07	1.21	1.20	1.24	1.18 ± 0.00
	200	1.03	0.97	1.08	1.23	1.08 ± 0.01
HBsAg:	3	1.16	1.24	1.19	1.12	1.18 ± 0.00
	33	1.14	1.18	1.19	1.20	1.18 ± 0.00
	100	0.86	0.99	0.96	0.97	0.95 ± 0.00
	200	0.75	0.87	0.86	0.91	0.85 ± 0.00

[a] PEC on average contained > 80% macrophages.
[b] MI < 0.8 represents significant inhibition (underlined).
[c] SEM, standard error of the mean.

Table 31 Total number of LMMI-tests with normal rhesus leucocytes mixed with normal guinea-pig peritoneal exudate cells (PEC) in the presence of antigens KLH, PPD, TB and HBsAg.[a]

Test antigens	Total no. of +ve LMMI (MI < 0.8) tests[b] (µg/ml)				
	3	33	100	200	Σ 3-200
KLH	3/22	1/22	4/21	1/15	9/80 (11%)
PPD	0/22	1/22	1/21	2/15	4/80 (5%)
TB	0/19	1/19	2/18	2/15	5/71 (7%)
HBsAg	0/9	0/2	0/10	2/10	2/38 (5%)
All antigens	3/72 (4%)	3/65 (5%)	7/70 (10%)	7/55 (13%)	20/269 (7%)

[a]Leucocytes and macrophages from both species were mixed as described before (Section 2.7.1).

[b]From figures 9a-d (Section 4.1.1).

In immunised monkeys the conversion rates of the mixed cell migration tests varied from one antigen to another. These rates were 50%, 51%, 59% and 79% for 3-200μg/ml HBsAg, PPD, TB and KLH respectively, making a cumulative mean rate of 60% (Section 4.1.3: table 17). Compared with the control unsensitised monkeys such conversion rates were highly significant ($P<0.005$ or <0.001). Therefore, in the studies reported here, the mixed cell migration test, originally described by Rajapakse and Glynn (1970) for detection of human hypersensitivity, was successfully adapted to detect the sensitivity of the rhesus monkey leucocytes.

It has been suggested that sequential, rather than a single, determination in the leucocyte migration test might provide a more significant index of cellular hypersensitivity against PPD in man (Ramsey et al, 1976). Here, sequential mixed cell migration tests in the immunised monkeys, showed that there was some variation in the onset and duration of the acquired LMMI-reactivity to an antigen. After immunisation the onset of reactivity varied from 2 to 5 weeks in different animals. The reactivity was picked up on 1 occasion ("transient"), or repeatedly for 2, 3, or 4 weeks ("lasting") (Section 4.1.2, figs. 11-14). Thus, this transient or lasting reactivity might mean that carrying out only one migration test after immunisation is of little value, since it may not detect positive responses. In contrast, carrying out the test at weekly intervals, between 2 to 5 weeks of immunisation, probably will detect the

reactivity making the LMMI test more meaningful. However, such different patterns of responses might also indicate that different immunised animals respond differently to the sensitising antigens. To determine whether repeated skin tests with an antigen induce inhibition of mixed cell migration, LMMI-tests were carried out in naive saline-treated rhesus monkeys, skin tested twice with KLH, PPD or HBsAg. Such an influence was considered unlikely in these experiments, since 30µg KLH and 50µg HBsAg did not significantly inhibit migration of the mixed cells in the LMMI-tests carried out with these antigens in these rhesus monkeys (Section 4.1.2: figs. 11a, 14a). In contrast, when 30µg PPD was used for the skin test in such a naive monkey the migration of cells seemed to be significantly inhibited by PPD, but not by TB (Section 4.1.2: figs. 12a, 13a). The 100µg/ml concentration of PPD used for the LMMI test was non-toxic (Section 8.2.1: tables 30-31), and TB was a more effective antigen for inhibition of migration (Section 4.1.1: figs. 10b cf 10c). It might be deduced from this that TB, rather than PPD, should have significantly inhibited migration of the mixed cells, if the skin test with PPD had induced true cellular hypersensitivity. However, this was not so and it may be concluded that skin tests do not seem to influence inhibition of mixed cell migration in these experiments.

The acquired cellular hypersensitivity, as indicated by LMMI-reactivity (MI<0.8) in the immunised rhesus monkey was antigen-specific (Section 4.1.3: table 17). The total number of positive LMMI tests carried out with 3-200µg/ml antigen in normal controls animals was

compared with those in the immunised monkeys. There was a highly significant difference between the individual rates of conversion for KLH P<0.001) for PPD (P<0.001) for TB (P<0.001) and for HBsAg (P<0.005), and the cumulative rate of conversion for all these antigens (P<0.001). Thus, the acquired cellular hypersensitivity can be said to be antigen-specific. It has already been demonstrated that the direct toxicity of antigens on the migration cells was minimal (Section 8.2.1). It is also known that the immunised animals were naive before immunisation (MI>0.8) (Section 4.1.2: figs. 11b-d, 12b-e, 13b-d, 14c-d). Thus, the antigen-specific cellular hypersensitivity must have resulted from active sensitisation of the immunised animals with FCA, with or without KLH or HBsAg.

To determine the true antigen-specificity of the LMMI-test in the immunised monkeys, tests may also be carried out with unrelated antigens. Such tests were only carried out in a few monkeys, but it was found that the LMMI-test was totally specific for tuberculin PPD or TB and HBsAg. In 3 monkeys immunised with FCA, with or without HBsAg, none of the tests non-specifically converted to an unrelated antigen (KLH) at any concentration used (Table 32). In contrast, some of the tests showed conversion to one or more sensitising antigens, at one or more concentration (Table 32). Thus like the direct or indirect macrophage migration test and leucocyte migration tests (David et al, 1964a; Rocklin, 1974), the direct LMMI test used here can assay for antigen-specific cellular hypersensitivity in this species.

Table 32 Antigen-specificity of LMMI-responses any time after immunisation of representative rhesus monkeys with FCA with or without HBsAg.[a]

Conc. of test antigen (µg/ml)		Migration indices (MI) in monkeys immunised with[b] FCA *(H33a)	FCA/HBsAg *(H42)	FCA/HBsAg *(H43)
KLH:	3	0.98	1.15	0.90
	33	0.80	1.10	0.91
	100	0.98	1.10	0.89
	200	1.15	ND	ND
PPD:	3	0.89	0.91	0.68
	33	0.88	0.86	0.55
	100	0.77	1.13	0.60
	200	0.79	ND	ND
TB:	3	0.78	ND	ND
	33	0.84	ND	ND
	100	0.63	ND	ND
	200	0.71	ND	ND
HBsAg:	3	ND	0.79	0.83
	33	ND	0.75	0.73
	100	ND	0.81	0.58
	200	ND	ND	ND

[a] LMMI-tests read after 20 hours of incubation (Section 2.7.1).
[b] MI < 0.8 underlined and significant (Section 2.7.1).
ND, not done; * Code of monkeys.

It was established that the LMMI-test under the conditions of these experiments was qualitatively a reliable *in vitro* technique of detecting antigen-specific cellular hypersensitivity in the actively sensitised monkeys. The acquired cellular hypersensitivity (LMMI:MI<0.8) did not seem to be significantly influenced by direct toxicity of an antigen in the LMMI-test or by repeated skin testing of monkeys. These findings, and the observation that the LMMI-test may also quantitively measure responses in the actively sensitised animals (Section 4.1.4: fig. 15), resulted in the application of this *in vitro* mixed cell migration test to monitor responses in rhesus monkeys injected with homologous dialysable transfer factor.

8.2.3 Acquisition and donor-specificity of LMMI-reactivity in transfer factor-treated monkeys: adoptive sensitisation (Tables 33-34).

Leucocyte or macrophage migration tests have successfully been used to detect conversion of cellular hypersensitivity to an antigen in patients treated with transfer factor (Section 1.2.1: table 3-6). Similarly a leucocyte migration test has been employed to monitor transfer of cellular hypersensitivity to PPD and HBsAg in chimpanzees treated with immune human dialysable transfer factor (Section 1.4.1: table 10; Trepo and Prince, 1976). Acquisition of reactivity to PPD and Leishmania has also been detected in guinea pigs treated with homologous dialysable transfer factor by a macrophage migration test (Section 1.4.1: table 10; Clinton and Magoc, 1976). In contrast, the mixed cell migration test has only once been

used in monitoring cellular hypersensitivity in one human treated with transfer factor (Arala-Chaves et al, 1977).

In this study, ten naive rhesus monkeys were treated with immune transfer factor. A total of 58 out of 97 (60%) of all LMMI tests, carried out with all antigens at all concentrations, showed reactivity (Section 4.2.1: figs. 18a-d; Section 4.2.3; table 19). Such cumulative rate of conversion was not significantly different from a total of 70 out of 105 (67%) in vitro leucocyte or macrophage migration tests, which converted to various microbial and tissue antigens in patients treated with human transfer factor by many investigators (Section 1.2.1: tables 3-6). The LMMI-test in the rhesus monkey, therefore, was apparently as good as the in vitro leucocyte and macrophage migration tests in man in detecting transfer of cellular hypersensitivity by the immune rhesus dialysable transfer factor. The individual cumulative rates of conversion were 49%, 52%, 76% and 90% for 3-200µg/ml TB, PPD, KLH and HBsAg respectively in rhesus monkeys treated with immune homologous dialysable transfer factor (Section 4.2.3: table 19). A comparable mixed leucocyte-macrophage migration test has most recently been used to detect transfer of cellular hypersensitivity to Candidin, PPD, and streptokinase-streptodornase in one transfer factor-treated patient with disseminated candiloma acuminatus (Arala-Chaves et al, 1977). This observation, therefore, lends preliminary support to the view that the LMMI-test developed in the rhesus monkey can probably be reliably adapted to monitor cellular hypersensitivity in transfer factor-

treated human subjects.

The onset of LMMI-reactivity in the recipient animals showed 2 different patterns. It occurred "early" (2-6 days) or "late" (7-14 days) (Section 4.2.2: figs. 22-26). These patterns seemed to be related to 3 factors. Table 33 shows that both the early and the late onset of reactivity to KLH PPD or TB occurred in monkeys converting after treatment with KLH-immune or FCA-immune transfer factor. Only an early onset of reactivity to HBsAg occurred in animals, which converted after treatment with HBsAg-immune transfer factor. Thus, the different patterns of onset of cellular hypersensitivity may be related to the type of test antigen or the antigen-sensitivity of the injected transfer factor. This table also shows that, among all the animals converting after treatment with comparably low (≅4.0 to 5.3 x 10^9 lymphocytes) or high doses of transfer factor (≅8.0 to 9.0 x 10^9 lymphocytes), the majority of the LMMI-tests converted late after treatment with the lower dose, but converted early after the higher dose. Thus, this indicated that the different patterns of the onset of cellular hypersensitivity might also be related to the dosage of the injected transfer factor. The table also shows that, regardless of the dose of dialysable transfer factor, among the early responders, the majority of the LMMI-tests (67%) converted after injection of monkeys with the water-dialysed and not the vacuum-dialysed (50%) transfer factor. In contrast, amongst the late responders, the majority of the LMMI-tests converted after the vacuum-dialysed (50%) and not the water-dialysed

Table 33. The different patterns of the onset of LMMI-reactivity in rhesus monkeys converting after administration of immune dialysable homologous transfer factor.[a]

Test antigens	Onset of LMMI-reactivity (MI < 0.8)[b]				
	"Early" (2 - 6 days)		"Late" (7 - 14 days)		
(100 µg/ml)*	Rh-TF_{WD}	Rh-TF_{VD}	Rh-TF_{WD}	Rh-TF_{VD}	
	Injected dose of Rh-TF_D ≡ 4-5.3x10^9 lymphocytes				
KLH	1		1	2	
PPD		1	1	1	6/10
TB	1	4/10 (40%)		1	(60%)
HBsAg	1				
	Injected dose of Rh-TF_D ≡ 8-9x10^9 lymphocytes				
KLH	1	1			
PPD		1	6/7		1/7
TB		1	(86%)		(14%)
HBsAg	2		1		
Total:	6/9 (67%)	4/8 (50%)	3/9 (33%)	4/8 (50%)	

[a] From section 4.2.2: figures 23-26.

[b] Rh-TF_{WD}, water-dialysed rhesus transfer factor; Rh-TF_{VD}, vacuum-dialysed rhesus transfer factor; Rh-TF_D, dialysable rhesus transfer factor.

*LMMI test for 1 monkey treated with HBsAg-immune Rh-TF_{WD} (Fig. 26a) was carried out with 1/25 dilution of HBsAg.

transfer factor. Thus, it was indicated that the different patterns of the onset of cellular hypersensitivity, in transfer-treated monkeys, might be related not only to the type of test antigen, but also to the dose and the method of dialysis of rhesus transfer factor.

The duration of the acquired LMMI-reactivity to KLH, PPD, TB and to HBsAg also varied in these experiments from one transfer factor to recipient monkey to another. The acquired reactivity was seen on one occasion ("transient") or repeatedly for 2 to 68 days after the onset of reactivity ("lasting") (Section 4.2.2). These patterns were influenced by 3 factors. Firstly, table 34 shows that both transient and lasting reactivities were seen for KLH, PPD, and TB in monkeys converting after administration of KLH-immune or FCA-immune transfer factor. Only a lasting reactivity occurred for HBsAg in animals treated with HBsAg-immune transfer factor. Thus, the duration of the acquired reactivity, like the time of onset of reactivity (Table 33), may be related to the type of test antigen or the antigen-sensitivity of the injected transfer factor. Secondly, among all the animals converting after administration of comparably low dose of transfer factor ($\simeq$4.0 to 5.3 x 10^9 lymphocytes), the majority of the tests showed lasting reactivity (56%), and not transient reactivity (46%). Among those receiving comparably high doses of transfer factor ($\simeq$8-9 x 10^9 lymphocytes), one half of all tests (50%) showed either transient or lasting reactivity to all antigens used. Thus, the high dose of the

Table 34. Duration of the acquired LMMI-reactivity in rhesus monkeys converting after administration of immune dialysable homologous transfer factor.[a]

Test antigens (100 µg/ml)*	Duration of LMMI-reactivity (MI < 0.8)[b] "Transient" (1 day) Rh-TF_{WD}	Rh-TF_{VD}	"Lasting" (2 - 63 days) Rh-TF_{WD}	Rh-TF_{VD}
	Injected dose of Rh-TF_D ≡ 4-5.3x10^9 lymphocytes			
KLH	1	2	1	
PPD		1		1
TB		1	1	
HBsAg			1	2
	5/11 (46%)			6/11 (56%)
	Injected dose of Rh-TF_D ≡ 8-9x10^9 lymphocytes			
KLH		1	1	
PPD		1	1	
TB		1	1	
HBsAg	ND			ND
	3/6 (50%)			3/6 (50%)
Total:	1/7 (14%)	7/10 (70%)	6/7 (86%)	3/10 (30%)

[a] From section 4.2.2: figs. 23-26.

[b] Rh-TF_{WD}, water-dialysed rhesus transfer factor; Rh-TF_{VD}, vacuum dialysed rhesus transfer factor; Rh-TF_D, dialysable rhesus transfer factor.

*LMMI-test for 1 monkey treated with HBsAg-immune Rh-TF_{WD} (Fig. 26a) was carried out with 1/25 dilution of HBsAg.

ND, not done.

injected transfer factor, unlike the low dose, did not seem to favour either the transient or the lasting pattern of the duration of the acquired cellular hypersensitivity in the recipient monkeys. Thirdly, amongst the responders with "transient" reactivity, the majority of the tests (70%) converted after injection of the vacuum-dialysed and not the water-dialysed transfer factor (14%). Conversely, among the responders with lasting reactivity, the majority of the tests (86%) converted after the water-dialysed, and not the vacuum-dialysed, transfer factor (30%). Therefore, the different patterns of duration of the acquired cellular hypersensitivity in transfer factor-treated animals seemed to be influenced not only by the test antigen, but also by the dose and type of dialysis of rhesus transfer factor. The acquired cellular hypersensitivity in the recipient monkeys seemed to be due to adoptive sensitisation by dialysable transfer factor. LMMI tests carried out before the administration of control or immune transfer factor revealed significant non-reactivity to KLH, PPD, TB or HBsAg (Section 4.2.2: figs. 19-26). This demonstrated that all the recipient monkeys were previously naive for the antigens used. Skin tests did not seem to significantly influence inhibition of the mixed cell migration because: (1) in all the animals which converted after treatment with the immune transfer factor preparations, the acquired LMMI-reactivity appeared before the first skin test with 30-50μg KLH, PPD or HBsAg, and (2) none of the recipients of the control transfer factor preparations showed LMMI-reactivity after

the first or subsequent skin tests. Even in the one animal, which converted after treatment with control transfer factor, the acquired LMMI-reactivity to KLH appeared 8 days before the first skin test (Section 4.2.2: fig. 19a). Therefore, dialysable rhesus transfer factor probably adoptively sensitised the recipient animals conferring cellular hypersensitivity LMMI-reactivity to an antigen.

The specificity of the *in vivo* activity of transfer factor towards some antigens has for long been a controversial issue in man and animals (Section 1.5). Based on the definitions of donor-specificity and nonspecificity (Section 8.1.2), in the experiments reported here the *in vivo* activity of rhesus transfer factor was donor-specific for KLH, PPD, TB and HBsAg. This was unequivocally indicated by the significant acquisition of LMMI-reactivity to specific antigens in animals treated with the immune and not the control normal or unrelated dialysable transfer factor (Section 4.2.3: table 19). In support of this work, Trepo and Prince (1976) suggested donor-specific transfer of leucocyte migration inhibition for PPD or HBsAg by dialysable transfer factor in the human to chimpanzee system (Section 1.4.2: table 10). Others have reported donor-specific transfer of MIF activity, indicated by direct or indirect inhibition of guinea pig macrophage migration by *Leishmania enrietti* in the guinea pig to guinea pig system (Clinton and Magoc, 1976), or by candida and streptokinase-streptodornase in the man to man system (Kirkpatrick and Smith, 1976). Hence, if dialysable rhesus

transfer factor acts without further antigen upon naive animals, it may do so by specifically "sensitising" a cell population concerned with initiation and/or production of MIF/LIF lymphokines.

Therefore, the LMMI test served as an appropriate *in vitro* technique for qualitatively following specific cellular hypersensitivity, not only in the actively immunised, but also in transfer factor-treated monkeys. In addition, there were indications that this test could also quantitatively measure responses in this species (Section 4.1.4: fig 15; section 4.2.4: fig 27).

8.3 Cellular hypersensitivity and the mitogenic or blastogenic factor (MF): the LT test as an *in vitro* indicator of cell-mediated immunity.

Several *in vitro* lymphocyte transformation tests, using peripheral blood or tissue lymphocytes, have been described in man and animals to assay cellular responsiveness specifically to an antigen or non-specifically to a mitogen like phytohaemagglutinin (Ling, 1968). Lymphocyte transformation is indicated in this study by significant *in vitro* incorporation of tritiated thymidine by lymphocytes stimulated by antigens (KLH, PPD, TB, HBsAg) or a mitogen (PHA).

Here, cellular hypersensitivity is defined as the *in vitro* reactivity induced by antigen in the lymphocyte transformation

test, an *in vitro* correlate of CMI. Experiments in guinea pigs and man showed that lymphocyte transformation can be induced by an antigen and it was suggested that this phenomenon depended upon the presence of delayed hypersensitivity specific for the immunising antigen (Mills, 1966; Oppenheim, 1968). Some investigators have even reported that the *in vitro* lymphocyte transformation may be a more sensitive technique for the detection of the *in vivo* delayed hypersensitivity than the skin test (Oppenheim, 1968).

Stimulation of lymphocyte transformation *in vitro* by PHA probably reflects the immunocompetence of T-cells *in vivo* (Reisenbuk and Ioks, 1974). In 1964, Robbins reported transformation of peripheral blood lymphocytes by PHA in man, and subsequently this was extended to laboratory animals like, monkeys, rabbits, guinea pigs, hamsters, rats and mice (Knight *et al*, 1965). Thus, *in vitro* transformation tests have traditionally been used to monitor, not only the specific cellular hypersensitivity or CMI to an antigen, but also the non-specific general cellular responsiveness to PHA. Both antigen-induced and PHA-induced transformation responses are thought to be mediated by soluble substances or lymphokines called the mitogenic or blastogenic factor(s).

In 1969, Dumonde *et al* showed that, sensitised lymph node cells from immunised guinea pigs, could be stimulated to produce lymphokine *in vitro* by incubation with a specific antigen, and not with an unrelated antigen. This lymphokine

was called mitogenic factor (MF) and it was suggested as a mediator of the *in vitro* transformation of unsensitised lymphocytes. In 1971, Wolstencroft reported that normal lymphocytes can also be activated with PHA to elaborate a mitogenic factor. It is unlikely that the cell-free activities, attributed to MF, MIF and skin reactive factors are attributable to the same molecule (Bray *et al*, 1976). It has been suggested that the mitogenic and other lymphokines may be involved in the expression and regulation of cell-mediated immunity. (Wolstencroft and Dumonde, 1970). Furthermore, it is likely that antigen and mitogen-induced *in vitro* lymphocyte transformation may be mediated by a mitogenic factor.

Lymphocytes from the rhesus monkey can also be stimulated by PHA or an antigen to undergo transformation *in vitro* (Ling, 1968). In 1970, Phillips *et al* showed that rhesus lymph node or spleen cells, separately or mixed, can specifically respond to the sensitising antigens in rhesus monkeys sensitised with FCA containing malaria parasite-antigens or sheep red blood cells. They reported that this transformation correlated with the delayed hypersensitivity. In 1971 and 1972, Mackler *et al* found antigen-induced transformation in delayed hypersensitivity in a monkey immunised with trinitrophenyl-KLH suspended in FCA and PHA-induced transformation in normal monkeys. Thus previous studies have shown that, in the rhesus monkey, lymphocytes can also transform *in vitro* by antigen or PHA to reflect specific delayed hypersensitivity or non-specific cellular responsiveness

respectively.

Against the above background, in these experiments a whole blood lymphocyte transformation technique (Section 2.7.2) was used to monitor cellular hypersensitivity to antigens KLH, PPD, TB or HBsAg or responsiveness to a mitogen PHA in normal, immunised and transfer factor-treated rhesus monkeys. A whole blood technique rather than a purified lymphocyte technique, was used because the assay procedure is simpler, less expensive, more rapid and uses very little blood. Transformation was quantified by liquid scintillation counting of tritiated thymidine uptake. Such radioactive quantification, rather than the other methods like morphological or autoradiographic evaluation, was used because it has been reported that, in man or rhesus monkey, tritiated thymidine uptake is more reproducible, simpler and quicker than the others (Ling, 1968; Schellekens and Eijsvoogel, 1968; Mackler et al, 1972). The LT-results obtained are shown above (Section 5) and the conversion rates for the acquisition of lymphocyte transformation and its specificity to KLH, PPD and/or TB and HBsAg together with its nonspecific stimulation by PHA are discussed below on the basis of the criteria used to indicate significant LT-reactivity (Sections 8.3.1 - 8.3.5).

8.3.1 Criteria for LT-reactivity to antigen or mitogen.

In these experiments a stimulation index of above 2.0 (Section 2.7.2) was used throughout as a significant indication of LT-

reactivity to antigen or PHA. This index was justified for the following reasons. Firstly, at this cut-off point the majority of the normal unsensitised rhesus monkeys showed no response to 1 to 100 µg/ml KLH, PPD, TB or HBsAg (Section 5.1.1, figs. 29a-d: SI<2.0), whilst majority of the actively immunised animals responded to 5-50 µg/ml of these antigens (Section 5.1.1, figs. 30a-d: SI>2.0). Secondly, an index of more than around 2.0 has been used by others, in man and non-human primates, to indicate presence of cellular hypersensitivity towards number of antigens (Junge et al, 1970; Baram et al, 1971; Maini et al, 1974; Steele et al, 1976). On the basis of this cut-off point, to validate the acquisition of cellular hypersensitivity to an antigen, the possible interference with LT-reactivity of previous sensitisation with an antigen, cross-reactivity of stimulants and/or potential mitogenicity of some antigens had to be considered.

It has been reported that LT-reactivity can occur to KLH in normal control human subjects (Curtis et al, 1970), or to PPD in normal control rhesus monkeys (Baram et al, 1971). Similarly, in the experiments reported here, some normal control rhesus monkeys showed positive LT-responses to KLH, PPD and HBsAg, but not to TB, at 2 or more concentrations (Section 5.1.1: figs. 29a-d). Such responses can be indicative of previous sensitisation of the animals with KLH, PPD or HBsAg, and/or of cross reactivity of these antigens with others, and/or potential mitogenicity of these antigens. KLH is isolated from a crustacean (Section 2.10.1) and it is unlikely that rhesus monkeys would have previously been sensitised.

In man KLH may be mitogenic because pre-immunisation studies in LT-tests have revealed dose-dependent *in vitro* response to this antigen (Curtis *et al*, 1970). KLH might also cross-react with some cell products in this test. Thus, since an increasing number of normal monkeys convert with the increasing *in vitro* concentration of KLH (Section 5.1.1: fig. 29a) it is likely that the LT-reactivity to this antigen in the normal animals, as in man (Curtis *et al*, 1970), resulted from the mitogenic activity of KLH.

In contrast to KLH, tuberculin PPD (Section 2.10.2) is a ubiquitous antigen and, therefore, normal monkeys could have easily experienced natural sensitisation with this antigen. In any event, sensitisation may have occurred because all the rhesus monkeys used had previously been screened for tuberculosis by skin tests with PPD by the importers. These considerations may explain the PPD-stimulated LT response seen in normal animals (Section 5.1.1: fig. 29b).

HBsAg is associated with the hepatitis B virus (Section 2.10.4) and can infect man and monkey (Zuckerman, 1975b; Zuckerman *et al*, 1975). Whether HBsAg cross reacts with other antigens or not is not clear, but this is a possibility because of its complex composition of lipids, polypeptides and some carbohydrates (Howard, 1975). Since there seemed to be a slight indication of a dose-response pattern in the normal animals (Section 5.1.1: fig. 29d), HBsAg may have been mitogenic, but to a lesser extent

than KLH (Section 5.1.1: figs. 29a cf 29d). Thus, the LT-test reactivity in normal animals was probably due to non-specific cross-reactivity and/or mitogenicity of KLH or HBsAg, and not due to true acquisition of cellular hypersensitivity to these antigens (Section 5.1.1: figs. 29a, d). In contrast, the responses to PPD probably resulted from previous sensitisation (Section 5.1.1: fig. 29b). When all antigens were taken together only 18 out of 243 tests (7%) were positive. As a result of such minimal non-specific reactivity in normal monkeys, the acquisition of LT-reactivity to an antigen in the actively immunised monkeys was considered significant.

8.3.2 Acquisition and antigen-specificity of LT-reactivity in immunised monkeys: active sensitisation (Table 35).

Adopting the above criteria for in vitro LT-reactivity (Section 8.3.1), in the immunised animals the conversion rates of lymphocyte transformation tests were 67%, 83%, 88% and 100% for 5-50 µg/ml TB, HBsAg, PPD and KLH respectively making a cumulative mean conversion rate of 83% (Section 5.1.3: table 21). Compared with the control normal animals, such conversion rates were highly significant ($P < 0.01$). A conversion rate of 22 out of 39 positive tests (57%) was observed by Baram et al (1971) for PPD-induced transformation in 22 BCG-immunised rhesus monkeys. A conversion rate of 19 out of 23 tests (83%) occurred in KLH-induced transformation seen by Curtis et al (1970) in 14 normal human subjects immunised with KLH. Thus, as in man, in this species the lymphocyte transformation test used, was a reliable index of cellular hypersensitivity to KLH, PPD, TB

and HBsAg. The soluble tuberculin PPD antigen seemed to be a more effective stimulant than the particulate TB antigen, since the former had a higher conversion rate (88%) than the latter (67%).

The acquisition of cellular hypersensitivity to KLH, PPD, TB and HBsAg was time-dependent. Sequential *in vitro* transformation tests were carried out before and after immunisation (Section 2.1: figs. 1b, d). Results showed that, in all but one animal tested with KLH (Section 5.1.2: fig. 31c), LT-reactivity to 10 µg/ml KLH, PPD, TB or HBsAg was absent before immunisation. However, after immunisation it appeared in the majority of cases 1 to 4 weeks later and lasted for 1 to 5 weeks (Section 5.1.2: figs. 31a, b, d, 32-34). Such time-dependent acquisition of cellular hypersensitivity, as indicated by LT-reactivity agree with similar observations of Baram *et al* (1971) for PPD in actively sensitised rhesus monkeys, and with those of Curtis *et al* (1970) for KLH in actively sensitised normal humans.

Skin tests with an antigen did not seem to produce positive *in vitro* transformation. In the majority of animals immunised with FCA with or without KLH, lymphocyte transformation tests converted to 10 µg/ml KLH, PPD or TB before the first skin test carried out with 30 µg KLH or 10-30 µg PPD (Section 5.1.2: figs. 31-33). However, the effect of the skin test on lymphocyte transformation with HBsAg, in animals immunised with FCA containing HBsAg, remained unknown, since significant transformation was not seen before the first skin test with 10-30 µg HBsAg (Section 5.1.2: fig. 34).

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In the actively sensitised animals, the mean intensity of the acquired maximum LT-reactivity varied from one antigen to another, and from one concentration to another (Section 5.1.1: figs. 30a-d). The acquired transformation response was always highest for KLH with mean stimulation indices of 38, 31 and 43 for 5, 10, 50 µg/ml KLH respectively (Section 5.1.1: fig. 30a). Baram et al (1971) showed that the mean transformation response to 80 µg/ml PPD, in all the tests carried out in their actively sensitised monkeys, was higher (Mean SI = 22) than that observed in these experiments (Section 5.1.1: fig. 30b, mean SI = 7.0, 8.0 and 7.0 for 5, 10 and 50 µg/ml PPD respectively). This lower degree of in vitro transformation response may be derived from the use of lower concentrations of PPD from a different source, and of a different transformation test system. A whole blood technique (Section 2.7.2) was used here, whilst Baram et al (1971) used cotton wool-filtered enriched lymphocyte cultures. The higher red blood cell and other cell contaminants in the whole blood transformation cultures used here may have been responsible for the lower PPD-stimulated response. It is unlikely that the presence of only red blood cells can affect the in vitro response of lymphocytes to a stimulant (Junge et al, 1970). Factors probably contributing more effectively to such reduced in vitro responses, to such PPD-stimulated whole blood cultures, are the larger proportions of granulocytes, monocytes and platelets present in a whole blood lymphocyte culture.

The cellular hypersensitivity induced by active immunisation of rhesus monkeys and indicated by LT-reactivity (SI > 2.0) was antigen-specific (Section 5.1.3: table 21). When the total number of

positive LT-tests carried out with 5-50 µg/ml antigen in normal control animals were compared with those in the immunised monkeys, there was a highly significant difference between the rates of conversion for KLH, PPD, TB and HBsAg taken separately or altogether ($P < 0.001$). Thus, the induced cellular hypersensitivity was considered to be antigen-specific. This was supported by tests carried out in the immunised animals using antigens unrelated to the sensitising antigens (Table 35). These tests were only carried out in a few representative monkeys, and indicated that the whole blood *in vitro* lymphocyte transformation used in these experiments seemed to be specific for PPD, TB and HBsAg (Table 35). In the 3 monkeys shown in this table, only 1 out of 10 tests non-specifically converted to an unrelated antigen KLH at 50 µg/ml, whilst 16 out of 24 (67%) tests converted to one or more sensitising antigens at various concentrations. Thus, rhesus monkeys can be actively sensitised with FCA, with or without KLH or HBsAg. Their lymphocytes can, under the conditions of these experiments, specifically respond to the sensitising antigen(s) by the *in vitro* whole blood transformation technique.

8.3.3 PHA-induced *in vitro* lymphocyte transformation in normal monkeys.

Lymphocyte transformation to PHA has been regarded as a measure of cellular responsiveness reflecting the integrity and immunocompetence of T-lymphocytes (Reisenbuk and Ioks, 1974). In these experiments 71 out of 75 of all tests (95%) carried out in normal monkeys showed significant responsiveness to 5-300 µg/ml PHA with

Table 35. Antigen-specificity of LT-responses three weeks after immunisation of representative rhesus monkeys with FCA with or without HBsAg.[a]

Conc. of test antigen (µg/ml)		Stimulation indices (SI) in monkeys immunised with[b] FCA *(H32)	FCA *(H33a)	FCA/HBsAg *(H42)
KLH:	5	1.2	1.5	1.4
	10	1.8	1.6	ND
	50	1.8	2.1	ND
	100	2.0	1.7	0.9
PPD:	5	2.3	2.1	1.7
	10	3.8	2.4	2.0
	50	3.5	2.3	1.1
	100	3.4	1.7	1.2
TB:	5	2.4	1.0	ND
	10	2.6	1.2	ND
	50	5.2	2.1	ND
	100	5.5	2.7	ND
HBsAg:	5	ND	ND	2.8
	10	ND	ND	3.0
	50	ND	ND	2.2
	100	ND	ND	0.6

[a]LT-tests carried out as described before (Section 2.7.2), except for monkey H42 where 0.03 ml blood, rather than 0.1 ml, was used per culture.

[b]SI > 2.0 underlined and significant (Section 2.7.2).

ND, not done; *Code of monkeys.

a good dose-response (Section 5.1.6: fig. 37). Such reactivity established the whole blood transformation test in these experiments as a reliable measure of *in vitro* cellular reactivity. This confirmed the findings of others (Mackler *et al*, 1972), who showed PHA-induced blastogenesis using purified rhesus peripheral lymphocytes in culture. However, Mackler *et al* (1972) could only demonstrate a significant dose-response in "roller" cultures and not stationary cultures, whereas here a dose-response was seen using stationary cultures. When the *in vitro* concentrations of PHA used were above the optimum, the degree of the mean response fell (Section 5.1.6: fig. 37). This probably indicated accumulation of potentially toxic metabolic products affecting the cells undergoing blastogenesis (Mackler *et al*, 1972), induction of suppressor factors and/or toxicity of PHA.

The wide range of reactivity to PHA at all concentrations in individual animals (Section 5.1.6: fig. 37) may result from the following. Different proportions of different contaminants like red blood cells and non-transforming leucocytes and/or the different immuno-competence of lymphocytes. These hypotheses can only be proved by the use of purified T- and B-lymphocytes in the test in much the same way as that used by Clot *et al* (1975). They reported that, in man, *in vitro* lymphocyte transformation by PHA can occur with only 10% T-cells added to B-cells, but not with B-cells alone. Thus, the experiments reported here show that, PHA-stimulated stationary whole blood cultures can be used to examine non-specific responsiveness of normal lymphocytes, presumed to be T-lymphocytes. As a result, such lymphocyte transformation tests with PHA were used to determine the

general state of cellular responsiveness in actively immunised monkeys.

8.3.4 Depression of PHA-induced lymphocyte transformation in immunised monkeys.

Using the optimum concentration of PHA, *in vitro* lymphocyte transformation tests were carried out with immune rhesus lymphocytes (Section 5.1.7: fig. 38). There was evidence for depression of mean reactivity 1 to 5 weeks after immunisation compared with the mean maximum reactivity before immunisation (Section 5.1.8: fig. 39). Such observations are, in contrast to the findings of others, who did not observe depression of lymphocyte transformation to PHA in KLH-sensitised normal human subjects (Curtis *et al*, 1970). Antibodies to PHA can inhibit the *in vitro* blastogenic activity of PHA (Ling, 1968). Thus, it is likely that in the immunised monkeys, the depression of the *in vitro* transformation to PHA might have been due to inhibition of blastogenesis by cross-reacting antibodies reacting with PHA *in vitro* and thereby possibly preventing good contact with cells. The depression effect could also have been due to a reduced efficiency in the production of mitogenic factor(s), probably mediating the PHA-induced *in vitro* lymphocyte transformation (Section 8.3). The last and simplest possibility is that the main population of the responsive cells are not in circulation.

The whole blood lymphocyte transformation test can be used to monitor cellular hypersensitivity to an antigen in actively sensitised monkeys (Section 8.3.2). It can also be used to detect general

cellular responsiveness to PHA in normal and immunised monkeys (Sections 8.3.3 and 8.3.4). As a result, this test was used to monitor antigen- and PHA-induced responses in transfer factor-treated monkeys.

8.3.5 Acquisition and non-specificity of LT-reactivity to antigen in transfer factor-treated monkeys.

The *in vitro* lymphocyte transformation test with KLH, PPD and HBsAg showed cumulative conversion rates of 5 out of 33 tests (15%) 2 to 27 days after treatment of recipients with immune dialysable transfer factor (Section 5.2.3: table 24). In contrast, Steele *et al* (1976) found a cumulative rate of conversion of 26 out of 40 tests (65%) to a battery of antigens for the lymphocyte transformation test after treatment of 9 animals of 3 species of other non-human primates with immune human dialysable transfer factor. Klesius and Fudenberg (1977) have reported a cumulative conversion rate of 28 out of 34 (80%) of lymphocyte transformation tests to PPD and Eimoria bovis in 17 recipient cattle treated with immune homologous transfer factor. Comparably high cumulative LT-conversion rates have been reported to some fungal antigens in transfer factor-treated patients (Section 1.2.1.1: table 3, cumulative conversion rate = 77%). However, in 1975, Zanelli and Adler (Section 1.4.1: table 9) reported no LT-conversion to PPD in 5 rhesus monkeys treated with immune rhesus transfer factor, whilst others reported conversion rates of only 10% to 40% for bacterial, viral, neoplastic tissue and other antigens in transfer factor-treated patients (Sections 1.2.1.2 - 1.2.1.3: tables 3-4; sections 1.2.2 - 1.2.3: tables 5-6). Thus, the species of the donor

and the recipient, the method of preparation of transfer factor, and the state of health of the recipients probably affect the conversion rate of the lymphocyte transformation test. The low rate of LT-conversion in transfer factor-treated monkeys reported here may have arisen from inhibition of blastogenesis by blocking factor(s). For example auto-antibodies or some antigen-antibody complexes could have covered potentially reactive lymphocytes, thereby preventing either antigen recognition and/or production of blastogenic factor. Alternatively, the LT-test was simply not adequate in detecting adoptive sensitisation of this species with dialysable transfer factor (Zanelli and Adler, 1975).

Despite the low cumulative rates of conversion, a higher individual LT-rate of conversion of 4 out of 9 tests (44%) was recorded for KLH in animals treated with immune transfer factor (Section 5.2.3: table 24). This response to KLH with the immune transfer factor did not seem to be related to the specificity of the donor(s), since a conversion rate to KLH of 2 out of 15 tests (13%) was also found with the control transfer factor (Section 5.2.3: table 24, KLH: $P > 0.2$). Therefore, some LT-tests converted to KLH after injection of rhesus monkeys with dialysable transfer factor, but the conversions were apparently non-specific. The potential mitogenicity of KLH probably influenced the LT-conversions in the recipient animals, since the rate of LT-reactivity to KLH in normal rhesus monkeys (Section 5.1.3: table 21) was high (16%) compared with the other antigens ($\leq$ 2%). Thus the acquisition of transformation reactivity to KLH (Section 5.2.3: table 24) may not have been due to adoptive sensitisation with transfer factor, but due to the potential

mitogenicity of KLH. Thus, the LT-test here does not seem to be an adequate *in vitro* test for following the cellular hypersensitivity responses to KLH in transfer factor-treated rhesus monkeys.

8.3.6 Elevation of PHA-induced *in vitro* lymphocyte transformation responses in transfer factor-treated monkeys.

The optimum dose of PHA in lymphocyte transformation tests in transfer factor-treated rhesus monkeys was the same as that for normal or immunised monkeys (100 µg/ml) (Section 5.1.6: fig. 37; section 5.2.7: fig. 38; section 5.2.6: fig. 44). Using this concentration of PHA an elevation of the mean *in vitro* transformation reactivity was found 2-17 days after injecting rhesus monkeys with dialysable homologous transfer factor (Section 3.2.7: fig. 45). Increased lymphocyte transformation responses to this mitogen have been observed by others in transfer factor-treated patients with an immunodeficiency disorder (Amman *et al*, 1974; Valdimarsson *et al*, 1974), or in dogs injected with canine transfer factor (Shifrine *et al*, 1976). Such results in rhesus monkeys may reflect a non-specific "adjuvant-like" activity present in dialysable transfer factor (Valdimarsson *et al*, 1974). Assuming that a mitogenic factor mediates the PHA-stimulated *in vitro* response, then the transfer factor may have stimulated a population of cells to elaborate more of this lymphokine. Alternatively more T-cells may have been recruited into the pool (Wybran *et al*, 1973), thereby increasing the *in vitro* reactivity to PHA. It is likely that T-cells rather than B-cells were the ones influenced by transfer factor, since in man it has been shown that PHA-stimulated *in vitro* transformation requires T-cells (Clot *et al*, 1975).

In the experiments here the LT-test did not emerge as a sensitive *in vitro* technique for monitoring cellular hypersensitivity to antigens in rhesus monkeys treated with transfer factor (Section 8.3.5). It could, however, detect the apparent non-specific "adjuvant-like" activity of transfer factor *in vivo*, indicated by the elevation of PHA-induced lymphocyte transformation (See above).

8.4. Association or dissociation of the different CMI-tests in immunised or transfer factor-treated monkeys (Table 36).

It has been reported that cell migration inhibition tests or lymphocyte transformation tests are *in vitro* correlates of the *in vivo* delayed hypersensitivity skin test to an antigen in sensitised man or animals (David *et al*, 1964b; Ling, 1968; Thor *et al*, 1968; Rajapakse and Glynn, 1970; Baram *et al*, 1971; Marsman *et al*, 1972). In support of these observations, table 36 shows that when all the LMMI- and DH-skin tests, LT- and DH-skin tests or LT- and LMMI-tests were taken together, with all the antigens, most of the paired tests (LMMI/DH, LT/DH, LT/LMMI) converted in the immunised monkeys (66% - 85%), but not in transfer factor-treated animals (0% - 33%). Comparison of such double-conversion of tests in immunised animals, with that in animals treated with the immune transfer factor, showed that the likelihood of these conversions was significantly greater in the former than in the latter (Table 36: $P < 0.005$). Therefore, it can be said that in the immunised monkeys the *in vitro* cellular hypersensitivity tests "associated" with each other (i.e. LT/LMMI) and with the *in vivo* delayed hypersensitivity (i.e. LMMI/DH, LT/DH).

Table 36. Relationship between the different tests (DH, LMMI, LT) of cell-mediated immunity in immunised and transfer factor-treated monkeys.

Rhesus injection with	Cumulative no. of double-conversion of tests/all tests					
	LMMI & DH[a]		LT & DH[b]		LT & LMMI[c]	
FCA ± KLH or HBsAg	41/63	(66%)	34/40	(85%)	8/11	(73%)
Immune Rh-TF_D	16/48	(33%)	0/22		1/11	(9%)
P for x^2	< 0.005		< 0.005		< 0.005	

[a]From sections 4.1.5, table 18 and 4.2.5, table 20.

[b]From sections 5.1.4, table 22 and 5.2.4, table 25.

[c]From sections 5.1.5, table 23 and 5.2.5, table 26.

In contrast, in transfer factor-treated monkeys double conversions mainly occurred in the LMMI/DH pair of tests. Compared to the immunised animals these conversions were not significant. Thus, the in vitro cellular hypersensitivity LMMI-test and the in vivo delayed hypersensitivity skin test (LMMI/DH) can be said to be "dissociated" from each other. This observation is in contrast to other reports suggesting an association of DH-skin test with a cell migration inhibition test in diseased patients injected with transfer factor (Section 1.2: tables 3 and 6) (Spitler et al, 1975; Arala-Chaves et al, 1977). Thus, such differences in the relationship of these tests might be indicative of species differences in adoptive sensitisation of transfer factor recipients for preferential lymphokine production, or different sensitivity of these tests in man and monkey.

In the immunised monkeys here, the association of the various test reactivities may indicate that the DH-skin test, probably indicative of the skin reactive factor(s) (Section 8.1); the in vitro LT-test, probably indicative of blastogenic factor(s) (Section 8.3) and the in vitro LMMI-test probably indicative of the MIF/LIF (Section 8.2) are comparably sensitive as assay systems for such lymphokines. It is also possible that these tests are indicative of different manifestations of the same lymphokine molecule. However, in the guinea pig, parallel bioassays using the three of these lymphokines against a standard preparation have indicated that they are heterogeneous (Bray et al, 1976). It is, therefore, likely that the so called skin reactive, blastogenic and MIF/LIF lymphokines are also heterogeneous in the rhesus monkey. By inference, therefore,

the dissociation of the LMMI- and DH-skin test reactivities in transfer factor-treated monkeys might be indicative of preferential lymphokine production.

Hence, table 36 indicates that in the immunised monkeys, the LMMI- and LT-conversion of tests associated with each other and with the DH-skin test conversion. In contrast, in transfer factor-treated monkeys, only the LMMI- and DH-skin tests, and not the others (LT/LMMI, LT/DH), seemed to convert together, but the LMMI-conversion apparently dissociated from the DH-skin test conversions.

8.5 Effects of different methods of preparation of rhesus dialysable transfer factor upon its *in vivo* activity.

For the *in vivo* study, dialysable human transfer factor has traditionally been prepared from heparinised peripheral blood leucocytes. Such cells are treated with deoxyribonuclease and magnesium sulphate, freeze-thawed seven to ten times and the extract of completely disrupted leucocytes is exhaustively dialysed into equal or 50 times greater volume of water (Lawrence and Al-Askari, 1971). Another method of preparation of human transfer factor, originally described by Arala-Chaves *et al* (1967) and later modified by Burger *et al* (1974), involves dialysis of blood leucocyte extracts into a vacuum ("ultrafiltration"). In all these methods the leucocyte dialysates containing transfer factor are subsequently freeze-dried, reconstituted in saline or water, and their biological activity tested by their ability to convert delayed hypersensitivity skin tests with different antigens. In the experiments presented

here, the dialysable rhesus transfer factor for the in vivo study was prepared by water-dialysis and vacuum-dialysis of leucocyte lysates, obtained from treated pooled lymph node and spleen cells (Sections 2.5.2, 2.5.4, 2.5.5, 2.5.7). The in vivo biological effect(s) of these preparations were monitored not only by the in vivo delayed hypersensitivity skin tests (Section 2.6), but also by the in vitro mixed leucocyte-macrophage migration and lymphocyte transformation tests (Section 2.7).

When comparable doses of dialysable rhesus transfer factor were administered into naive rhesus recipients, the water-dialysed transfer factor, prepared from actively immunised donor(s) (Section 3.1.2: figs. 5b, 6, plates 10a-b), did not transfer delayed hypersensitivity skin test reactions to KLH, PPD or HBsAg (Section 3.2.4: fig. 8). Similar observations in this species were reported by Zanelli and Adler (1975). However, in most instances reported by others in the human to human, human to rhesus, or rhesus to rhesus system, the water-dialysed transfer factor did transfer skin test reactivity to an antigen (Lawrence et al, 1963; Maddison et al, 1972; Gallin and Kirkpatrick, 1974; Littman et al, 1978). In this work, the absence of systemic transfer of skin test reactivity to an antigen with water-dialysed transfer factor may be because lower doses of KLH and PPD were used for skin testing recipients of the water-dialysed transfer factor, rather than the recipients of the vacuum-dialysed transfer factor. Assuming this was not the case, then other factors may have played a role. The preparations may have not contained component(s) responsible for transferring delayed hypersensitivity skin test reactivity. These

component(s) were somehow inactivated either during the long and laborious preparation procedures or after injection by the recipients. The skin test conversion activity may have been present, but it was so weak that the recipient animals did not respond strongly enough for the skin tests to pick up the delayed hypersensitivity responses. Finally the skin tests may have been carried out at times when transferred delayed hypersensitivity had not yet developed or had already disappeared. Zanelli and Adler (1975) did not, at first, succeed in systemically transferring skin test reactivity to PPD, from rhesus to rhesus or rhesus to cynomolgus monkeys, with water-dialysed transfer factor prepared by the standard method of Lawrence and Al-Askari (1971). However, they succeeded after slightly modifying the preparative method to include gentle rupturing of rhesus blood leucocytes with mechanical shearing and subsequent dialysis into tissue culture medium. On the other hand, Gallin and Kirkpatrick (1974) modified the standard technique of dialysable transfer factor preparation by separating the human blood leucocytes by leukophoresis to prepare a water-dialysed transfer factor for the transfer of delayed hypersensitivity skin test reactions to mumps and other antigens in the rhesus monkey system. It is possible that similar or other small modifications in the preparation of water-dialysed transfer factor could have helped to obtain skin test-active dialysable rhesus transfer factor in these experiments.

The majority of the transfer factor was prepared by vacuum-dialysis (Section 2.5.7). Such vacuum-dialysed rhesus transfer factor, at one or more doses, did transfer delayed hypersensitivity skin test

reactions to KLH, PPD or HBsAg in some rhesus monkey recipients (Section 3.2.2: fig. 7, plates 13a-b; section 3.2.4: figs. 8a-b). Such positive results agreed with those of others, who also used vacuum-dialysed transfer factor, prepared from human blood leucocytes, to transfer delayed hypersensitivity skin tests to various antigens in man (Arala-Chaves et al, 1967; Burger et al, 1974). It is possible that vacuum-dialysis, rather than water-dialysis, of lysed rhesus leucocytes from pooled lymph node-spleen cells was capable of dialysing out transfer factor component(s) responsible for the transfer of skin test-reactivity to the antigens used under the conditions of these experiments.

In these experiments, both water-dialysed and vacuum-dialysed immune rhesus transfer factor transferred mixed cell migration reactivity to KLH, PPD, TB and HBsAg in some recipient animals (Section 4.2.2: figs. 23-26). However, the mean intensity of LMMI-responses in animals treated with immune transfer factor was stronger with the water-dialysed than with the vacuum-dialysed material (Section 4.2.4: fig. 27). Thus, in these recipients the LMMI-responses also seemed to be influenced by the method of dialysis of transfer factor.

Unlike some investigators (Section 1.4.1: table 9; Zanelli and Adler, 1975), others (Section 1.4.1: table 9; Maddison et al, 1972) have reported conversion of lymphocyte transformation in tests to antigens in rhesus monkeys treated with water-dialysed rhesus transfer factor. In this work, lymphocyte transformation tests were carried out only in the recipients of vacuum-dialysed, and not water-dialysed transfer factor. The conversion of tests seemed to be mainly

limited to the potential mitogenicity of KLH (Section 5.2.1: fig. 41a; section 8.3.5). Therefore, although here the influence of the method of dialysis of transfer factor could not be determined upon the lymphocyte transformation tests, it did seem to affect the delayed hypersensitivity skin tests and the mixed cell migration tests.

8.6 Non-specific *in vitro* activity of human or rhesus dialysable transfer factor.

It has been reported that dialysable human transfer factor can specifically sensitise non-immune lymphocytes, or non-specifically augment transformation of previously sensitised cells to an antigen *in vitro* (Section 1.3: table 7). To test these findings and extend them to the rhesus monkey, both rhesus and human dialysable transfer factor preparations were employed with "recipient" human lymphocyte or leucocyte cultures and KLH or PPD as described before (Section 2.8).

In contrast to previous reports (Section 1.3.1: Adler *et al*, 1970; Ascher *et al*, 1974; Palmer and Smith, 1974; Sharma *et al*, 1977) there was no indication that positive transfer factor could transfer antigen sensitivity to unsensitised "recipient" human leucocytes *in vitro* (Section 6.1: figs. 47-48). It is possible that the rhesus transfer factor could not cross the species barrier to transfer antigen-responsiveness to naive human lymphocytes *in vitro*. However, Baram and Condoulis (1970) could not transfer KLH-sensitivity to naive rhesus lymphocytes *in vitro* with rhesus dialysable transfer factor, although they successfully transferred such sensitivity with

non-dialysable transfer factor. This suggested that the latter may be more effective *in vitro*. Preliminary experiments carried out during this work revealed no such transfer of KLH-sensitivity to unsensitised human lymphocytes by non-dialysable rhesus transfer factor (Personal observations).

It was demonstrated that both rhesus and human dialysable transfer factor preparations can, non-specifically augment, or suppress KLH- and/or PPD-stimulated lymphocyte transformation *in vitro* (Section 6.1: fig. 48, table 27; section 6.2: fig. 49, table 28). These results confirmed and extended those of others, who have reported that dialysable human transfer factor, or fractions of it, can augment antigen-stimulated lymphocyte transformation, as indicated by increased incorporation of tritiated thymidine *in vitro* (Hamblin, 1975) (Section 1.3.1, table 7: Ascher and Andron, 1976; Burger *et al*, 1976a, b; Hamblin *et al*, 1976a; Salaman, 1976; Littman *et al*, 1977). Some of these investigators have also observed suppression of *in vitro* transformation in cultures containing antigen and whole or fractions of human dialysable tranfer factor (Burger *et al*, 1976a; Hamblin *et al*, 1976a).

Specificity of the *in vitro* activity, as indicated by lymphocyte transformation tests, has always been a controversial issue. It has been found that the human transfer factor may or may not be donor-specific *in vitro* (Section 1.3.1: table 7) (Mazaheri *et al*, 1977). Some have even suggested that the non-specific effect(s) may be masking the specific activity (Arala-Chaves *et al*, 1976). These experiments showed that the rhesus dialysable transfer factor, prepared

from donors sensitised or not sensitised to KLH, were similarly effective in augmenting cultured "recipient" human lymphocyte transformation responses to KLH (Section 6.1: fig. 48, table 27). The human dialysable transfer factor, prepared from Mantoux negative donors, augmented PPD-induced transformation of human lymphocytes *in vitro* (Section 6.2: fig. 49, table 28). Thus, in agreement with the observations of many with the human dialysable transfer factor, it was shown that the augmentation of antigen-induced lymphocyte transformation was not related either to the KLH-sensitivity or PPD-sensitivity of the rhesus or human transfer factor donors respectively (Hamblin, 1975) (Section 1.3.1: Ascher and Andron, 1976; Burger *et al*, 1976a, b; Hamblin *et al*, 1976a; Salaman, 1976; Littman *et al*, 1977).

It has been found that the degree of the *in vitro* augmentation by human dialysable transfer factor is related to antigen sensitivity of the "recipient" cells *in vitro* (Hamblin, 1975). This has been repeatedly confirmed with the human dialysable transfer factor (Burger *et al*, 1976b; Hamblin *et al*, 1976a), but never with the rhesus monkey dialysable transfer factor. The findings of this work supports this view in the rhesus monkey. It was found that the degree of augmentation of KLH- and/or PPD-induced lymphocyte transformation by rhesus or human dialysable transfer factor was significantly related to the antigen-sensitivity of the "recipient" lymphocytes *in vitro* (Section 6.3: fig. 50a, $r = +0.7$ $P < 0.001$; fig. 50c, $r = +0.6$ $P < 0.05$, fig. 50d, $r = 0.7$ $P < 0.05$). Thus, such augmenting effects of rhesus and human dialysable transfer factor preparations can be said to be "recipient-specific" (Arala-Chaves

and Fudenberg, 1976). In this thesis, in agreement with the observations of others (Hamblin, 1975; Burger et al, 1976b; Hamblin et al, 1976a, c), the degree of augmentation of KLH- and/or PPD-induced lymphocyte transformation by rhesus dialysable transfer factor increased with the increasing antigen-sensitivity of the "recipient" cells in vitro (Section 6.3; figs. 50a, c). However, in contrast to these findings and those of Hamblin (1975) and Hamblin et al (1976a, c), the degree of the augmenting activity to PPD by the human dialysable transfer factor decreased, with the increasing degree of "recipient" sensitivity to this antigen (Section 6.3: fig. 50d).

Within the current framework of the theories of transfer factor activity (Section 1.7), the absence of transfer of KLH-sensitivity to naive lymphocytes in vitro by rhesus transfer factor (i.e. absence of adoptive sensitisation in vitro) does not support the view that transfer factor is an "informational derepressor", a "minireceptor" or a metabolised but highly immunogenic "superantigen" (Sections 1.7.1 - 1.7.3). However, it is possible that the recipient-specific augmenting effect by rhesus or human dialysable transfer reflects the immunologically non-specific adjuvants promoting the activity of specifically sensitised lymphocyte populations to antigen (Bloom, 1973) (Section 1.7.4).

Such evidence of an "adjuvant-like" activity has been said to be reinforced (Hamblin et al, 1976c) by the demonstration that transfer factor can augment lymphocyte transformation responses to phytohaemagglutinin and/or pokeweed mitogen in vitro (Burger et al,

1976b; Hamblin *et al*, 1976b). This may be related to the *in vivo* augmenting activity of transfer factor, indicated by the elevation of PHA-induced LT-responses detected here in monkeys (Section 8.3.6) or by others in humans and dogs (Amman *et al*, 1974; Valdimarsson *et al*, 1974; Shifrine *et al*, 1976). In this work, however, the direct relationship between the *in vitro* and the *in vivo* activities of transfer factor could not be determined, since the same transfer factor was not used throughout these experiments. Others have presented evidence that the *in vitro* augmenting effect of human dialysable transfer factor cannot be correlated to its apparent donor-specific *in vivo* activity in man. Littman *et al* (1977) reported that a fraction of human transfer factor with the *in vitro* augmenting activity did not transfer skin test reactivity *in vivo* and, that the fraction with this *in vivo* activity was inactive *in vitro*. Furthermore, whilst the *in vivo* activity of transfer factor is traditionally thought to be derived from lymphocytes, the *in vitro* augmenting activity has been derived from pure polymorphs (Littman *et al*, 1977) and other cells, like L-cells and BHK-cells (Hamblin *et al*, 1976c). In order to determine the nature and biological specificity of dialysable transfer factor, and to relate the various *in vivo* and *in vitro* activities to one another, and to its clinical effects, purified fractions of dialysable transfer factor should in the future be used in all experiments.

8.7 Sephadex G-25 fractionation properties of rhesus or human dialysable transfer factor.

It has been reported that dialysable transfer factor contains various

in vivo and *in vitro* biological activities whose relationship to one another and to the apparent beneficial clinical effects of administration of transfer factor, is not yet understood (Sections 1.2 - 1.4). The components responsible for these effects have never been unequivocally located or isolated in a pure form, and their biological specificities have always been controversial (Sections 1.5 - 1.6). It has been reported that the human dialysable leucocyte transfer factor contains polypeptides and polynucleotides, hypoxanthine, uracil, adenine, guanine, cytosine, and many others as yet unidentified substances (Baram *et al*, 1966; Lawrence, 1974; Burger *et al*, 1976a; Kirkpatrick *et al*, 1976; Krohn *et al*, 1976a, b; O'Dorisio *et al*, 1976; Tomar *et al*, 1976; Krohn *et al*, 1977; Littman *et al*, 1977; Wilson *et al*, 1977b).

In this study, crude concentrated dialysable leucocyte transfer factor from man or monkey were fractionated on columns of Sephadex G-25 (Section 2.9). A preliminary attempt was made to compare some of their fractionation properties. These were indicated by: (1) the absorption spectra of the crude rhesus preparations (Section 7: fig. 54), (2) the elution profiles of the human and rhesus preparations (Section 7: figs. 51-53), (3) the E_{260}/E_{280} absorption ratios, (4) the V_e/V_t elution ratios, and (5) the partition coefficient values of each separated fraction (Section 7: table 29). No attempt was made here to test the various fractions *in vivo* or *in vitro* or to chemically characterise them, but experiments can be designed in the future for such determinations.

Different number of elution peaks (2 to 14) have been separated by

other workers when human or rhesus monkey dialysable transfer factor was fractionated on Sephadex G 10, G 25, G 200 or DEAE-cellulose (Baram and Mosko, 1962; Lawrence et al, 1963; Baram et al, 1966; Arala-Chaves et al, 1967; Gottlieb et al, 1973; Neidhart et al, 1973; Zuckerman et al, 1974; Reymond and Grob, 1975; Baram and Condoulis, 1976; Burger et al, 1976a; Kirkpatrick et al, 1976; Krohn et al, 1976a, b, 1977; Wilson et al, 1977a, b). In the experiments reported here, Sephadex G-25 fractionation of dialysable transfer factor, prepared from normal human or rhesus blood leucocytes or from pooled rhesus lymph node and spleen leucocytes, revealed 9, 10 and 11 elution peaks respectively (Section 7: figs. 51-53). The type of chromatography columns, the size of the columns, the elution buffer and the method of preparation and dialysis of transfer factor are amongst the many factors which influence the number of elution peaks. In this work, one factor influencing the difference in the number of peaks between the human (Section 7.1.1: fig. 51) and the rhesus preparations (Section 7.1.1: figs. 52-53) was probably the longer length of column(s) used for the latter {2 (1.6 x 90 cms)}, compared with the former {1 (1.6 x 90 cms)}. This is supported by similar observations of Baram et al (1966). They found that when human cell-free extract, containing dialysable transfer factor with skin test converting activity, was fractionated on Sephadex G-25, the number of elution peaks increased from about 4 to 10 by increasing the column length from 2 x 45 cms to 2 x 95 cms.

The E_{260}/E_{280} ratios for the different fractions of the dialysable transfer factor preparations varied with the individual peaks (Section 7: table 29). It has been suggested that this indicates

different compositions (Baram and Mosko, 1965). The relatively high and low ratios are indicative of derivatives of nucleic acids and protein respectively (Mr. Brian Ellis, 1978, personal communications). Here, the most revealing findings are that 2 of the peaks from the human material making up the fractions F and G, and 1 peak from the rhesus blood material, making up fraction B VII, had very high ratios (Section 7, table 29: $E_{260}/E_{280} > 4 < 7$). In contrast, none of the peaks from rhesus lymph node-spleen cells showed such a high ratio (Section 7, table 29: $E_{260}/E_{280} < 2$). High ratios, not necessarily in the same magnitude, have been associated with a nucleotide material, notably hypoxanthine (Neidhart et al, 1973; Kirkpatrick et al, 1976; O'Dorisio et al, 1976; Tomar et al, 1976). Thus, in this work, the human and rhesus blood material, but not the rhesus lymph node and spleen material, probably contained nucleotide-rich fraction(s).

It has been reported that, the hypoxanthine-rich fractions of the human dialysable transfer factor, on Sephadex G-25, can elute after the total bed volume at about 1.25 V_t (Neidhart et al, 1973; Zuckerman et al, 1974; Kirkpatrick et al, 1976; Wilson et al, 1977b). In view of this, it is likely that amongst the high ratio fractions from the human and rhesus preparations, the human fraction G (Section 7, table 29: $H\text{-}TF_{VD}$, E_{260}/E_{280} 6.54) eluting at 1.28 V_t and the rhesus fraction B VII (Section 7, table 29: $Rh\text{-}TF_{VD/BI}$, E_{260}/E_{280} 4.39) eluting at 1.28 V_t were also hypoxanthine-rich fractions. This was later found to be consistent with the observation that a commercially prepared hypoxanthine also eluted in the same region of these columns (Hamblin, 1978: personal communications). The

hypoxanthine-rich fraction of human dialysable transfer factor is said to contain the skin converting capacity (Neidhart et al, 1973; Zuckerman et al, 1974; Kirkpatrick et al, 1976; Wilson et al, 1977b), but hypoxanthine alone does not seem to be responsible for it (Kirkpatrick et al, 1976; Wilson et al, 1977b). If the hypoxanthine-rich fractions were to contain the skin converting activity, this should reside in the human fraction G (Section 7, table 29: $H\text{-}TF_{VD}$) or in the rhesus fraction B VII (Section 7, table 29: $Rh\text{-}TF_{VD/BI}$).

The molecular weight of the human dialysable transfer factor component(s), with the capacity to convert skin tests from negative to positive, has been estimated to be less than 10,000 on the basis of both dialysis (Lawrence, 1974) and column chromatography (Arala-Chaves et al, 1967; Gottlieb et al, 1973). Here, in agreement with the evidence from other laboratories (Neidhart et al, 1973; Reymond and Grob, 1975; Krohn et al, 1976a, b, 1977), the partition coefficient and elution ratios indicated that all the preparations contained gel adsorbing (Section 7, table 29: $K_{av} > 1$, $V_e/V_t > 1$) and non-adsorbing substances (Section 7, table 29: $K_{av} < 1$, $V_e/V_t < 1$). Thus, the molecular weight of the various fractions, including the hypoxanthine-rich ones, could not have been determined by the region of elution of the various fractions.

Ultraviolet absorption spectra of preparations of crude concentrated human dialysable transfer factor has been reported to have a peak absorption at 255nm wavelength, indicating the presence of components derived from nucleic acids (Kirkpatrick and Gallin, 1974).

Ultraviolet absorption spectra were determined for the two crude monkey preparations (Section 7: fig. 54) and both preparations revealed peak absorption between 250-260nm, probably indicating presence of substances derived from nucleic acids. This absorption was stronger in the rhesus material from blood (Section 7: fig. 54a) than that from lymph node and spleen cells (Section 7: fig. 54b), suggesting that the concentration of substances derived from nucleic acids was greater in the former than in the latter. This might be a reflection of the different number of lymphocytes and polymorphs found in the rhesus blood and lymph node-spleen leucocytes, from which the transfer factor preparations were made (Section 7.1.1).

Therefore, Sephadex G-25 column chromatography indicated that normal human and the rhesus dialysable transfer factor preparations were of heterogeneous composition and separable into 9 to 11 fractions. Only the human or the rhesus preparation from the blood leucocytes, apparently contained a hypoxanthine-rich fraction eluting at 1.28 V_t, whilst these preparations and that from the rhesus lymph node and spleen cells contained gel adsorbing and non-adsorbing substances. No attempt was made to determine the molecular weight of the different fractions or their biological activities, but experiments may be designed to find out the possible in vivo and in vitro effects of these fractions. However, dialysable transfer factor contains a wide range of small molecular weight substances, like histamine, prostaglandin, serotinin, all of which may be pharmacologically active (Hamblin, 1978). Thus, in studying the various in vitro and in vivo biological phenomena, interferences from artefacts and relevance of these to the various activities of dialysable transfer factor must be considered.

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9. CONCLUSIONS

Studies of cell-mediated immune responses in rhesus monkeys, either actively immunised with FCA, with or without KLH or HBsAg, or treated with homologous dialysable transfer factor, led to the following conclusions.

9.1 Antigen-specific cell-mediated immunity in actively sensitised rhesus monkeys (Table 37).

To determine antigen- specificity of CMI responses in immunised rhesus monkeys and establish the appropriateness of the tests (DH, LMMI, LT) used to monitor these responses in this species, 22 normal and 17 actively immunised animals were investigated. Of the former, 2 were tested with the DH-skin test, all with the LMMI test and 17 with the LT-test. Of the latter, all were tested with the DH-skin test, 12 with the LMMI-test and 8 with the LT-test.

It might have been presumed that none of the tests in the normal control animals, but all of the tests in the immunised animals, would have shown antigen-reactivity. However, table 37 shows that none of the DH-skin tests, but some of the LMMI and LT-tests, were positive in normal animals. In the LMMI-test, reactivity (MI<0.8) was detected for all antigens (Section 4.1.3: table 17, KLH, PPD, TB, HBsAg). This reactivity did not seem to be associated with the direct toxicity of the antigens upon the migrating indicator cells, since migration of normal guinea pig macrophages alone or mixed with normal rhesus leucocytes, produced only 2 out of

Table 37. Summary of cumulative results of delayed (DH) and cellular hypersensitivities (LMMI, LT) in actively immunised and transfer factor-treated monkeys.

Monkeys	Cumulative rates of test conversions to all antigens		
	DH[a]	LMMI[b]	LT[c]
	Active sensitisation		
Normal[d] ("controls")	0/5 (0%)	20/269 (7%)	9/162 (6%)
Immunised[e] (FCA ± Ag)	60/60 (100%)	64/107 (60%)	50/60 (83%)
P for χ^2 d vs e	< 0.001	< 0.001	< 0.01
	Adoptive sensitisation		
Control[f] $Rh\text{-}TF_D$	4/18 (22%)	6/52 (12%)	2/37 (5%)
Immune[g] $Rh\text{-}TF_D$	17/32 (53%)	58/97 (60%)	5/33 (15%)
P for χ^2 f vs g	> 0.05	< 0.001	> 0.3

[a] DH histology results from section 3.1.3, table 13; section 3.2.3, table 16.

[b] LMMI results from section 4.1.3, table 17; section 4.2.3, table 19.

[c] LT results from section 5.1.3, table 21; section 5.2.3, table 24.

[d] Monkeys treated with saline or nothing.

[e] Monkeys immunised with FCA ± KLH or HBsAg according to protocols (Section 2.1, figs. 1a-d).

[f] $Rh\text{-}TF_D$ prepared from normal unsensitised monkeys or monkeys immunised with unrelated antigen(s) (Section 2.2.1.,table 11).

[g] $Rh\text{-}TF_D$ prepared from monkeys sensitised to specific antigen(s) (Section 2.2.1, table 11).

64 (3%) positive macrophage migration tests and 20 out of 269 (7%) positive mixed cell migration tests (Section 8.2.1: tables 30-31). Thus, it seems more likely that other reasons like cross-reactivity of these antigens with others, to which the monkeys may have been sensitised, must be used to explain these positive results. In the LT-test, reactivity (SI>2) in normal animals was detected only to PPD, KLH and HBsAg (Section 5.1.3: table 21). The reactivity to KLH seemed to be associated with the potential mitogenicity of this antigen (Section 8.3.1). Thus, in normal animals non-specific reactivity to antigen was either absent (Tables 37: DH) or minimal (Table 37: LMMI, LT).

In contrast, the table shows that, in the immunised monkeys, all the DH-skin tests and the majority of the LMMI and LT-tests showed reactivity. This reactivity was related to active sensitisation of the animals by immunisation with FCA, with or without KLH or HBsAg (Sections 8.1.1, 8.2.2, 8.3.2). Active sensitisation was indicated by all the three tests as follows. Firstly, the normal saline-injected normal control animals showed no reactivity to any of the skin test antigens whereas all the immunised animals did (Section 3.1.3: table 13, KLH, PPD, HBsAg). Secondly, none of the immunised animals tested showed LMMI-reactivity to any antigen before immunisation (Section 4.1.2: figs. KLH: 11b-d, PPD: 12b-e, TB: 13b-d, HBsAg: 14c-d). Thirdly, only 1 out of 16 immunised animals tested showed LT-reactivity before immunisation, and this was to the potentially mitogenic KLH (Section 5.1.2: fig. 31c). Hence, immunisation of rhesus monkeys with

FCA, with or without KLH or HBsAg, led to active sensitisation indicated *in vivo* by the DH-skin test reactivity to antigen, and *in vitro* by the LMMI- or LT-reactivity to antigen.

Two methods are potentially available for examining antigen-specificity of the active sensitisation. Firstly comparison of the test results in the normal with those in the immunised animals, and secondly comparison of the results of tests carried out with related and unrelated antigens in only the immunised animals. In this work, DH-skin tests were not carried out with an antigen unrelated to the sensitising antigen(s), and the LMMI- and LT-tests with unrelated antigens, were only carried out in a few animals (Sections 8.2.2: table 32; section 8.3.2: table 35). Thus, to determine antigen-specificity of active sensitisation, the test results of the normal animals were compared with those of the immunised ones. This demonstrated that, reactivity in the DH, LMMI and LT-tests was significantly greater in the immunised than in the normal monkeys, indicating antigen-specificity (Table 37: top half, P for x^2 : DH<0.001, LMMI<0.001, LT<0.01).

In conclusion, table 37 shows, not only that active sensitisation of rhesus monkeys was apparently antigen-specific, but also that all the three tests (DH, LMMI, LT) seemed appropriate for monitoring CMI responsiveness in this species. The DH-skin test emerged as the most appropriate test. In addition further analysis of the results showed that in actively sensitised animals the antigen-induced LT- and LMMI- reactivities associated with each other and

with the antigen-induced DH-skin test reactivity (Section 8.4: table 36). Furthermore, compared with the normal animals prior to immunisation, in the immunised animals there was a depression of PHA-induced LT-reactivity, suggesting suppression of general cellular responsiveness in these monkeys.

9.2. Donor-specific and non-specific *in vivo* activities of rhesus dialysable transfer factor (Table 37).

To determine the specificity of *in vivo* activity of dialysable transfer factor in the rhesus monkey model, and establish the appropriateness of the various tests (DH, LMMI, LT) in monitoring CMI responses to selective antigens, 12 normal animals were treated with dialysable rhesus transfer factor. The cumulative number of control animals monitored with DH, LMMI and LT tests was 5. Of these, 2 received normal control transfer factor, whilst 3 others received control transfer factor not related to the test antigens. In contrast, 10 test monkeys received immune transfer factor, prepared from rhesus monkeys immunised with FCA, with or without KLH or HBsAg. Of these 9 were monitored with the DH- and LMMI-tests and 6 with the DH, LMMI and LT-tests. The responses in all 10, however, were followed with at least two of these tests.

Assuming that the *in vivo* specificity of transfer factor in this species was exclusively related to antigen-reactivity of the donor ("Donor-specific"), then none of the tests in animals

treated with the so called control transfer factor should have shown reactivity. In contrast, under the same conditions, all the tests in animals treated with immune transfer factor should have shown antigen reactivity.

Table 37 shows that, in DH-skin tests carried out in control animals 4 tests were positive. Of these one occurred to PPD at the first skin test, after transfer factor administration, and 3 to KLH and HBsAg at the second skin test (Section 3.2.3: table 16). The PPD-reactivity may have been related to pre-exposure or priming of the recipient with tuberculin by Mantoux tests carried out by the suppliers previous to transfer factor administration. In addition, this so called normal control transfer factor was prepared from an animal which had itself been previously Mantoux tested and, therefore, also primed with antigen (Section 8.1.2). In contrast, in test animals given immune transfer factor, amongst the 17 positive DH-skin tests shown in the table, 15 occurred to KLH, PPD and HBsAg at times when the recipients had already been primed with these antigens (Section 3.2.3: table 16). Comparison of the cumulative DH-results showed that the likelihood of reactivity after treatment of animals with the immune transfer factor was not sifnificantly different from those after the control transfer factor (Table 37: $P>0.0.5$). This meant that the rhesus dialysable transfer factor did not seem to adoptively transfer specific CMI *de novo*. Thus, the *in vivo* specificity of rhesus dialysable transfer factor remains equivocal, and DH-skin test did not seem to be an appropriate test for monitoring antigen-specific

CMI-responsiveness in transfer factor-treated animals.

In LMMI-tests carried out in control animals, the table shows that 6 tests were positive. Out of these 4 were due to KLH and 2 to HBsAg (Section 4.2.3: table 19). Reasons for such non-specific reactivity were not known, but both these antigens are complex and may have been cross-reactive with other substances or antigens. In contrast, in test animals treated with immune transfer factor, the 58 positive LMMI-tests included reactivity to KLH, PPD, TB and HBsAg (Section 4.1.3: table 17). Comparison of the cumulative LMMI-results showed that antigen-reactivity, after treatment of monkeys with the immune transfer factor, was significantly greater than that after control transfer factor (Table 37: $P<0.001$). Dialysable rhesus transfer factor may therefore act by specific adoptive "sensitisation" of a cell population concerned with initiation and/or production of MIF/LIF lymphokines (Section 8.2.3). The LMMI-test therefore indicates that the specificity of rhesus transfer factor was related to antigen-sensitivity of the donor ie. "donor-specific".

In LT-tests carried out in control animals, table 37 shows that 2 tests were positive. These were due to KLH (Section 5.2.3: table 24). In animals treated with immune transfer factor, out of the 5 positive LT-tests 4 were due to KLH and 1 to PPD (Section 5.2.3: table 24). Thus, in this test, KLH was predominantly responsible for the LT-reactivity in the transfer factor-treated animals. Comparison of the cumulative LT-results

showed that the likelihood for antigen-reactivity after treatment of monkeys with the immune transfer factor was not significantly different from that after control transfer factor (Table 37: P>0.3). Whilst the positive results may be interpreted as non-specific "adjuvant-like" activity of rhesus transfer factor, it is more likely that the non-specific LT-reactivity in transfer factor recipients was related to potential mitogenicity of KLH (Section 8.3.1).

Therefore, table 37 shows that rhesus dialysable transfer factor can adoptively sensitise naive animals to specific antigens. Such donor-specific in vivo activity of transfer factor could only be demonstrable by the LMMI-test, and apparently not by the DH-skin test or the LT-test. Further analysis of the results from animals treated with the immune transfer factor demonstrated that the antigen-induced in vitro LMMI-reactivity seemed to be dissociated from DH-skin test reactivity (Section 8.4). This might be indicative of different sensitivity of these tests in the recipients, or preferential lymphokine production. Furthermore, compared with the normal animals prior to administration of transfer factor, there was an elevation of LT-reactivity in the recipients. This possibly reflected a non-specific "adjuvant-like" activity also present in the dialysable rhesus transfer factor.

9.3. Recipient-specific in vitro augmenting activity of rhesus and human dialysable transfer factor preparations (Table 38).

The results of in vitro lymphocyte transformation studies of human or rhesus dialysable transfer factor (Section 6) are summarised in

table 38. The following conclusions can be drawn. (a) The rhesus dialysable transfer factor could not apparently transfer antigen-sensitivity to naive human lymphocytes *in vitro* in any of the tests carried out. (b) The rhesus and human dialysable transfer factor preparations augmented antigen-stimulated lymphocyte transformation *in vitro* (Rh-TF_{DM}: augmenting rate =92%); H-TF_{DM}: augmenting rate = 77%). (c) The rhesus and human dialysable transfer factor preparations suppressed antigen-stimulated lymphocyte transformation *in vitro* (suppression rate: Rh-TF_{DM}= 8%, H-TF_{DM}= 23%). (d) The *in vitro* augmenting and suppression effects of the rhesus dialysable transfer factor preparation crossed the species barrier, because they were assayed with transformation tests using human lymphocyte or leucocyte culutres. (e) The *in vitro* augmenting activities of rhesus and human dialysable transfer preparations, regardless of the antigen-specificity of donors, reflected the degree of antigen-sensitivity of the "recipient" cells in culture i.e. "recipient-specific" (Section 6.3: Rh-TF_{DM} and KLH; $n=24$, $r=+0.7$, $P<0.001$; Rh-TF_{DM} and PPD: $n=12$, $r=+0.6$, $P<0.005$; H-TF_{DM} and PPD: $n=11$, $r=-0.7$, $P<0.05$).

These results suggest that the *in vitro* assay of transfer factor did not show transfer of antigen-sensitivity with rhesus dialysable transfer factor, but that rhesus and human dialysable transfer factor preparations had non-specific augmenting and suppressing activities *in vitro*.

Table 38. Summary of cumulative results of in vitro activities of rhesus and human medium-dialysed transfer factor preparations (TF_{DM}).

In vitro activities (LT-response)	Test antigen (2-100 µg)	Cumulative rates of +ve tests (0.25 - 1.0 ml TF_{DM})				
		Rh-TF_{DM}[a] antigen -ve	Rh-TF_{DM}[b] antigen +ve	SUM	H-TF_{DM}[c] antigen -ve	SUM
Transfer rate	KLH[d]	0/3	0/3	0/6 (0%)	ND	
Augmentation rate	KLH[e]	10/12	14/15	36/39 (92%)	3/6	14/18 (77%)
	PPD[f]	ND	12/12		11/12	
Suppression rate	KLH[e]	2/12	1/15	3/39 (8%)	3/6	4/18 (23%)
	PPD[f]	ND	0/12		1/12	

[a] Medium-dialysed rhesus transfer factor prepared from monkeys sensitised to unrelated antigen(s) (Section 2.5.6).

[b] Medium-dialysed rhesus transfer factor prepared from monkeys sensitised to specific antigen(s) (Section 2.5.6).

[c] Medium-dialysed human transfer factor prepared from normal Mantoux negative donors (Section 2.5.6).

[d] From section 6.1, fig. 47; [e] from section 6.1, fig. 48, table 27; [f] from section 6.2, fig. 49, table 28.

9.4 Biochemical heterogeneity of rhesus and human dialysable transfer factor preparations.

Preliminary biochemical analysis of transfer factor indicated that normal human and monkey dialysable transfer factor preparations can be separated on column(s) of Sephadex G-25 into 9 to 11 fractions (Section 7.1.1: figs. 51-53). Some of the fractions contain materials that adsorb to the gel, while others contain non-adsorbing substances. A hypoxanthine-rich fraction, with a high absorption ratio and eluting at 1.28 V_t, was indicated in preparations derived from human and monkey blood (Section 7.1.1; table 29: H-TF_{VD}, fraction G, E_{260}/E_{280} = 6.54; Rh-$TF_{VD/Bl}$, fraction B VII, E_{260}/E_{280} = 4.39) (Section 8.7). Such a fraction was not indicated in the preparation from monkey lymph node and spleen cells (Section 7.1.1, table 29: Rh-$TF_{VD/LSI}$) (Section 8.7).

Without rigorous biochemical analysis in the future, it would be difficult to be certain of the exact chemical composition of dialysable transfer factor, prepared from any species or cell source. More biochemical studies must be carried out with both crude human and rhesus monkey dialysable transfer factor preparations, and their purified fractions, in an attempt to identify and characterise the various component substances. Therefore, as an extension of the work here, appropriate <u>in vivo</u> and <u>in vitro</u> experiments, and extensive biochemical studies, can be designed, using fractions of rhesus and/or human dialysable

transfer factor preparation, in an attempt to locate and chemically characterise the fractions with various specific and/or non-specific activities.

ABBREVIATIONS

Ag	Antigen
^{o}C	Degree Centigrade
CO_2	Carbon dioxide
Ci/mM	Curie per milli-mole
CMI	Cell-mediated immunity
Conc	Concentration
CPM	Counts per minute
CsCl	Caesium chloride
DH	Delayed hypersensitivity
DNA	Deoxyribonucleic acid
DNCB	Dinitrochlorobenzene
DNFB	Dinitrofluorobenzene
DPM	Disintegrations per minute
Σ	Sum
et al	Et alia (and others)
EMEM	Eagle's minimum essential medium
E_{260}	Extinction coefficient at 260 nm
E_{280}	Extinction coefficient at 280 nm
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
Fig	Figure
g	Gravity
gm	Gramme
HBsAg	Hepatitis B surface antigen
hr	Hour
H_3T	Tritiated methyl thymidine
H-TF	Human transfer factor
MF	Mitogenic factor
NS	Non-specific
O.T.	Old tuberculin
P	Level of significance (<0.05, significant)
PHA	Phytohaemagglutinin
PPD	Purified protein derivative of tuberculin

PL	Purified lymphocyte culture
Rh-TF	Rhesus monkey transfer factor
$Rh\text{-}TF_D$	Rhesus monkey dialysable transfer factor
$Rh\text{-}TF_{VD}$	Vacuum-dialysed rhesus monkey transfer factor
$Rh\text{-}TF_{WD}$	Water-dialysed rhesus monkey transfer factor
$Rh\text{-}TF_{DM}$	Medium-dialysed rhesus monkey transfer factor
SEM	Standard error of the mean
SI	Stimulation index
SRF	Skin reactive factor
SK-SD	Streptokinase-Streptodornase
TB	Particulate tubercle bacilli (killed)
TF	Transfer factor
TF_D	Dialysable transfer factor
TF_{ND}	Non-dialysable transfer factor
T.U.	Tuberculin unit
U/ml	Units per milli-litre
V_e	Elution volume
V_o	Void volume
V_t	Total bed volume
x^2	Chi squared test with Yate's correction.

APPENDIX: test materials

Acetic Acid

Type: glacial
Code: 27013
From: BDH Co. Ltd.,
Poole,
England.

Ammonium Bicarbonate

Code: 10302
From: BDH Co. Ltd.

Ampoules

Type: 5 ml - 20 ml volumes.
Use: to store freeze-dried dialysable leucocyte transfer factor.
From: Adelphi (Tubes) Ltd.,
20-21, Duncan Terrace,
London, N.1.
837-2959/9459

Bung

Type: silicone rubber
Size: No. E27; to fit Buchner Flask: 33 mm diameter
From: ESCO (Rubber) Ltd.,
14-16 Gt. Portland Street,
London, W1N 5AB,
England.

Caesium chloride (CsCl)

Type: analar, 168.36 M.Wt.
From: BDH Co. Ltd.

Capillaries

Code: 1604
Type: Micro-haematocrit centrifuge tubes (plain: 59 µl).
From: Gelman-Hawksley Ltd.,
12 Peter Road,
Lancing,
Sussex,
England.

Cover Slips

Type: round, glass
Code: No. 1
Size: 22 mm diameter
From: Chance Propper Ltd.,
Smethwick,
Waley,
England.

Crucible

Type: nickel
Code: CW-310
Size: 35 mm x 35 mm
From: Gallenkamp
P.O. Box 290,
Technico House,
Christopher Street,
London, EC2P 2ER,
England

Crystal Violet (counting dye)

Type: crystals
Code: 34024
From: BDH Co. Ltd.
Preparation: Stock solution (0.1% cv)

Crystal Violet (cv) ..... 0.1 gm
Acetic acid (3%) ..... 100 mls

Mixed and filtered through Whatman filter paper to remove c.v. sediments. Stored at room temperature. Cells were diluted 1/20 as follows and counted in an improved Neubaur haemocytomer:

Eagles MEM	0.9 mls
c.v. (0.1%)	1.0 ml
cell suspension	0.1 ml

Deoxyribonuclease (DNase: pure)

Type: from bovine pancreas
Code: DCLIIS; 11 mg vials
Store: +4°C
From: Cabrian Chemicals Ltd.,
Suffolk House,
George St.,
Croydon CR9 3QL,
England
Preparation: Stock solution: 1 mg/ml

DNa'se (pure) .....11 mg
Non-pyrogenic distilled water.11 mls

Mixed and sterilised by filtration through 0.22 μ millipore filter. Stored at -20°C in 1 ml aliquots.

Deoxyribonuclease I (DNa'se I: crude)

Type: from beef pancreas
Code: DN-25; 10 mg vials
Store: +4°C
From: Sigma London Chemical Co. Ltd.,
Norbiton Station Yard,
Kingston-Upon-Thames,
Surrey KT2 7BH
England.

preparation: Stock Solution: 1 mg/ml

DNa'se I .....10 mg
Non-pyrogenic distilled water.10 mg

Mixed sterilised by filtration through 0.22 μ millipore filter. Store at -20°C in 1 ml aliquots.

Dextran

Type: Grade A powder; 200,000 - 275,000 M.W.
From: Fisons Laboratory Reagents,
Loughborough,
Leicestershire,
England.

Preparation: (a) Stock dextran: 6% solution

Dextran ..... 6 gms
Sterile Normal Saline ..... 100 mls

Mixed and sterilised through 0.45 μ millipore stored at -20°.

(b) Stock dextran: 1.5% solution

6% Dextran ..... 1 vol.
Hank's solution ..... 3 vols.

Mixed and stored at +4°C.

Deoxyribonuclease I (DNa'se I: crude)

Type: from beef pancreas
Code: DN-25; 10 mg vials
Store: +4°C
From: Sigma London Chemical Co. Ltd.,
Norbiton Station Yard,
Kingston-Upon-Thames,
Surrey KT2 7BH
England.

preparation: Stock Solution: 1 mg/ml

DNa'se I .....10 mg
Non-pyrogenic distilled water.10 mg

Mixed sterilised by filtration through 0.22 μ millipore filter. Store at -20°C in 1 ml aliquots.

Dextran

Type: Grade A powder; 200,000 - 275,000 M.W.
From: Fisons Laboratory Reagents,
Loughborough,
Leicestershire,
England.

Preparation: (a) Stock dextran: 6% solution

Dextran ..... 6 gms
Sterile Normal Saline ..... 100 mls

Mixed and sterilised through 0.45 μ millipore stored at -20°.

(b) Stock dextran: 1.5% solution

6% Dextran ..... 1 vol.
Hank's solution ..... 3 vols.

Mixed and stored at +4°C.

Dialysis tube

Type: Visking tube
Size: 8/32 mm
From: Scientific Instruments Ltd.,
1 Leeke Street,
London W.C.1.,
England.

Eagle's Minimum Essential Medium (EMEM)

Type: Stock EMEM x10 with Earle's salts but without sodium bicarbonate.
Code: 1-013M
Vols: 500 ml bottles
Store: +4°C
From: Flow Laboratories (U.K.),
P.O. Box 17,
Second Avenue Industrial Estate,
Irvine KA12 8NB,
Scotland.
Preparation: Stock EMEM (1X)

EMEM (10X)	50 mls
$NaHCO_3$ (7.5%)	13.4 mls
L-glutamine (200 mM)	5 mls
Benzyl Penicillin (4×10^5 Units)	0.5 ml
Streptomycin Sulphate (4×10^5 µg/ml)	0.025 ml
Distilled water	430 mls

Prepared on day of each experiment and used with uninactivated horse serum (10-20%) for migration tests, and used without serum for lymphocyte transformation tests done for normal, actively and adoptively sensitised Rhesus monkeys.

Fibre Glass Filter

Grade: GF/A
Size: 2.5 cms
From: Whatman Lab. Sales Ltd.,
Springfield Mill,
Maidstone,
Kent ME14 2LE,
England.

Ficoll

Type: 400,000 M.W.; powder
Store: +4°C or room temperature
From: Pharmacia Ltd.,
Box 175,
S-75104,
Uppsala 1,
Sweden.

Preparation: Stock (6%)

Ficoll ..... 6 gms
Distilled water ..... 100 mls

Stirred while gradually adding powder. Stored at +4°C.

Ficoll/Triosil mixture

Preparation: Stock solution

Triosil (45.5%) ..... 10 vols
Ficoll (6%)..... 24 vols

Mixed and sterilised by filtration through 0.45 μ millipore, stored at +4°C and used on same day. Used to separate lymphocytes from peripheral blood leucocytes. Ratio of Ficoll/Triosil mixture to leucocytes was 1:2 respectively.

Flask (glass)

Type: Buchner
Size: 500 mls
Code: 1170/06
From: Jobling Laboratory Division,
Stone,
Staffordshire, STT5 OBG
England.

Formaldehyde

Type: 37% - 41% concentrated
Code: 284216 N
From: BDH Chemicals Ltd.

Formal Saline

preparation: 10% Stock Solution
Formaldehyde ..... 1 vol.
Saline (0.85% NaCl) ..... 9 vols

Made 1 litre stock solutions and stored at room temperature. Used as fixative to store rhesus monkey skin biopsies before despatch to Miss Rosemary Ellis for cutting and staining with Cole's Haematoxylin and Eosin.

Flasks

Type: round bottom, glass
Size: 1 litre
From: Gallenkamp

Freeze Dryer

(a) Model: EF03
From: Edwards High Vacuum Plant,
BOC Ltd.,
Manor Royal,
Crawley,
Sussex,
England.

(b) Model: Virtis 10-MR-ST
From: Techmation Ltd.,
58 Edgware Way,
Edgware,
Middlesex, HB88JP,
England.

Freund's Incomplete Adjuvant (FIA)

Type: 10 ml ampules
From: Difco Laboratories,
Detroit 1,
Michigan,
U.S.A.

Glutamine (200 mM)

Type: L-glutamine
Code: 6-134C; 50 mls volumes.
Store: -20^{o}C
From: Flow Laboratories Ltd.,
P.O. Box 17,
Victoria Park,
Heatherhouse Road,
Irvine KA 128NB,
Scotland.

Guinea Pig (Hartley Albino)

Weight: mean = 800 grams
Sex: female
From: Tuck and Sons Ltd.,
Laboratory Animal Breeding Station,
Rayleigh,
Essex,
England.

Haemocytometer

Type: improved Neubauer,
Code: MD-210
From: Gallenkamp.

Hank's Balanced Salt Solution (HBSS)

Type: Packs A and B.
Code: BR 19.
Store: +4°C
From: Oxoid Ltd.,
London SE1 9HF,
England

Preparation: Stock Solution x1

Packs A and B were each dissolved in 100 mls of deionised water and stored at +4°C as 10x stock solutions. Before each set of experiments, however, the following 1x solution was made.

Solution A (10 x) ..... 100 mls
Solution B (10 x) ..... 100 mls
Deionised H_2O ..... 800 mls

Mixed and autoclaved at 121°C for 15 minutes. Cooled to room temperature and 25 mls of sterile 1.4% sodium bicarbonate solution were added (see sodium bicarbonate). Stored at 4°C in aliquots of 200 mls for pepitoneal washings of oil-injected guinea pigs and preparation of 1.5% dextran solution.

Guinea Pig (Hartley Albino)

Weight: mean = 800 grams
Sex: female
From: Tuck and Sons Ltd.,
Laboratory Animal Breeding Station,
Rayleigh,
Essex,
England.

Haemocytometer

Type: improved Neubauer,
Code: MD-210
From: Gallenkamp.

Hank's Balanced Salt Solution (HBSS)

Type: Packs A and B.
Code: BR 19.
Store: +4°C
From: Oxoid Ltd.,
London SE1 9HF,
England

Preparation: Stock Solution x1

Packs A and B were each dissolved in 100 mls of deionised water and stored at +4°C as 10x stock solutions. Before each set of experiments, however, the following 1x solution was made.

Solution A (10 x) ..... 100 mls
Solution B (10 x) ..... 100 mls
Deionised H_2O ..... 800 mls

Mixed and autoclaved at 121°C for 15 minutes. Cooled to room temperature and 25 mls of sterile 1.4% sodium bicarbonate solution were added (see sodium bicarbonate). Stored at 4°C in aliquots of 200 mls for pepitoneal washings of oil-injected guinea pigs and preparation of 1.5% dextran solution.

Harvesting Apparatus

Type: for lymphocyte transformation harvesting.
Buchner Flask code: (see above).
Apparatus: Glass funnel, code: XX1002533
Metal grid, code: -
Metal Clip, code: XX1002503
Complete set, code: XX1002530
From: Millipore S.A.
46 Bis Rue Louis Bleriot,
78530 Buc,
France.

Heparin (freeze-dried: mucous)

Type: preservative-free
Conc.: 20,000 U/vial
Store: +4°C
From: BDH Laboratory Reagents.
Preparation: Stock Solution; 1000 U/ml

Heparin (1 vial) ..... 20,000 Units
Non-pyrogenic saline ..... 20 mls

Mixed and sterilised by filtration through 0.22 μ Millipore and stored at +4°C in 5 ml aliquots.

Heparin (solution: mucous)

Type: preservative-free
Conc.: 1000 Units/ml
Store: +4°C
From: Weddel Pharmaceuticals Ltd.,
London, EC1A 9H7,
England.

Hepatitis B Surface Antigen (HBsAg)

Stock purified HBsAg type ad was prepared by 2 methods:

a) Caesium chloride method

A modified technique of Gerin et al was used. HBsAg protein from serum of a healthy chronic carrier was precipitated with polyethylene glycol or PEG solution (5_1 V/V: final concentration of PEG was 5.5%). This was left at +4°C overnight. The precipitate was collected and resuspended in 0.01 M Tris buffer pH 7.4 (1/12th volume or original mixture). 1 ml of HBsAg-rich precipitate was loaded onto 5 ml CsCl gradient (1.1, 1.2, 1.3 gms/ml density: W/W in Tris buffer). Loaded gradient was immediately spun for 18 hours at 35,000 r.p.m. at +4°C. Aliquots of 0.1 ml were collected and tested with Wellcome haemagglutination test for HBsAg. Aliquots, with positive results at 1/512 dilutions, were pooled and dialysed for 24-48 hours in 3 x 5 litre changes of phosphate buffered saline at 4°C. Protein concentration of purified HBsAg was determined by measurement of extinction at 280 nm in Unican Spectrophonometer and stored at -20°C. Hepatitis B surface antigen banded at 1.175 to 1.223 CsCl density.

b) Sucrose gradient method

Human serum, from a healthy chronic carrier of HBsAg, was spun at 25,000 r.p.m. in SW27.1 rotor for 18 hours in a Sorvall OTD-2 ultracentrifuge at +4°C. Supernatant was decanted and HBsAg-rich pellet resuspended in 1-2 mls of 0.01M Tris buffer (Sigma: pH 7.4). 1 ml suspension of this pellet was loaded onto 15 ml Sucrose gradient (60%, 40%, 20% solution: W/W in Tris buffer). After 6 hours at +4°C one millilitre fractions were tested as above for HBsAg and pooled.

In this case 2 mg/ml stock solution was made in Eagle's MEM and stored in 1 ml aliquots at -20°C. Hepatitis B surface antigen floccule was seen in a layer between 40-60% sucrose solution.

Immunogen: Freund's Complete Adjuvant (FCA)

preparation: FCA; 3 mg/ml TB

FIA .....10 mls
TB (finely ground) ..30 mg

Mixed under pressure using a syringe and suspension used to actively immunise rhesus monkeys.

Immunogen: Hepatitis B surface antigen in Freund's complete adjuvant

Preparation: HBsAg/FCA

HBsAg (2 mg/ml: purified ..... 1 vol.
FCA (6 mg TB/ml) ..... 1 vol.

Thoroughly mixed under pressure using a syringe and used on same day to immunise rhesus monkeys. Immunogen contained 1 mg HBsAg and 3 mg particulate TB.

Immunogen: Keyhole limpet haemocyanin in Freund's complete adjuvant.

Preparation: KLH/FCA

KLH (2 mg/ml or 200 µg/ml) ..... 1 vol.
FCA (6 mg TB/ml) ..... 1 vol.

Thoroughly mixed under pressure using a syringe and used on same day (or stored at +4°C) for active immunisation of rhesus monkeys. Immunogen contained 1 mg/ml or 100 µg/ml KLH and 3 mg particulate TB.

"Ketalar" (Ketamine hydrochloride)

Code: PLR 0018/5118
Conc.: 50 mg/ml; 10 ml vials
Store: Dark and cool place
From: Parke-Davis Co.,
Pontypool,
South Wales,
U.K.

Keyhole Limpet Haemocyanin (KLH)

Type: Grade B/1 gm in 65% saturated ammonium sulphate slurry containing 46.9 mg/ml protein.
Code: 374811
From: Calbiochem Ltd.,
Thorpe House,
King Street,
Hereford HR4 9BQ,
England.

Preparation: Stock solution: 4.9 - 5.2 mg/ml

To remove ammonium sulphate, 2 mls KLH slurry (≡ 100 mg KLH) was diluted to 50 mls with non-pyrogenic saline. This was concentrated over an Amicon PM10 Ultrafiltration membrane at +4°C. The retentate was diluted to approximately 60 mls with saline and reconcentrated 3 times over the same membrane. The washed retentate was made up to 5 mls with saline. This stock solution now contained less than 20 µg/ml ammonium sulphate and 4.9 or 5.2 mg/ml protein. The actual protein concentration of KLH used was determined by measurement of extinction at 280 nm (E_{280} 1.8 ≡ 1 mg/ml).

Methanol

Type: analar
Code: 10158
From: BDH Co. Ltd.

Migration Plates

Type: 12 wells/plate
Code: 132
From: Sterilin,
43-45 Broad Street,
Teddington,
Middlesex, TW11 8QZ,
England.

Millipore Filters

Types: 22 μ, 45 μ
Size: 10-50 mm diameter
From: Millipore S.A.

Minimum Essential Medium - S (MEM - S)

Type: tissue culture medium for suspension cultures (1X); without glutamine.
Code: 138; 500 ml bottles
Store: +4°C
From: Gibco-Biocult
Washington Road,
Abbetsinch Industrial Estate,
Paisley, PA3 4ER
Scotland
Preparation: MEM - S/glutamine

. MEM - S (1X) ..... 100 mls
L-glutamine (200 mM) ..... 1 ml

Made on same day as experiments. Used with 1% glutamine in suspension of leucocytes or lymphocytes for *in vitro* tests with dialysable leucocyte transfer factor. However, used without glutamine in dialysis of rhesus monkey or human leucocyte lysates for preparation of medium-dialysed transfer factor materials.

Needles (Sterile, disposable)

Sizes and Types:	Large:	19 G 1½: 40/11
	Medium:	21 G 1½: 40/8
	Small:	25 G ⅝: 16/5
From:	Yale Microlance,	
	Becton Dickinson Co. Ltd.,	
	Dun Laoghaire,	
	County Dublin,	
	Ireland.	

Old tuberculin

Type:	1/100 O.T. solution ≡ 100 TU/0.1 ml
From:	Evans Medical,
	C/O Vestrics Ltd.,
	P.O. Box 21,
	Lockfield Avenue,
	Enfield, Middlesex,
	England.

Paraffin Oil

Type:	Light liquid - specific gravity 0.865 to 0.890
Code:	29834
From:	BDH Co. Ltd.

Penicillin

Type: Benzyl Penicillin (sodium) B.P.
Conc.: 600 mg (1,000,000 Units)/vial
From: Glaxo Laboratories Ltd,
Greenford, Middlesex,
England.
preparation: Stock; 4 x 10^5 Units/ml

Benzyl penicillin ..... 1 vial
Distilled water ..... 2.5 mls

Mixed on day of experiment and stored at +4°C not longer than 1 week for use in preparation of EMEM 1X.

Phosphate Buffered Saline (PBS)

Type: tablets
From: Oxoid Ltd.
preparation: Stock solution

Tablet 10
Distilled water ..... 1000 mls

Mixed and stored at 4°C.

Phytohaemagglutinin (PHA)

Type: Reagent grade: freeze dried ampules
Code: HA15
From: Wellcome Reagents Ltd.,
Beckenham, BR3 3BS,
England.
preparation: Stock solution: 1 mg/ml

PHA ..... 1 ampule
*EMEM ..... 40 mls

Mixed and sterilised by filtration through 0.22 μ millipore.
Stored in 1 ml aliquots at -20°C.

* EMEM = tissue culture medium without horse serum.

Planimeter

Model: Sliding bar and fixed index.
From: W.F. Stanley Co. Ltd.,
Avery Hill Road,
New Eltham,
London SE9,
England

POPOP (2-4-methyl-5-phenyl-oxazolyl-benzene)

Store: in dark
From: Koch-Light Laboratories Ltd.,
Colnbrook,
Bucks,
England.

PPO (2,5-diphenyloxazole)

From: Koch-Light Laboratories Ltd.

Polyethylene glycol (PEG)

Type: laboratory grade
Code: P-2263
From: Sigma Chemical Co.,
P.O. Box 14508,
St. Louis,
Mo. 63178,
U.S.A.

Purified Protein Derivative (PPD)

Type: neutralised freeze-dried powder of human tuberculin.
Code: 292

From: Tuberculin Section,
Ministry of Agriculture, Fisheries and Food,
Central Veterinary Laboratory,
New Haw, Weybridge,
Surrey, England

preparation: Stock PPD: 1 mg/ml

PPD .....100 mg
*EMEM .....

Mixed and placed in 37°C water-bath for a few hours.
Sterilised by filtration through 0.22 μ millipore filter.
Stored in 1 ml aliquots at -20°C.
*EMEM = tissue culture medium without horse serum

Rhesus monkey (Macaca mulatta)

Sex: Male and Female
Weight: 3.5 - 8.0 Kg
From: Shamrock Farms (G.B.) Ltd.,
Upper Horton Farm,
Small Dole,
Nr. Henfield,
Sussex, England.

"Safety Cabinet"

Type: Laminar Flow
Size: 6 foot
From: Microflow Ltd.,
Fleet Mill,
Minley Road,
Fleet, Aldershot,
Hampshire, GU13 8RD,
England

Scintillation Counter (β-counter)

Type: Spectro/matic and Coru/matic 200.
From: Tracerlab Instrument Division
2 Riverdene Industrial Estate,
Molesey Road,
Hersham, Surrey KT12 4RG
England

Scintillation Fluid

preparation: For β-scintillation counting

PPO ..... 4 gms
POPOP ..... 0.1 gm
Toluene ..... 1 litre

Mixed and placed in 37°C waterbath overnight. Stored in dark at room temperature.

Scintillation Vials

a) Type: Plastic
From: Griffiths and Nielson Plastics Ltd.,
Huffwood Trading Estate,
Brookers Road,
Billingshurst,
Sussex, England

b) Type: Glass
From: Packard Instruments Ltd.,
Caversham Bridge House,
13-17 Church Road,
Caversham,
Berks R64 7AA,
England

Sephadex G-25

Type: Medium or dry particle diameter of 50-150 μ
Fractionation
Range: 1000-5000 M.W.
From: Pharmacia Fine Chemicals Ltd.
preparation: Fractionation columns

Sephadex beads were swollen overnight at room temperature in ammonium bicarbonate-acetic acid buffer pH 7.4 ("volatile" buffer). One to two chromatographic columns of 1.6 x 90 cms each were poured at room temperature. Columns were equilibrated before use by washing with 3 bed volumes of buffer at +4°C using a flow rate of 20 mls per hour.

Serum (horse)

Type: preservative-free, uninactivated
Code: HS38
Store: -20°C
From: Wellcome Reagents Ltd.

Sieve

Type: nickel; hemispherical
Size: to fit matching crucibles (see crucible)
From: R. Cadish and Sons,
Arcadia Ave.,
Regents Park Road,
Finchley, N3
London, England

Sodium Biocarbonate (7.5% W/V)

Code: 7-041D
Vol: 100 ml bottles
Store: +4°C
From: Flow Laboratories Ltd.

Spetrophotometer

Type: Unicam SP500 series 2.
From: Pye-Unicam Ltd.,
Cambridge,
England.

Streptomycin

Type: Streptomycin sulphate B.P.
Conc.: 1,000,000 µg/vial
From: Glaxo Laboratories Ltd.
Preparation: Stock; 4×10^5 µg/ml

Streptomycin Sulphate ..... 1 vial
Distilled water ..... 2.5 mls

Mixed on day of experiment for immediate use and stored at $+4^{o}C$ not longer than 1 week for subsequent use in preparation of EMEM 1X.

Stopcock grease

Type: 50 gms tubes
Use: to grease migration plates
From: Dow Corning Ltd.,
Barry,
Glamorgan,
U.K.

Sucrose ($C_{12}H_{22}O_{11}$)

Type: from sugar cane, analar, 342.30 M.Wt.
From: BDH Co. Ltd.

Syringes

Types: 1-50 mls
From: Plastipak,
Becton Dickenson Co. Ltd.,
Dun Laoghaire,
Co. Dublin,
Ireland

Toluene

Type: analar; $C_6H_5CH_3$ = 92.14
Size: 2.5 litre bottles
From: BDH Chemicals Ltd.

Test-Tubes (glass)

Type: rimless: for in vivo experiments.
Size: 5" x ½"
From: Gallenkamp

Test-Tubes (plastic)

Type: Disposable, round bottom: for in vitro experiments.
Size: 100 x 14/15 mm
From: Primax Plastic,
Weasenham Lane,
Wisbech,
Cambs.
England.

Preparation: Washing

Tubes were placed into 2% hydrochloric acid overnight. Rinsed twice under tap water for a few hours, and finally washed a few times with deionised water. They were dried, loosely capped and sterilised in the hot oven at 160°C for 2 hours. Sterile capped tubes were used for lymphocyte transformation in the in vitro study of dialysable leucocyte transfer factor.

Test-Tube Caps (metal)

Type: plain
Code: TTA3
From: Oxoid Ltd.

Trichloroacetic Acid (TCA)

Type: analar/$CCl_3.COOH$ = 163.39
From: BDH Chemicals Ltd.
CAUTION: Poison
Preparation: Stock solution 5%

TCA ..... 5 gms
Distilled Water ..... 100 mls

Mixed and stored at $4^{o}C$.

Triosil (or "Hypaque")

Type: 5 x 20 mls or 10 x 50 mls ampules
Store: $+4^{o}C$ in dark
From: Vestric Co. Ltd.,
Runcorn,
Cheshire,
U.K.
Preparation: Stock (45.5%)

Triosil ..... 20 mls
Distilled Water ..... 24 mls

Stock solution stored in foil-wrapped dark bottle at $+4^{o}C$.

Tris buffer

Preparation: Stock Solution (0.01M, pH 7.4)

Trizma (HCl) ..... 1.322 gms
Trizma (Base) ..... 0.194 gms
Distilled Water ..... 1.0 l

Made up in 1 litre volumetric flask and stored at $+4^{o}C$.

Tritiated Methyl Thymidine (H_3T)

Type: aqueous solution; 1 ml vials
Conc.: 1 mCi/ml
Specific
Activity: 5-19 Ci/mM
From: The Radiochemical Centre,
Amersham, England.
Preparation: a) Stock Solution (100 μCi/ml)

H_3T (1mCi/ml) ..... 1 ml
Non-pyrogenic saline ... 9 mls

Mixed and stored at +4°C until before each set of experiments
This was diluted further as follows:

b) Stock solution (10μCi/ml)

H_3T (100 Ci/ml) .....1 vol
*EMEM (1 x without serum).9 vols

0.1 ml (= 1μCi) of this concentration (10μCi/ml) was used for pulsing lymphocyte transformation tests in experiment for *in vivo* study of dialysable leucocyte transfer factor.
* For *in vitro* study EMEM was replaced by MEM-S.

Tubercle bacilli (particulate TB)

Type: heat killed freeze-dried mycobacteria
From: Tuberculin Section,
Ministry of Agriculture, Fisheries and Food,
England.
Preparation: Stock TB: 1.5 mg/ml

TB (ground, autoclaved for
15 mins/15 lbs p.s.i) . 150 mgs
*EMEM ..... 100 mls

Thoroughly mixed and stored at +4°C in 1 ml aliquots.

* EMEM = tissue culture medium without horse serum.

Tritiated Methyl Thymidine (H_3T)

Type: aqueous solution; 1 ml vials
Conc.: 1 mCi/ml
Specific
Activity: 5-19 Ci/mM
From: The Radiochemical Centre,
Amersham, England.
Preparation: a) Stock Solution (100 μCi/ml)

H_3T (1mCi/ml) ..... 1 ml
Non-pyrogenic saline ... 9 mls

Mixed and stored at +4°C until before each set of experiments
This was diluted further as follows:

b) Stock solution (10μCi/ml)

H_3T (100 Ci/ml) .....1 vol
*EMEM (1 x without serum).9 vols

0.1 ml (= 1μCi) of this concentration (10μCi/ml) was used for pulsing lymphocyte transformation tests in experiment for in vivo study of dialysable leucocyte transfer factor.
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Tubercle bacilli (particulate TB)

Type: heat killed freeze-dried mycobacteria
From: Tuberculin Section,
Ministry of Agriculture, Fisheries and Food,
England.
Preparation: Stock TB: 1.5 mg/ml

TB (ground, autoclaved for
15 mins/15 lbs p.s.i) . 150 mgs
*EMEM ..... 100 mls

Thoroughly mixed and stored at +4°C in 1 ml aliquots.

* EMEM = tissue culture medium without horse serum.

Trizma (Base, HCl)

Types: a) Base: 121.1 m.wt., No. T-1503
b) HCl: 158.0 m.wt., No. T-3253

Grade: reagent

From: Sigma Chemical Co.

Universal (sterile)

Code: 128C, plastic

Type: plain label, 30 mls volume

From: Sterilin.

'Volatile' fractionation buffer (pH 7.4)

Type: 0.02M ammonium bicarbonate/acetic acid pH 7.4

Preparation: For fractionation purposes

Amm. Bicarb. (analar) ..... 7.9 gms
Acetic Acid (2M:analar) .... 2.9 gms

Made up to 5 litres with deionised water and stored at +4°C.

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TRANSMISSION OF HEPATITIS B TO THE RHESUS MONKEY

A.J. Zuckerman, G. Scalise, M.R. Mazaheri, Jenny Kremastinou, C.R. Howard and K. Sorensen

ABSTRACT

The rhesus monkey does provide a suitable laboratory animal model for studying hepatitis B virus and for investigating various aspects of interaction between this infectious agent and the host. This species of non-human primate, however, offers a less sensitive animal model for hepatitis B infection than the chimpanzee.

There have been many attempts to transmit hepatitis B virus to non-human primates and these studies have yielded, until recently, equivocal or negative results. The finding of hepatitis B surface antigen and antibody in the serum of a small proportion of chimpanzees, orangutans and gibbons renewed interest in the possibility that these primates might serve as a suitable experimental model for hepatitis B.

Hepatitis B surface antigen and surface antibody have been detected in 6-12% of captive chimpanzees when tested by relatively insensitive techniques. Most of the animals appear to be healthy carriers of the antigen. Hepatitis B surface antibody was found in a significant number of captive non-human primates when sensitive techniques such as passive hemagglutination and radioimmunoassay had been used. Antibody was detected in the chimpanzee, orangutan, gibbon, baboon, Celebes ape, patas monkey, vervet, several species of macaque, mangabey and langur and in a number of species of New World monkey. Antibody was found in about 50% of chimpanzees examined but in less than 10% of most Old World and New World monkeys (5, 7). It appears that much of the difficulty which had been experienced in the past was due to the unknown susceptibility of these animals before experimentation and the relatively mild nature of the infection.

Recent studies have shown that although hepatitis B in chimpanzees is consistently mild, as shown by modest serum transaminase enzyme elevations without jaundice or overt signs of illness, the serological responses are identical to those seen in man and further that susceptible chimpanzees are almost as

sensitive to infection with hepatitis B virus as man (1). However, the practical obstacles to the use of chimpanzees for studying infection with hepatitis B virus include the strictly limited availability and expense of these non-human primates.

Transmission of hepatitis B to the rhesus monkey

London *et al.* (1972) reported the successful serial transmission in rhesus monkeys (*Macaca mulata*) of an infectious agent, which stimulated an antibody response to hepatitis B surface antigen. The infection was inapparent and it was not associated with biochemical evidence of liver damage nor with histological changes in the liver. Hepatitis B surface antigen was demonstrated in low titre and transiently in some of the sera by solid-phase radioimmunoassay. The antibody responses measured by radioimmunoprecipitation and passive hemagglutination were similar in pattern to those observed in man following either natural or artificial infection with hepatitis B virus. Successful transmission was achieved through five serial passages in rhesus monkeys. Although the rhesus monkey is less susceptible to human hepatitis B virus, it does provide a more accessible animal species for the experimental study of this infection.

Zuckerman (1972) postulated that the high prevalence of hepatitis B antigen in many tropical countries may be related to changes in the immunological response associated with a background of repeated parasitic infection and in particular to the immunosuppressive effect of malaria. An extension of this hypothesis was the induction of chronic infection with *Plasmodium inuei* in the rhesus monkey in an attempt to enhance the susceptibility of this species of non-human primate to hepatitis B virus.

Malaria and hepatitis B infection in the rhesus monkey

Sera from young rhesus monkeys were screened for hepatitis B surface antigen and hepatitis B surface antibody by solid-phase radioimmunoassay before they were admitted to the study. The animals were divided into small groups consisting of uninoculated controls, those infected chronically with *Plasmodium inuei*, a group inoculated intravenously with 0.25 ml of a well-characterized serum known to have induced clinical post-transfusion hepatitis in man and containing hepatitis B surface antigen subtype *adw*, and a small number of animals inoculated with the same hepatitis B infective serum after chronic infection with malaria was fully established.

Serum samples were tested for hepatitis B surface antigen and antibody by solid-phase radioimmunoassay. Results for antigen were considered positive only after neutralization with specific human antibody. Liver biopsies were examined histologically and sections were also stained for cytoplasmic hepatitis B surface antigen after Shikata *et al.* (1974). Finally, cellular hypersensitivity was examined by the mixed leucocyte-macrophage migration test (3) whole blood lymphocyte transformation and by intradermal tests and skin biopsy. The results of the studies of cell-mediated immunity will be the subject of another report.

Hepatitis B surface antigen was detected in the serum of four out of six rhesus monkeys previously infected with malaria. The antigen was demonstrated in one animal on the day of inoculation, presumably due to the antigen present

in the original inoculum, and the antigen was subsequently found repeatedly in the serum of this subject for 45 days. Surface antibody was first detected 12 days after inoculation and it has persisted in the serum so far for 18 months. The presence of circulating hepatitis B antigen-antibody complexes was confirmed by electron microscopy. In another monkey the antigen was first detected 18 days after inoculation and it persisted for 30 days. An antibody response followed 54 days after inoculation. In the third animal the antigen was first found 4 days after inoculation and it remained in the circulation for 45 days without a detectable antibody response. Antigen was detected for one day only 25 days after inoculation in another animal, again without an antibody response (Figure 1). There was no detectable antigen or antibody in the remaining two monkeys in this group.

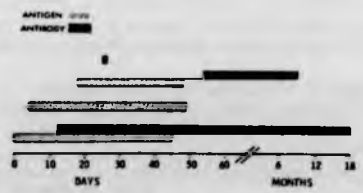

Fig. 1. HB_sAg and anti-HB_s response in rhesus monkeys infected with malaria and hepatitis B.

Hepatitis B surface antigen was not found by radioimmunoassay in any of four animals inoculated with hepatitis B only. An antibody response was elicited in only one of these rhesus monkeys, 54 days after infection, and the antibody has persisted for some months.

Serum containing hepatitis B antigen was collected from two of the successfully infected rhesus monkeys and 1ml was administered intravenously to three animals previously infected chronically with *Plasmodium inuei*. Hepatitis B surface antigen was detected in the serum of one of these animals 49 days after inoculation, but an antibody response has not been observed. Further passages are in progress.

Infection with hepatitis B in these rhesus monkeys was subclinical, specific histological changes in the liver were not seen and the surface antigen was not detected in the liver by staining with orcein or with aldehyde fuchsin.

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ACQUISITION OF MIXED CELL MIGRATION REACTIVITY BY ACTIVELY SENSITISED AND TRANSFER-FACTOR TREATED RHESUS MONKEYS.

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Summary and introduction

A convenient test of cellular hypersensitivity is described for studying the response of rhesus monkeys to the injection of transfer factor. Dextran-prepared monkey leucocytes (>50% lymphocytes) are mixed with guinea pig peritoneal exudate cells (PEC) in the ratio 70% PEC to 30% leucocytes. The mixed cell population is packed in capillaries and migrated in 12-well disposable chambers using 20% horse serum: Eagles MEM. Monkeys actively sensitised with haemocyanin (KLH) and Freund's complete adjuvant (FCA) exhibited strong delayed hypersensitivity reactions when skin tested two and four weeks later with both PPD and KLH and gave mixed cell migration responses and lymphocyte transformation responses to 10-100 µg/ml of KLH, PPD or particulate TB. However, when dialysable transfer factor, prepared from similarly sensitised monkeys (RH-TF), was injected into unsensitised recipients,the animals acquired mixed-cell migration reactivity towards the three antigens at times when their skin tests and lymphocyte transformation responses remained negative. In the rhesus monkey the mixed cell migration system appears to provide an index of lymphocyte reactivity whereby adoptive sensitisation with RH-TF can be detected in the absence of delayed hypersensitivity or lymphocyte transformation responses.

During the past years, occasional laboratories have reported "persuasive" evidence (1) of DTF in the rhesus monkey. In view of the recent interest in giving DTF in chronic active hepatitis, and of confirmation that the rhesus monkey is susceptible to Hepatitis-B infection (2), we have begun to study cellular immune responses and adoptive sensitisation with TF in this species. The problems of skin testing for low levels of delayed hypersensitivity are well recognised in the monkey; and the purpose of this study was therefore to develop in vitro test systems of cellular hypersensitivity which might be used to follow the response of rhesus monkey recipients to the injection of dialysable transfer factor (RH-TF) from actively sensitised animals.

Methodology and design

Our experiments consist of studying the acquisition and specificity of delayed hypersensitivity (DH), mixed leucocyte macrophage migration (LMMI) reactivity and lymphocyte transformation (LT) responses in rhesus monkeys actively immunised with haemocyanin (KLH) in Freund's complete adjuvant (FCA) and in rhesus monkeys injected with RH-TF.

Active immunisation of 4-8 kg male or female animals was done by giving a single set of I/M and I/D injections of 100 µg, 250 µg or 1mg KLH emulsified in FCA (containing 3 mg heat-killed H37Rv tubercle bacilli).. Skin tests were by intradermal injection of 10-30 µg KLH or PPD in 0.1 ml NaCl into the anterior aspect of the chest and after clinical measurement of induration diameter at 24 and 48 hours, skin test sites were biopsied for study of lymphocyte infiltration. Lymphocyte transformation tests were done in glass tubes by minituarised whole blood culture in which 0.1 ml of blood was added to 0.9 ml Eagle's MEM containing KLH, PPD, particulate tubercle bacilli (heat killed H37Rv) or reagent grade PHA in the concentration range 5-100 µg/ml. Cultures were maintained at 37° in 5% CO_2:air for 5 days; 0.1 ml (1 µCi) ^{3}H-thymidine (15-18 Ci/mM) was then added and cultures were harvested 18 hours later for scintillation counting. LT results were expressed as incremental dpm (^{3}H-thymidine uptake) in the presence of antigen or PHA corrected for baseline dpm of control cultures maintained in the absence of stimulant. For the LMMI test, 10 ml quantities of heparinised (50 U/ml) blood were mixed with half volumes of dextran (Mwt 250,000:final concentration 0.5%). After sedimentation of RBC for 30-60' at 37°, leucocytes were centrifuged from the plasma layer, thrice washed with 20% horse serum (HS):Eagles MEM and suspended at 1.5 x 10^7 cells/ml (about 50% lymphocytes) in this medium at room temperature. An equal volume of washed oil-induced guinea pig peritoneal exudate cells (at 3 x 10^7/ml) in 20% HS-MEM was then added and the cell mixtures were quickly migrated from single capillary tubes placed in individual wells of a 12-well sterile migration chamber unit. Wells were filled with 20% HS:MEM containing antigens (KLH, PPD, TB) at concentrations from 3 to 200 µg/ml. Migration ratios related to medium alone were evaluated after 20 hours at 37° in a 5% CO_2:air incubator.

Dialysable transfer factor (RH-TF) was prepared from pooled lymph node and spleen cells obtained 4-6 weeks after sensitisation of donor monkeys. Lymphoid cells were washed once; cell pellets were freeze thawed 10 times with 100 µg

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DNase/10^8 lymphocytes; and RH-TF was obtained by vacuum dialysis at 4^o and subsequent freeze-drying of the dialysate. Recipient naive monkeys were first examined several times by LMMI and LT to ensure absence of cellular hypersensitivity towards KLH, PPD and TB; and to ensure that LT responses to PHA were normal. For injection, RH-TF from 3-9 x 10^9 donor mononuclear cells were dissolved in 20-30 ml volumes of NaCl and the material was distributed subcutaneously over the back and intramuscularly in all four upper limbs of individual recipient monkeys.

Results: (a) Acquisition of cellular immune responsss following active immunisation (Tables 1-3)

TABLE 1 DELAYED HYPERSENSITIVITY* FOLLOWING ACTIVE IMMUNISATION OF RHESUS MONKEYS

	Skin test* diameter at 2w				Skin test* diameter at 4w			
	KLH		PPD		KLH		PPD	
	30µg	10µg	30µg	10µg	30µg	10µg	30µg	10µg
Monkey No. 16	27	25	31	26	31	24	21	16
Monkey No. 17	20	23	36	33	11	<3	21	<3

*(average diameter of 24 hr-induration in mm when skin tested 2w and 4w after sensitisation with 100µg KLH in FCA)

Monkeys actively immunised with KLH in FCA developed delayed hypersensitivity (DH: Table 1), lymphocyte transformation (LT:Table 2) and mixed cell migration reactivity (LMMI:Table 3) towards KLH, PPD and particulate TB. DH responses were all well marked by 2 weeks and were still present 6 weeks after sensitisation; LT and LMMI responses varied widely from animal to animal but were generally not present until the second week of sensitisation. The histology of positive (>3mm induration) DH responses showed widespread infiltration of all dermal layers with lymphocytes, macrophages and polymorphs, the latter being prominent in the more intense reactions. The pattern of acquisition and duration of *in vitro* (LT/LMMI) reactivity could not be predicted from a knowledge of the skin reactions, and in individual animals, LT and LMMI responses did not run parallel with each other. LT responses to PHA fell sharply after immunisation (Table 2).

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TABLE 2 LYMPHOCYTE TRANSFORMATION RESPONSES FOLLOWING ACTIVE IMMUNISATION OF RHESUS MONKEYS

Test AG and conc.(μg/ml)		^{3}H-thymidine incorporation (mean Δdpm/culture) before sensn.	after sensitisation 1w	2w	3w	4w	5w
Monkey No. 16							
KLH	50	129	300	225	681	3284	728
	10	100	170	820	626	1885	284
PPD	50	-24	61	122	105	84	129
	10	-16	35	157	117	74	239
TB	50	-77	-30	78	99	448	40
	10	77	-52	68	67	-24	334
PHA	25	8182	3904	793	247	394	402
Monkey No. 17							
KLH	50	30	-51	972	174	262	-19
	10	15	164	191	60	750	341
PPD	50	-34	-45	379	490	62	72
	10	4	-116	280	233	86	193
TB	50	-15	-84	111	180	94	69
	10	-10	-77	-29	182	140	70
PHA	25	5013	100	964	229	342	301

TABLE 3 MIXED CELL MIGRATION REACTIVITY (LMMI) FOLLOWING ACTIVE IMMUNISATION OF RHESUS MONKEYS

Test AG and conc. (μg/ml)		Migration index (medium control = 1.00) before sensn.	after sensitisation 1w	2w	3w	4w	5w
Monkey No. 16							
KLH	100	1.05, .87	.94	.97	.67	.97	.94
	33	1.12, .87	.80	.89	.71	.88	.93
PPD	100	1.11, .87	.89	1.00	.83	.77	.90
	33	1.03, .95	.96	1.14	.87	.84	.92
TB	100	.89,1.19	.80	1.00	.52	.79	.82
	33	1.04,1.27	.87	1.02	.80	1.15	1.00
Monkey No. 17							
KLH	100	1.06, .92	1.10	.74	.53	.80	1.19
	33	1.06,1.00	.98	.71	.58	.78	1.08
PPD	100	.98,1.04	1.04	.97	.65	.67	.90
	33	1.11, .99	.96	.98	.63	.62	1.13
TB	100	.91, .82	.89	.76	.38	.71	1.02
	33	.89, .86	.81	.90	.54	.73	1.10

Results: (b) Acquisition of cellular immune responses following injection of RH-TF (Tables 4-7)

TABLE 4 RESULTS OF REPEATED SKIN TESTS FOR DELAYED HYPERSENSITIVITY IN CONTROL AND TF-INJECTED MONKEYS

Skin test AG and dose	Diameter 24-hr induration (mm) at different times after first TF injection		
	13 days	27 days	34 days
Monkey No. 35 ('normal' TF):-			
KLH(50µg)	<3	<3	<3
PPD(50µg)	<3	<3	<3
NaCl(0.1 ml)	<3	<3	<3
Monkey No. 36 (KLH + FCA TF):-			
KLH(50µg)	<3	7	9.5
PPD(50µg)	<3	11.5	13
NaCl(0.1 ml)	<3	<3	<3

TABLE 5 LYMPHOCYTE TRANSFORMATION RESPONSES FOLLOWING INJECTION OF RH-TF INTO RHESUS MONKEYS

Test AG and conc. (µg/ml)		^{3}H-thymidine incorporation (mean Δdpm/culture) before TF	days after TF 6	13	20	34
Monkey No. 35 ('normal' TF):-						
KLH	50	80	-3	0	12	45
	10	64	-4	21	16	109
PPD	50	60	-20	-23	16	33
	10	37	-24	-2	10	66
TB	50	7	-13	-15	3	22
	10	42	3	15	10	40
PHA	25	2524	19,670	11,256	7,373	14,649
Monkey No. 36 (KLH+FCA TF):-						
KLH	50	74	-9	12	-11	24
	10	62	-6	29	-37	30
PPD	50	5	-36	-32	-22	16
	10	22	-23	-21	-13	-7
TB	50	11	11	36	-15	-1
	10	50	-2	22	-12	4
PHA	25	2024	905	1275	1301	2514

Tables 4–6 show an illustrative experiment with two monkeys (No. 35, No, 36). 'Control' monkey 35 received RH-TF from 2.4×10^9 non-immune lymph node and spleen cells on day 0; a repeat injection on day 3; and 20 ml NaCl on day 20. Monkey 36 received 4.5×10^9 'immune' (KLH + FCA) spleen and lymph node cell RH-TF on days 0, 3 and 20. Both monkeys were skin-tested on days 13, 27 and 34 (see Table 4). The data shows that the control monkey failed to react in all tests with the exception of LMMI to the highest concentration of KLH on day 6. In contrast, the 'test' monkey acquired LMMI reactivity (Table 6) on day 6 which was lost by day 13 and later, despite repeated skin tests and a further injection of RH-TF. Even on day 13, DH responses in this monkey were negative. However, positive DH responses were obtained on days 27 and 34 (Table 4); these raise the question of whether RH-TF was acting to facilitate the selective development of DH by repeated skin testing. LT responses following RH-TF were uniformly negative to the antigens; PHA responses behaved differently in the two animals.

TABLE 6 MIXED CELL MIGRATION REACTIVITY (LMMI) FOLLOWING RH-TF INJECTION INTO RHESUS MONKEYS*

Test AG and conc. (μg/ml)		Migration index (medium control = 1.00)					
		before TF	days after TF				
			6	13	20	27	34
Monkey No. 35 ('normal' TF):-							
KLH	100	.90,1.03	.75	1.24	1.21	1.48	.86
	33	.96,1.25	.80	1.29	1.45	1.36	.90
PPD	100	.96,1.05	.81	1.14	1.10	1.06	.83
	33	.96,1.15	1.07	1.04	1.28	.81	.91
TB	100	1.11, .99	.86	1.14	ND	ND	.90
	33	.96, .96	.94	1.07	ND	ND	.92
Monkey No. 36 (KLH+FCA TF):-							
KLH	100	.94,1.04	.74	1.08	1.17	.89	1.00
	33	.85,1.01	.88	1.07	.96	.74	.90
PPD	100	.90, .96	.65	1.05	.86	.89	.99
	33	1.00,1.04	.58	.99	.82	.81	.92
TB	100	.88, .82	.60	.85	.78	.79	.96
	33	.96,1.04	.79	.89	.74	.77	.91

* (for TF schedule see text)

Table 7 shows that different patterns of LMMI reactivity were acquired by two different recipient animals (no. 19, No. 27) receiving a single injection of RH-TF (KLH + FCA) from another pool at two different doses (8 and 4 x 10^9 cell-equivalents). Neither of these monkeys had acquired DH responses when skin tested on days 7 and 14. The circumstances which govern the early (2-3 day) or later (7-14 day) acquisition of LMMI reactivity in RH-TF recipients are not yet defined.

TABLE 7 DIFFERENT PATTERNS OF ACQUISITION OF LMMI REACTIVITY AFTER RH-TF INJECTION*

Monkey and test AG (ug/ml)		Migration index (medium = 1.00) before TF	days after TF 2-3	7	12-14	43	70
Control No. 18 (NaCl only):-							
KLH	100	1.16	.78	.97	.83	-	1.20
PPD	100	1.00	1.02	.83	.73	-	1.02
TB	100	1.13	.90	1.25	.99	-	1.07
KLH+FCA TF (No.19): 8 x 10^9:-							
KLH	100	1.07	.77	.46	.48	-	0.73
PPD	100	.87	.66	.47	.59	-	1.17
TB	100	.88	.80	.55	.59	-	0.77
KLH+FCA TF (No.27): 4 x 10^9:-							
KLH	100	.99	1.30	-	.51	1.11	-
	33	.94	1.07	-	.61	.99	-
TB	100	.87	1.19	-	.85	1.16	-
	33	.80	1.26	-	.90	1.04	-

* (for TF and skin test schedule see text)

Discussion and conclusions

Our limited experience of RH-TF and of the active induction of CMI in the rhesus monkey leads us to the following interim conclusions: (1) that DH is easy to demonstrate following active immunisation but that it seems inappropriate for following the response to RH-TF; (2) that LT responses develop after active immunisation but not after RH-TF; and (3) that the LMMI test, probably indicative of lymphokine production, emerges as the most useful of the three tests for studying the CMI-response of recipients of RH-TF. This leads to the suggestion that if RH-TF acts without further

antigen upon naive animals, it may do so by preferentially 'sensitising' a cell population concerned with initiating or producing MIF-lymphokine rather than with the production of skin-reactive or mitogenic lymphokines. We know that these three lymphokines differ in parallel bioassay in the guinea pig (3) and it is reasonable to suppose that they differ also in the rhesus monkey. The results support the view that DTF may initiate or promote adoptive sensitisation for selective lymphokine production (4) and indicate that the LMMI test is worthy of further exploitation in this context in the rhesus monkey.

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Immunotherapy of Viral Infections With Transfer Factor

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It has been reported that dialysable leucocyte extract preparations, thought to contain transfer factor, may be used therapeutically for the treatment of a variety of immunodeficiency syndromes. Clinical and laboratory studies have suggested that such preparations, in addition to transferring specific cellular immunity may also contain non-specific adjuvant activities. Attempts at immunotherapy of viral infections are described against a background of current research on the biological and biochemical properties of leucocyte dialysates.

Key words: dialysable leucocyte extract, viral immunotherapy

INTRODUCTION

Landsteiner and Chase (1942) reported that delayed hypersensitivity skin reactions to simple chemical compounds could be transferred from sensitized to unsensitized guinea pigs with live peritoneal exudate cells, but not with heated cells or serum, thereby distinguishing cell-mediated from humoral antibody-mediated responses. Extending this work to man, Lawrence (1949, 1952) described the passive or adoptive cellular transfer of both local and systemic cutaneous delayed hypersensitivity to microbial antigens, using viable blood leucocytes. In contrast to the guinea pig, where skin reactions could only be demonstrated for up to 5 days posttransfer, it was reported that in man transferred skin

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reactivity persisted from 3 months to as long as 2 years. This observation was inconsistent with the view that transferred delayed hypersensitivity lasted only as long as the donor cells remained viable. Furthermore, similar transfers could be achieved when peripheral blood leucocytes were disrupted either with distilled water, or freeze-thawing in the presence or absence of ribonuclease and deoxyribonuclease (Lawrence 1954, 1955). Such methods of disruption did not apparently cause loss of transfer activity. Since DNase and RNase did not seem to affect the transfer of delayed hypersensitivity, the mechanism of transfer did not appear to depend on the presence of highly polymerized DNA or RNA. In addition, only small amounts of intact or disrupted cells (0.1—0.4 ml of packed white cells) relative to a large recipient mass were required to transfer cutaneous sensitivity. It was, therefore, concluded that the transfer component might be a self-replicating molecule.

Further studies (Lawrence and Pappenheimer, 1956) implied that the transfer extract was not immunogenic and that transferred cutaneous delayed hypersensitivity was not a function of humoral immunity. The term transfer factor was therefore introduced to describe the material in the leucocyte extracts responsible for the adoptive transfer of cell-mediated immunity. It was subsequently shown that transfer factor could be extracted without loss of activity from the bulk of the disrupted leucocytes by exhaustive dialysis into distilled water and lyophilisation (Lawrence et al., 1963). The term dialysable transfer factor was therefore reserved for the unidentified factor or factors contained in the crude dialysate which were capable of transferring delayed cutaneous hypersensitivity (Lawrence, 1969; Lawrence and Al-Askari, 1971). It has subsequently been suggested that the term dialysable leucocyte extract (Valdimarsson, 1975; Arala-Chaves et al., 1976) should be used to describe the total crude extract of leucocytes, which has been used to treat diseases where there is a defect in cellular immunity. It is believed that the transfer factor component of dialysable leucocyte extract may reconstitute specific cellular immunity and, therefore, be responsible for the therapeutic efficacy of leucocyte dialysates. However, there is now evidence that such material may also possess nonspecific adjuvant activities which may play an important role in immunotherapy.

Specificity

Clinical observations. Clinical and immunological observations on the acquisition of delayed type hypersensitivity following the administration of transfer factor have led to the concept that transfer factor converts nonimmune to immune lymphocytes in vivo. Thus, it has been repeatedly reported that previously unresponsive recipients acquire delayed type skin reactions to antigens to which the donor was positive (Rapaport et al., 1960; Kirkpatrick and Smith, 1976). However, there are reports that recipients may acquire skin test reactivities which were not demonstrable in the donor (Levin et al., 1971; Spitler et al., 1972; Griscelli, 1975). It has also been shown that not all positive skin reactions demonstrable in the donor may be transferred to the recipients (Kirkpatrick and Smith, 1976). Other alterations in cellular-immune responsiveness, which do not apparently relate to donor specificity, have been reported in recipients. These include altered mixed lymphocyte culture responsiveness (Dupont et al., 1974), increased responsiveness to phytohaemagglutinin in vitro (Arala-Chaves et al., 1974; Valdimarsson et al., 1974) and increases in the numbers of circulating T-cells as shown by increased sheep red blood cell rosetting (Wybran et al., 1973). These observations in man have led to the concept that dialysable leucocyte extract, in addition to conferring antigen responsiveness, may also possess nonspecific adjuvant properties. Further support for this hypothesis has been

gained from laboratory studies designed to test dialysable leucocyte extract activity in vivo in animals and in vitro.

In vivo and in vitro test system for transfer factor. Much of the scepticism which surrounded the early work on transfer factor resulted from the inability to demonstrate the transfer phenomenon in animals. Recently, however, studies in nonhuman primates, guinea pigs, and other animals have demonstrated that some of the properties of leucocyte extracts described in man may be reproduced in animals.

In nonhuman primates successful transfer of delayed hypersensitivity using viable leucocytes and leucocyte extracts has been reported from monkey to monkey (Zanelli and Adler, 1975) and from man to monkey (Steele et al., 1976a). Immunological specificity was implied by the observation that recipients never converted to antigens to which the donor did not react in vivo.

In guinea pigs transfer of delayed hypersensitivity has proved extremely difficult (Bloom and Chase, 1967). However, recent studies have shown that guinea pigs primed with antigen gave delayed skin reaction following injection of dialysable leucocyte extract (Welch et al., 1976). Whilst exposure to antigen is a prerequisite for the demonstration of subsequent delayed hypersensitivity skin reacitons, the role of donor specificity in eliciting such responses has yet to be fully determined.

In vitro tests for transfer factor have also provided evidence for both specific and nonspecific activities in dialysable leucocyte extract preparations (Hamblin, 1977). It has been reported that human and monkey dialysable leucocyte extracts nonspecifically augment antigen and mitogen induced lymphocyte transformation (Hamblin et al., 1976a, b; Mazaheri, unpublished). However, evidence has also been presented that such transformation test systems may also be used to detect the specific conversion of nonimmune to immune lymphocytes (Arala-Chaves et al., 1976).

Thus in vivo and in vitro test systems for transfer factor lend support to the clinical observations that dialysable leucocyte extract contains both specific and nonspecific activities. However, the relative importance of the specific and nonspecific activities of dialysable leucocyte extract for the treatment of disease have yet to be delineated. The biochemical properties of dialysable leucocyte extract have therefore been investigated in an attempt to determine which components of the extract are responsible for the alterations in cellular immune function in vivo and in vitro.

Biochemical Properties of Dialysable Leucocyte Extract

Using the transfer of delayed hypersensitivity as the criteria for assessing potency, the following properties of transfer factor were described by Lawrence (1974). The material is present in dialysable or ultrafiltrable extracts of disrupted leucocytes and therefore has a molecular weight of less than 10,000. It is not immunogenic, it is not an immunoglobulin, and it is separable from histocompatability antigens. The activity is not destroyed by repeated freeze-thawing, and it is not inactivated by DNase, RNase, and trypsin. It is stable for months to several years at −20°C or freeze-dried at 4°C.

Fractionation of dialysable leucocyte extract has been attempted in order to locate its specific and nonspecific components. Sephadex column chromatography has shown that the material is of heterogenous composition and can be separated into a number of fractions (Baram et al., 1966; Arala-Chaves et al., 1967; Gottlieb et al.,1973; Zuckerman et al., 1974; Burger et al., 1976a). Several publications report that one of the fractions,

rich in hypoxanthine, is associated with the ability to transfer delayed skin reactions (O'Dorisio et al., 1976; Tomar et al., 1976; Kirkpatrick et al., 1976). Others have produced evidence for fractions which nonspecifically stimulate weak immune reactivity in vivo (Krohn et al., 1976), and nonspecifically augment lymphocyte transformation in vitro (Burger et al., 1976b, Littman et al., 1977). More biochemical studies are required to relate these activities to each other and to the therapeutic efficacy of dialysable leucocyte extract.

Immunotherapy of Viral Infections

The concept that transfer factor could restore defective cell-mediated immunity as expressed by the acquisition of delayed-type skin reactions resulted in its therapeutic use in the treatment of a wide variety of acquired and congenital immunodeficiency diseases (Lawrence, 1974; Fundenberg et al. 1974). Therapeutic benefit from treatment with dialysable leucocyte extract has been reported for many disseminated intracellular infections, including viral infections (Zuckerman, 1975; Heim et al., 1976).

The initial attempts at immunotherapy of viral infections employed viable leucocytes, presumed to contain transfer factor. Thus Kempe (1960) treated a young boy suffering from progressive vaccinia infection with peripheral blood or lymph node leucocytes, and by transplantation of sensitized lymph nodes form vaccinated immune donors. The progression of the lesions was arrested and delayed hypersensitivity to inactivated vaccinia virus was conferred. Similarly O'Connell et al. (1964) treated an elderly patient suffering from severe progressive vaccinia. Intramuscular and subcutaneous injections of specific immune leucocytes were given on 2 occasions about 2 months apart. Acquisition of cutaneous delayed hypersensitivity to vaccinia antigen and termination of the infection were reported.

These initial studies were extended to the use of dialysable leucocyte extract thought to contain transfer factor. Drew et al. (1973) treated a patient with disseminated herpes zoster with dialysable leucocyte extract prepared from a pool of 27 healthy donors who demonstrated immunity to varicella-zoster in vitro. The patient, a 44-year-old man with stage IV B Hodgkin's disease and defective cell-mediated immunity was not treated with immunosuppressive drugs or corticosteroids in the 3 months preceding therapy. Following 2 subcutaneous injections of dialysable leucocyte extract the vesicular lesions completely healed. Moulias et al. (1973) treated 2 children with "giant-cell" measles pneumonia with dialysable leucocyte extract from immune donors. The patients responded with improvement and regression of the pulmonary lesions. Positive in vitro leucocyte migration inhibition responses to measles virus were reported after transfer, indicating improved cell-mediated immunity to the virus. Moulias et al. (1973) also treated 7 children with sub-acute sclerosing panencephalitis (SSPE), a degenerative disease associated with measles virus infection. Clinical improvement was seen in 2 out of the 7 children although 5 out of 7 showed conversion of the leucocyte migration inhibition test to measles antigen in vitro. Vandvik et al. (1973) treated a 16-year-old boy with SSPE with dialysable leucocyte extract from donors with measles immunity. The patient improved clinically and developed delayed hypersensitivity to various antigens and a selective reduction in measles antibody titre in the cerebrospinal fluid and serum. Rytel et al. (1975) treated a renal transplant patient with cytomegalovirus retinitis with dialysable leucocyte extract. Inactivation of the natural inflammatory lesions was seen. The patient also acquired delayed skin reaction and in vitro inhibition of leucocyte migration.

Attempts have been made to treat patients with chronic active hepatitis with transfer factor. There was no clinical improvement in a 29-year-old patient with chronic active hepatitis who received, over a period of 8 weeks, 17 injections of dialysable leucocyte extract twice weekly (Tong et al., 1976). Each injection was derived from 5×10^8 leucocytes from a donor who previously had acute hepatitis B, and showed lymphocyte reactivity to hepatitis B surface antigen in vitro. Clinical and biochemical improvement was not observed. However, the transfer of cell-mediated immunity was demonstrated by the appearance of delayed hypersensitivity to mumps and streptokinase-streptodornase and by in vitro lymphocyte responses to hepatitis B surface antigen. It was suggested that clinical improvement might have been seen with increased quantity, frequency, and duration of treatment. Kohler et al. (1974) treated a healthy persistent carrier of hepatitis B surface antigen with lymphocytes from an individual who had recovered from hepatitis B 8 months previously. Within 24 hours of the administration of lymphocytes, there was an increase in the titre of the surface antigen and of serum aspartate transaminase levels, suggesting liver cell damage. It was presumed that the development of cell-mediated immunity to hepatitis B was reflected in the injury of the virus-laden liver cells.

Another patient, a 4-month-old infant, who acquired hepatitis B from her mother at birth was given dialysable leucocyte extract prepared from the mother's leucocytes on 2 occasions. The recipient promptly developed an increase in the titre of hepatitis B surface antigen and elevation of serum transaminase activity. Subsequently, however, the liver function tests returned to normal and the titre of hepatitis B surface antigen decreased by 95%.

Jain et al. (1975) treated 3 patients with dialysates prepared from healthy donors and donors who had recovered from hepatitis B infection. One patient with hepatitis B surface antigen-positive chronic active hepatitis and cirrhosis did not respond to "normal" dialysable leucocyte extract, but injection of "specific" dialysable leucocyte extract resulted in a 2-fold increase in T lymphocytes, as measured by sheep red blood cell rosetting. The second patient, also did not respond to "normal" dialysable leucocyte extract, but showed a transient increase in transaminase activity. This suggested stimulation of cell-mediated immunity with a resulting hepatic damage. The third patient suffered from antigen-positive active chronic hepatitis complicated by primary liver cancer. This patient was being treated with corticosteroids and did not respond to either "normal" or "specific" dialysable leucocyte extract. There was no change in the serum titre of hepatitis B surface antigen in any of the patients.

The above accounts of the therapeutic use of transfer factor reflect the anecdotal nature of the work. A major criticism of transfer factor therapy has been the lack of properly controlled clinical trials. Since many diseases go through natural cycles of relapse and remission, and patients may be treated with other therapeutic agents, it is often difficult to assess the clinical benefit of treatment with transfer factor. Schulman et al. (1976) initiated a small, double-blind controlled trial with dialysable leucocyte extract. Four patients with chronic active hepatitis received dialysable leucocyte extract from pools of donors who had recovered from hepatitis B and A, while 4 others received normal saline. The results indicate that all 4 patients receiving dialysable leucocyte extract showed biochemical evidence of improvement, with a fall in serum transaminase activity. In 3 of the patients it was considered that there was also histological improvement, but hepatitis B surface antigen remained in the serum. The patients responding to dialysable leucocyte extract have remained in remission for periods of 9 months to 1½ years. None of the 4

recipients of normal saline improved. Based on these observations it was considered that dialysable leucocyte extract may be useful for the management of chronic hepatitis B infection (Schulman, 1977; personal communication).

It has been reported that transfer factor is a safe therapeutic agent. However, reactivation associated with reconstitution of cellular immunity may in itself produce deleterious side-effects.

Moulias et al. (1973) reported that regression of the pulmonary lesions in the children with measles pneumonia was associated with an initial inflammatory response. In addition, Drew et al. (1973) reported that their patient with Hodgkin's disease and disseminated herpes zoster developed fever and intense erythema around the vesicles following injections of dialysable leucocyte extract. Finally, it has been suggested that the increases in serum transaminase levels in chronic active hepatitis after the administration of dialysable leucocyte extract may reflect liver damage due to cellular immune reactions against the virus-laden liver cells (Kohler et al., 1974). Therefore, therapy with dialysable leucocyte extract should be used with caution for the treatment of disseminated intracellular infections.

Many of the problems relating to the treatment of infectious diseases could be clarified with an animal model. Several reports exist which suggest that transfer factor may be used to protect animals from infection. Thus Steele et al. (1976b) demonstrated that marmosets could be protected from fatal herpes simplex virus type 1 infection using dialysable leucocyte extract prepared from a human donor with marked cellular immunity to this virus. The protection was reported as being specific since it was ineffective against another member of the herpes group of viruses, herpes virus saimuri. Trepo and Prince (1976) treated hepatitis B virus carrier chimpanzees with leucocyte from donors sensitive to hepatitis B surface antigen and/or PPD. Systemic and local transfer of donor specific skin reactivity and leucocyte migration inhibition to both antigens were demonstrable in recipient monkeys. The development of delayed hypersensitivity to hepatitis B surface antigen in the chimpanzees was not accompanied by serious liver damage.

This review has summarized the immunotherapy of viral disease with transfer factor, a term which refers to "specific" transfer of delayed hypersensitivity, mainly in man. However, a more appropriate term, dialysable leucocyte extract, has been used here since this term embraces all the emerging specific and non-specific activities of leucocyte extracts observed clinically in man, experimentally in animals and in vitro studies. The treatment of a variety of viral infections with dialysable leucocyte extract has strongly suggested that an improvement in cellular immunity is often clinically beneficial. Controlled clinical trials are required to determine the role of dialysable leucocyte extract in the management of viral infections. Such trials, together with continued laboratory studies, will in due course relate the biological and biochemical activities of dialysable leucocyte extract to its usefulness as a therapeutic agent.

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