# STUDIES ON THE HUMAN IMMUNE RESPONSE TO TETANUS TOXOID

by

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## ABSTRACT

Two aspects of the human immune response to tetanus toxoid were investigated, viz: the kinetics of the response of pregnant females to tetanus toxoid immunization with respect to the immunity conferred on their babies; and the in vitro lymphocyte response to tetanus toxoid of reactors to tetanus vaccination.

Variables affecting the techniques for measuring the humoral immune response to tetanus toxoid were determined, and a method for antitoxin titration was devised which required only a small volume of serum. Antitoxin determinations in cord sera collected at delivery indicated the relation between timing of tetanus toxoid injections in the mother and the development of an acceptable level of protection in the babies. Analysis of the cord/maternal antitoxin ratios showed that active transport of maternal antitoxin to the foetus can occur, and a possible mechanism for this is discussed. The transplacental passage of tetanus toxoid itself was investigated by the use of an indirect immunofluorescence technique for the detection of tetanus-specific IgM in cord sera. No specific IgM was found in the cord sera, however, but assays for tetanus-specific IgM in maternal sera indicated that this immunoglobulin is often produced in response to second injections of tetanus toxoid.

Lymphocyte reactivity in vitro was determined by lymphocyte transformation in the presence of tetanus toxoid and PHA, as measured by the uptake of tritiated thymidine. Only a few of the severe reactors showed any evidence of an increased in vitro response to tetanus toxoid, but a clearcut inverse relation between age and lymphocyte reactivity to tetanus toxoid was demonstrated. Such an age-related decline in lymphocyte reactivity to a specific antigen in vitro has not been previously reported apparently. The relevance of this finding to current immunological theories of ageing is discussed.

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## INTRODUCTION

For studies on the human immune response to vaccines, tetanus toxoid is, in many ways, an ideal antigen. A major advantage is that the immune response to this antigen is not complicated by the presence of antibodies resulting from clinical or subclinical injections - at least, there is no evidence for this in man. Furthermore, tetanus toxoid is a relatively pure antigen whose effects can be evaluated biologically in terms of toxin-neutralising antibodies as well as by immunochemical methods. Studies on aspects of humoral immunity in the field of prevention of neonatal tetanus by maternal immunization, and on cellular immunity in reactors to tetanus toxoid immunization are reported here.

The true incidence of neonatal tetanus is unknown in many areas of the world, but Bytchenko (1966) estimates that up to one-third of all cases of tetanus occur in neonates. According to Miller (1972), tetanus during the first month of life accounts for one-quarter to one-half of all tetanus deaths. Newborn babies, therefore, constitute the largest single group requiring protection from tetanus.

In the type of developing, tropical, rural community in which tetanus neonatorum is most prevalent, the direct and logical approach to its prevention, i.e. by improving standards of hygiene in the delivery and care of newborn babies is not the most practicable. Non-cooperation in training programmes on the part of the traditional midwives, and the general economic, social and cultural characteristics of the populations concerned are largely responsible for making such an approach unworkable (Barten, 1969; Chen, 1974; Yunes et al., 1974). The alternative means of prevention by prenatal immunization of the mothers with consequent passage of protective antitoxin to their babies is the more effective and economical. Of course, widespread immunization programmes are not without their own problems, some of which have

been specified by MacLennan et al. (1965) as scarcity of staff; limited communication; and the difficulties of keeping an illiterate population to a schedule of appointments with vaccinators. Such difficulties and restrictions make it all the more important that reliable guidelines for efficient immunization procedures be provided, based on a determination of the parameters governing antitoxin production in the mothers and its transport to their babies.

Several field trials have been conducted on the prevention of neonatal tetanus by maternal immunization, the results of which have verified the general effectiveness of the procedure, and indicated some of the limitations. It has been shown that, while two or three suitably spaced injections of fluid tetanus toxoid in the mothers are capable of preventing or significantly reducing the incidence of neonatal tetanus (Schofield, Tucker & Weathrook, 1961), immunization with adsorbed toxoid is more effective at providing the protective level of 0.01 unit per ml of tetanus antitoxin in cord sera (Suri, Dhillon & Grewal, 1964). In both these studies, three injections were found to be more effective than two. Suri et al. (1964) reported also that none of the 19 subjects given a single dose of adsorbed toxoid less than 30 days before delivery gave cord blood titres of 0.01 unit per ml or more. MacLennan et al. (1965) confirmed the superiority of adsorbed toxoids over the fluid type particularly in terms of the persistence of the antibody formed. Oil-adjuvant toxoids produced even more persistent titres, but also gave unacceptable side-effects.

In a double-blind controlled field trial, Newell et al. (1966) showed that two or three injections of 10 Lf of adsorbed toxoid effectively eliminated tetanus neonatorum for a period of five years. In this study, two injections, given six weeks apart, were found to be as effective as three injections in preventing tetanus among the 341 babies born to mothers immunized during pregnancy or up to five years before. In a similar group of 347

women who received no toxoid, 27 cases of tetanus occurred. One injection did not reduce the incidance of tetanus significantly. The results of serological investigations on the group (Newell et al., 1971) confirmed the finidngs of the clinical trial in that 90% of the subjects who received two doses of toxoid had protective titres for up to five years. It was also observed that antitoxin titres were directly related to the interval between injections, and inversely related to age.

Stanfield, Gall & Bracken (1973) examined the effects of various vaccine schedules, dosages of toxoid, and types of adjuvants in an effort to reduce the number of injections required to achieve protection with the ultimate goal of providing an effective one-dose schedule. The best single-dose schedule tested was 100 Lf of an adsorbed toxoid containing the quaternary ammonium adjuvant "Arquad 2HT "which achieved protective levels of antitoxin in 81% of the mothers when the injections were given at least 60 days before delivery. These workers also established the fact that the transfer of antitoxin to the foetus is more effective and rapid after two doses of toxoid than it is after a single dose.

Recently, Dhillon & Mahon (1975) reported on their investigations into the effect of varying the time interval between two injections of 5 Lf of adsorbed toxoid over periods ranging from 4 to 16 weeks. The recommendation emanating from the study was th . the two injections should be given not less than 12 weeks apart with the second at least 4 weeks from delivery, although it was conceded that this schedule is not always feasible.

Since the findings indicate that the timing of maternal immunization may play an important role in determining the level of protection conferred, a study was undertaken to determine the relation between the timing of the tetanus toxoid injections in the mother and the presence of protective antitoxin titres at delivery in the babies. Specifically, the questions to be answered were how long before delivery must the first of a two-dose schedule of tetanus toxoid injections be given in order to provide a reasonable

probability of protection; and how late in pregnancy might a single dose of toxoid be effective. To provide the answers, a series of pregnant women were injected at different times during gestation with two doses of a standard commercially available adsorbed toxoid with the usual six weeks interval between injections, and maternal and cord bloods were collected at delivery and subsequently titrated for antitoxin content.

It is Gill's hypothesis (1973) that antigens such as tetanus toxoid can also cross the placenta, and stimulate the foetus. Leiken & Oppenheim (1971) and Cramer, Kunz & Gill (1974) claim that exposure of the foetus to antigen in this manner can just as readily sensitize the foetus as render it partially tolerant, and cite as evidence for sensitization the altered immune reactivity of the foetus for antigens - i.e. aside from infectious agents - to which the mother has been exposed intentionally or accidentally during pregnancy. The determination of the transplacental passage of tetanus toxoid depends on the production of a specific immune response by the foetus. It has been shown that the capacity for immune responsiveness is acquired fairly early in foetal development (van Furth, Schuit & Hijmans, 1965; Prindull, 1974), and it is well known that various congenital infections result in the production of measurable amounts of foetal IgM (Eichenwald & Shinefield, 1961; Saver, 1969), so it may be expected that such a foetal response will occur in the presence of tetanus toxoid with production of tetanus-specific IgM. Hence, the presence of specific IgM in cord sera was investigated as a means of testing Gill's hypothesis.

Investigations into these aspects of the humoral immune response to tetanus toxoid require the measurement of tetanus antitoxin and a method for detecting the IgM component of the antitoxin. However, existing methods for the titration of tetanus antitoxin by toxin neutralization do not provide a means of conducting assays on small volumes of serum with accuracy and

sensitivity. The need for such a method is real since in those developing countries where tetanus is a problem the collection of adequate volumes of test samples is also a problem.

In response to this need, therefore, a method for titrating tetanus antitoxin by toxin neutralization was investigated which conserved serum by making comparisons on 0.3 ml serum samples against 0.3 ml volumes of an equine standard diluted to a concentration of ten times that required to be assayed in the test sample. The technique was designed to measure from 0.001 unit per ml of antitoxin up to any higher concentration in twofold serum dilutions. The influence of such variables as the body weight of the mice used in the toxin neutralization test was also investigated.

To avoid the necessity for the large number of mice that the titration of tetanus antitoxin by toxin neutralization in twofold serum dilutions would otherwise entail, a prior assessment of the antitoxin content is made by the indirect haemagglutination technique. This technique can also be used as a reliable screening procedure for the presence of antitoxin because of its high sensitivity. A systematic investigation of the variables affecting the indirect haemagglutination test for tetanus antitoxin was undertaken with a view to attaining maximum sensitivity in the test system by the application of optimal conditions. The use of 2-mercaptoethanol in the diluent as a means of inactivating the IgM component of the tetanus antitoxin, and so improving the correlation between the toxin neutralization and indirect haemagglutination tests, was also investigated.

Despite the high sensitivity of the indirect haemagglutination technique, the requirement for a fourfold difference in titres for significance which is necessitated by its inherent variability limits its value as a method for detecting small amounts of specific IgM on the basis of the difference between titres before and after treatment of the serum with 2-mercaptoethanol.

Zimmerman & Hill (1969) encountered this problem when they attempted to demonstrate the foetal production of streptococcal antibody by haemagglutination. They found that the titres obtained with M protein-sensitized sheep cells after treatment of the test sera with 2-mercaptoethanol differed in every instance, but only by one twofold dilution. Hence, the method chosen for the detection of tetanus-specific IgM was one based on immunofluorescence since immunofluorescence techniques have been applied successfully to the detection of specific IgM in such neonatal infections as congenital toxoplasmosis (Remington, 1969) and congenital syphilis (Scotti, Logan & Caldwell, 1969). In the development of a suitable indirect immunofluorescence technique, comparisons were made using polymerized tetanus toxoid and tetanus toxoid-coated Sepharose beads as antigens, and steps were taken to ensure that a high degree of specificity was achieved in the reaction system.

Accounts of the methods for measuring total antitoxin by toxin neutralization and haemagglutination, and for detecting IgM antitoxin by immunofluorescence are given in Chapters 1, 2 and 3 respectively. Chapter 4 embodies the results of the application of these methods to the assay of sera obtained from mothers and their babies at delivery following injection of the mothers with tetanus toxoid at different times during gestation.

Investigation into the cell-mediated response to tetanus toxoid was prompted by the report of White et al. (1973) that a 0.9% incidence of reactions to primary injections of tetanus toxoid occurred in a population of industrial workers. Reactors on first injection suggest a mechanism of delayed type hypersensitivity, and, although their occasional occurrence had been described previously (Levine, Ipsen & McComb, 1961; Griffith, 1966; Kittler et al., 1966), reactions to tetanus toxoid immunization are usually attributed to and associated with the formation of antigen-antibody complexes due to relatively high serum antitoxin concentrations (Edsall, 1959; Levine, Ipsen & McComb,

1961; Griffith, 1966; Edsall et al., 1967).

Various other associations have also been reported, e.g. the incidence of reactions to tetanus toxoid is higher in females than in males (Griffith, 1966; Relihan, 1969; White et al., 1973), and increases with age in adults (McComb & Levine, 1961; Levine, Ipsen & McComb, 1961; Relihan, 1969; White et al., 1973). Reactions are more common after subcutaneous than after intramuscular injections (Edsall, 1959; Relihan, 1969), and a reduction in the tetanus toxoid dosage does reduce the incidence (McComb & Levine, 1961; Relihan, 1966; White et al., 1973). Facktor, Bernstein & Fireman (1973) reported a very high incidence of mixed reactions - immediate allergic, Arthus-type, and delayed type hypersensitivity - in 70 apparently normal individuals given an intracutaneous injection of tetanus toxoid. The delayed type hypersensitivity reaction occurred in 76% of these subjects. Obviously, the high incidence of reactions under these circumstances has little relevance to the clinical situation, but it does indicate that the delayed type of hypersensitivity reaction, generally associated with T cell reactivity, can occur in reactions to tetanus toxoid vaccinations.

A method for measuring cell-mediated immunity to tetanus toxoid in vitro, viz: lymphocyte transformation in the presence of tetanus toxoid, as determined by the uptake of tritiated thymidine, was used for assessing the degree of lymphocyte reactivity in the reactor group. The general reactivity of the lymphocytes to PHA was also determined. The objectives of the study were to determine whether there was any correlation between specific or general lymphocyte reactivity and the occurrence of reactions; and whether the association of reactions with the female sex and with advancing age in adults would be reflected in the in vitro situation. The study is described in Chapter 5.

# CHAPTER 1

## THE TITRATION OF TETANUS ANTITOXIN BY TOXIN NEUTRALIZATION

Tetanus antitoxin is generally measured in biological terms by using the mouse as the indicator of any excess toxin which remains after mixtures of toxin and antitoxin interact. Titres obtained by such a method give a measure of the biologically active form of antitoxin.

In principle, a test dose of toxin is determined in relation to a known amount of Standard Tetanus Antitoxin, and the concentration of antitoxin in an unknown serum sample is then estimated from the dilution of that serum which gives the same end-point as the Standard Antitoxin with this test dose. In practice, the existing assay techniques require a relatively large volume of serum for the determination of low concentrations of tetanus antitoxin.

Because of the difficulties involved in the collection of adequate serum volumes, a method for tetanus antitoxin determination by toxin neutralization was developed with a reduced sample volume requirement. The technique is simple, and the results obtained compare favourably with, or exceed, those of other methods in terms of sensitivity, precision and accuracy, provided that the weight range of the mice used is restricted and standardized, and that the conditions of interaction of toxin and antitoxin are controlled.

## MATERIALS AND METHODS

Toxins. Two batches of tetanus toxins, designated No.954 and No.956, were obtained from the Rijks Instituut of the Netherlands. A third sample of toxin, lot No.682-C2, was supplied by Wellcome Research Laboratories. Details of the source, production, characteristics, and suppliers of these toxins are given in Appendix (I).

Toxins in the freeze-dried state were weighed, dissolved in 1% peptone water of pH 7.2-7.4 (Oxoid Ltd., Southwark Bridge Road, London), and filtered through a Gelman type GA-6 triacetate

membrane filter (Gelman Hawksley Ltd., 12 Peter Road, Lancing, Sussex). The toxin solutions were then distributed in small volumes and stored at -30°C. In general, this procedure follows the recommendations of the WHO Expert Committee on Biological Standardization (1965) for the preparation of materials to serve as reference reagents.

Standard Antitoxins. A sample of the Third British Standard for Tetanus Antitoxin, provided by the Medical Research Council, was used. In some experiments, a sample of the U.S. Standard Tetanus Antitoxin was also included for comparison. Both are equine standards. Details are given in Appendix (ii).

Diluent. All dilutions for the toxin neutralization test were performed in sterile 1% peptone water (Oxoid), adjusted to a pH of 7.2-7.4 (Ipsen, 1942).

Mice. Swiss mice of strain "Theiler's Original" were obtained from A. J. Tuck & Son Ltd., Animal Research Breeders, Rayleigh, Essex. Mice of either sex and of weight range 14-17 g (av.15-16 g) at the time of injection were used. To reduce nervousness and so facilitate their handling, the mice were allowed a day's settling-in period before injection.

Determination of the test dose levels of toxin.

Definitions. The L+/100 test dose of toxin is the least amount of toxin which, when combined with 0.01 unit of standard antitoxin in the dose per mouse, causes death of all mice injected by the end of 96 hours. Similar definitions apply to the L+/1000 and L+/4000 toxin doses with 0.001 and 0.00025 unit of Standard Antitoxin respectively.

The Lp/10000 test dose of toxin is the least amount of toxin which, when combined with 0.0001 unit of Standard Antitoxin in the mouse dose, causes a 2+ degree of paralysis in the injected legs after five days. With this degree of paralysis, the mouse shows an obvious disability of the hind limb and limping in free movement, i.e. the limb is functional but its activity is impaired.

L+/1000 determinations. Preliminary experiments are conducted to estimate the approximate L+/1000 dose of toxin, and a series of toxin dilutions then prepared with the estimated L+/1000 dose at about the mid-point for further testing against the Standard Tetanus Antitoxin. The least amount of toxin in an 0.2 ml volume

which reacts with 0.001 unit of Standard Antitoxin in 0.1 ml to give death of all mice after 96 hours is determined. Hence dilutions of 5 x estimated dose level of toxin per ml and 10 x 0.001 unit of Standard Antitoxin per ml are mixed in the ratio of 2 volumes: 1 volume to give the required concentrations in the 0.3 ml aliquots which are injected into mice. E-MIL, A grade pipettes (James A. Jobling & Co., Ltd., Glamorgan) are used for the preparation of dilutions, since the 5 x and 10 x concentration factors tend to magnify small errors in volume measurement.

At the time of their distribution into cages, the mice are marked by means of a suitable coding system, e.g. coloured dyes near the heads or tails, to represent the sequence of dilutions to be tested. Generally, five toxin dilutions are tested using eight mice per dilution. Therefore, a total volume of 2.4 ml of toxin-antitoxin mixture is required. This is provided by mixing 1.8 ml of the 5 x L+/1000 dose per ml of toxin with 0.9 ml of the 0.01 unit per ml of Standard Antitoxin. After mixing, the toxin and antitoxin are allowed to interact at room temperature (approx. 20°C) for one hour. Volumes of 0.3 ml of the mixture are then injected into the right thigh muscles of the mice. An operator working alone can inject the mice with ease, accuracy and speed by using an ordinary hair roller to hold the animal (Peel & Horwood, 1975). A single, mounted, corked roller may be used as shown in figure 1, or a series of rollers may be joined by adhesive tape, and the mouse thereby provided with a means of returning directly to its cage when the injection procedure has been completed. Deaths are recorded 96 hours after injection, and the end-points determined. Titrations of the L+/4000 and L+/100 doses are usually also conducted on each batch of diluted toxin to establish that the L+ values are in proportion to the unitage of Standard Antitoxin used.

Determination of the minimum lethal dose (MLD) of toxin.

The MLD is defined as the least amount of toxin that causes death of all mice injected at the end of 96 hours. It is determined by a method similar to that used for the L+ determinations except

Fig. 1 Intramuscular injection of the leg of a mouse. A corked hair-roller provides a convenient and inexpensive means of holding the animal.

that diluent is substituted for antitoxin.

Procedure for serum titrations.

The toxin dose level at which the titration of an unknown serum is conducted, as well as the actual range of serum dilutions tested, are pre-determined on the basis of the tetanus antitoxin concentration as estimated by the indirect microhaemagglutination technique. Usually an end-point for the toxin neutralization test can be obtained by testing a range of four or five serum dilutions using one or two mice per dilution.

Twofold dilutions of each test serum are prepared from the second of a series of tubes with a 100 microlitre Oxford sampling pipette and sterile, disposable plastic tips (The Boehringer Corporation Ltd., Bell Lane, Lewes, East Sussex). Four or five dilutions are then selected for testing, and the lower dilutions are discarded. Undiluted serum (0.3 ml) is delivered to the first tube of the series if required. A volume of 0.6 ml of toxin diluted to contain 5 x L+/1000 test dose per ml, is added to each tube. This gives a total volume of 0.9 ml in each tube of which 0.3 ml is injected into each of one or two mice after one hour. The volume injected thus represents one-third of the total volume of the contents of each tube, equivalent to the test dose of toxin (in 0.2 ml) and 0.1 ml of serum or diluted serum.

Controls consisting of the toxin test dose with the corresponding concentration of Standard Antitoxin and the toxin test dose with twice this concentration of Standard Antitoxin are included. For a valid titration, the former should result in the death of all animals injected by the end of 96 hours, while the latter should protect the mice from death. When titrating at the Lp/10000 toxin level, an additional control consisting of toxin only is included to give a 4+ paralytic end-point for comparison with the 2+ paralytic end-point.

If undiluted serum fails to protect both mice, i.e. if death occurs in both, then the serum has an antitoxin concentration equal to or less than the lowest detectable antitoxin titre of the test range. This varies according to the toxin dose level of the test. If serum diluted 1:2 fails to protect while undiluted serum protects at least one of the mice, then the antitoxin titre is greater than the lowest level of the range and equal to or less

than the next higher amount. Since the amount of antitoxin in 0.1 ml of an unknown serum is measured against the toxin test dose, the actual antitoxin content per ml of the original serum is obtained by multiplying this amount by a factor of 10.

Tables 1 and 2 provide details of the protocol used for the titation of antitoxin at the L+/100 and Lp/10000 toxin levels. Tables 3 and 4 illustrate the interpretation of the readings for each of these test levels. Table 5 sets out the relationship between the volume of serum available and the minimal antitoxin titres that can be determined for different test dose levels of toxin.

## EXPERIMENTS AND RESULTS

### L+/1000 and L+/4000 determinations.

Examples of L+/1000 and L+/4000 determinations are given in tables 6 and 7. Average weights (± standard deviation) of the mice used were 15.42 ± 0.66g and 15.92 ± 0.97g for L+/1000 and L+/4000 respectively. Results indicate that the L+/1000 and L+/4000 values are in the ratio of 4:1 as expected. The 95% confidence limits for these determinations are 5.13 µg and 5.21 µg for the L+/1000 dose; and 1.21 µg and 1.29 µg for the L+/4000 dose. The same toxin gave an MLD of 0.35 ± 0.04 µg for mice of weight 16.04 ± 1.21g over six experiments. Therefore, the L+/4000 dose contains 3-4 MLD's while the L+/1000 dose contains 15-16 MLD's. This difference probably accounts for the fact that the coefficient of variation is 4.0% for the L+/4000 assays and only 0.97% for the L+/1000 assays.

### Ratios of L+ values for different toxins.

Three different batches of toxins were assayed for L+ levels (table 8). Ratios of the L+/4000, L+/1000 and L+/100 values for these toxins were 1:4:10, a necessary condition for their use at different dose levels in the titration of antitoxin. The constancy of the ratios also illustrates the stability of the toxin under the conditions of storage, and the consistency of the procedures used for the determination of L+ values. The reproducibility of an L+ determination is particularly well demonstrated for Dutch toxin No.954 where eight repeated assays of L+/1000 gave the

TABLE 1

Titration of tetanus antitoxin in sera at the L+/100 toxin dose level[a]

| Vol. of toxin diluted in 1% peptone buffer to 5 x L+/100 dose/ml | i Vol. of diluted standard antitoxin / ii Vol. of diluted sera | Total vol. per tube | Vol. injected per mouse | i Units of standard antitoxin injected / ii Vol. of serum dilutions injected | Results 96h after injection |
|---|---|---|---|---|---|
| | | I CONTROLS | | | |
| 0.6 ml (i.e. 3 x L+/100) | [b]0.3 ml of 0.1 u/ml (i.e. 0.03 unit) | 0.9 ml | 0.3 ml (i.e. L+/100) | 0.01 unit | Death in all mice |
| 0.6 ml | 0.3 ml of 0.2 u/ml (i.e. 0.06 unit) | 0.9 ml | 0.3 ml | 0.02 unit | Survival of all mice with symptoms of tetanus |
| | | II TEST | | | |
| 0.6 ml (i.e. 3 x L+/100) | 0.3 ml of undiluted serum (1/1) | 0.9 ml | 0.3 ml | 0.1 ml of 1/1 dilution | Death or survival[c] |
| 0.6 ml | 0.3 ml of serum diluted 1/2 etc. | 0.9 ml | 0.3 ml | 0.1 ml of 1/2 dilution | |

[a]This protocol is adapted for testing at lower toxin dose levels with lethal end-points by simply diluting the toxin and standard antitoxin to the concentrations required.

[b]These volumes are usually doubled to allow for the injection of four mice per control; generally one or two mice are used per test serum dilution.

[c]See Table 3 for interpretation.

TABLE 2

Titration of low titres of tetanus antitoxin at the Lp/10000 toxin test dose level

| Vol. of toxin diluted in 1% peptone buffer to 5 x Lp/10000dose/ml | i Vol. of diluted standard antitoxin or diluent<br>ii Vol. of diluted sera | Total vol. per tube | Vol. injected per mouse | i Units of standard antitoxin injected<br>ii Vol. of serum dilutions injected | Results 120h after injection |
|---|---|---|---|---|---|
| | | I CONTROLS | | | |
| [a]0.6 ml (i.e. 3 x Lp/10000) | [a]0.3 ml of 0.001 u/ml (i.e. 0.0003 unit) | 0.9 ml | 0.3 ml (i.e. 1 x Lp/10000) | 0.0001 unit | Paralysis 2+ |
| 0.6 ml | 0.3 ml of 0.002 u/ml (i.e. 0.0006 unit) | 0.9 ml | 0.3 ml | 0.0002 unit | Paralysis 1+ |
| 0.6 ml | 0.3 ml of diluent | 0.9 ml | 0.3 ml | - | Paralysis 4+ |
| | | II TEST | | | |
| 0.6 ml | 0.3 ml of undiluted serum (1/1) | 0.9 ml | 0.3 ml | 0.1 ml of 1/1 dilution | Paralysis graded 0-4+[b] |
| 0.6 ml | 0.3 ml of serum diluted 1/2 etc. | 0.9 ml | 0.3 ml | 0.1 ml of 1/2 dilution | |

[a]These volumes are usually doubled to allow for the injection of four mice per control.

[b]See Table 4 for interpretation.

TABLE 3

Interpretation of end-point readings in the assay of tetanus antitoxin at the L+/100 test dose level

| Serum dilutions: | 1/1 | 1/2 | 1/4 | Antitoxin titres u/ml |
|---|---|---|---|---|
| Readings 96h after injection: | $2_D$[a] | $2_D$ | $2_D$ | = or < 0.1 |
| | $1_D + 1_S$ | $2_D$ | $2_D$ | >0.1 = or <0.2 |
| | $2_S$[b] | $2_S$ | $2_D$ | >0.2 = or <0.4 |

[a] $2_D$ = death of both mice.

[b] $2_S$ = survival of both mice.

TABLE 4

Interpretation of end-point readings in the assay of tetanus antitoxin at the Lp/10000 test dose level

| Serum dilutions: | 1/1 | 1/2 | 1/4 | Antitoxin titres u/ml |
|---|---|---|---|---|
| | $^{2}P_{4}$[a] | $^{2}P_{4}$ | $^{2}P_{4}$ | <0.001 |
| Readings 120h after injection: | $^{2}P_{2}$ | $^{2}P_{3}$ | $^{2}P_{4}$ | 0.001 |
| | $^{2}P_{1}$ | $^{2}P_{2}$ | $^{2}P_{3}$ | 0.002 |
| | $^{2}P_{1}$ | $^{2}P_{2}$ | $^{2}P_{2}$ | 0.004 |

[a] Results are expressed in the form $^{x}P_{y}$ where x is the number of mice showing paralytic symptoms of grade y 120 hours after injection.

TABLE 5

Relationship between the volume of serum available for testing and the minimal antitoxin titre that can be determined for different test dose levels of toxin.

| VOLUME OF SERUM | TEST DOSE LEVEL OF TOXIN | LOWEST DETECTABLE ANTITOXIN TITRE |
|---|---|---|
| | | u/ml |
| 0.6 ml | L+/100 | 0.1 |
| | L+/1000 | 0.01 |
| | L+/4000 | 0.0025 |
| | Lp/10000 | 0.001 |
| 0.3 ml | L+/100 | 0.2 |
| | L+/1000 | 0.02 |
| | L+/4000 | 0.005 |
| | Lp/10000 | 0.002 |

TABLE 6

L+/1000 determinations for Dutch tetanus toxin batch No.956 with the Third British Standard Antitoxin

| DOSE OF TOXIN | EXPERIMENT NUMBER | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| µg | | | | | | |
| 5.4 | [a]8/8 | 8/8 | 8/8 | 8/8 | 8/8 | 8/8 |
| 5.3 | 8/8 | 8/8 | 8/8 | 8/8 | 8/8 | 8/8 |
| 5.2 | *8/8 | 8/8 | *8/8 | 8/8 | *8/8 | *8/8 |
| 5.1 | 6/8 | *8/8 | 7/8 | *8/8 | 7/8 | 7/8 |
| 5.0 | 7/8 | 6/8 | 7/8 | 7/8 | 6/8 | 6/8 |

[a]Results are presented as the number of mice dead at 96 hours/number of mice injected.

*Indicates the L+/1000 end-points. Mean ± s.d. for L+/1000 = 5.2 ± 0.05µg.

TABLE 7

L+/4000 determinations for Dutch tetanus toxin batch No.956 with the Third Standard Antitoxin (British)

| DOSE OF TOXIN | EXPERIMENT NUMBER | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| µg | | | | | | |
| 1.4 | [a]8/8 | 8/8 | 8/8 | 8/8 | 8/8 | 8/8 |
| 1.3 | *8/8 | 8/8 | *8/8 | *8/8 | 8/8 | 8/8 |
| 1.2 | 6/8 | *8/8 | 4/8 | 6/8 | *8/8 | *8/8 |
| 1.1 | 5/8 | 6/8 | 5/8 | 2/8 | 4/8 | 6/8 |
| 1.0 | 3/8 | 7/8 | 2/8 | 0/8 | 0/8 | 3/8 |

[a]Results are presented as the number of mice dead at 96 hours/number of mice injected.

*Indicates the L+/4000 end-points. Mean ± s.d. for L+/4000 = 1.3 ± 0.05µg.

TABLE 8

Constancy of the ratios of the L+/4000, L+/1000 and L+/100 values for three preparations of tetanus toxins.

| TOXIN | TEST DOSE LEVELS[a] | | |
|---|---|---|---|
| | L+/4000 | L+/1000 | L+/100 |
| | | ml x $10^{-4}$ | |
| British No.682 C2 | [b] 0.6 ± 0.1 (4) | 2.4 ± 0.4 (4) | 24 ± 0.1 (4) |
| | | µg | |
| Dutch No.954 | 1.1 ± 0.05 (15) | 4.3 ± 0 (8) | 44 ± 0.6 (4) |
| Dutch No.956 | 1.3 ± 0.08 (6) | 5.2 ± 0.05 (6) | N.D.[c] |

[a] The test dose levels were usually determined with the Third British Standard Antitoxin. Where U.S. Standard Antitoxin was used (Lot E114 from N.I.H.), no significant differences were found so results were combined.

[b] (n) is the number of separate experiments for each determination. A range of seven to eight toxin dilutions were tested for each experiment using eight (sometimes four) mice per dilution. Results are expressed as the mean ± s.d. for n experiments.

[c] Not determined.

same value of 4.3 µg.

Importance of the diluent.

pH of diluent. The original L+/1000 and L+/4000 determinations for Dutch toxin No.954 did not give values in accord with the expected ratio of 4:1. Furthermore, it was found that the results varied depending on whether dilutions for the L+/1000 or L+/4000 levels were injected first into mice when both were determined in the one experiment. Variation according to the order of injections suggested that the time of contact of the toxin-antitoxin mixtures before injection was at least partly responsible for the discrepancies observed. Subsequently, it was discovered that the diluent used in these experiments had a pH value of 6.4 instead of 7.2-7.4. Apparently, the acidity of the 1% Evan's peptone that was added to Oxoid phosphate buffered saline (Appendix iii) for the preparation of this diluent was sufficient to exceed its buffering capacity. When this was discovered, experiments were designed to define more precisely the relationship between an unfavourable pH and the time of contact of toxin-antitoxin mixtures prior to injection. Results of such experiments (tables 9, 10 and 11) show that the deleterious effect of a diluent of pH 6.4 is, indeed, time-dependent; an increase in the time of contact of toxin-antitoxin mixtures before injection causes an increase in the apparent value of L+/4000 with a diluent of pH 6.4 but not with a diluent of pH 7.4. If the results of all three experiments are combined, and the number of deaths at pH 6.4 and at pH 7.4 are compared, a significant difference at the 0.01 significance level can be shown by the Chi-squared test ($p < 0.01$).

A diluent of pH 6.4 also caused an increase in the estimated MLD values with prolonged time of standing before injection e.g. after 2 hours standing, the value was 0.3 µg; after 3 hours standing, the value was 0.4 µg. This suggests that the increase in exposure with toxin-antitoxin mixtures is the result of slow inactivation of the excess toxin in those mixtures.

The weights of all mice used in these experiments were recorded, and the mean weight and standard deviation for each group calculated. None of the means was less than 14 g or greater than 16 g, and the standard deviations were similar in magnitude for all

TABLE 9

Variation in the value of L+/4000 determinations on Dutch tetanus toxin batch No.954 with diluent of pH6.4 and prolonged toxin-antitoxin contact time prior to injection into mice.

| TIME OF CONTACT OF MIXTURES BEFORE INJECTION | L+/4000 DETERMINATIONS[a] | |
|---|---|---|
| | Diluent of pH7.4 (Control) | Diluent of pH6.4 |
| h. | ml | ml |
| 1 | 1.1 ± 0.04 | 1.1 ± 0.04 |
| 1½ - 2 | 1.1 ± 0.05 | 1.3 ± 0.07 |

[a]Five separate experiments were performed for each L+/4000 determination given in the table. Each determination involved the testing of a range of five toxin doses with four mice per dose. Four of the determinations were performed with the Third British Standard Antitoxin: the other with a U.S. Standard Tetanus Antitoxin (Lot E114 from N.I.H.). Results are expressed as the L+/4000 end-point ± s.d. for five experiments.

TABLE 10

Effect of toxin-antitoxin contact times of one and two hours on L+/4000 determinations with diluent of pH6.4

| DOSE OF TOXIN | CONTACT TIME OF ONE HOUR BEFORE INJECTION | | CONTACT TIME OF TWO HOURS BEFORE INJECTION | |
|---|---|---|---|---|
| | Diluent of pH7.4 (Control) | Diluent of pH6.4 | Diluent of pH7.4 (Control) | Diluent of pH6.4 |
| µg | | | | |
| 1.4 | [a]4/4 | 4/4 | 4/4 | 4/4 |
| 1.3 | 4/4 | 4/4 | 4/4 | *4/4 |
| 1.2 | 4/4 | 4/4 | 4/4 | 1/4 |
| 1.1 | *4/4 | *4/4 | *4/4 | 3/4 |
| 1.0 | 2/4 | 3/4 | 1/4 | 1/4 |
| 0.9 | 0/4 | 1/4 | 0/4 | 0/4 |

[a] Results are presented as the number of mice dead at 96 hours/number of mice injected.

* Indicates the L+/4000 end-points.

TABLE 11

Effect of toxin-antitoxin contact times of one, two and three hours on L+/4000 determinations with diluent of pH6.4

| DOSE OF TOXIN | CONTACT TIME OF ONE HOUR BEFORE INJECTION | | CONTACT TIME OF TWO HOURS BEFORE INJECTION | | CONTACT TIME OF THREE HOURS BEFORE INJECTION | |
|---|---|---|---|---|---|---|
| | Diluent of pH7.4 (Control) | Diluent of pH6.4 | Diluent of pH7.4 (Control) | Diluent of pH6.4 | Diluent of pH7.4 (Control) | Diluent of pH6.4 |
| µg | | | | | | |
| 1.4 | [a]4/4 | 4/4 | 4/4 | 4/4 | 4/4 | 4/4 |
| 1.3 | 4/4 | 4/4 | 4/4 | 4/4 | 4/4 | *4/4 |
| 1.2 | 4/4 | 4/4 | 4/4 | *4/4 | 4/4 | 2/4 |
| 1.1 | *4/4 | 4/4 | *4/4 | 2/4 | *4/4 | 0/4 |
| 1.0 | 3/4 | *4/4 | 3/4 | 1/4 | 2/4 | 1/4 |
| 0.9 | 2/4 | 2/4 | 1/4 | 2/4 | 1/4 | 0/4 |
| 0.8 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 |

[a] Results are presented as the number of mice dead at 96 hours/number of mice injected.

* Indicates the L+/4000 end-points.

groups.

Protein concentration of diluent. High protein concentrations do not affect the in vivo activity of tetanus toxin (table 12). This is obvious for the results obtained with the toxin dose giving a paralytic end-point. That the same is also true for the toxin dose giving a lethal end-point can be seen more readily if the death times are expressed in a numerical form e.g. by the use of Ipsen's scoring system (1955) viz:

| | Assumed score |
|---|---|
| Death in less than 2 days | 0 |
| Death in 3 to 4 days | 2 |
| Death in 5 to 7 days | 1 |

The sum of the individual responses for each group, graded according to this system, indicate that there is no significant difference between the groups.

Importance of the body weight of mice.

The influence of mouse weight on responses to toxin-antitoxin mixtures at the L+/1000 level and to toxin alone was investigated. The experiments were designed so as to ensure that the injections were performed randomly once the groups of mice of different weight ranges had been selected. To accomplish this, all mice were allocated numbers made relevant to their colour coding, and the order of injections determined by reference to a table of random digits (Campbell, 1967).

Figure 2 represents the scatter of deaths recorded at regular observation periods for different weight groups in the assay of L+/1000 for Dutch toxin No.954. Figure 3 shows the number of deaths in each weight group 96 hours after injection - the usual time interval for reading lethal end-points. If the deaths are expressed as proportions, the proportion dead for groups I, II, III and IV are 1.0, 0.6, 0.3, and 0.1 respectively. Statistical treatment of the results according to the method for comparison of several proportions as described by Armitage (1971) shows that the differences are higly significant ($p < 0.001$).

Results of readings taken 96 hours and 120 hours after the injection of toxin alone are given in figure 4. In order to provide a sensitive indicator of the influence of mouse weight, a

TABLE 12

The in vivo activity of tetanus toxin in diluents containing high concentrations of protein

| DOSE OF TOXIN | TYPE OF END-POINT | RESULTS IN MICE | | |
|---|---|---|---|---|
| | | Diluent containing 1% peptone (Control) | Diluent containing 8% peptone | Diluent consisting of normal rabbit serum |
| µg | | | | |
| 0.02 | Degree of paralysis | $^{7}P_{4}$[a] + $^{3}P_{3}$ | $^{7}P_{4}$ + $^{3}P_{3}$ | $^{6}P_{4}$ + $^{4}P_{3}$ |
| 0.2 | Death (Ipsen's score)[c] | $^{7}D_{5}$[b] + $^{3}D_{6}$ (30) | $^{2}D_{4}$ + $^{3}D_{5}$ + $^{4}D_{6}$ + $^{1}D_{7}$ (28) | $^{4}D_{4}$ + $^{2}D_{5}$ + $^{4}D_{6}$ (26) |

[a] Results are expressed in form $^{x}P_{y}$ where x is the number of mice showing paralytic symptoms of grade y 120 hours after injection.

[b] Results are expressed in form $^{x}D_{y}$ where x is the number of mice dead y days after injection.

[c] Ipsen's score is the sum of the individual responses in each group graded according to the day of death (Ipsen, 1955).

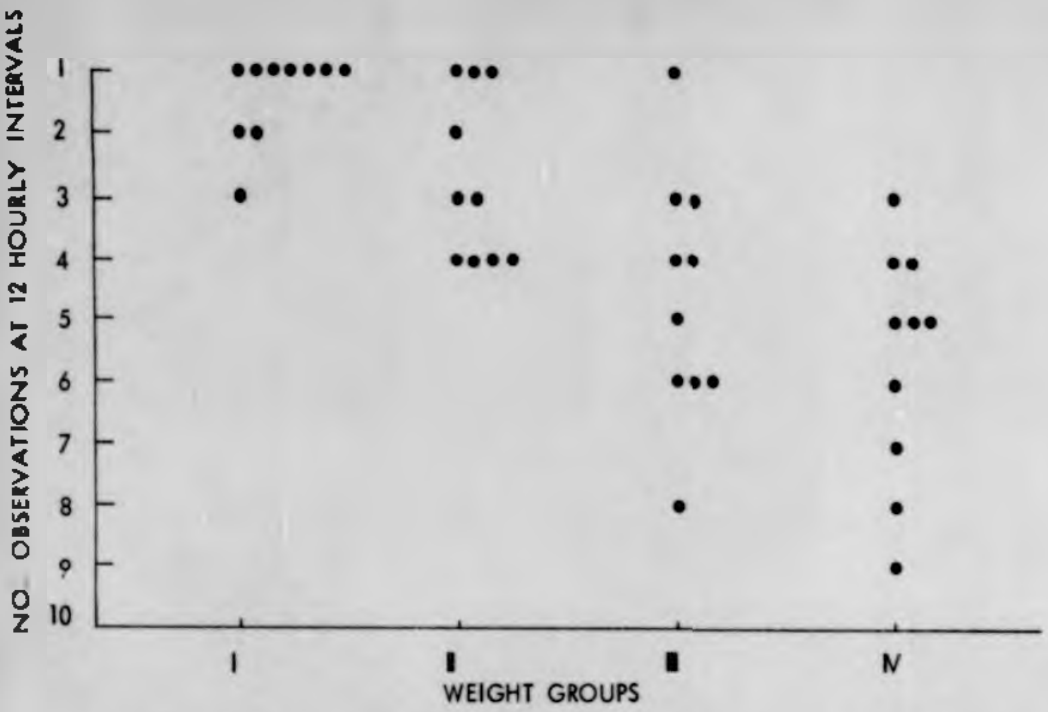


Fig. 2 Distribution of deaths resulting from the injection of toxin-antitoxin mixtures at the L+/1000 level of Dutch toxin No.954 into mice that are grouped according to weight. Each point represents the death of one animal. Deaths were recorded every 12 hours starting from 08.00 on the third day after injection - the first day on which deaths occurred. The mean weights ± s.d. for the groups are: Group I, 14.45 ± 0.44g; Group II, 18.05 ± 0.69g; Group III, 22.5 ± 1.27g; Group IV, 25.85 ± 0.63.

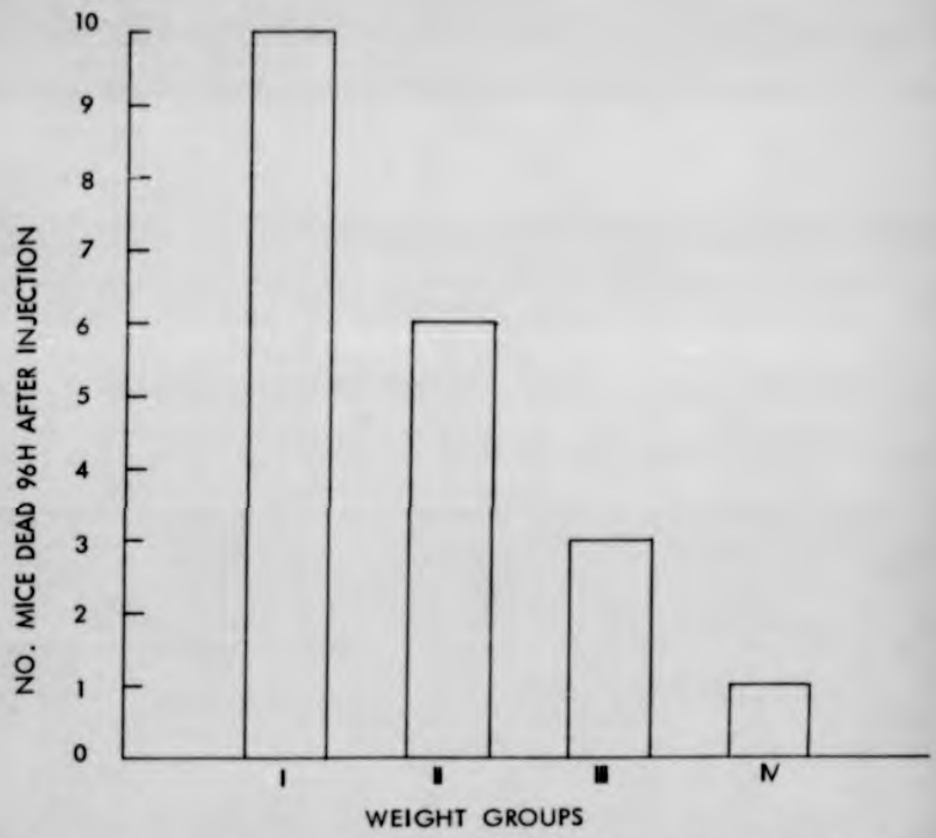


Fig.3 Influence of mouse weights on the L+/1000 determination of Dutch toxin No.954. Mean weights ± s.d. for the four groups of ten mice are 14.45 ± 0.44g, 18.05 ± 0.69g, 22.15 ± 1.27g and 25.85 ± 0.63g in order I to IV. Of these, only Group I gives the end-point.

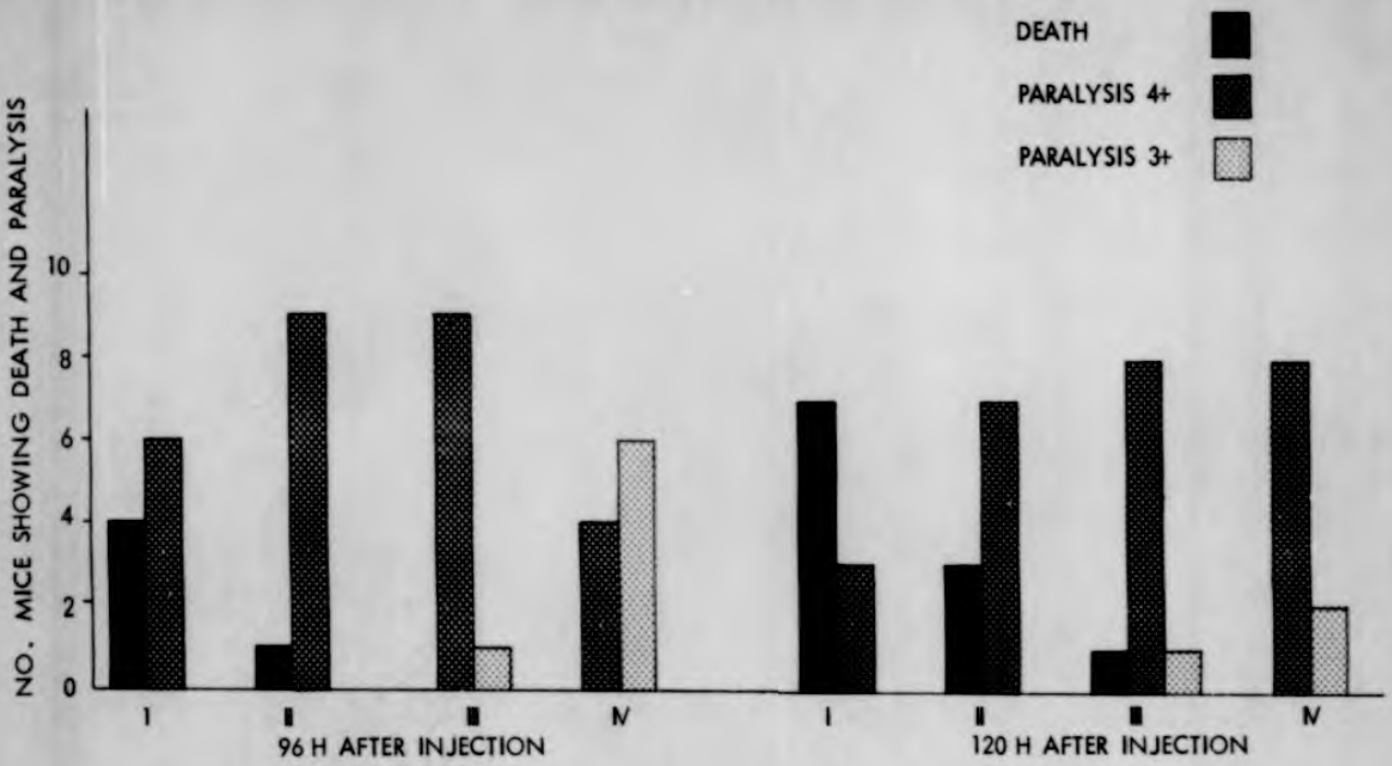


Fig.4 Influence of mouse weights on the response to 0.2 µg doses of Dutch toxin No.954. This toxin concentration gives both death and paralysis over the weight ranges tested. The mean weights ± s.d. for the groups are: Group I, 12.84 ± 0.69g; Group II, 16.67 ± 0.49g; Group III, 20.22 ± 0.86g; Group IV, 25.09 ± 0.63g.

toxin dose was chosen that produced an effect on the borderline between advanced paralysis and death. It is clear that, as mouse weight increases, the number of deaths in mice decreases while the number of mice with advanced paralysis increases.

Assays at the Lp/10000 toxin dose level.

Degree of paralysis. Symptoms of local tetanus in the hind limbs of mice, produced by the intramuscular injection of toxin or toxin-antitoxin mixtures, were assigned scores from 0 to 4+ according to the scheme proposed by Mellanby et al. (1968) for assessing the reduction in paralysis produced by gangliosides in experimental tetanus in mice. Paralysis of grades 2+, 3+ and 4+ are illustrated in figures 5, 6 and 7. A 1+ degree of paralysis is not represented since the stiffness in the injected limb with this grade is detectable only when the mouse is suspended by its tail or by close observations of its movements.

Assessments of paralysis on the same mice by two observers working independently are given in table 13. As might be expected, no significant differences occurred in the evaluation of paralysis of grades 2+ and higher, but variation is evident in the region of 0, +/- and 1+ where differences are difficult to define and detect. Therefore, a 2+ degree of paralysis was chosen as end-point.

Time of readings. It is clear that the full development of paralysis may take five days (table 14). Observations made seven days after injection usually gave the same results as those made at five days; but, sometimes, there was an actual decrease in the extent of paralysis between five and seven days. Hence, a five-day interval from injections to readings was adopted for the paralytic end-point of the Lp/10000 test dose level.

Attempts to extend the sensitivity of antitoxin assays.

Suckling mice of average weight 5.4 g were investigated along with a control group of average weight 15.1 g for possible use in the titration of antitoxin at the Lp/40000 and Lp/100000 levels. However, despite the pronounced effect of mouse weight on susceptibility to toxin already demonstrated, the suckling mice did not respond to a significantly greater degree. Apart from that, the actual procedure of injecting limbs of mice weighing as little as 5 g presented practical difficulties.

Fig. 3 Mouse showing a 2+ degree of paralysis of the left leg. Although the limb is used regularly, its movements are perceptively awkward and there is obvious limping.

Fig. 6 Mouse with J+ paralysis of the right leg. The affected limb is held in an abnormal position and, although still moveable, it is used infrequently. The tail is deflected towards the side of inoculation.

Fig. 7 Mouse with 4+ paralysis of the right leg. The injected limb is rigid and permanently deformed as the result of severe paralysis. The tail shows marked deflection.

TABLE 13

Independent scoring of paralytic end-points by two observers

| GRADES OF PARALYSIS | TOTAL NO. OF MICE OBSERVED | DISCREPANCIES IN GRADINGS |
|---|---|---|
| | | per cent |
| 3+ & 4+ | 190 | 4.7 |
| 2+ & 3+ | 141 | 5.0 |
| 1+ & 2+ | 108 | 7.4 |
| 1+ & +/- & 0 | 55 | 30.9 |

TABLE 14

Observations supporting the adoption of a five-day interval between the injection of mice and the scoring of results for the paralytic end-points of the Lp/10000 toxin test dose level.

| OBSERVATION PERIOD | NUMBER OF MICE SHOWING ONE SCORE DIFFERENCE IN PARALYSIS OVER THE OBSERVATION PERIOD[a] | |
|---|---|---|
| | No. with increased paralysis | No. with decreased paralysis |
| From 4 to 5d | 28 | 1 |
| From 5 to 7d | 1 | 19 |

[a] Mice giving variations in the region 0 to 1+ were excluded from the figures listed because of the difficulty of assessing minimal degrees of paralysis. With these mice excluded, the total number observed was 130.

Procedure for the evaluation of precision and accuracy of the toxin-neutralization method.

The antitoxin content of two samples of human sera was assayed repeatedly by toxin neutralization at selected toxin dose levels in order to provide a measure of the precision and accuracy of the method. One of the test materials consisted of dilutions of "Tetagam", a preparation of human tetanus immunoglobulin (Appendix iv); the other was a pooled sample of approximately twenty human antisera collected about 28 days after primary injections of tetanus toxoid. Dilutions of the former were assayed at the toxin test dose levels of L+/100, L+/1000, L+/4000 and Lp/10000; the latter was assayed at the Lp/10000 level.

Before these experiments were undertaken, however, a more exact evaluation of the antitoxin concentration of a Tetagam test sample was obtained by the use of a scheme which gave dilutions for testing at close intervals within the limits of the pre-determined range of antitoxin values for the sample (Appendix v). This was deemed necessary since it seemed logical to suppose that a greater variability in results would occur if the actual antitoxin concentration approached one or other of the limits of the range dilimating the titre than if it happened to be around the mid-point of that range. The procedure showed that a sample of Tetagam which had previously resulted in survival of all mice at a 1:2 dilution and death of all mice at a 1:4 dilution when assayed at the L+/1000 dose level (i.e. an antitoxin concentration of >0.02 and = or <0.04 u/ml) actually produced death in all animals at dilutions slightly higher than the mid-point of this range, viz: at 1:3.15 and 1:3.53 in two separate assays. Hence, a 10 x concentration of this sample was used for testing at the L+/100 toxin dose level and 1 1:10 dilution for testing at the Lp/10000 level.

Results of ten separate assays on the test samples and dilutions are shown in table 15. Accuracy is indicated by the extent to which the antitoxin titres for the Tetagam dilutions, tested at four different toxin dose levels, are in proportion to the dilution factors; and precision is measured by the reproducibility of results obtained for antitoxin titrations at the different dose level with multiple determinations (Whitby, Mitchell

TABLE 15

Evaluation of the accuracy and precision

of the toxin neutralization test

| TEST SAMPLE | TEST DOSE LEVEL | ANTITOXIN TITRE u/ml | FREQUENCY |
|---|---|---|---|
| Human antitoxin (Tetagam)[a] | L+/100 | >0.4 = or <0.8 | [c]10/10 |
| Tetagam diluted 1:10 | L+/1000 | >0.02 = or <0.04 | 10/10 |
| Tetagam diluted 1:10 | L+/4000 | >0.02 = or <0.04 | 10/10 |
| Tetagam diluted 1:100 | Lp/10000 | 0.004 | 10/10 |
| Pooled early human antisera[b] | Lp/10000 | 0.004 | [d]8/10: 9/10 |

[a]See Appendix (iv).

[b]Antisera were collected about 28 days after primary injections of tetanus toxoid.

[c]Results are presented as the number of assays giving the titres listed/number of assays conducted.

[d]Read independently by two observers - one recorded a value of 0.002 u/ml once in ten readings; the other recorded this value twice in ten readings.

& Moss, 1967; Vikeläde, Bechgaard & Magid, 1974). A high degree of accuracy and precision is evident. Two deviations from the ideal occurred, viz: a consistent inaccuracy in the L+/100 estimations of a twofold dilution step; and a lack of reproducibility in the Lp/10000 assays of pooled early human antisera for one or two readings which also differed to the extent of a single twofold dilution step. Such a deviation in titrations of twofold serial dilutions is generally regarded as being of no significance.

Of a total of ninety pairs of mice injected in the L+ assays, only one pair showed a discrepancy with respect to death or survival four days later. This indicates that one mouse only should suffice for testing each dilution in routine L+ determinations.

## DISCUSSION

It is apparent that the weight range of the mice used in the toxin neutralization test should be restricted and defined whether the end-point is based on death or paralysis. The critical importance of the body weight of the animals in the toxin neutralization test does not seem to have been previously recognized, and certainly has not been previously stressed. The much-quoted procedure of Glenny & Stevens (1938), for example, does not mention the weight of the mice used. Of the published methods that do include some reference to the weight of the mice, only *Chen (1959) states that - for a paralytic end-point - larger mice are less susceptible to the action of toxin.

That the mouse weight would exert some influence on the outcome over a range of 13 g to 26 g was not entirely unexpected, but it is the extent of the effect that is noteworthy and impressive. Moreover, the total range of weights investigated was not unrealistic in that the lower limit represented the 13-14 g weight

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* The author is grateful to Miss Mona Bo Lo for translating this article from the original Chinese.

range stipulated by Chen (1959), while the upper limit represented the 20-25 g weight range used by Taylor & Moloney (1960). Despite the clear-cut evidence for the determining effect of weight of mice on the responses obtained in the toxin neutralization test, however, the use of suckling mice did not provide a means of extending the range of assays to even lower toxin test dose levels. An obvious explanation may be that the difference between the average weight of the suckling mice (approx. 5 g) and that of the control group (approx. 15 g) was just not sufficient to permit assays to be conducted at toxin dose levels as low as Lp/40000 and Lp/100000. Aside from that, however, the observed difference in the responses of the two groups did not seem to parallel the weight difference.

Dilutions for the toxin neutralization test are performed in a diluent containing protein, e.g. peptone, which serves the function of stabilizing the toxin. The protective action of peptone was first recognized by Condrea and Poenaru (1933), and subsequently confirmed by Ipsen (1942). Ipsen showed that, because non-specific serum proteins possess the same stabilizing effect, undiluted sera appear relatively weaker than diluted sera unless protein is included in the diluent. The probable mechanism of this type of stabilization is that, since inert molecules are just as likely to be removed or destroyed by adsorption to the container or by surface forces as are toxin molecules, the probability of the latter being lost can be minimized by the addition of a large excess of the former (Jarma, 1951; Lavina, Ipsen & Mc Comb, 1961). Results of the experiment on the effect of increased peptone concentration on toxin activity indicated that the concentration of peptone generally used (i.e.1%) exceeds that of toxin doses causing paralysis and death to such an extent that the use of an increased peptone concentration made no difference. On the other hand, the increased peptone concentration did not reduce toxicity by non-specific binding either. In these respects, serum proteins behaved similarly.

It is axiomatic that the diluent should preserve and not harm the toxin. Nevertheless, in early experiments for L+ determinations, the pH produced by the addition of peptone to

buffer caused a slow destruction of the toxin, evident only with an increase in the time of exposure before injection. This experience points to the need for careful control of the conditions of interaction of toxin and antitoxin.

Control of the conditions of testing and, more importantly, of the body weight of the mice used is probably responsible for the high degree of accuracy and precision that was found for the L+ titrations of high-titred human antitoxin (Tetagam), and for the concordance of results obtained in the pairs of mice injected with the same dilutions. Furthermore, the use of a well-defined end-point for the Lp/10000 assay, instead of the mere presence or absence of symptoms as in previous methods, probably accounts for the high degree of reproducibility obtained with the assays on low-titred early human antisera and the dilution of Tetagam tested at this level. Whether the deviations which did occur in the assays of early human antisera are due to low avidity or error in the assessment of paralytic end-points is uncertain, but the limited extent and low frequency of the deviations indicate that they are of no real significance or practical consequence.

Techniques currently available for the titration of tetanus antitoxin may be conveniently divided into three groups. The first comprises methods based on the reading of death times, as exemplified by Ipsen (1942), and used also by Kyselová, Maly and Valachovská (1968). In these techniques, the toxin dose has a relatively high value e.g. L+/200. The methods are economical as regards the materials required, but the type of end-point necessitates frequent observations - e.g. nine readings over a five-day period with Ipsen's method - and the use of an elaborate mathematical scheme for the calculation of the results. Furthermore, Ipsen's basic system of serum dilutions yields only four titre values, viz: 0.02, 0.125, 0.8 and 5.0 unit per ml. Other titres, including those less than 0.02 u/ml, are calculated by extrapolation, and the application of correction factors to compensate for such variables as the deviation from expected death times in the control group. Although Kyselová et al. maintain that their end-points have the advantage of being quantitative rather than quantal, this is not strictly true since the readings of deaths in mice must be restricted to certain times of observation, albeit

frequent.

The representative method of the second group of techniques is that of Glenny & Stevens (1938). Similar methods are used by Wilkens & Tasman (1959), Eckmann (1963), and Barile, Hardegree & Pittman (1970). In these procedures, end-point readings are based on death at the end of a defined period of time after injection - usually four days - and the interpretation and calculation of results is simple. Toxin test dose levels are generally in the vicinity of L+/1000. The major disadvantage of the techniques in this group is the relatively large volume of serum required for titrations of low antitoxin concentrations. For example, in the method of Barile et al., 1 ml of undiluted serum is required for the estimation of the single titre, 0.001 u/ml, and extra serum is needed for the preparation of dilutions for the assay of higher titres. Where a twofold serial dilution is used, at least 2 ml of serum is required (Wilkens & Tasman, 1959). Eckmann's method calls for a serum volume of 1.5 ml to cover the range of titres between 0.005 and 0.5 u/ml. In the method of Glenny & Stevens (1938), sera are divided into two groups based on an initial estimate of their antitoxin content i.e. whether greater than or less than 2 u/ml. When a serum has less than 2 u/ml of antitoxin, subsequent assays are performed using constant volumes of 0.1 ml of serum and reduced amounts of toxin. This means that a serum volume of 1.2 ml would be required for the determination of titres from 0.001 u/ml to 2 u/ml in twofold dilution steps.

A low sample volume requirement is the commendable feature of the third group of methods as used by Chen et al. (1956); Chen (1959); and Taylor & Moloney (1960). However, the method of Taylor & Moloney has little to recommend it otherwise, as only three antitoxin titres are determined, and the lowest detectable titre is as high as 0.02 u/ml. A major shortcoming of Chen's technique is his use of an end-point based on the neutralization of symptoms of tetanus. As the differentiation of minimal degrees of paralysis and complete neutralization is influenced significantly by variations in personal perception of the observers, detection of this type of end-point is difficult

and inaccurate. Furthermore, results obtained with the method are likely to be affected appreciably by the avidity of the test sera, i.e. that property of sera which determines their firmness of combination with toxin (Glenny & Barr, 1932). Generally, sera collected early in the immune response are non-avid, so tend to dissociate from combination with toxin. Because of this tendency, relatively more antitoxin is required at low toxin concentrations to force the amount of free toxin below the neutralization level (Jerne, 1951); and a toxin-antitoxin mixture which should give absence of symptoms (Lo) as end-point may become sufficiently diluted after injection into an animal to produce paralysis (Glenny et al. 1932). Chen recognizes the limitations imposed by avidity on the accuracy of his method, but contends that the impossibility of obtaining a theoretically correct antitoxin value with a small volume of serum and a low concentration of toxin must be accepted. However, it is of interest that, in contrast to the findings of Barile et al. (1970) and of the studies reported here, Chen's toxin did not give L+ values in proportion to the unitage of antitoxin even when that antitoxin was an avid equine standard - probably as a result of the uncertainty inherent in the recognition of an end-point based on complete neutralization.

What are the criteria for the evaluation of a toxin neutralization technique? The chief points of interest, as put forward by Chen (1959) and van Ramshorst (1971), may be combined as follows:

1. Minimum antitoxin titre that can be determined;
2. Amount of serum required for testing;
3. Simplicity in the technical procedures, and in the interpretation and calculation of results;
4. Economy in terms of time, labour and materials;
5. Precision and accuracy.

A toxin neutralization technique should be capable of measuring titres lower than 0.01 u/ml, the generally accepted "protective threshold" (McComb, 1964), as there are indications that the actual protective level may be less than this value (Newell et al., 1971). Only an accumulation of data on the

incidence of tetanus in the presence of low concentrations of circulating antibodies will be of value in defining the protective level more precisely. Furthermore, since antitoxin titres estimated three or four weeks after a single injection of tetanus toxoid are usually less than 0.01 u/ml and sometimes almost negligible (MacLennan et al., 1973; Cohen & Leussink, 1973), a sensitive method of antitoxin assay is required for monitoring the primary immune response, particularly in studies on the effects of host factors which may cause immunosuppression.

The amount of serum needed for testing depends on the minimum antitoxin titre to be determined; or, more specifically, on whether or not the determination of the minimum titre requires undiluted serum. In the technique presented here, 0.6 ml of serum is required for the titration of antitoxin from 0.001 u/ml, but as little as 0.3 ml of serum will suffice where a minimum titre of 0.002 u/ml is acceptable.

The method presented also has the advantages of simplicity and economy. Its simplicity is a consequence of its design, notably the use of a straight-forward open-ended twofold scheme for the preparation of serum dilutions; clear-cut end-points of death or 2+ (50%) paralysis that require only a single reading; and an uncomplicated method for the calculation of antitoxin titres. In addition, the speed and ease of the technical manipulations are promoted by the use of microlitre sampling pipettes for the preparation of dilutions, and of a simple but effective device for restraining mice during the injection procedure. Substantial savings in mice, time and expense are realized by reducing the number of serum dilutions to be tested on the basis of a prior assessment of the antitoxin concentration by an indirect microhaemagglutination technique - an essential part of the antitoxin titration - and by the use of one mouse only per test dilution in assays based on toxin doses giving lethal end-points. The method also has a high degree of precision and accuracy.

# CHAPTER 2

## THE TITRATION OF TETANUS ANTITOXIN BY INDIRECT HAEMAGGLUTINATION

Methods for the measurement of tetanus antitoxin by indirect haemagglutination generally use unfixed sheep or horse red cells (Stavitsky, 1954; Tasman, van Ramshorst & Smith, 1960; Levine et al. 1960; Levine & Wyman, 1964a; Levine, Wyman & McComb, 1967; Chatterjee, 1964) or sheep cells fixed by formalin (Galazka & Abgarowicz, 1967; Hardegree et al., 1970; Kyselova, Libich & Srbová, 1970). The fixed or unfixed erythrocytes are treated with tannic acid (Boyden, 1951), and sensitized by exposure to tetanus toxoid. Titration of antisera is performed in a macro or micro-haemagglutination system by haemagglutination inhibition (Fulthorpe, 1957, 1958, & 1959; Tasman et al., 1960); or, more commonly, by testing directly for the agglutination of sensitized cells by dilutions of the antisera.

Aldehydes other than formaldehyde have been recommended as fixing agents for indirect haemagglutination techniques on the grounds that their use results in a saving of time and effort and a more stable or satisfactory end-product (Ling, 1961; Bing, Weyand & Stavitsky, 1969). For similar reasons, chromic chloride has been advocated as a coupling agent to replace tannic acid (Jandl & Simmons, 1957; Gold & Fudenberg, 1967; Faulk & Houba, 1973). Modifications that have been proposed for the purpose of avoiding the necessity for prior absorption of sera by sheep cells include the replacement of absorbed normal rabbit serum in the diluent by polyvinylpyrrolidone (Bordues & Graber, 1953) and of sheep erythrocytes by erythrocytes from a different species (Greenwood, 1970) or latex particles (Jouja, 1965). Nelson (1973) did apply glutaraldehyde-fixed human group O cells to which tetanus toxoid had been coupled by chromic chloride to the assay of tetanus antitoxin, but he was concerned only with the screening of relatively high-titred plasma (3u/ml) for the production of prophylactic human anti-tetanus globulin, and not with the development of a technique of optimal sensitivity based on a knowledge of the influential variables.

When used in conjunction with the toxin neutralization test, the main value of the indirect haemagglutination technique is as a screening procedure for the presence of tetanus antitoxin, and as an indicator of the range of serum dilutions to be tested by toxin neutralization. As with any screening procedure, high sensitivity is

desirable. In order to obtain coated cells of high sensitivity, therefore, systematic comparative tests were undertaken to determine the optimal conditions for fixing, tanning and coating gluteraldehyde-fixed sheep red cells.

The use of aldehydes other than formaldehyde as fixing agents; chromic chloride as coupling agent; polyvinylpyrrolidone (PVP) instead of absorbed normal rabbit serum in the diluent; and human group O erythrocytes, chick cells and latex particles as substitutes for sheep cells was also evaluated. In addition, the inclusion of 2-mercaptoethanol (2-ME) in the diluent as a simple means of removing heterophile agglutinins (IgM), and of improving the correlation between titres estimated by the indirect haemagglutintion and toxin neutralization techniques was investigated.

## MATERIALS AND METHODS

Erythrocytes. Sheep cells in Alsever's solution were obtained commercially (Wellcome Reagents Ltd., Wellcome Research Laboratories, Beckenham, Kent). The cells were usually fixed within a day or two of receipt, but could be fixed satisfactorily after storage for periods of up to two weeks at 4°C. Group O cells were collected in ACD anti-coagulant (Appendix vi) from human volunteers. Chick cells were collected in Alsever's solution.

Latex particles. Bacto-Latex (0.81) was obtained from Difco Laboratories (P.O.Box 14B, Central Avenue, West Molesey, Surrey).

Tetanus toxoid. Batch V.G.T. No.6, of purity 1666 Lf/mg protein N and concentration 1000 Lf/ml, was supplied by the Rijks Instituut of the Netherlands (Appendix vii).

Diluent. Oxoid phosphate buffered saline (PBS) containing 0.5% heat-inactivated normal rabbit serum (NRS) was used as suspension medium for the storage of sensitized cells, and as diluent for the preparation of antiserum dilutions in the haemagglutination titration. The rabbit serum was first absorbed with an equal volume of washed, packed sheep cells, and stored in small volumes at -20°C until required. To prevent bacterial contamination, a 1:10000 concentration of Thiomersal (Eli Lilly & Co., Ltd., Basingstoke, England) was added to the NRS-PBS diluent.

Microtitre equipment. Disposable polystyrene microtitre titration plates with U-shaped wells were used together with the metal micro-diluters and disposable micropipettes of the Cooke Microtiter System (Dynatech Laboratories Ltd., Daux Road, Billingshurst, Sussex).

Antisera. For most of the experiments, an initial 1:2 and 1:3 dilution of the same diluted sample of human antitoxin was titrated along with a sample of the British Standard Antitoxin containing 0.1u/ml. In some experiments, titrations were performed on rabbit antisera collected ten days after an injection of 2 ml of adsorbed tetanus toxoid of concentration 40 Lf/ml.

Chemical reagents. (a) 50% glutaraldehyde (B.D.H. Chemicals Ltd., Poole, England); (b) formaldehyde (B.D.H.); (c) pyruvic aldehyde (Koch-Light Laboratories Ltd., Colnbrook, Bucks, England); (d) tannic acid (B.D.H.); (e) chromic chloride, $CrCl_3 6H_2O$ (B.D.H.); (f) polyvinylpyrrolidone (May & Baker Ltd., Dagenham, England); (g) 2-mercaptoethanol (B.D.H.).

General procedure for the sensitization of sheep cells.

Glutaraldehyde fixation. Initially, the technique used was essentially the same as that of Bing et al., (1969). Blood cells from a selected sheep are washed three times in isotonic saline (0.85% v/v = 0.15M) or until the supernatant is clear and colourless. The packed cells are then diluted to a 2% suspension with 1% glutaraldehyde in a salt solution consisting of:

| | |
|---|---|
| NaCl (0.15M) | 9 vol. |
| $Na_2HPO_4$ (0.15M) adjusted to pH8.2 with | |
| $KH_2PO_4$ (0.15M) | 1 vol. |
| Dist. water | 5 vol. |

The mixture is placed in an ice-bath for 30 min, and shaken occasionally during the period. After fixation, the cells are centrifuged, and washed two or three times with saline, then two or three times with distilled water. A 10% suspension of the fixed cells is prepared in Oxoid PBS containing 1:10000 Thiomersal, and the suspension is stored at 4°C.

After the optimal conditions for cell sensitization had been determined, it was found that the procedure for glutaraldehyde fixation could be simplified, without loss of sensitivity, by using saline alone

with the phosphate buffer of pH8.2 in the salt solution, and by fixing at room temperature (approx. 20°C) for 30 minutes.

Tanning procedure. Equal volumes of 10% glutaraldehyde-fixed cells and freshly prepared 1:60000 tannic acid are mixed and placed in a water bath at 37°C for 15 minutes. The mixture is shaken periodically. The cells are then centrifuged, washed once with saline, and resuspended in 0.15M phosphate buffer of pH6.4 at a concentration of 10%

Sensitization with tetanus toxoid. Equal volumes of 10% fixed, tanned red cells in buffer of pH6.4 and tetanus toxoid at a concentration of 50 Lf/ml (approx.0.2 mg/ml) are placed in a water bath at 37°C for 60 minutes. The mixture is shaken periodically. The sensitized cells are then centrifuged, washed three times with 0.5% NRS-PBS diluent, and stored at 4°C as a 10% suspension in diluent containing 1:10000 Thiomersal.

Assay of tetanus antitoxin by the micro-haemagglutination technique.

*Preparation of antisera and cell suspension.* Antisera for titration are inactivated at 56°C for 30 minutes and absorbed with an equal volume of washed, packed sheep cells at room temperature for 15 to 30 minutes.

For the preparation of the cell suspensions, sensitized and non-sensitized fixed cells are diluted to an 0.5% concentration in the NRS-PBS diluent according to their haematocrit values.

Titration procedure. Twofold serial dilutions of test sera and Standard Antitoxin are prepared in 0.5% PBS-NRS diluent using Cooke disposable micropipettes for addition of the diluent in 0.025 ml volumes and 0.025 ml metal microdiluters for serial dilution of the antisera. The diluters are pre-wet to ensure accurate filling by capillary action, and are subsequently emptied and checked for delivery volumes on a delivery tester. Excess fluid is removed from the outside of the microdiluters by rolling them on a dry paper towel. The microdiluters are filled by contact with the surface only of the serum samples, and serial dilutions then prepared by rotating the diluters back and forth at the rate of four times per second for a period of eight seconds in each successive well (Cooper, Bowie & Owen, 1972).

For the test, 0.025 ml volumes of 0.5% suspensions of sensitized cells are added to the wells containing dilutions of antisera.

For controls, non-sensitized fixed cells are added to 1:2 dilutions of the test sera to check for the presence of non-specific agglutinins; and sensitized and non-sensitized cells are added to wells containing diluent only to check for auto-agglutination. Plastic disposable plates may carry a static charge which tends to pull drops, not fully formed, into the wells; the charge is removed by wiping the under-surface of the plates with a damp cloth. After addition of the cells, the contents of the wells are mixed by holding the plates in the palm of one hand and tapping gently with the other about ten times on each side of the plate.

Reading and interpretation of end-points. The haemagglutination reactions are graded as follows:-

4+ = mat of cells covering the entire area of the bottom of the well.

3+ = mat of cells covering a slightly reduced area of the well.

2+ = mat of cells with a narrow band of unagglutinated cells around the periphery.

1+ = mat of cells covering a reduced area with a wider peripheral band of unagglutinated cells.

+/- = small mat of cells restricted to the central portion of the well.

- = discrete ring of cells.

The highest dilution of an antiserum giving a 1+ agglutination reaction is taken as the end-point dilution. By convention, figures expressing end-point dilutions do not take into account the dilution caused by the addition of erythrocyte suspension. The antitoxin concentration of an unknown serum may be calculated in terms of "haemagglutinin units" (HU) on the basis of an assumption that the end-point of a test serum has the same unitage as that of a standard antitoxin giving a 1+ agglutination reaction. For a valid titration, there must be no agglutination of non-sensitized cells by the antiserum, or of sensitized or non-sensitized cells in diluent alone.

Results of investigations described here are expressed in two ways, viz: as reciprocals of the highest serum dilutions giving 1+ reactions, and in the form of scores representing the mean value of the sum of the agglutination reactions in each well, graded from 0 to 4,

for a single titration. The value of the score is that it provides a means of differentiating those antisera which happen to have the same end-points but which vary in their degree of haemagglutinating activity.

If a fourfold difference in titre is the criterion demanded for real significance, then responses to small changes (of arbitrary magnitude) in the variables tested are not always significant. Nevertheless, a trend towards the optimal is usually evident. Moreover, the validity of these trends is supported by the consistency of results obtained when each experiment, performed in duplicate, was repeated using a fresh sample of blood from the same animal. Furthermore, the titration of two serial dilutions prepared from a 1:2 and a 1:3 initial dilution of the same antiserum gives more information than is usually provided by titration of a single two-fold series with its relatively large inherent error. Specificity of the haemagglutination reactions was established by the demonstration of complete absence of agglutination when small amounts of tetanus toxoid were added to antitoxin dilutions before the addition of sensitized cells (Stavitsky, 1954), i.e. neutralizing amounts.

## EXPERIMENTS AND RESULTS

### Determination of the variables affecting sensitization of sheep red cells.

Source of sheep cells. Results of several experiments investigating the suitability of red cells from different sheep for tetanus antitoxin assay by indirect haemagglutination are summarized in table 16. Considerable variation is evident. Consequently, a single sheep - No.16 - was selected as cell donor for all subsequent experiments. The non-specific agglutination shown by cells from sheep No.145 could be eliminated by the use of an increased concentration of normal rabbit serum in the diluent.

Optimal conditions for tanning. Preliminary checkerboard titrations in which both the concentrations of tannic acid and of tetanus toxoid were varied indicated that the former influenced the sensitivity of the final cell preparation as much as did the latter. Table 17 shows

TABLE 16

Variation in antitoxin titres with tetanus toxoid-sensitized erythrocytes from different sheep.

| SHEEP NUMBER[a] | RECIPROCAL OF HAEMAGGLUTINATION TITRE[b] | | | | |
|---|---|---|---|---|---|
| | Human antitoxin | Rabbit antitoxin | Third British Standard Antitoxin (5u/ml) | U.S. Standard Antitoxin (5u/ml) | Wellcome equine antitoxin (5u/ml) |
| 3 | 128 | NT[d] | 0-128 | 0-128 | 512 |
| 4 | 64 | NT | 0-64 | 0-128 | 256 |
| *16 | 256 | 256 | 512-1024 | 512 | 512-1024 |
| 69 | 128 | 128 | 128-512 | 512 | 512 |
| 145 | NSA[c] | NSA | NSA | NSA | NSA |
| 3005 | 64-128 | 128 | 512 | 512 | 512 |

[a] All samples of sheep cells were obtained from Wellcome Laboratories and were tested, after sensitization, as 1% suspensions.

[b] The end-point is the highest serum dilution giving an agglutination reaction of 1+. Results presented as the range of titres obtained in four experiments, i.e. for eight titrations on each antiserum.

[c] Non-specific agglutination.

[d] Not tested.

* Indicates the sheep cells chosen for use.

TABLE 17

Effect of concentration of tannic acid on antitoxin titres

| INITIAL TANNIC ACID CONCENTRATION | RECIPROCAL OF IHA TITRE FOR HUMAN ANTITOXIN[a] | SCORE[b] |
|---|---|---|
| 1:5000 | 16-32 | 9 |
| 1:10000 | 32-64 | 10 |
| 1:20000 | 64 | 14 |
| *1:40000 | 64-128 | 15 |
| *1:80000 | 128 | 16 |
| 1:160000 | 64-128 | 13 |
| 1:320000 | 32-64 | 12 |
| 1:640000 | 16-32 | 9 |

[a] Results are presented as the range of titres obtained in two experiments, i.e. for four titrations.

[b] The score is the mean value of the sum of the agglutination reactions in each well, graded from 0 to 4, for four determinations.

* An average value of 1:60000 was chosen for use.

the effect of different concentrations of tannic acid on the sensitivity of the system. Other variables of the tanning procedure investigated were the temperature of the process (table 18); the duration of exposure of fixed cells to tannic acid (table 19); and the nature of the medium in which the fixed cells were suspended during tanning (table 20). Results indicate that a 1:60000 dilution of tannic acid mixed with an equal volume of 10% glutaraldehyde fixed sheep cells in PBS at 37°C for 15 minutes provides optimal conditions. Continuous agitation during the tanning procedure did not enhance the sensitivity of the system.

Optimal conditions for sensitization. Results of experiments on the effect of such variables as concentration of sensitizing tetanus toxoid; time of exposure to tetanus toxoid; pH; and temperature on the sensitizing procedure are given in tables 21, 22, 23 and 24 respectively. The findings indicate that an initial tetanus toxoid concentration of about 50 Lf/ml (0.2 mg/ml) mixed with an equal volume of 10% tanned cells in buffer of pH5.6-6.4 for 60 minutes at a temperature of 56°C provides optimal conditions. However, although titres were higher after sensitization at 56°C, a temperature of 37°C was generally used for convenience. Continuous agitation during the process of sensitization had no effect on sensitivity.

Importance of the concentration of sensitized cells. It is obvious that the concentration of sensitized cells has a pronounced effect on the titres obtained (table 25). Sensitivity increases significantly as cell concentration decreases. However, the haemagglutination reactions tend to become more difficult to read with reduced cell numbers, and this imposes a lower limit to the cell concentration which is practicable.

Comparison of methods for aldehyde fixation.

Initial comparisons of the final sensitivity of sheep cells fixed by glutaraldehyde according to the procedure of Bing et al., (1969) and by formaldehyde according to Caizman (1960) showed that the former gave cell preparations of greater sensitivity (table 26). Subsequent investigations included cells fixed by the simple formalinization procedure of Galazka & Abgarowicz (1967) and by

TABLE 13

Effect of variation in the temperature of the tanning process

| TEMPERATURE | RECIPROCAL OF TITRES AND SCORES[a] | | |
|---|---|---|---|
| | Human antitoxin diluted 1:3 | Human antitoxin diluted 1:2 | Third British Standard Antitoxin (0.1u/ml) |
| Room temperature (approx. 20°C) | 16 (8) | 16-32 (10) | 64 (11) |
| *37°C | 16-32 (10) | 32-64 (14) | 64 (12) |
| 56°C | 16-32 (8) | 32-64 (9) | 64 (6) |

[a] Results are presented as the range of titres obtained in two experiments (four titrations) with (n) representing the mean value of the sum of the scores for the agglutination reactions.

* Indicates the temperature chosen for the tanning process.

TABLE 19

Effect of duration of exposure to tannic acid

| DURATION OF EXPOSURE TO 1:60000 TANNIC ACID | RECIPROCAL OF TITRES AND SCORES[a] | | |
|---|---|---|---|
| | Human antitoxin diluted 1:3 | Human antitoxin diluted 1:2 | Third British Standard Antitoxin (0.1u/ml). |
| min | | | |
| 5 | 64 (13) | 32-64 (10) | 64 (12) |
| 10 | 64-128 (14) | 64 (14) | 128 (14) |
| *15 | 32-64 (11) | 64-128 (14) | 128 (13) |
| 20 | 32-64 (12) | 64 (14) | 64-128 (11) |
| 30 | 32-64 (13) | 64 (14) | 64-128 (11) |
| 60 | 32-64 (13) | 64 (14) | 64-128 (11) |

[a]Range of titres for two experiments (four titrations): (n) = mean score.

*Indicates adequate time of exposure to tannic acid.

TABLE 20

Comparison of Saline and Oxoid Phosphate Buffered Saline (pH approx. 7.3)[a] as suspending media for the glutaraldehyde-fixed cells during the tanning process.

| TEST ANTISERA | RECIPROCAL OF TITRES | |
|---|---|---|
| | Saline suspension. | P.B.S. suspension. |
| Human antitoxin No.1 | 64-128 | 128 |
| " " No.2* | 1024 | 4096 |
| " " No.3 | 256 | 512 |
| " " No.4* | 512 | 2048 |
| Third British Standard Antitoxin (5u/ml) | [b]256 | 256 |
| U.S. standard antitoxin (5u/ml) | 256 | 256 |
| Wellcome equine antitoxin (5u/ml) | 256 | 512 |

[a] See appendix iii

[b] Titres for the Standard Antitoxins are lower than usual because a 1% suspension of sensitized sheep cells was used in this particular experiment.

* Indicates antisera giving a significant four-fold difference in titres.

TABLE 21

Effect of concentration of sensitizing tetanus toxoid on antitoxin titres

| TETANUS TOXOID CONCENTRATION | | RECIPROCAL OF TITRES AND SCORES[a] | | |
|---|---|---|---|---|
| Initial | Final | Human antitoxin diluted 1:3 | Human antitoxin diluted 1:2 | Third British Standard Antitoxin (0.1u/ml). |
| Lf/ml | | | | |
| 5 | 2.5 | 2 (1) | 4 (2) | 4 (2) |
| 10 | 5 | 8-16 (6) | 16 (7) | 16 (7) |
| 20 | 10 | 16-32 (9) | 32 (10) | 32-64 (11) |
| *40 | 20 | 32-64 (11) | 64-128 (15) | 128 (14) |
| *80 | 40 | 32-64 (12) | 64 (14) | 64-128 (15) |
| 120 | 60 | 32 (11) | 32-64 (13) | 64-128 (15) |
| 160 | 80 | 16 (9) | 32-64 (12) | 128 (14) |
| 200 | 100 | 16 (9) | 32 (12) | 128 (15) |
| 500 | 250 | 16 (9) | 16-32 (9) | 64-128 (14) |

[a] Range of titres for two experiments (four titrations): (n) = mean score.

* An average value of 50 Lf/ml initial concentration was adopted for use.

TABLE 22

Effect of duration of exposure to sensitizing tetanus toxoid

| DURATION OF EXPOSURE TO 50 Lf/ml TETANUS TOXOID | RECIPROCAL OF TITRES AND SCORES[a] | | |
|---|---|---|---|
| | Human antitoxin diluted 1:3 | Human antitoxin diluted 1:2 | Third British Standard Antitoxin (0.1u/ml). |
| min | | | |
| 5 | 16 (5) | 16 (6) | 2 (2) |
| 10 | 16 (8) | 16 (9) | 2 (2) |
| 15 | 16 (8) | 16 (9) | 8 (3) |
| 20 | 16 (9) | 32 (11) | 8 (4) |
| 30 | 32 (10) | 64 (15) | 32 (6) |
| *60 | 64 (16) | 64 (18) | 64 (14) |
| 120 | 64 (17) | 64 (18) | 64 (15) |

[a] Titres given with (n) = mean score.

* Indicates adequate time of exposure to tetanus toxoid.

TABLE 23

Effect of pH on the sensitizing process

| pH | RECIPROCAL OF TITRES AND SCORES[a] | | |
|---|---|---|---|
| | Human antitoxin diluted 1:3 | Human antitoxin diluted 1:2 | British Standard Antitoxin (0.1u/ml). |
| 5.6 | 64 (13) | 128 (17) | 256 (18) |
| *6.4 | 64 (13) | 128 (17) | 128 (17) |
| 7.2 | 32 (12) | 128 (15) | 64 (15) |
| 8.0 | 16 (9) | 128 (15) | 64-128 (15) |

[a] Range of titres for two experiments (four titrations): (n) = mean score.

* Indicates the pH selected for the sensitizing process.

TABLE 24

Effect of variation in the temperature of the sensitizing process

| TEMPERATURE | RECIPROCAL OF TITRES AND SCORES[a] | | |
|---|---|---|---|
| | Human antitoxin dilution 1:3 | Human antitoxin dilution 1:2 | Third British Standard Antitoxin (0.1u/ml). |
| Room temperature (approx. 20°C) | 16-32 (10) | 32-64 (13) | 16-32 (10) |
| 37°C | 32-64 (13) | 64 (15) | 128 (19) |
| 56°C | 128 (19) | 128-256 (21) | 256 (20) |

[a] Range of titres for two experiments (four titrations): (n) = mean score.

TABLE 25

Variation in antitoxin titres with concentration of sensitized cells

| INITIAL CONCENTRATION OF SENSITIZED CELLS | RECIPROCAL OF TITRES AND SCORES[a] | | |
|---|---|---|---|
| | Human antitoxin diluted 1:3 | Human antitoxin diluted 1:2 | Third British Standard Antitoxin (0.1u/ml). |
| 0.1% | 1024-2048 (29) | 2048-4096 (32) | 4096 (36) |
| 0.2% | 512-1024 (28) | 512-1024 (26) | 2048 (35) |
| 0.5% | 64-128 (18) | 128 (21) | 256 (21) |
| 1.0% | 16-32 (8) | 64 (9) | 64 (13) |
| 2.0% | 8 (6) | 16 (8) | 16 (9) |
| 3.0% | 4 (5) | 4 (8) | 8 (7) |

[a] Titres given are the results of one experiment (two titrations) with (n) = mean score: three other experiments gave similar results.

TABLE 26

Comparison of the activity of formaldehyde-fixed[a] and glutaraldehyde-fixed sheep cells after sensitization with different concentrations of tetanus toxoid.

| TETANUS TOXOID CONCENTRATION (Initial Lf/ml) | RECIPROCAL OF TITRES AND SCORES[b] | | | | | |
|---|---|---|---|---|---|---|
| | Formaldehyde fixation | | | Glutaraldehyde fixation | | |
| | Human antitoxin diluted 1:2 | Human antitoxin diluted 1:3 | Rabbit antitoxin | Human antitoxin diluted 1:2 | Human antitoxin diluted 1:3 | Rabbit antitoxin |
| 20 | 16 (15) | 16 (9) | 128 - 256 (22) | 32 (25) | 32 (22) | 512 - 2048 (45) |
| 40 | 32 (22) | 16 (18) | 256 - 512 (40) | 64 (31) | 32 (25) | 1024 - 2048 (54) |
| 60 | 32 (21) | 16 - 32 (19) | 256 - 1024 (46) | 64 (27) | 32 (25) | 1024 - 2048 (49) |

[a]Formaldehyde fixation was performed according to the procedure of Csizmas (1960).

[b]Range of titres for two experiments (four titrations): n = mean score.

pyruvic aldehyde (Ling, 1961). Results indicate that there is no difference in the final sensitivity of sheep cells fixed by the simple method of Galazka & Abgarowicz and those fixed by the more technically elaborate procedure of Calzman, but that the sensitivity of sheep cells fixed by glutaraldehyde or pyruvic aldehyde is higher (table 27). Furthermore, the use of cells fixed by glutaraldehyde or pyruvic aldehyde resulted in less variable titres for human antitoxin on repeated processing. Otherwise, the fixed cells were equivalent, and could not be differentiated on the basis of criteria used by Ling (1961), e.g. lysis during preparation; microscopic appearance; tendency to spontaneous agglutination; stability towards water and mechanical stability.

Comparison of chromic chloride and tannic acid as coupling agents.

Sheep cells, with and without prior treatment by activated papain, were sensitized by exposure to tetanus toxoid in the presence of an 0.1% (w/v) solution of chromic chloride in saline for 4-5 minutes. (Gold & Fudenberg, 1967; Nelson, 1973). Both the enzyme-treated and untreated cells gave virtually the same titres and scores for human and equine antitoxins as did sheep cells to which tetanus toxoid had been linked by tannic acid.

Glutaraldehyde as both fixing and coupling agent.

Cell preparations for which glutaraldehyde was used as both fixing and coupling agents, based on the techniques of Avrameas, Taudou & Chuilon (1969) and Onkelinx et al., (1969), gave titres for human antitoxin not significantly different from those obtained when glutaraldehyde-fixed cells were coupled to tetanus toxoid by tannic acid. However, the scores were low, i.e. the agglutination reactions were of poor quality, and the sensitized cells did not react with the equine standards.

Use of Oxoid phosphate buffered saline (PBS) containing polyvinyl-pyrrolidone (PVP) as diluent.

PVP did not prove to be an effective substitute for absorbed normal rabbit serum (NRS) in the diluent. Diluents of PBS containing PVP at a concentration of 0.35% as used by Borduas & Grabar (1953) and at a higher concentration of 1.5% were tested in parallel with

TABLE 27

Comparison of the sensitivity of sheep cells fixed by different methods

| ALDEHYDE FIXATIVE | METHOD USED | RECIPROCALS OF TITRES AND SCORES[a] | |
|---|---|---|---|
| | | Human antitoxin | Third British Standard Antitoxin (0.1u/ml) |
| Formaldehyde | Galazka & Abgarowicz (1967) | 16 - 128 (13) | 16 - 32 (9) |
| Formaldehyde | Csizmas (1960) | 16 - 128 (15) | 16 - 32 (10) |
| Pyruvic aldehyde | Ling (1961) | 32 - 64 (15) | 64 - 128 (13) |
| *Glutaraldehyde | Bing, Weyand & Stavitsky (1969) | 64 - 128 (17) | 64 - 128 (13) |

[a]Range of titres for two experiments (four titrations): n = mean score.

*Indicates the fixative chosen.

0.5% NRS-PBS. Only the latter prevented total agglutination.

Use of substitutes for sheep erythrocytes.

Human group O erythrocytes. Three different batches of human group O cells were investigated, but all showed a tendency towards spontaneous agglutination after sensitization. The tendency was not counteracted by an increase in the concentration of normal rabbit serum, previously absorbed with human group O cells, from 0.5% to either 1 or 2% in the diluent. A single attempt at sensitizing human group O erythrocytes by the method of Nelson (1973) produced cells of low sensitivity.

Chick erythrocytes. Since only one in twenty of the Nigerian sera tested by Greenwood (1970) possessed heterophile antibodies, a batch of cells from this species was also sensitized and tested against human and equine antitoxins. The agglutination reactions were uniformly weak or negative, however.

Latex particles. Different dilutions of the Difco suspension of latex particles (1.5%) were sensitized with tetanus toxoid of concentrations ranging from 10 to 1000 Lf/ml in buffers of PBS, borate-saline (Salomon & Tew, 1968) and glycine (Kende, 1969). None of the resulting preparations consistently produced agglutination reactions in accord with reasonable expectations and the haemagglutination results, e.g. assays performed on samples collected before and after booster doses of tetanus toxoid sometimes gave the same titres when those obtained by the haemagglutination technique were significantly different. Washing the sensitized latex particles by centrifugation to remove any excess tetanus toxoid did not improve the results.

Use of a diluent containing 2-mercaptoethanol (2-ME).

Adeniyi-Jones (1957) and Greenwood (1970) have shown that the agglutinating activity of heterophile agglutinins can be eliminated by treatment with 2-ME. Newell et al. (1971) have pointed out that the IgM content of test sera is probably responsible for the observation that IHA titres usually exceed TN titres when the former are estimated in haemagglutinin units by comparison with equine standards which lack IgM. Furthermore, the fact that the ratios of IHA:TN titres of volunteers given vaccines of different concentrations of tetanus toxoid and adjuvants were higher in subjects receiving the

less antigenic vaccines and that all ratios fall significantly with the later bleedings (Edsall, personal communication) suggests that the haemagglutinating antibody responsible for the discrepancy was IgM. Therefore, the use of a diluent containing 2-ME, a disulphide bond-reducing agent that reduces IgM but not IgG (Deutsch & Morton, 1957), was investigated as a simple means of simultaneously avoiding the necessity for the absorption procedure and of obtaining haemagglutination titres that correlate more closely with those determined by toxin neutralization.

For the inactivation of the heterophile agglutinins, dilutions of sera were prepared in the usual manner in diluents consisting of 0.5% NRS-PBS plus 2-ME to a final concentration of 0.1M and of NRS-PBS alone. The haemagglutination trays were then placed in a moist chamber at 37°C for a period of 30 minutes before addition of the sensitized cells. As the presence of 2-ME had no adverse effect on the appearance of the haemagglutination reactions, sera were titrated without prior dialysis and without the addition of iodoacetamide. The function of the latter is to prevent re-aggregation of the IgM when mercaptoethanol is removed, so its use did not seem to be indicated in the system being investigated. The choice of 30 minutes as a suitable period of exposure to the action of 2-ME was based on the results of titrations conducted on sera collected from rabbits early in the primary immune response to tetanus toxoid. From these titrations, a period of 30 minutes appeared to be adequate for reduction of the 2-ME-sensitive antibody because no further reduction in titres occurred with a longer period of exposure (table 28). Twenty-four Gambian sera were tested against fixed, non-sensitized sheep cells in diluents with and without 2-ME. Of the twenty-three sera which possessed agglutinins at a titre of 1:4 or greater, only ten (i.e. approx 50%) gave negative titres in the presence of 2-ME. Hence the addition of 2-ME to the diluent used for the preparation of serum dilutions does not provide a reliable means of avoiding the need for serum absorption.

More success was obtained with the use of 2-ME in the diluent as a means of improving the correlation between haemagglutination and toxin neutralization titres. Titrations conducted on

TABLE 28

Determination of the length of time required

for reduction of IgM by 0.1M 2-mercaptoethanol (2-ME) at 37°C

| DURATION OF EXPOSURE | RECIPROCAL OF TITRE | | | |
|---|---|---|---|---|
| | Rabbit antitoxin No.1 [a] | | Rabbit antitoxin No.2 [a] | |
| | Without 2-ME | With 2-ME | Without 2-ME | With 2-ME |
| min | | | | |
| 5 | 1024-2048 | 128 | 128-256 | 64 |
| 15 | 2048 | 32-64 | 256 | 32-64 |
| *30 | 1024-2048 | 32 | 256 | 32 |
| 60 | 1024-2048 | 32 | 128-256 | 32 |

[a] Antisera were collected from two rabbits ten days after primary injections of 80 Lf of adsorbed tetanus toxoid.

* Indicates adequate time of exposures.

forty-one human antisera in the presence and absence of 2-ME showed that the presence of 2-ME in the diluent resulted in IHA titres that correlated more closely with TN titres overall, although it made no difference for some sera. The forty-one sera comprised a composite group collected at various stages of the immune response to tetanus toxoid, i.e. after one, two or three injections of toxoid; and included a small group of antisera collected during a primary or early secondary response to tetanus toxoid by Ugandan patients with Tropical Splenomegaly Syndrome, a condition characterized by an abnormally high total serum IgM concentration of more than 4 mg/ml. These antisera were supplied by, and studied in collaboration with, Dr. John L. Ziegler now at N.I.H., Bethesda, Maryland.

The relation between the IHA and TN titres of the forty-one sera, with the latter estimated in the presence and absence of 2-ME, is shown in figures 8 and 9 respectively. For these comparisons, upper limits only of the titre range as determined by toxin neutralization are taken; since, with the toxin neutralization technique used, the actual titre of an antiserum is greater than the lower limit of the range quoted but may be less than or equal to the upper limit. The respective regression equations and correlation coefficients (r) for these results are:

$Y = 0.93x + 0.11$; $r = 0.84$

where Y is the TN titre and x is the IHA titre with 2-ME

and $Y = 0.89x - 0.04$; $r = 0.81$

where Y is the TN titre and x is the IHA titre without 2-ME

Although the IHA titres tend to correspond better with the TN titres in the presence of 2-ME than in its absence, there was no significant difference between the slope of either regression line and the ideal line by t tests. However, it can be seen that the regression coefficients are closer to the ideal value of 1 when assays are performed in the presence of 2-ME. Furthermore, titrations of antisera from the selected Ugandan subjects with high serum IgM levels did show that the use of a diluent containing 2-ME can significantly reduce haemagglutination titres to values that approach or equal those obtained by toxin neutralization (Table 29).

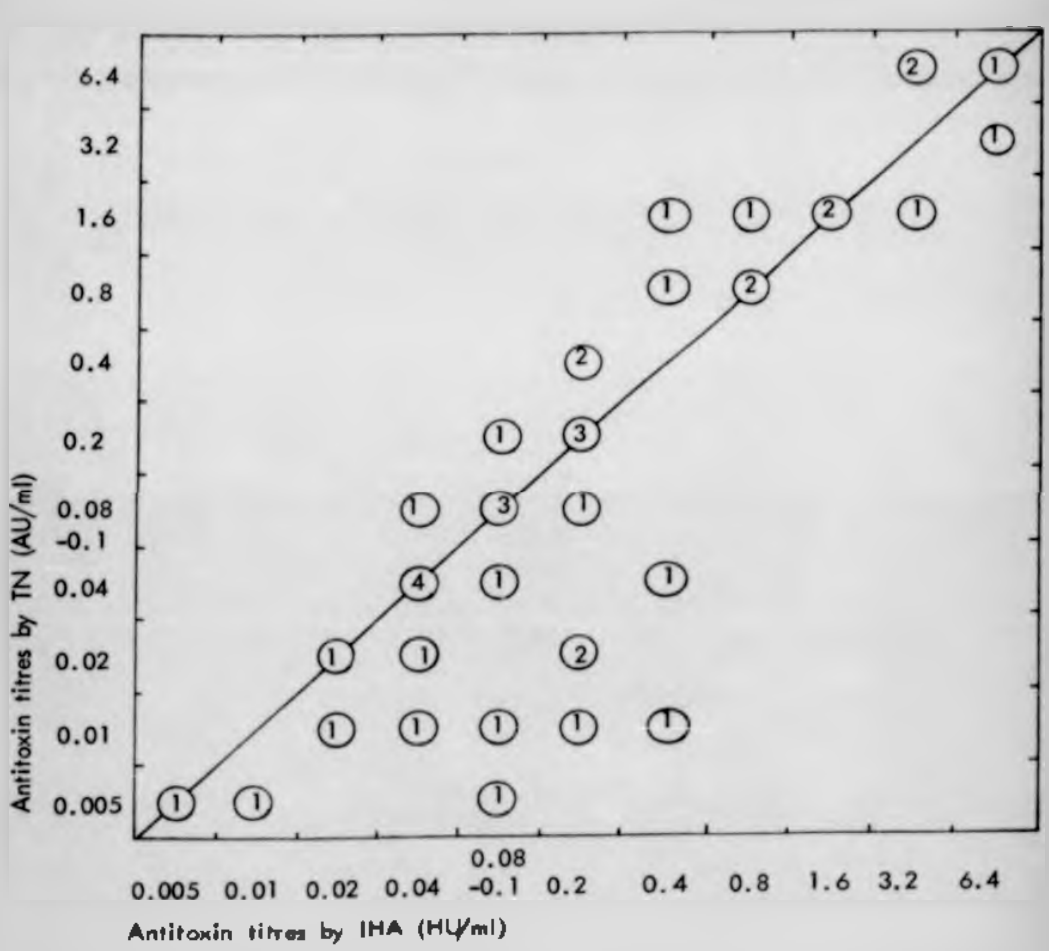


Fig. 8 Tetanus antitoxin values by haemagglutination (HU/ml) in the presence of 2-mercaptoethanol plotted against antitoxin values obtained by toxin neutralization (AU/ml). Numbers in circles represent frequency of titres at particular levels.

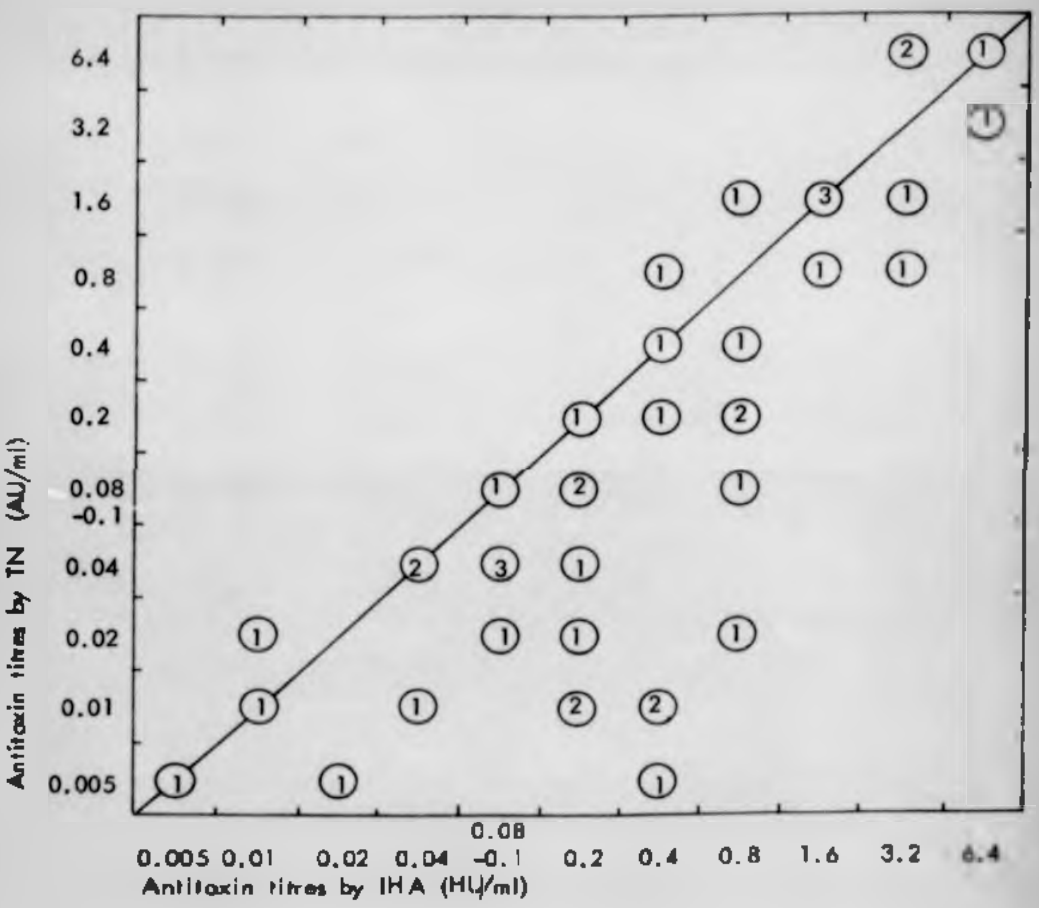


Fig. 9 Tetanus antitoxin values by haemagglutination (HU/ml) plotted against the antitoxin values obtained by toxin neutralization (AU/ml). Numbers in circles represent frequency of titres at particular levels.

TABLE 29

Reduction in haemagglutination titres of antisera from patients with Tropical Splenomegaly Syndrome by use of a diluent containing 2-mercaptoethanol.

| SAMPLE NUMBER | SERUM IgM CONCENTRATION[a] | IHA TITRE | IHA TITRE WITH 2-ME | FOLD DECREASE IN IHA TITRE WITH 2-ME | TN TITRE | RATIO HU:AU WITH 2-ME |
|---|---|---|---|---|---|---|
| | mg/ml | HU/ml [b] | HU/ml | | AU/ml [c] | |
| 1 | 4.6 | 0.64 | 0.16 | 4 | 0.16 | 1.0 |
| 2 | 4.6 | 0.08 | 0.04 | 2 | 0.04 | 1.0 |
| 3 | 4.5 | 0.02 | 0.01 | 2 | 0.005 | 2.0 |
| 4 | 4.4 | 0.64 | 0.16 | 4 | 0.32 | 0.5 |
| 5 | 5.4 | 0.02 | 0.01 | 2 | = or <0.0025 | N.D[d] |
| 6 | 4.7 | 0.16 | 0.04 | 4 | 0.02 | 2.0 |

[a] Total serum IgM concentrations were estimated by means of Tri-Partigen Immunodiffusion Plates (Behringwerke).

[b] Haemagglutinin units per ml calculated by reference to a standard antitoxin.

[c] Antitoxin units, as determined by toxin neutralization, are expressed as AU/ml to distinguish them from HU/ml.

[d] Not determinable.

# DISCUSSION

A systematic investigation of the variables affecting the application of glutaraldehyde-fixed sheep cells to the assay of tetanus antitoxin by the indirect haemagglutination technique was undertaken for the purpose of defining the optimal conditions for the assay. As a screening procedure, the indirect haemagglutination test has the advantage of being potentially highly sensitive; this potential may be realized by the use of optimal conditions for processing the cells and conducting the assays. Furthermore, in the course of this investigation it was observed that the problems of weak agglutination reactions and long prozones with equine antitoxins, first reported by Fulthorpe (1957), were largely overcome by the use of erythrocytes processed optimally. Since it is an accepted and convenient practice to express haemagglutination titres for tetanus antitoxin in terms of "haemagglutinin units" (HU) instead of end-point dilutions by comparison of these dilutions with those given by a standard equine antitoxin in the same system (Levine & Wyman, 1964b; Newell et al., 1971), any reduction in the haemagglutinating activity of the standard antitoxins will falsely inflate the HU values. Hence the improved reactivity of the equine standards with cells sensitized under optimal conditions also leads to the estimation of tetanus antitoxin in haemagglutinin units which correlate more closely with the titres obtained by toxin neutralization. This is obviously of value where the haemagglutination results are used as guides to the selection of antitoxin dilutions for testing by toxin neutralization, and even more important when tetanus antitoxin titrations are determined by the indirect haemagglutination test alone.

Principal factors governing the final sensitivity of glutaraldehyde-fixed sheep cells are the concentration of tannic acid and tetanus toxoid used in processing the cells, and the concentration of sensitized cells used in the titrations. In general, conditions are not as important as concentrations, but they can have a considerable influence. Variations in the time and temperature of

exposure of tanned cells to tetanus toxoid, for example, resulted in significant differences in the sensitivity of the coated cells. Another major factor influencing sensitivity is the source of sheep cells, hence a single animal should be used as cell donor. For all these variables, differences of more than fourfold in titre occurred with either the human or equine antitoxins over the range of variable tested. Other variables causing a difference equal to fourfold in titre were the time and temperature of the tanning process, and the pH of the tanning and sensitizing procedures.

Of course, the apparent importance of a variable depends to a large extent on the interval widths and range of values chosen for testing, and especially on the values arbitrarily selected as defining the limits of the range. Obviously, a fourfold difference in titre is more likely to occur where a wide range is tested. In this study, however, the values and range of variables investigated were generally restricted to those recommended by various authors for tetanus antitoxin assay by the haemagglutination technique with fresh or formalinized sheep cells. It may be concluded, then, that the concentration of sensitized cells is the most important variable under the usual conditions of assay since variations in cell concentration had the most pronounced effect on titres. The actual cell concentration chosen for use represented a compromise between sensitivity and readability. An 0.2% concentration is highly sensitive, but an 0.5% concentration produces more readable end-points while still retaining sufficient sensitivity for the detection of tetanus antitoxin at levels lower than those normally detected by the toxin neutralization test. This is supported by the fact that only two antisera out of a total of about seven hundred sera tested by both methods gave a positive TN result in the absence of an IHA reaction.

Detailed studies on the influence of variations in the procedures of fixing, tanning and sensitizing sheep cells on final sensitivity have not been previously reported for the tetanus antitoxin system; although it is known that, for indirect haemagglutination techniques, the concentrations and conditions of importance vary with the sensitizing antigen (Daniel, Weyand & Stavitsky, 1963; Hirata & Brandriss, 1967). However, Fulthorpe (1959) stated that the concentration of sensitizing tetanus toxoid was of importance, and

also demonstrated that the concentration of sensitized cells had a decisive effect on the titres obtained.

The need to use blood from a single sheep for consistent and comparable results in the titration of diphtheria and tetanus antitoxins by haemagglutination was stressed by Galazka & Abgarowicz (1967). Evidently, the erythrocytes are not merely inert carriers as might be imagined. Indeed, in their investigations of various protein and polysaccharide antigens with aldehyde-fixed erythrocytes, Hirata & Brandriss (1967) found differences of as much as thirtyfold in titres when batches of cells from different rabbits were tested. They attributed the variation to differences in the surface characteristics of the blood cells which, in turn, vary with the physiologic state of the animals. Similarly, Hoq et al. (1971) reported significant differences in the ability of blood cells from different sheep to absorb heterophile agglutinins from samples of human sera. In view of this finding, the possibility of sheep cells being "low reactors" should be considered whenever difficulties arise in the complete absorption of serum samples prior to testing.

Fixed erythrocytes are used in preference to unfixed erythrocytes in the haemagglutination technique because of their resistance to lysis; stability on storage; and ease in handling. Coupling agents, in particular, tend to damage unfixed blood cells, and the resulting haemolysis may interfere with subsequent titrations (Onkelinx et al., 1969). Furthermore, Fulthorpe (1957) and Kyselova et al. (1970) found that the results of titrations conducted with fixed cells are generally more reliable and more reproducible than those conducted with unfixed cells.

From amongst the published methods for fixing cells by aldehydes, the glutaraldehyde-fixation procedure of Bing et al. (1969) was chosen initially, and then subsequently applied successfully in a simplified form. The method stipulates a fixation period of only 30 minutes as compared to the 18-24 hour time period usually employed for fixation by formaldehyde, and was shown here to have the additonal advantages of giving more sensitive cells and more

reproducible end-points. Glutaraldehyde can act as a coupling agent by linkage to free amino acids of antigens (Avrameas et al., 1969); and, although its coupling activity was not sufficiently powerful to allow it to be used for that purpose in the technique described in this study, it is likely that the increased sensitivity of cells fixed by this bifunctional compound is related to its coupling ability.

With regard to the fixing of sheep cells by formaldehyde, a comparison of sensitized cell preparations fixed by the frequently-cited method of Caizmas (1960) and the much simpler procedure of Galaszka & Abgarowicz (1967) indicated that fixation by the more complicated procedure of Caizmas offers no advantages. Moreover, Herbert (1967) found that titres obtained with cells fixed by the Caizmas method were significantly affected by storage for nine months at 4°C in contrast to those obtained with cells fixed by simple formalinization procedures. The sensitized glutaraldehyde-fixed cells which were prepared by the method described here maintained their sensitivity for at least six months when stored at 4°C in 0.5% NRS-PBS diluent containing 1:10000 Thiomersal.

The use of tannic acid as a coupling agent was first described by Boyden (1951); the use of chromic chloride for this purpose was first described by Jandl & Simmons (1957). The latter has been advocated as being preferable to the former on the grounds that the coupling procedure is less complicated and less time-consuming (Gold & Fudenburg, 1967). However, when sensitised cells which had been coupled to tetanus toxoid by tannic acid and by chromic chloride were compared, no difference was detected in the sensitivity of the final cell preparations, and nor was there any difference of practical consequence in the times required for coupling or in the ease of performance of the coupling procedures. Further, if the results obtained with chromic chloride are not always reliable unless a buffer such as piperazine is present - as claimed by Pearson (1974) - then the tanning procedure has the advantage of greater simplicity. A tannic acid solution for coupling is prepared simply by dissolving the acid in distilled water or saline. Tannic

acid tends to precipitate if prepared in Oxoid PBS (Appendix iii), but it is this tendancy which probably accounts for the greater sensitivity of cells suspended in PBS during the tanning process where precipitation onto the cells would be expected to promote the reaction.

As a result of his studies on the uptake of $^{131}$I-NRS by cells already sensitized with antigen, Shinjiri (1964) proposed that the most probable mechanisms of stabilization by 1% NRS is the blocking of unoccupied sites that would otherwise tend to cause non-specific adherence. Certainly, the addition of a protein stabilizer to the diluent is necessary to avoid non-specific agglutination; and an increase in the concentration of the protein will often prevent the non-specific agglutination that occasionally persists at lower concentrations of the stabilizer. Paradoxically, however, the 0.5% NRS-PBS diluent used in early experiments of the study reported here *actually caused non-specific* agglutination because of bacterial contamination. The ease with which the 0.5% NRS-PBS diluent was susceptible to contamination had not been appreciated initially. Replacement of the normal rabbit serum by polyvinylpyrrolidone (PVP) was investigated not only as a means of avoiding the procedure of absorption of the NRS but also for the purpose of providing a diluent less favourable to bacterial growth. PVP did not prove to be a useful substitute for absorbed normal rabbit serum, however, and the problem of bacterial contamination was overcome by the addition of Thiomersal to a final concentration of 1:10000.

The absorption of serum samples by washed, packed sheep cells for removal of heterophile agglutinins is a time-consuming, serum-consuming, and tedious procedure. For these reasons, various substitutes for sheep cells in the haemagglutination technique were investigated. However, neither human group O cells, chick cells, nor latex particles provided an acceptable alternative. It is of interest that the non-specific agglutination reactions which occurred with the glutaraldehyde-fixed human cells were also reported by Ali-Khan (1974) for human cells fixed by this method.

Another approach that was tried in an effort to avoid the necessity for serum absorptions was the use of a diluent containing 2-mercaptoethanol for the preparation and treatment of the serum dilutions. The procedure was effective in removing heterophile agglutinins from only half of the sera tested, however. Such a result is not easily reconciled with those of Adeniyi-Jones (1967) and Greenwood (1970) who reported that the heterophile agglutinins for sheep cells were susceptible to inactivation by 2-ME, but these workers did subject their test sera to alkylation by iodoacetamide and purification by dialysis - procedures not justified in a method designed to provide a simple alternative to absorption.

The simple technique of using 2-ME in the diluent did result in some improvement in the correlation between haemagglutination and toxin neutralization results. Differences of as much as four or five-fold between IHA and TN titres for individual serum samples have frequently been reported (Surjan & Nyerges, 1962; Chatterjee, 1964; Levine & Wyman, 1964b); and, indeed an IHA:TN titre ratio of as much as twenty was found for early bleedings from subjects who received the tetanus toxoid preparation of lowest antigenicity in the study on vaccines containing different concentrations of tetanus toxoid and adjuvant (Edsall, personal communication). Presumably, the inactivation of IgM by the use of 2-ME in the haemagglutination technique helps reduce the differential. More importantly, its use results in a more valid assessment of the protective level of antitoxin since Ourth & Edsall (1972) showed that the IgM component of rabbit tetanus antitoxin was not capable of neutralizing toxin to any significant extent.

Nevertheless, it was observed that, for individual sera, the presence of 2-ME in the haemagglutination test sometimes made no difference to the discrepancy between IHA and TN titres. This indicates that the difference in activity of the IgM component of tetanus antitoxin in the two systems is not the only factor responsible for the discrepancies observed. It is probable that differences in the inherent sensitivities of these two methods to

other classes of antibody; to antibodies of different avidities; and, possibly, to cross-reacting antibodies are also responsible.

The major advantages of the indirect haemagglutination technique for the determination of tetanus antitoxin are its high sensitivity and economy in terms of time, effort and animals. Because the technique requires no special materials or animal house facilities, it is often used alone for the assay of tetanus antitoxin. Such a course of action is reasonable where the results of comparative group studies are more important than accurate estimates of antitoxin in individual serum samples. However, the savings in terms of time, effort and animals apply whether the haemagglutination technique is used alone or in association with the toxin neutralization technique, and are so important and substantial that the haemagglutination test may be considered an essential preliminary to the toxin neutralization test.

Results of investigations conducted here on variables affecting the assay of tetanus antitoxin by haemagglutination using glutaraldehyde-fixed sheep cells led to the application of optimal conditions for preparing the sensitized cells and conducting the assays. This resulted in a technique of optimal sensitivity capable of measuring as little as 0.001 haemagglutinin unit per ml, since the haemagglutination end-point for 0.1 u/ml of the Third British Standard Antitoxin is usually 1:128. The fact that a negative haemagglutination reaction in a serum invariably meant a negative toxin neutralization result attests to the high sensitivity of the haemagglutination technique and its usefulness as a reliable screening procedure.

Various modifications of the technique, designed to increase speed and reduce the technical manipulations, were also investigated. The use of glutaraldehyde as fixative was advantageous in these respects, but replacement of tannic acid by chromic chloride was not indicated. Attempts to avoid the need for absorption of serum samples prior to testing did not prove to be successful; but, since the technique developed here was applied mainly to the assay

of cord blood samples which do not possess heterophile agglutinins (Adeniyi-Jones, 1967), there was little motivation for making further efforts in this direction. Nevertheless, the necessity for prior absorption of serum samples remains a shortcoming of the technique, and means of avoiding it deserve further study.

The enhanced reactivity of equine standard antitoxins with optimally sensitized cells and the use of 2-mercaptoethanol in the haemagglutination test resulted in haemagglutination titres which correlate more closely with those determined by toxin neutralization. Hence, despite the discrepancies between the IHA and TN titres which continue to occur, especially at the low antitoxin concentrations, the indirect haemagglutination technique described here does provide a method of measuring tetanus antitoxin with a high degree of sensitivity and with a useful degree of validity.

# CHAPTER 3

## DETECTION OF THE IgM COMPONENT OF TETANUS ANTITOXIN BY IMMUNOFLUORESCENCE

The testing of the hypothesis that the transplacental passage of antigens such as tetanus toxoid can occur with the consequent production of specific IgM by the foetus requires a method for the detection of very small amounts of tetanus-specific IgM. An immunofluorescence technique for the identification of the various immunoglobulin classes of human tetanus antitoxin - including IgM - has been developed by Hernandez, Just & Bürgin-Wolff (1973). In their method, suspensions of agarose-bound tetanus toxoid were exposed to test sera, then to rabbit antisera to human IgM, IgA or IgG followed by fluorescein-labelled goat anti-rabbit immunoglobulin. More recently, a polymer of tetanus toxoid has been used as slide antigen for the assessment of tetanus antitoxin by an indirect immunofluorescence technique (Ourth et al., 1975).

Both forms of antigen were investigated here in a technique in which the antigens were exposed to test sera then to FITC-conjugated goat antisera to human IgM and IgG which had been rendered specific by absorptions with polymerized purified heterologous immunoblobulins. To increase the sensitivity of the test system, the bulk of the IgG was removed from the test antisera by prior absorption of the samples with protein A, the purpose being to reduce the competition by specific IgG for the limited number of antigenic sites available to specific IgM. Two antisera containing tetanus-specific IgM and IgG as well as antiserum containing specific IgG only were used in the development and testing of the technique. These antisera were used to demonstrate the absorption of specific IgG by protein A, and the specificity of the indirect immunofluorescence technique for the detection of IgM.

## MATERIALS AND METHODS

Agarose beads. Sepharose 4B and a sample of a freeze-dried preparation of CNBr-activated Sepharose 4B (Batch No.3538) were obtained from Pharmacia Fine Chemicals Ltd. (Uppsala, Sweden , available from Pharmacia (G.B.) Ltd., Paramount House, 75 Uxbridge Road, London). The agarose spheres range in diameter from 40 μm to 190 μm (av.110 μm) in the swollen state. Each 1 g of the freeze-dried product yields about 3 ml of gel when reconstituted.

Tetanus toxoid. Batch V.C.T. No.6 was used (Appendix vii).

FITC-labelled antisera to human IgG and IgM. Conjugates of goat antisera, labelled with fluorescein isothiocyanate (FITC), as well as polymerized purified IgG and IgM from human sera, were supplied by Dr. A. B. MacDonald, Harvard School of Public Health, 665 Huntington Avenue, Boston, Massachusetts. The methods used for the isolation and purification of these immunoglobulins and for the preparation of the specific antisera have been described by Barenfanger & MacDonald (1975). Briefly, the goat antisera were rendered highly specific for human IgG and IgM by absorption with purified heterologous Ig classes which had been polymerized by a modification of the procedure of cross-linkage by ethyl chloroformate of Avrameas & Ternynck (1967). The immunoglobulins used for absorption in the polymerized state were initially isolated by methods designed to give pure Ig classes uncontaminated by other proteins, and were further purified by absorption with $F(ab)_2^*$ by affinity chromatography. The increased specificity of the absorbed conjugated goat antisera, as compared with unabsorbed material, was demonstrated by immunofluorescence with polymerized Ig classes as slide antigens.

Precipitating antiserum to human IgM. Samples of sheep anti-human IgM (Code No.SH15-01-P04) and horse anti-human IgG (Code No. PH16-04-P14) were obtained from Organon Teknika Ltd. (Crowthorn, London Road, Morden, Surrey).

Human tetanus antitoxin. One of the test samples consisted of a pool of antisera collected in the primary or early secondary immune response of patients with Tropical Splenomegaly Syndrome, a condition characterised by an abnormally high total serum IgM concentration; another was composed of about twenty individual serum samples collected about 28 days after primary injections with tetanus toxoid. Both contain IgM specific for tetanus toxoid as indicated by the significantly different titres obtained when these antisera were titrated by the indirect haemagglutination technique for measuring tetanus antitoxin in the presence and absence of 2-mercaptoethanol. The third test serum consisted of a highly diluted sample of "Tetagam" (Appendix iv) containing specific IgG only.

Bacterial strains for protein A. Two strains of Staphylococcus aureus were obtained from the National Collection of Type Cultures, namely the Cowan I strain (NCTC No.8530) which produces protein A, and the Wood 46 strain (NCTC No.10345) which is known not to produce protein A.

Purification of the tetanus toxoid.

Before polymerization or attachment to Sepharose beads, the tetanus toxoid was purified by filtration on a Sephadex G-200 (Pharmacia) column (Khaustova, Ugleva & Rozhdestvenskaya, 1970). Volumes of 2 - 3 ml of tetanus toxoid were passed through a bed of approx. 30 cm of swollen Sephadex gel in a 2.6 x 40 cm column (Type K 26/40, Pharmacia). The gel was equilibrated in Oxoid PBS of pH 7.3 (Appendix iii), and the fractions were eluted in the same buffer under an operating pressure of 15 cm of water, and collected in 3 ml volumes. The first major protein peak was located by means of the optical density readings of the fractions at 280 nm on a Unicam SP 1700 Ultraviolet Spectrophotometer. Eluates constituting this peak were combined and concentrated by ultrafiltration with a B-15 type of Amicon filter (Amicon Ltd., 57 Queen's Road, High Wycombe, Bucks). After concentration, the protein content was estimated by the technique of Lowry et al. (1951).

The identity of the purified concentrated tetanus toxoid was checked by immunodiffusion against tetanus antitoxin of equivalent Lf value. The test was carried out on a microscope slide covered with a layer of medium consisting of 0.8% Ionagar No.2 (Oxoid Ltd.)

in barbitone buffer (Appendix viii). The circular wells were cut by a small cork borer. Results were read after the reaction had been allowed to develop for 48 hours in a moist chamber at room temperature (approx. 20°C).

Purity of the tetanus toxoid was tested by immuno-electrophoresis. Again, the supporting medium was 0.8% Ionagar No.2 in barbitone buffer on a microscope slide. After the application of the tetanus toxoid at a concentration of about 100 Lf/ml, 4-5 volts per cm were applied for a period of 45 minutes in the presence of a buffer of the same composition as that used for the preparation of the agar gel. Antitoxin of concentration 50 Lf/ml was then added to the trough, and the results read after 48 hours.

Polymerization of tetanus toxoid.

The method of Ourth et al. (1975) was followed. Tetanus toxoid at a concentration of 20-30 mg/ml was adjusted to a pH of 5.1 with 0.2M acetate buffer. Ethyl chloroformate (B.D.H.) was added at the rate of 0.1 ml per 100 mg of toxoid protein, and the mixture stirred by a magnetic stirrer for 24 hours at room temperature. The polymer was then washed several times on a glass filter of porosity 3 with 0.1M ammonium chloride solution of pH 7.4; dialysed in 0.1M borate saline buffer (BSB) of pH 8.0 at 4°C for 24 hours; and washed repeatedly in BSB. It was stored in small volumes of BSB at -20°C.

Polymerization of anti-IgM and anti-IgG.

The technique used was based on that of Avrameas & Ternynck (1967). Ethyl chloroformate (B.D.H.) was added to 2 ml volumes of sheep precipitating antiserum to human IgM and horse precipitating antiserum to human IgG in 10 ml beakers at a concentration of 0.5 ml of ethyl chloroformate per 1000 mg of protein. The mixtures were stirred gently by a magnetic stirrer for 15 minutes, and allowed to stand for one hour at room temperature. After formation, the polymers were washed thoroughly by successive centrifugation with Oxoid PBS; 0.1% sodium carbonate solution; PBS; 0.2M glycine-HCl buffer of pH 2.2; and PBS.

Preparation of tetanus toxoid-coated Sepharose beads.

Activation by cyanogen bromide. Sepharose was activated for protein coupling with CNBr by a modification of the method of Axén, Porath & Ernback (1967) as used by Deelder & Ploem (1974). Four ml of sedimented agarose beads were washed with distilled water on a glass filter of porosity 3 to remove bacteriostatic agents. The beads were then re-suspended in an equal volume of distilled water, and a solution of 1 g of CNBr (Koch-Light Laboratories) in 20 ml of distilled water was added to the Sepharose suspension in a well-ventilated fume cupboard. The mixture was stirred gently by means of a magnetic stirrer for 6-10 minutes during which time the pH was maintained at about 11 by the addition of 2N sodium hydroxide. Finally, the activated beads were washed on a glass filter with distilled water and Oxoid PBS.

Coupling of tetanus toxoid to the activated beads. The required concentration of purified tetanus toxoid in a volume of 4 ml was added to a 4 ml suspension of the activated beads. The coupling reaction was carried out under the conditions described for attachment of tetanus toxoid to Sepharose by Hernandez et al. (1973) viz: with adjustment of the pH to 10 by sodium hydroxide, and with stirring of the suspension overnight at 4°C by end-over-end rotation.

Deactivation of the tetanus toxoid-coated beads. An equal volume of 2M ethanolamine (B.D.H.) in 0.05M bicarbonate buffer of pH 10 was added to the tetanus toxoid-coupled beads, and the suspension stirred for one hour at room temperature. The product was washed in three cycles each consisting of a wash with 0.1M acetate buffer containing 1M NaCl at pH 4 followed by a wash with 0.1M borate buffer containing 1M NaCl at pH 8. It was washed finally in Oxoid PBS, and then stored in a solution of 0.02% sodium azide in Oxoid PBS.

Preparation of tetanus toxoid-coated beads from freeze-dried CNBr-activated Sepharose.

Before use, the commercially activated Sepharose beads

were washed on a glass filter with $10^{-3}$M HCl, in accord with the manufacturer's directions, for the removal of the dextran and lactose stabilisers. About 10 mg of purified tetanus toxoid was mixed with about 3 ml of the swollen gel from 1 g of freeze-dried material in a 0.1M sodium bicarbonate buffer of pH 8.3 containing 0.5M NaCl (5 ml), and the mixture was rotated end-over-end at 4°C overnight. Unbound material was washed away with the coupling buffer, and any remaining activated groups were deactivated by exposure to 1M ethanolamine (B.D.H.) at pH 8 for 12 hours. Non-covalently adsorbed protein was then removed by washing alternatively with acetate and borate buffers.

Absorption of IgG in test sera by protein A.

Cultivation of the staphylococcal strains. The Cowan I (protein A-positive) and the Wood 46 (protein A-negative) strains of Staphylococcus aureus were cultivated on a solidified form of the medium of Arvidson, Holme & Wadström (1971) designed to promote the production of extracellular proteins by staphylococci (Appendix ix). Layers of this medium on the flat sides of large bottles were inoculated with broth cultures of the bacterial strains. After incubation of the cultures at 37°C for 24 hours, the staphylococci were harvested and washed in Oxoid PBS.

Processing of the cultures. The harvested cultures were exposed to 3% formalin in PBS for a period of 30 minutes at room temperature, and were washed three times in PBS (Lind & Mansa, 1968). The formalin-treated suspensions were then heated in a water bath at 80°C for 15 minutes; and, after additional washing, they were stored as 20% suspensions in PBS at 4°C.

Absorption procedure. For use, the staphylococcal suspensions were distributed in small test tubes (35 mm x 8 mm), and washed once with Oxoid PBS. Equal volumes of 1:4 dilutions of sera were absorbed with the sediments at room temperature for 30 minutes, and the bacteria then separated by centrifugation at 1500 g for 30 minutes (Ankerst et al., 1974).

General procedure for immunofluorescence with polymerized tetanus toxoid as slide antigen.

Preparation of smears of polymerized tetanus toxoid for immunofluorescence staining. Microscope slides with multiple test areas, each of about 7-8 mm diameter, are prepared by positioning small buttons on the slides, and spraying them with a preparation of a water-repellent polymer of tetrafluoroethylene (Chemplast Inc., obtainable from Marshall-Howlett Ltd., 293 Main Road, Sidcup, Kent). The use of such slides saves time, labour and materials (O'Neill & Johnson, 1970).

To each plaque a 10 µl volume of the polymer suspension is added by means of an adjustable 5-50 µl Finnpipette (Buckley Membranes Ltd., Chequers Pde., Prestwood, Great Missenden, Bucks). The smears are air-dried, then fixed in acetone for ten minutes.

Immunofluorescence staining by the indirect method. In general, the procedure follows the recommendations of Johnson & Holborow (1973). The protein A-absorbed test sera and known negative control sera (1:4 dilutions) are applied in 10 µl volumes to the plaques of fixed polymer. Saline only is applied to one plaque as a control for reaction between polymer and conjugate. The slides are then placed in a moist chamber to minimize evaporation and left at room temperature for 30 minutes. At the end of this reaction period, the antisera are removed by gentle rinsing with Oxoid PBS, and the slides then transferred to a metal carrier supported over a magnetic stirrer in a bath of buffered barbitone saline of pH 7.2 (Appendix x). After 30 minutes of washing, the slides are taken from the bath, and excess washing fluid is removed from the slides by paper tissues to prevent uncontrolled dilution of the conjugate. Care is taken to avoid drying of the test areas, however, as local concentrations of salts may produce artifacts.

A suitable dilution of the antiglobulin conjugate is applied to each polymer plaque in a 20 µl volume, and the slides are returned to the moist chamber for 30 minutes. At the end of this time, the slides are placed in the bath for washing for 1-2 hours. The stained preparations are then mounted in a mountant (Appendix xi) of pH 8.5 - a pH which is more suited to the fluorescence emission of FITC than is the pH of 7.1 so frequently

used (Heimer & Taylor, 1974).

General procedure for immunofluorescence with tetanus toxoid-coated Sepharose beads as antigen.

Use of microtitre equipment for immunofluorescence staining of coated Sepharose by the indirect method. Volumes of 25 µl of a suspension of tetanus toxoid-coated beads containing $10^5$ beads per ml (as determined on a counting chamber) and 25 µl of the protein A-absorbed test sera and known negative control sera (1:4 dilutions) are added to V-shaped wells of disposable polystyrene microtitre plates of the Cooke Microtiter system (Dynatech Laboratories Ltd.). Saline only is added to one well. Disposable Cooke micropipettes (25 µl) or a Finnpipette adjusted to a 25 µl delivery volume are used for the additons. The wells are covered with sealing tape to prevent evaporation and the plates are placed on a rotary plate mixer (Luckham Ltd., Victoria Gardens, Burgess Hill, Sussex) for 30 minutes.

At the end of this time, the sealing tape is removed from the wells, and the plates are loaded into centrifuge plate carriers (Microtiter Code No.M18E, Dynatech Laboratories Ltd.) for washing of the beads by centrifugation. The beads are washed three times in 0.65M sodium solution (Capel, 1974) by mixing on the rotary plate shaker for 5 minutes then centrifuging at 100 g for 5 minutes. After each centrifugation, the supernatant fluid is removed by simply inverting the plate and shaking once; this procedure does not result in any significant loss of beads.

A volume of 25 µl of buffered barbitone saline is added to each well followed by 25 µl of a suitable dilution of anti-globulin conjugate in 4% bovine serum albumin in the same buffer (Deelder & Ploem, 1974). The contents of the wells are mixed once more on the rotary plate shaker for 30 minutes after which time the beads are washed by centrifugation four or five times with 0.65M sodium chloride solution. Finally, the beads are transferred to test areas on multispot slides by Pasteur pipettes, and mounted for examination.

Fluorescence microscopy. Specimens are examined on a Reichert Zetopan fluorescence microscope equipped with a dark field condenser; a HBO 200 high pressure mercury vapour lamp; an E2 type exciter filter; and a UV-Sperr, yellow-green absorption filter.

The fluorescent patterns of the particulate polymers are graded as follows:-

4+ = whole particle fluorescences.

3+ = rim of particle fluorescences; central portion shows a mixture of fluorescent parts and bright pink-yellow background.

2+ = thin rim of fluorescence around the particle; central portion shows a greenish fluorescence of low intensity, or is dull pink in colour.

1+ = particle shows slight greenish fluorescence.

0 = particle is dull red in colour.

Fluorescence in shades of yellow-green ranging from brilliant to pale is observed with the tetanus toxoid-coated Sepharose. Beads giving a negative reaction are faintly gray in colour. Figures 10 and 11 show fluorescence of the tetanus toxoid polymer and tetanus toxoid-coated Sepharose beads respectively.

## EXPERIMENTS AND RESULTS

Testing of goat conjugates.

Estimation of the fluorescein: protein ratios. The labelling ratios of the FITC-conjugated goat anti-human IgG and anti-human IgM were estimated from the optical densities of the conjugates at 495 nm (corresponding to the peak absorbance of FITC-conjugated to protein) and at 280 nm (Johnson & Holborow, 1973). Results are given in table 30. Conjugates with F/P ratios of under 1 or over 4 are unsatisfactory (Capel, 1974).

Investigation of the presence of free fluorochrome. Unbound fluorochrome in the conjugate can cause non-specific staining (Chadwick & Nairn, 1960). Its presence was investigated in the goat conjugates

Fig. 10 Fluorescence of tetanus toxoid polymer. The polymerized tetanus toxoid has been incubated with human tetanus antitoxin and with an FITC-labelled goat anti-human conjugate.

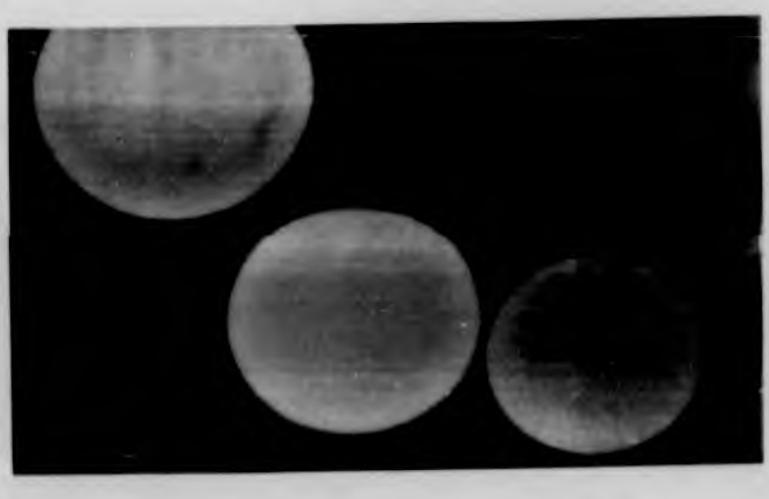

Fig. 11 Fluorescence of tetanus toxoid-coated Sepharose beads. The beads have been incubated with human tetanus antitoxin and with an FITC-labelled goat anti-human conjugate.

TABLE 30

Characteristics of the FITC-conjugated goat antisera used for the indirect immunofluorescence technique

| PROPERTY | CONJUGATED GLOBULINS | |
|---|---|---|
| | Anti-human IgM | Anti-human IgG |
| Optical density at 280 nm (1:40 dilution) | 0.202 | 0.230 |
| Optical density at 495 nm (1:40 dilution) | 0.096 | 0.112 |
| Fluorescence: protein ratio ($O.D_{280}$: $O.D_{495}$) | 2.10 | 2.05 |
| Free fluorochrome | Absent | Absent |

[a]Optical densities were read on a Unicam SP 1700 Ultraviolet Spectrophotometer.

by the simple procedure described by Johnson & Holborow (1973). Microscope slides, coated with a slurry of Sephadex G-25 were arranged at an angle of inclination of about 10-20° between the open halves of Petri dishes. Filter paper wicks were attached to the ends of the slides, and the upper dishes were filled with Oxoid PBS. Once the flow of PBS was established, drops of conjugate were applied to the upper ends of the slides, about 1 cm from the edge. Any unbound dye in the conjugate remains at the point of application where it can be seen after a period of 15-20 minutes during which time the labelled protein migrates several cm from that point. The absence of free fluorochrome in the goat conjugates was confirmed (table 30).

Determination of suitable test dilutions of the conjugates.

Dilutions of goat anti-human IgM were tested against polymerised IgM and polymerized IgG as slide antigens (table 31). A 1:100 dilution stained the IgM polymer without staining the IgG polymer so this dilution was considered suitable for use for the specific detection of IgM in antisera by the indirect immunofluorescence technique. A suitable test dilution for goat anti-human IgG was established similarly.

Comparison of different preparations of tetanus toxoid-coated Sepharose beads.

The tetanus toxoid used for coating the Sepharose beads was pure and homogeneous, as judged by the single lines of precipitate which formed between toxoid and tetanus antitoxin in the techniques of immunodiffusion and immunoelectrophoresis

Two different concentrations of tetanus toxoid were coupled to 4 ml volumes of activated Sepharose beads viz: 7.5 mg of toxoid in a 4 ml volume and 15 mg of toxoid in a 4 ml volume. There was no discernible difference between the resulting preparations when observed for fluorescence after exposure to tetanus antitoxin and conjugated antiserum, however. In contrast, coated beads prepared from the freeze-dried, CNBr-activated Sepharose were insensitive and unsatisfactory as the usual finding with positive specimens was a few fluorescing beads interspersed amongst many negative beads.

TABLE 31

Determination of the dilution of FITC-conjugated goat anti-human IgM that reacts specifically with polymerized purified IgM

| Dilution of anti-human IgM | FLUORESCENCE WITH SLIDE ANTIGENS | |
|---|---|---|
| | Polymerized IgM | Polymerized IgG |
| 1:20 | 4+ | 2+ |
| 1:50 | 3+/4+ | 1+ |
| *1:100 | 2+ | - |
| 1:200 | 1+ | - |
| 1:500 | +/- | - |

* Dilution used for the indirect immunofluorescence test.

Efficiency of absorption of tetanus-specific IgG by protein A.

The extent of absorption of tetanus-specific IgG resulting from a single exposure to the protein A-containing Cowan 1 strain of *S. aureus* was investigated for two antisera - one containing a relatively high concentration of specific IgG, i.e. approx. 5 u/ml by toxin neutralization method; the other containing a low concentration of tetanus-specific IgG (i.e. approx. 0.1 u/ml) as well as tetanus-specific IgM.

As indicated by the results presented in table 32, the single absorption procedure was not effective in removing all detectable specific IgG from the antiserum of relatively high titre, although it did remove the specific IgG from the low-titre antiserum. No absorption of the specific IgM of the latter by protein A was evident, and nor was there any absorption of either specific immunoglobulin class by the negative Wood 46 strain.

Detection of tetanus-specific IgM by the indirect immunofluorescence technique and specificity of the method.

Two samples of test antisera containing tetanus-specific IgM (as indicated by their source and their IHA titres in the presence and absence of 2-mercaptoethanol) and a highly diluted sample of a high-titred antiserum containing tetanus-specific IgG only were tested for the presence of specific IgM by the indirect immunofluorescence technique using polymerized tetanus toxoid and tetanus toxoid-coated Sepharose beads as antigens. Results were essentially the same for both forms of antigen, and are given in table 33.

Antigen specificity. Antigen specificity of the reaction was indicated by the inhibition of immunofluorescence that occurred when the test samples were incubated twice with an equal volume of polymerized tetanus toxoid for 2 hours at 37°C prior to testing.

Immunoglobulin class specificity. Selection of the dilution of conjugate used in the test was based on its specificity for IgM as determined by reaction with polymerized IgM and absence of reaction with polymerized IgG. The specificity of its action was further supported by the fact that prior incubation of test antisera containing specific IgM with polymerized anti-IgM for 2 hours

TABLE 32

Absorption of the IgG component of tetanus antitoxin by protein A as indicated by the indirect immunofluorescence technique with tetanus toxoid polymer as antigen.

| Test antisera | Goat anti-human conjugate (1:50 dilution) | FLUORESCENCE WITH TETANUS TOXOID POLYMER | | |
|---|---|---|---|---|
| | | Unabsorbed antisera | Antisera absorbed with Cowan I strain (protein A) | Antisera absorbed with Wood 46 strain (negative) |
| Pooled antisera from TSS patients [a] | Anti-IgG | 2+/3+ | - | 2+/3+ |
| | Anti-IgM | 2+/3+ | 2+/3+ | 2+/3+ |
| Tetagam [b] | Anti-IgG | 3+ | [c] 1+ | 3+ |
| | Anti-IgM | - | - | - |

[a] Antisera collected during a primary or early secondary immune response from patients with Tropical Splenomegaly Syndrome (IgM >4 mg/ml).

[b] See Appendix (iv).

[c] Absorption reduced but did not remove all the specific IgG from this antiserum of 5 u/ml antitoxin concentration.

TABLE 33

Detection of the IgM class of tetanus antibody by the indirect immunofluorescence technique with tetanus toxoid polymer and tetanus toxoid-coated Sepharose as antigens [a]

| Test antisera | Goat anti-human conjugate (1:50 dilution) | REACTION WITH TETANUS TOXOID ANTIGENS | | | |
|---|---|---|---|---|---|
| | | Unabsorbed antisera | Antisera absorbed with Cowan I strain (protein A) | Antisera abosrbed with polymerized anti-IgM | Antisera absorbed with polymerized tetanus toxoid |
| Pooled antisera from TSS patients [b] | Anti-IgM | + | + | - | - |
| Pooled early human antisera [c] | Anti-IgM | + | + | - | - |
| Tetagam [d] | Anti-IgM | - | - | - | - |
| Tetagam | Anti-IgG | + | [e] + | + | - |

[a] Both forms of antigen gave the same results but with different degrees of fluorescence sometimes hence positive reactions only listed.

[b] See table 32

[c] Antisera collected about 28 days after primary injections with tetanus toxoid.

[d] See Appendix (iv).

[e] See table 32

at 37°C eliminated the immunofluorescence reaction with conjugated anti-IgM, while prior incubation of the antisera with polymerized IgG did not interfere with this reaction.

## DISCUSSION

Achieving a high degree of specificity whilst retaining a high degree of sensitivity generally presents a problem with immunofluorescence reactions. Efforts to remove non-specific staining and cross-reacting antibodies by absorption and dilution of conjugated antisera frequently result in a loss of sensitivity in the test system especially as the resulting diminished intensity of the fluorescent staining makes interpretation by the usual method of subjective evaluation liable to uncertainty and error.

In the technique developed here, emphasis was placed on specificity of the immunofluorescence test for the detection of tetanus-specific IgM at the expense of a loss in intensity of the fluorescent staining reactions. Nevertheless, the results obtained were in accord with expectations - at least for the two specific IgM-containing antisera tested - when readings were made with no foreknowledge of the identity of the test samples.

The specificity of the reaction of FITC-labelled goat anti-human IgM for the IgM component of tetanus antitoxin was accomplished, tested and confirmed by the use of proteins polymerized with ethyl chloroformate based on the procedure of Avrameas & Ternynck (1967). Polymerized purified IgG, IgM and IgA were used for absorption of the conjugates; polymerized purified IgG and IgM were used as slide antigens for the determination of test dilutions of the conjugates; and polymerized tetanus toxoid and precipitating anti-IgM and anti-IgG were used for confirmation of the specificity of the reactions of the conjugates. When antisera are absorbed with polymerized antigens or antibodies, no dilution of the sera occurs and no immune complexes are formed. The problem with the latter is that they may dissociate and produce staining which might be interpreted as specific (Johnson & Holborow, 1973),

so their absence is an advantage.

The relatively high dilution of the conjugated IgM required to give a specific reaction with the IgM slide antigen produced a staining reaction of low intensity. The removal of IgG by protein A absorption was introduced, therefore, as a means of increasing the sensitivity of the test system by reducing the competition for antigenic sites available to IgM. Protein A, a cell wall constituent of many strains of S. aureus, combines non-specifically with the Fc region of IgG (Forsgren & Sjöquist, 1966). It does not react with $IgG_3$ (Kronvall & Williams, 1969); but, since this subclass constitutes only 5.3% of the total human IgG (Morell et al., 1972), the lack of reaction is of no practical consequence. Lind and Manss (1968) showed that, while a single absorption of undiluted sera with an equal volume of a protein A-containing S. aureus produced a considerable decrease in the IgG content of rabbit serum, two absorptions were much more effective in that they left only trace amounts of IgG as determined by immunoelectrophoretic analysis. Despite this, only one absorption of the 1:4 dilutions of test sera with equal volumes of protein A-containing staphylococcal suspensions was carried out; and, although the single absorption procedure was not capable of removing all specific IgG from an antiserum of relatively high antitoxin concentration, it did reduce the specific IgG of an antiserum of relatively low antitoxin concentration to a level beyond that detectable by the indirect immunofluorescence technique. Hence, it seems reasonable to assume that the absorption procedure with protein A removes enough of the IgG to promote the sensitivity of the reaction system by increasing the probability of interaction between specific IgM and the antigenic sites of tetanus toxoid.

Attachment of tetanus toxoid to Sepharose beads is relatively simple. The procedure consists of activation of the beads by cyanogen bromide; coupling of the tetanus toxoid to the activated beads; and deactivation of any residual activated groups (Axén et al., 1967; Axén & Ernback, 1970; Cuatrecasas, 1970). In agreement with the reports of van Dalen, Knapp & Ploem (1973)

and of Deelder & Ploem (1974), the freeze-dried commercially activated Sepharose beads were found to be unsatisfactory as substitutes for freshly activated beads. The probable explanation for this is that the activation of the freeze-dried product is not sufficiently homogeneous for microscopic observations and measurements (van Dalen et al., 1973). However, as the technique for coupling tetanus toxoid to agarose is so simple, there is no need to rely on a supply of commercially activated material.

The use of tetanus toxoid-coated Sepharose beads as substrates in the indirect immunofluorescence technique offered definite advantages over the use of tetanus toxoid polymer as substrate. Firstly, since the fluorescence was confined to large, well-defined spheres, it was easy to detect with assurance. In contrast, the presence of small pieces of fluorescent or negative material or a faint greenish colouration in the tetanus toxoid polymer often caused confusion. Secondly, the degree of fluorescence of the beads was relatively easy to assess because of the uniformity of the distribution of the fluorescence and its gradual reduction in intensity from grades 4+ to 1+. Mixtures of fluorescing and non-fluorescing portions in the tetanus toxoid polymers, on the other hand, made results difficult to evaluate.

Both the polymerized tetanus toxoid and the Sepharose-bound tetanus toxoid showed some tendency towards low-grade non-specific staining despite the use of a dilution of anti-IgM conjugate that reacted only with polymerized purified IgM as slide antigen. However, the problem of non-specific staining in the Sepharose system can be circumvented by the use of 0.65M sodium chloride solution as washing fluid (Capel, 1974), and of 4% bovine serum albumin in the conjugate diluent (Deelder & Ploem, 1974). Capel's recommendation of an 0.65M sodium chloride solution for washing is based on the results of testing a range of salt solutions for reduction of non-specific fluorescence due to non-specific ionic interactions. Blumberg et al. (1972) also pointed out the importance of the ionic concentration of solutions in the

prevention of non-specific binding of proteins by CNBr-activated Sepharose. The presence of 4% BSA in the conjugate diluent apparently prevents the attachment of conjugate itself to antigen-bound beads which tends to occur at high serum dilutions. Its inclusion was based on the observation by Daelder & Ploem (1974) that a column of antigen-coated beads bound serum albumin as well as specific antibody from mouse serum.

With regard to methodology, the application of microtitre techniques and equipment to the indirect immunofluorescence technique with Sepharose- bound tetanus toxoid proved to be of great value. A large number of antisera can be accommodated on each plate, and the use of a simple procedure for washing the beads in the plates significantly reduced the number of manipulations involved in their testing. Aside from these considerations, the usual savings in materials associated with microtitre techniques apply. A combination of methods for the automatic measurement of the fluorescence emission of the beads by microfluorometers (van Dalen et al., 1973; Daelder & Ploem, 1974) and for the automatic processing of the antisera by modern microtitre equipment could lead to routine procedures for testing large numbers of sera by immunofluorescence techniques for antibodies to tetanus toxoid or any other antigen coupled to agarose spheres.

# CHAPTER 4

## INVESTIGATIONS INTO THE RELATION BETWEEN TETANUS IMMUNIZATION OF FEMALES AT DIFFERENT TIMES DURING GESTATION AND THE TRANSPLACENTAL PASSAGE OF IMMUNITY

The time requirements for production of tetanus antitoxin by the mother and its transfer to the foetus at a concentration sufficient to provide protection were investigated by immunizing groups of pregnant women at different stages of gestation with a standard preparation of adsorbed tetanus toxoid, and assaying cord and maternal blood samples collected at delivery for antitoxin content. Both a two-dose and a one-dose schedule of injections were used.

With the two-dose schedule, injections were administered at the usual time interval of six weeks where the first injection was given early enough in the pregnancy to permit this regimen; otherwise the time interval between injections was three to four weeks. The purpose of the single-dose study was to provide more information on the extent to which a single dose of a standard, commercially available vaccine may confer protection, and the time necessary to achieve this.

Titrations of tetanus antitoxin in cord and maternal sera were performed by the techniques of indirect haemagglutination and toxin neutralization as described in Chapters 1 and 2. The method for the detection of the IgM class of tetanus antitoxin by immunofluorescence, described in Chapter 3, was applied to selected cord sera and paired maternal-cord sera to determine whether tetanus toxoid itself crosses the placenta. It was anticipated that the presence of tetanus-specific IgM in cord blood only would provide evidence for the transplacental passage of tetanus toxoid

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* The help of Mr. Z. Walc-Pokrzywnicki in titrating most of these sera and compiling the titration results is gratefully acknowledged.

and consequent stimulation of the foetal immune system, whereas the presence of specific IgM in both maternal and cord bloods would indicate either the passage of small amounts of maternal tetanus-specific IgM; or, more probably, contamination of cord blood by maternal blood. The latter is not an infrequent occurrence, but can usually be detected by the concomitant and substantial amounts of maternal IgA that are also introduced into the cord blood (Koronas et al., 1969). However, specific IgM was detected only in the maternal blood samples.

## METHODS AND MATERIALS

Subjects. Pregnant women from 16 weeks gestation with a history of no previous tetanus immunization formed the study group. The investigations were conducted in collaboration with Dr. S. T. Chen, Department of Paediatrics, University of Malaya, Kuala Lumpur, Malaysia; and the pregnant woman involved in the study were those attending the antenatal clinic at the University Hospital, Kuala Lumpur.

Immunization procedure. Lots Nos.80944, 81067, 82376 and 88474 of standard preparations of tetanus toxoid adsorbed to aluminium hydroxide from the Wellcome Laboratories were used. Each dose of 0.5 ml contained 20 Lf of tetanus toxoid. For the two-dose schedule, injections were given six weeks apart when the first was administered before the 32nd week of gestation, and three to four weeks apart if the first injection was administered after the 32nd week.

Collection of serum samples. Maternal and cord bloods were collected at delivery, and stored at 4°C overnight. The sera were then removed from the clots, transferred to other containers, and stored at -20°C. Blood and serum containers were labelled with the same information, viz: registration number; family name of mother and baby; date of collection of the samples; and source of the samples, i.e. whether from the mother or the baby. When a sufficient number of samples had been collected, they were packed in dry ice, and air-freighted to the London School of Hygiene and Tropical Medicine, where they were stored at -20°C

pending the serological investigations.

Titrations of serum samples for tetanus antitoxin.

Preliminary titrations of the sera by the indirect haemagglutination technique permitted the identification of samples with no antitoxin, and the subsequent assay of samples with antitoxin by toxin neutralization on the basis of only five twofold serum dilutions. After a number of the antisera had been assayed, it became evident that the toxin neutralization titres for paired maternal and cord samples rarely differed by more than one twofold dilution step. Antitoxin titrations by the indirect haemagglutination technique were then restricted to cord sera. This procedure obviated the need for prior absorption of the serum samples because cord sera do not possess heterophile agglutinins for sheep cells (Adeniyi-Jones, 1967). Furthermore, it also abrogated the indications for addition of 2-mercaptoethanol to the diluent as a means of inactivating the haemagglutinating activity of the IgM component of tetanus antitoxin since cord sera would not be expected to contain significant amounts of this class of antibody.

The toxin neutralization assays were usually conducted at the L+/100 or L+/4000 toxin test dose levels using one mouse per test dilution, but some selected sera were titrated at the Lp/10000 dose level. A suitable "working week" protocol for the titration of tetanus antitoxin in maternal and cord serum samples by the techniques of indirect haemagglutination and toxin neutralization is given in Appendix xii.

Detection of the IgM component of tetanus antitoxin.

Tetanus toxoid-coated Sepharose beads were exposed to 1:4 dilutions of the sera for testing followed by FITC-labelled anti-human IgM sera in microtitre haemagglutination plates. After each exposure, the beads were washed thoroughly in the plates as previously described (Chapter 3). The conjugated antiserum used had been rendered specific for human IgM by absorption and dilution; and any tendency towards non-specific staining in the

test system was counteracted by the use of 0.65M sodium chloride as wash fluid (Capel, 1974), and 4% bovine serum albumin as diluent for the conjugate (Daalder & Ploem, 1974).

Controls for the indirect immunofluorescent technique included the substitution of saline for serum (negative); the inclusion of a serum sample with no tetanus antitoxin (negative); and the inclusion of a pooled sample of antisera collected during the primary or early secondary immune response of patients with Tropical Splenomegaly Syndrome, and known to contain tetanus-specific IgM (positive). In addition, the dilution of anti-human IgM used was tested against polymerized purified human IgG (negative), and polymerized purified human IgM (positive).

## EXPERIMENTS AND RESULTS

### Investigation into the reliability of the assay of antisera by toxin neutralization.

As a check on the precision and accuracy of the procedure used for tetanus antitoxin titration, thirty-two antisera were titrated by two different workers at different times. In only two instances did the results differ, and then the difference was only one twofold dilution. This attests to a high degree of reliability in the tetanus antitoxin titrations.

### Determination of the relation between time of first or only injection of the mothers and the presence of protective antitoxin titres in cord sera.

Titration results for analysis. About one hundred of the mothers were tested for evidence of prior tetanus immunization by assaying for tetanus antitoxin in blood samples collected before the first injection. Of these, approximately 12% did show some level of tetanus antitoxin which indicates prior tetanus immunization despite the history of no such immunization. In view of this finding, a conservative selection of the results obtained for antitoxin titrations in the group without pre-immunization antitoxin determinations was undertaken. All titration results of 6.4 unit per ml and above were rejected on the basis that such high titres could not

with certainty be attributed to a two-dose immunization schedule with a standard vaccine. Moreover, the cut-off at 6.4 unit per ml also eliminated a proportion of the total which was comparable to the percentage of positive pre-immunization sera. Titres of more than 0.01 unit per ml up to 28 days after a single injection were also disregarded since titres greater than 0.01 unit do not usually occur under such circumstances with toxoids of normal potency (MacLennan et al., 1973; Cohen & Laussink, 1973). This selection process resulted in about 75% of the titration results for sera without pre-immunization antitoxin screening being retained for analysis.

Two-dose schedule. The relationship between the time of first injections of the mothers and the presence of at least 0.01 unit per ml of antitoxin - the generally accepted protective level (McComb, 1964) - in cord sera is expressed as proportions and percentages of the total number of cord sera tested for ten-day time intervals in figure 12 and table 34 respectively. Results indicate that the two-dose schedule conferred protection in about 50% of the babies for the 50 to 59 and 60 to 69 day intervals from first injection of the mothers, and that this percentage increased to about 70 to 80% for the 70 to 79 and 80 to 89 day intervals. Even after 90 days from the first of the two injections, some babies were not protected (figure 12), probably because their mothers fall into the "low responder" category seen in all immunization studies.

One-dose schedule. Results of the antitoxin titrations on cord sera with the single-dose schedule of maternal immunization (figure 13) indicated that, with one exception out of 56, no protection occurred earlier than 60 days after injection. Thereafter, the numbers are too few to draw any significant conclusions.

Determination of the relation between time of first or only injection of the mothers and the concentration of antitoxin in cord sera.

Two-dose schedule. The relationship between the time of first injection of the mothers in ten-day intervals and the geometric mean titre of tetanus antitoxin in the cord sera is given in table 35. For the calculation of the means, titres that fell within the

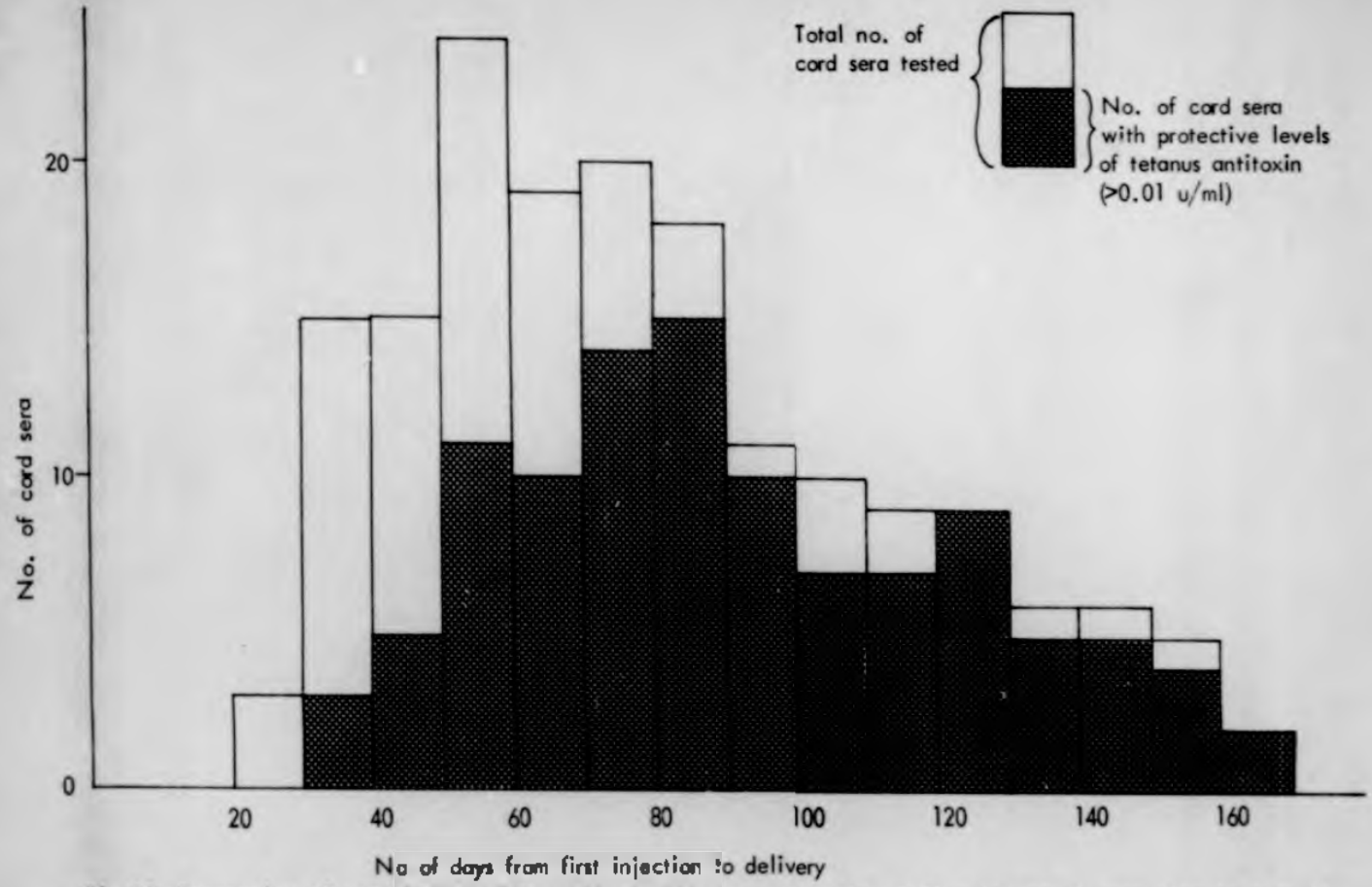


Fig.12 Proportion of cord bloods with antitoxin titres greater than 0.01 unit per ml according to time in ten-day intervals elapsing from first injection of the mothers to delivery: two dose schedule.

TABLE 34

Relation between time of first injection of mothers and the percentage of cord bloods with antitoxin titres greater than 0.01 unit per ml at delivery: two-dose schedule.

| TIME IN TEN-DAY INTERVALS FROM FIRST INJECTION TO DELIVERY | PERCENTAGE OF CORD BLOODS WITH PROTECTIVE LEVELS OF ANTITOXIN |
|---|---|
| 20-29 | 0 |
| 30-39 | 20 |
| 40-49 | 33 |
| 50-59 | 46 |
| 60-69 | 53 |
| 70-79 | 70 |
| 80-89 | 83 |
| 90-99 | 91 |
| 100-109 | 70 |
| 110-119 | 78 |
| 120-129 | 100 |
| 130-139 | 83 |
| 140-149 | 83 |
| 150-159 | 80 |
| 160-169 | 100 |

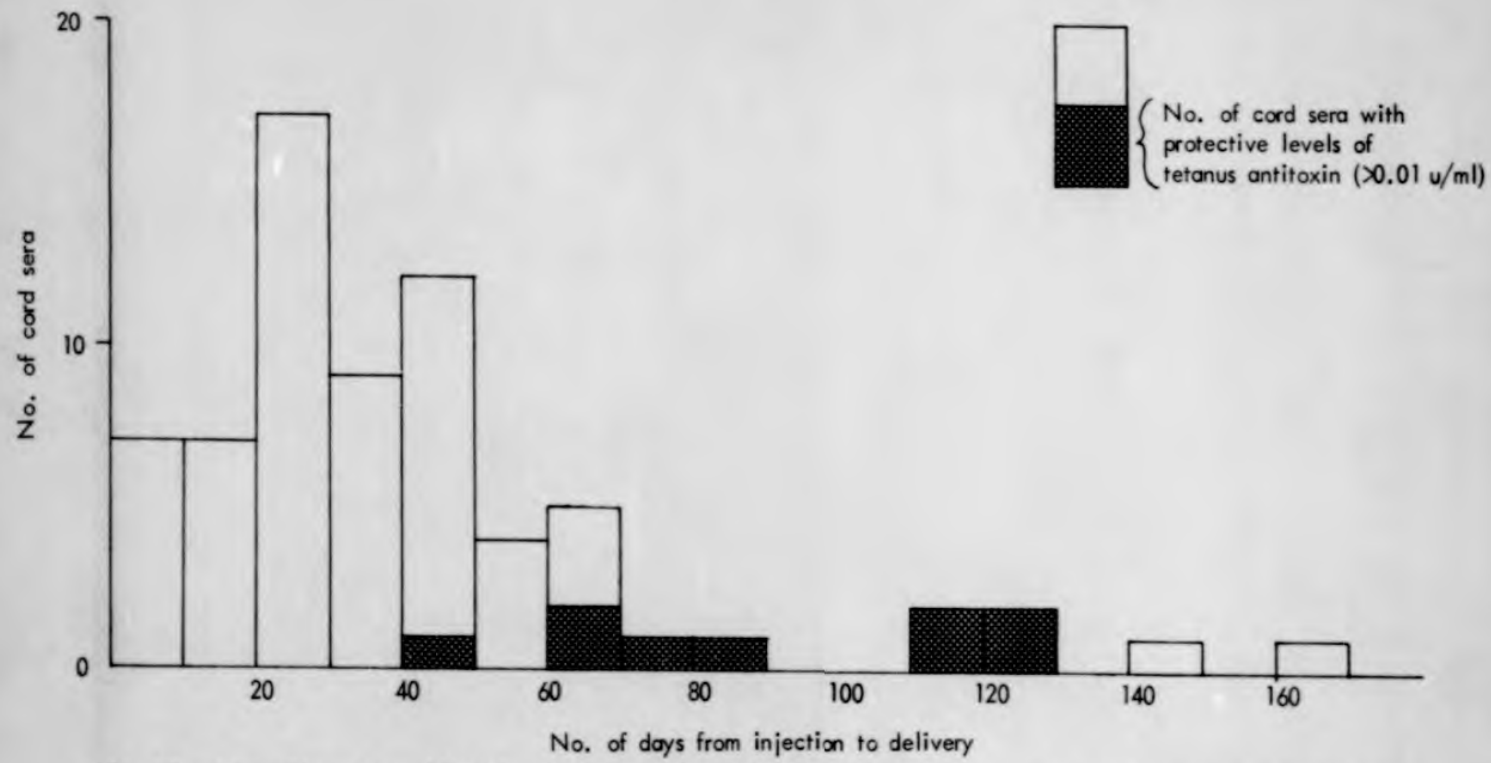


Fig. 13 Proportion of cord bloods with antitoxin titres greater than 0.01 unit per ml according to time in ten-day intervals elapsing from injection of the mothers to delivery: one dose schedule.

TABLE 35

Relation between time of first injection of mothers and the antitoxin titres of cord bloods at delivery: two-dose schedule.

| TIME IN TEN-DAY INTERVALS FROM FIRST INJECTION TO DELIVERY | ANTITOXIN TITRES OF CORD BLOODS | | |
|---|---|---|---|
| | No.with = or <0.0025 unit per ml | No.with >0.0025 unit per ml | Geometric mean titres |
| 20–29 | 2 | 1 | 0.003 |
| 30–39 | 12 | 3 | 0.004 |
| 40–49 | 8 | 8 | 0.02 |
| 50–59 | 5 | 20 | 0.03 |
| 60–69 | 5 | 14 | 0.03 |
| 70–79 | 4 | 16 | 0.09 |
| 80–89 | 2 | 16 | 0.09 |
| 90–99 | 1 | 10 | 0.26 |
| 100–109 | 2 | 8 | 0.12 |
| 110–119 | 2 | 5 | 0.08 |
| 120–129 | 0 | 9 | 0.36 |
| 130–139 | 0 | 6 | 0.37 |
| 140–149 | 0 | 6 | 0.19 |
| 150–159 | 0 | 4 | 0.2 |
| 160–169 | 0 | 2 | 0.18 |

category of = or <0.0025 unit per ml were assumed to have a value of 0.00125 unit per ml in accord with the practice of Newell et al. (1971).

As shown in the table, the geometric mean titres for the 20 to 29 and 30 to 39 day intervals, which marks the period of the primary immune response, were well below the protective level. However, a significant increase in the geometric mean titre occurred in the 40 to 49 day interval with the administration of the second injection early in that interval. From 70 days after first injection, the mean titre rose sharply to a value of 0.09 unit per ml in keeping with the 70% level of protection conferred at this time.

One-dose schedule. Because of the large number of sera with titres less than or equal to 0.0025 unit per ml, the calculated geometric mean titres for the one-dose schedule are not realistic. Nevertheless, the results (table 36) support the contention that no protection is likely before 60 days. In the 60 to 69 day interval, the five subjects gave a mean of 0.01 unit per ml.

Cord/maternal antitoxin ratios and their dependence on the time interval from the second injection to delivery.

Where maternal as well as cord sera were assayed, the cord/maternal antitoxin ratios could be determined. These fell into three groups, i.e. less than one, equal to one, and greater than one. The distribution within the groups was unusual in that there happened to be an equal number in each. The cord/maternal ratio of greater than 1 actually equalled 2 as the difference between the maternal and cord titres was exactly one twofold dilution in every instance. Most of the paired maternal and cord sera giving cord/maternal ratios less than 1 also differed by a single twofold dilution, but there were two exceptions - one pair with a fourfold difference and one pair with an eightfold difference.

Inspection of the time intervals in days from injections to delivery for the three cord/maternal ratio groups in table 37 indicates an association between the ratios and the times from second injection to delivery. This impression is supported by the results of a one-way analysis of variance on the differences between

TABLE 36

Relation between time of injection of mothers and the antitoxin titres of cord bloods at delivery: one-dose schedule.

| TIME IN TEN-DAY INTERVALS FROM INJECTION TO DELIVERY | ANTITOXIN TITRES OF CORD BLOODS | |
|---|---|---|
| | No. in group | Geometric mean titre |
| 0–9 | 7 | 0.002 |
| 10–19 | 7 | 0.002 |
| 20–29 | 17 | 0.001 |
| 30–39 | 9 | 0.002 |
| 40–49 | 12 | 0.002 |
| 50–59 | 4 | 0.003 |
| 60–69 | 5 | 0.01 |
| 70–79 | 1 only | 1.6 |
| 80–89 | 1 only | 0.4 |
| 110–119 | 2 | 0.13 |
| 120–129 | 2 | 0.04 |
| 140–149 | 1 only | = or <0.01 |
| 160–169 | 1 only | = or <0.0025 |

TABLE 37

Relation between the cord/maternal anritoxin ratios and the time intervals in days from injections to delivery.

| CORD/MATERNAL RATIO | INTERVAL IN DAYS [a] FROM INJECTIONS TO DELIVERY | |
|---|---|---|
| | First injection | Second injection |
| C/M <1 | 86.58 ± 38.43 | 27.16 ± 25.47 |
| C/M = 1 | 87.17 ± 31.79 | 36.52 ± 18.92 |
| C/M >1 | 100.41 ± 33.07 | 61.41 ± 34.98 |

[a]Results are expressed as the mean number of days ± s.d. for each group of 17.

TABLE 38

Presence of tetanus-specific IgM antibodies in maternal sera of paired maternal-cord samples as indicated by the indirect immunofluorescence test.

| | INTERVAL IN DAYS FROM INJECTIONS TO DELIVERY | | TOXIN-NEUTRALIZING TETANUS ANTIBODIES | | TETANUS-SPECIFIC IgM ANTIBODIES | |
|---|---|---|---|---|---|---|
| No. | First Injection | Second Injection | Maternal serum | Cord serum | Maternal serum | Cord serum |
| | | | u/ml | | Presence at 1:4 serum dilution | |
| 1 | 54 | 12 | 0.2-0.4 | 0.2-0.4 | + | - |
| 2 | 36 | 8 | 0.8-1.6 | 0.4-0.8 | + | - |
| 3 | 77 | 2 | 0.02-0.04 | 0.01-0.02 | + | - |
| 4 | 44 | 13 | 0.4-0.8 | 0.2-0.4 | - | - |
| 5 | 48 | - | 0.1-0.2 | 0.04-0.08 | - | - |
| 6 | 88 | 46 | 0.1-0.2 | 0.1-0.2 | + | - |
| 7 | 53 | 11 | 0.8-1.6 | 0.4-0.8 | +/- | - |
| 8 | 37 | 9 | Not determined | 0.02-0.04 | - | - |
| 9 | 28 | - | Not determined | Not determined | - | - |
| 10 | 35 | 11 | Not determined | 0.4-0.8 | + | - |

the times from injections to delivery for the three ratio groups. A significant difference ($p<0.005$) was found for times from the second injections only.

Because of the size of the standard deviations and their tendency to increase with the mean for the number of days from second injection to delivery in the three groups, an analysis of variance was also conducted by computer using $\log_e$ and square root transformations of the numbers of days. Results of the analysis by computer supported the significance of the differences.

Detection of tetanus-specific IgM in maternal sera by immunofluorescence.

Details of the antisera selected for testing and the results of testing for the presence of tetanus-specific IgM are given in table 38. Six of the maternal antisera (60%) from the two-dose injection schedule were positive, but no specific IgM was found in any of the cord sera. In addition to the cord sera listed in table 38, a further ten samples were tested; but, again, there was no evidence for the presence of tetanus-specific IgM in any of them. The two samples of maternal antisera tested after one dose of tetanus toxoid were also negative.

## DISCUSSION

Defining what constitutes an acceptable level of protection in terms of the percentage of cord bloods with protective antitoxin titres at delivery as a criterion for interpretation of data presents a problem. A 100% protection rate would be too stringent a requirement for a standard two-dose injection schedule of a standard tetanus toxoid, and one which does not take into account the existence of "low responders". Stanfield, Gall & Bracken (1973) proposed that a 70 to 80% protection rate in the mothers at delivery may be approaching acceptable levels on the basis that even the mothers with less than 0.01 unit per ml of antitoxin in the groups with this percentage of protected women

possessed some antitoxin which would offer some protection to their babies in about one third of cases.

Of course, "protection" in babies, which is defined as a maternal antitoxin titre of 0.01 unit per ml or greater, is a less demanding criterion for success in any vaccination programme. It is based on the extrapolation from the observation by MacLennan et al. (1965) that no tetanus occurred in any of five babies with antitoxin titres less than 0.01 unit per ml born to mothers with antitoxin titres in the protective range. While it may well be true that the actual protective titre of antitoxin is less than the generally accepted protective titre (Newell et al., 1971), extrapolation from only five subjects does not seem to justify the acceptance of a lower standard for determining protection in the newborn at present.

In studies reported here, a titre of at least 0.01 unit per ml in the cord sera was considered the criterion for protection. This means that the 70 and 83% protection rates which were observed for the 70 to 79 and 80 to 89 day intervals respectively are more significant than are equivalent percentages of protective levels based on maternal titres only. The 70th day seems to be critical, in the sense that a first injection given to mothers before the 70th day from delivery, and followed by the usual injection six weeks later, would be expected to confer a degree of protection approaching acceptable levels in their babies, while a first injection given after this time would offer no better than a protection rate of about 50%.

In spite of the different means of gauging protection, however, the 91% rate obtained here with a two-dose schedule of 20 Lf of adsorbed toxoid for the 90 to 99 day interval from the first injection was comparable with those found by Stanfield et al. (1973) for the two-dose schedules of 10 Lf, 20 Lf and 40 Lf of adsorbed toxoids given at least 60 days from the last injection, i.e. 90 days from the first injection with the one month interval between injections.

Results of the single dose schedule indicate that the protection conferred for up to 60 days following immunization is practically negligible. This finding agrees with and extends that of Suri, Dhillon & Grewal (1964) who reported that none of the nineteen mothers receiving a single injection of 5 Lf of adsorbed toxoid less than 30 days before delivery gave protective levels of antitoxin in the cord blood of their babies. In the study reported here, only one out of fifty-six mothers gave a protective cord level when injected with a 20 Lf dose of adsorbed toxoid less than 60 days before delivery. Beyond 60 days, results are too few to establish just how early in pregnancy a single dose might be effective - but the study has shown that it must be at least earlier than 60 days.

When paired maternal and cord antisera are assayed on the basis of twofold serum dilutions, the resulting titres seldom differ by more than one twofold dilution. Of the 51 paired antisera tested here, for example, only two cord sera gave antitoxin titres that were less than the corresponding maternal antitoxin titres by more than one twofold serum dilution. This raised the question of the significance of the twofold differences. In the series tested, paired maternal and cord antisera were always assayed by toxin neutralization at the same toxin test dose level e.g. either at L+/100 or L+/1000 or L+/4000. This means that the variation of a one twofold dilution which may occur when assays of dilutions of the same serum sample are conducted at different toxin test dose levels, as previously shown in Chapter 1, is not pertinent. Moreover, as also shown previously, repeated titration of the same antiserum at the same toxin test dose level gives a remarkably constant antitoxin titre. Hence, a twofold difference in antitoxin titres between maternal and cord sera probably does represent a valid difference in antitoxin concentrations.

An examination of the cord/maternal antitoxin ratio groups of less than 1, equal to 1, and greater than 1, in terms of the number of days elapsing from first and second injections to delivery for each group, indicated that there was an association between the three ratio

groups and the number of days from second injection. An analysis of variance on the means and variances of the number of days from second injection for the three groups showed that the variance between the groups was significantly greater than the variance within each group which supported the relation indicated. This approach leads to the conclusion that the value of the cord/maternal antitoxin ratio is directly related to the time elapsing from the second injection to delivery; and, more significantly, provides evidence for an active mechanism for the transplacental passage of antitoxin against a concentration gradient in the later stages of the interval from the second injection to delivery. The findings of Stanfield et al. (1973) differ from the ones reported here in that their average cord/maternal antitoxin ratio was less than 1. However, Suri et al. (1964) obtained a value of about 1 for cord/maternal antitoxin ratios after two and three injections of tetanus toxoid; and, in other fields, cord/maternal ratios are frequently reported to be greater than 1 (Barr, Glenny & Randall, 1949; Murray, Calman & Lepine, 1950; Toivanen, Mäntyjärvi & Hirvonen, 1968). From the results obtained for the investigation into cord/maternal ratios, it appears that not only is maternal antitoxin transferred with increasing efficiency after the second injection as compared to the first (Stanfield et al., 1973), but also it is transferred with progressively increasing efficiency throughout the period from the second injection; until, finally, it is transported against a concentration gradient.

No evidence was found to support the suggestion of Gill (1973) that antigens such as tetanus toxoid may also cross the placenta. Of course, this does not exclude the possibility of such an occurrence, especially in view of the balance of technical factors that operate in the indirect immunofluorescence technique which was used for the detection of any specific foetal IgM resulting from tetanus toxoid stimulation. In general, where the immunofluorescence test is positive, results are of value provided that the specificity has been established: where the test is

negative, conclusions cannot be made because of the limitations of sensitivity. It may be that small amounts of tetanus toxoid do pass the placenta, but the magnitude of the IgM response by the foetus is not sufficient to be detected by the technique used.

Nevertheless, the immunofluorescence technique was sensitive enough to detect tetanus-specific IgM in maternal sera apparently. The results obtained for the maternal sera are in accord with the interesting and rather unusual findings of Hernandez, Just & Bürgin-Wolff (1973) who reported that the formation of transient IgM antitoxin was very common - even in obviously immune individuals with measureable amounts of IgG antitoxin - after re-vaccination, but not so common after the first vaccination. Hernandez et al. (1973) found that 11 out of 24 antisera (66%) tested after revaccination within six months to two years of vaccination produced tetanus-specific IgM. In the investigation conducted on the maternal sera, a comparable percentage (60%) were found to contain specific IgM after variable intervals from the second vaccination given six weeks after the first vaccination. On the other hand, only one in four persons given first injections produced specific IgM in the series tested by Hernandez et al. (1973), and neither of the two maternal antisera tested after a single injection showed evidence of the presence of specific IgM: admittedly, the numbers tested after primary injections are few. It seems therefore that the immune response to primary, secondary, and booster doses of tetanus toxoid does not follow the classical stereotype as far as the production of IgM is concerned.

# CHAPTER 3

## INVESTIGATIONS INTO THE RELATION BETWEEN LYMPHOCYTE REACTIVITY AND THE OCCURRENCE OF REACTIONS TO TETANUS TOXOID VACCINATION

Sensitized peripheral lymphocytes, in the presence of specific antigen, undergo blast transformation with an increase in the rate of synthesis of DNA. More pronounced transformation is produced in lymphocyte cultures in the presence of non-specific mitogens such as phytohaemagglutinin (Nowell, 1960). The extent of activation of the lymphocytes is usually measured by determining the incorporation of radioactive thymidine into their DNA (Dutton & Eady, 1964; Caron et al., 1965).

The response of lymphocytes from a group of subjects who developed reactions to tetanus toxoid vaccination as compared to a control group showing no such reactions was investigated by measuring the uptake of tritiated thymidine by lymphocytes cultured from the subjects of each group in the presence of tetanus toxoid antigen. Response to phytohaemagglutinin was investigated simultaneously to provide information on general T cell reactivity (Davies et al., 1968) for comparison with the specific reactivity. Furthermore, since the response to phytohaemagglutinin involves the majority of peripheral lymphocytes whereas that to specific antigens involves from only 0.5 to 5% (Oppenheim, Leventhal & Marsh, 1968), the former provides an assay system for lymphocyte reactivity that is far less subject to variation because of the large differential between the activity of stimulated and unstimulated (control) cells, and because errors due to sampling variation are negligible. This means that variables affecting the response of lymphocytes to phytohaemagglutinin can be investigated quantitatively and controlled so as to give optimal assay conditions. Results obtained in the quantitative evaluation of the variables were compared with those reported by others as a check on general methodology; and, more particularly, on the simplified procedure used for the harvesting and processing of the lymphocyte cultures. The PHA response, as measured under optimal conditions, was also used to assess possible

detrimental effects of storage on lymphocytes: such storage was necessary because the location of the test group precluded immediate processing.

## MATERIALS AND METHODS

Study populations. The test group consisted of employees of the British Leyland (Austin-Morris) Ltd. factory at Cowley, Oxford, who developed reactions to the tetanus toxoid vaccines used in the immunization procedures described by White et al. (1973). The age range was from 24 to 59 years, but the majority were in the 30 to 39 and 40 to 49 decades. Only two of the test group were probable reactors to first injections: the rest had reacted to second or booster doses; or, where immunization histories were uncertain, had produced antitoxin titres indicative of secondary responses.

The control group comprised laboratory workers who had been immunized with tetanus toxoid, without any adverse reactions, within a year of sampling. In this group, ages ranged from 21 to 67 years with the majority in the 20 to 29 year range, and several over 50 years. Although the age range for the control group was considerably greater than that for the test group, the mean age for the two populations was not significantly different.

Phytohaemagglutinin (PHA). Purified freeze-dried PHA (Wellcome Reagents Ltd.) was reconstituted in Oxoid PBS (Appendix iii); distributed in small volumes; stored at -20°C; and used immediately once thawed. A single batch (Lot No.KB805) was used for all tests.

Tissue culture media and culture tubes. Tissue culture media and additives such as foetal calf serum were usually obtained from Gibco Bio-Cult Diagnostics Ltd., Washington Road, Sandyford Industrial Estate, Paisley, Renfrewshire, Scotland. Sterile plastic tissue culture tubes, 125 mm x 16 mm, with screw caps (Falcon Ref.No.3033) were also purchased from Gibco Bio-Cult Diagnostics Ltd.

Tritiated thymidine. (Methyl-$^{3}$H) thymidine was supplied in aqueous solution by the Radiochemical Centre, Amersham. Two batches were used viz: one of specific activity 18.4 Ci/mmol (Batch No.92); the other of specific activity 17 Ci/mmol (Batch No.100).

Purification of tetanus toxoid antigen

Batch V.G.T. No.6 of tetanus toxoid (Appendix vii) is free of preservatives, and showed no lymphocyte toxicity in lymphocyte stimulation tests (Nagel, personal communication). It was purified by fractionation on Sephadex G-200 (Pharmacia); concentrated by ultrafiltration in a B-15 type of Amicon filter (Amicon Ltd.); and its protein concentration was estimated by the technique of Lowry et al. (1951). The identity and purity of the antigen was tested by immunodiffusion and immuno-electrophoresis against tetanus antitoxin of suitable concentration. With each of these methods, only a single line of precipitation developed.

Testing of foetal calf serum (FCS) for the presence of tetanus antitoxin.

Since the serum of cattle may contain low concentrations of naturally-acquired tetanus antitoxin (Wilson & Miles, 1955), each batch of heat-inactivated FCS was checked for the presence of tetanus antitoxin by the toxin neutralization test. The testing was conducted at the Lp/10000 test dose level on undiluted serum and on a serum dilution of 1:2, using four mice per test. At this test dose level, the minimum titre that can be determined is 0.001 u/ml. However, since the end-point for the Lp/10000 assay is based on a 2+ degree of paralysis, even lower levels of antitoxin can be detected - although not actually measured - by comparison of the degree of paralysis developing in the test group with the 4+ degree of paralysis of the control group which receive toxin only. One batch of FCS from Gibco Bio-Cult (Batch No.K148202) did give 3+ reactions in three of the four mice tested with an undiluted sample; this batch was avoided.

Assessment of the transformation response of lymphocytes to PHA and tetanus toxoid.

Collection of blood samples. A volume of 15-20 ml of blood is collected in preservative-free heparin (B.D.H.) at a concentration sufficient to give 20-30 units of heparin per ml of blood. The heparinized blood is then added to an equal volume of S-MEM (Suspension) medium containing antibiotics and HEPES buffer (Appendix xiii) for preservation of the lymphocytes if storage is necessary; or to any tissue culture medium if the sample is to be processed immediately.

Isolation of lymphocytes. Lymphocytes are isolated by a procedure based on that of Perper, Zee & Mickelson (1968). Volumes of 30 ml of a mixture of 24 parts of 9% Ficoll (Pharmacia) and 10 parts of 34% Triosil 440 (Nyegaard & Co., Norway, obtainable from Vestric & Co., Ltd., Runcorn, Cheshire) are added to large sterile test tubes of dimensions 170 mm x 26 mm i.d. (approx. 100 ml capacity). The blood, diluted in an equal volume of S-MEM (Suspension) medium or other medium, is layered on the surface of the Ficoll-Triosil mixture. Centrifugation at 400 g for 30 minutes then separates the lymphocytes as a white layer at the interface of the plasma-medium and Ficoll-Triosil layers.

Washing and counting of lymphocytes. The lymphocyte layers are transferred to large test tubes of the same type by Pasteur pipettes, and are washed twice in Eagles MEM with antibiotics (Appendix xiv), and once in complete RPMI-1640 medium supplemented with antitoxin-free FCS and containing antibiotics and HEPES buffer (Appendix xv). For the first wash, the cells are subjected to 500 g for 20 minutes in order to ensure their recovery from contaminating Ficoll-Triosil mixture. Subsequently, the cells are sedimented by 400 g for 10 minutes.

The washed lymphocytes are diluted in complete RPMI-1640 medium and then in eosin solution for counting and for checking of viability (Appendix xvi). A yield of approximately 60-70% of the number of lymphocytes theoretically available is obtained from the

blood samples, and the viability is usually 100% as estimated by dye exclusion. On average, contamination by red blood cells is about 5%, and granulocytes are seen only rarely.

Lymphocyte culture in the presence of stimulants. Suspensions of lymphocytes are prepared in complete RPMI-1640 medium at twice the final required concentration, and 0.5 ml aliquots of the suspensions are distributed into tissue culture tubes. The stimulants, PHA and tetanus toxoid, are also diluted in the same medium to twice the final concentration, and likewise added to the tubes in 0.5 ml volumes. Generally, duplicate cultures are prepared for each test concentration of PHA or tetanus toxoid, and cultures consisting of lymphocytes and medium only are always included for the determination of the rate of spontaneous lymphocyte transformation.

Tubes containing the cultures are incubated in an atmosphere of air plus 5% carbon dioxide with their screw caps slightly loosened. The lymphocytes are incubated for a total period of 72 hours in the presence of PHA, and for 6 days in the presence of tetanus toxoid.

Pulsing and harvesting of cultures. Each culture tube is pulsed with 1uCi of tritiated thymidine in 0.1 ml of medium 6 hours before the end of the incubation period for PHA, and 16 hours before the end of the incubation period for tetanus toxoid. Additions of the radioactive compound are made with a Finnpipette (Buckley membranes Ltd.) and sterile disposable tips (Boehringer Corporation Ltd.). At the same time, the cells are resuspended by shaking.

At the end of the incubation periods, aliquots of 150 ul of the mixed contents of each tube are transferred to each of two Whatman 2.5 cm glass fibre discs, type GF/C (W. & R. Balston Ltd., Springfield Mill, Maidstone, Kent). Figure 14 shows the discs supported on a base made from two layers of plastic sheeting between which circular discs of lead (obtainable commercially as "curtain weights") are fixed by a suitable epoxy resin adhesive such as "Araldite". Coloured markers on one side of the base

Fig.14 Lead-weighted, plastic-based pin-board supporting discs with culture samples. The samples are processed by transferring the unit through photographic developing dishes containing the appropriate solutions.

serve to identify the unit, and provide a reference point for indicating the sequence of samples.

Processing of samples. After the samples have dried, the discs are transferred on the weighted plastic pin-board in sequence through 22.5 cm x 16.5 cm photographic developing dishes (Paterson Products Ltd., 32 Bedford Row, London, W.1.) containing 10% trichloracetic acid, Oxoid PBS (Appendix iii), diluted ethanol (approx. 70%) and absolute ethanol. The pin-boards are left for about ten minutes in each solution.

Scintillation counting. The discs are dried overnight on the plastic pin-board in an incubator. Each is then transferred to a glass scintillation vial (Johnsen & Jorgensen Ltd., Herringhan Road, London, S.E.7) containing 5 ml of scintillation fluid. The scintillation fluid consists of 4 g/litre of *PPO (Koch-Light Laboratories Ltd.) and 0.1 g/litre of **dimethyl POPOP (Koch-Light Laboratories Ltd.) in a base of toluene (B.D.H.). Care is taken to avoid exposure of the vials to fluorescent light because the resulting induced phosphorescence may be transmitted to the scintillators (Price, 1973).

The emissions from each vial are counted for periods of ten minutes on a Tracerlab ICN Coru/matic 200 Scintillation Counter. Since counts on the tritium standard are constant, and there is relatively constant quenching by the channels ratios method, counts per minute are not converted to disintegrations per minute. Average counts per minute per ml of the unstimulated cultures are subtracted from those of the stimulated cultures, and the results usually expressed as $\log_{10}$ of the difference.

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* PPO = 2,5-diphenyloxazole.

** dimethyl POPOP = 1,4-Di-(2-(4-methyl-5-phenyloxazolyl))-benzene.

## EXPERIMENTS AND RESULTS

Determination of the major variables affecting the response of lymphocytes to phytohaemagglutinin.

Initially, a concentration of 0.5 x $10^6$ lymphocytes per ml of culture was used in the test system along with an incubation period of 48 hours and a pulse of 1 µCi/ml for four hours. As the concentration of each variable giving a maximal response was determined, its use at that concentration was adopted in subsequent experiments.

Concentration of PHA. Figure 15 shows the effect of concentration of PHA on the response under the conditions described above. A concentration of 1 µg/ml of PHA was chosen for the investigations which followed, although the results obtained with PHA of concentrations 0.5 µg/ml and 1 µg/ml differed only slightly.

Concentration of lymphocytes. The effect of lymphocyte concentration in both the PHA-stimulated and unstimulated control cultures was investigated. A concentration of 1 x $10^6$ lymphocytes per ml gave a clear-cut maximum (figure 16). The slightly reduced response in unstimulated cultures of concentration 0.25 x $10^6$ lymphocytes per ml as compared to unstimulated cultures of 1 x $10^6$ lymphocytes per ml is probably of no real significance.

Duration of culture. Results (figure 17) indicate that the uptake of labelled thymidine by the stimulated cultures increased at a constant rate for two days followed by a further increase, at a somewhat reduced rate, to reach a maximum on the third day. In the unstimulated cultures, a constant rate of increase in thymidine uptake occurred for up to three days.

Concentration of tritiated thymidine. A concentration of 2 µCi per ml of tritiated thymidine gave a higher count than did 1 µCi per ml (figure 18). However, because of the high cost of tritiated thymidine, a concentration of 1 µCi per ml of culture was adopted for use.

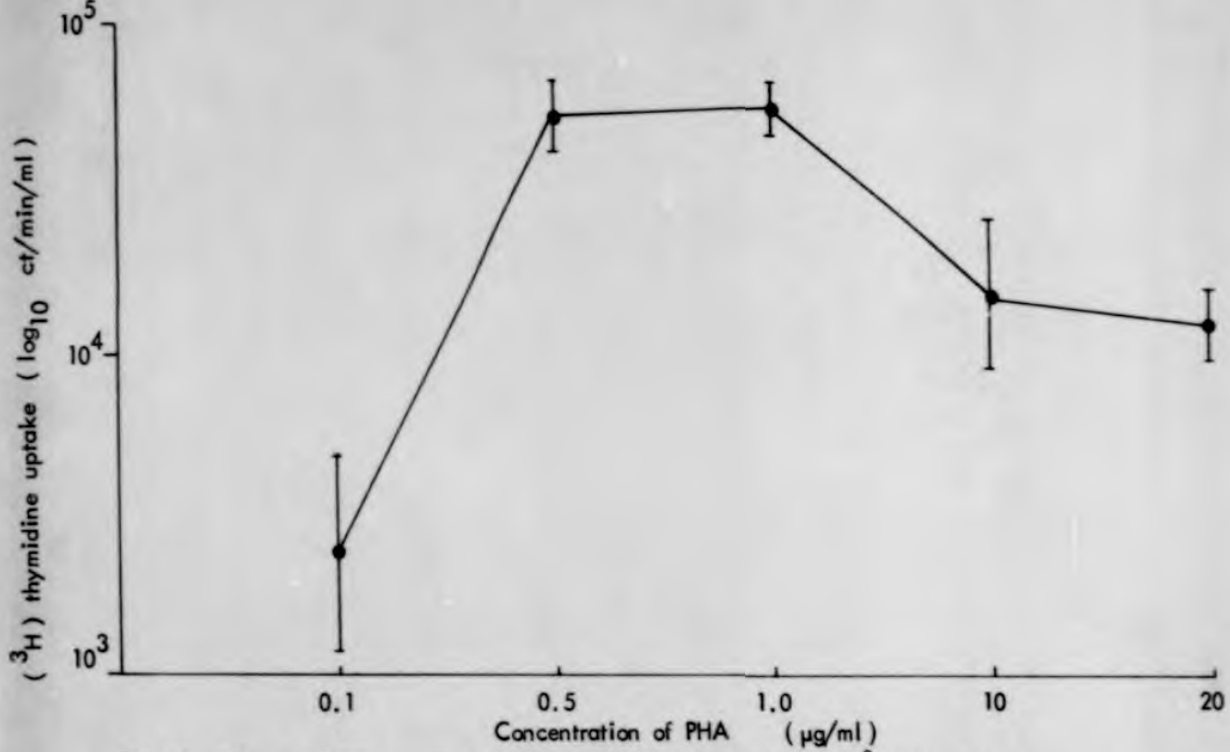


Fig.15 Effect of PHA concentration on the incorporation of ($^3$H) thymidine .
Each point represents the mean response ± s.e. mean of lymphocytes from six donors.

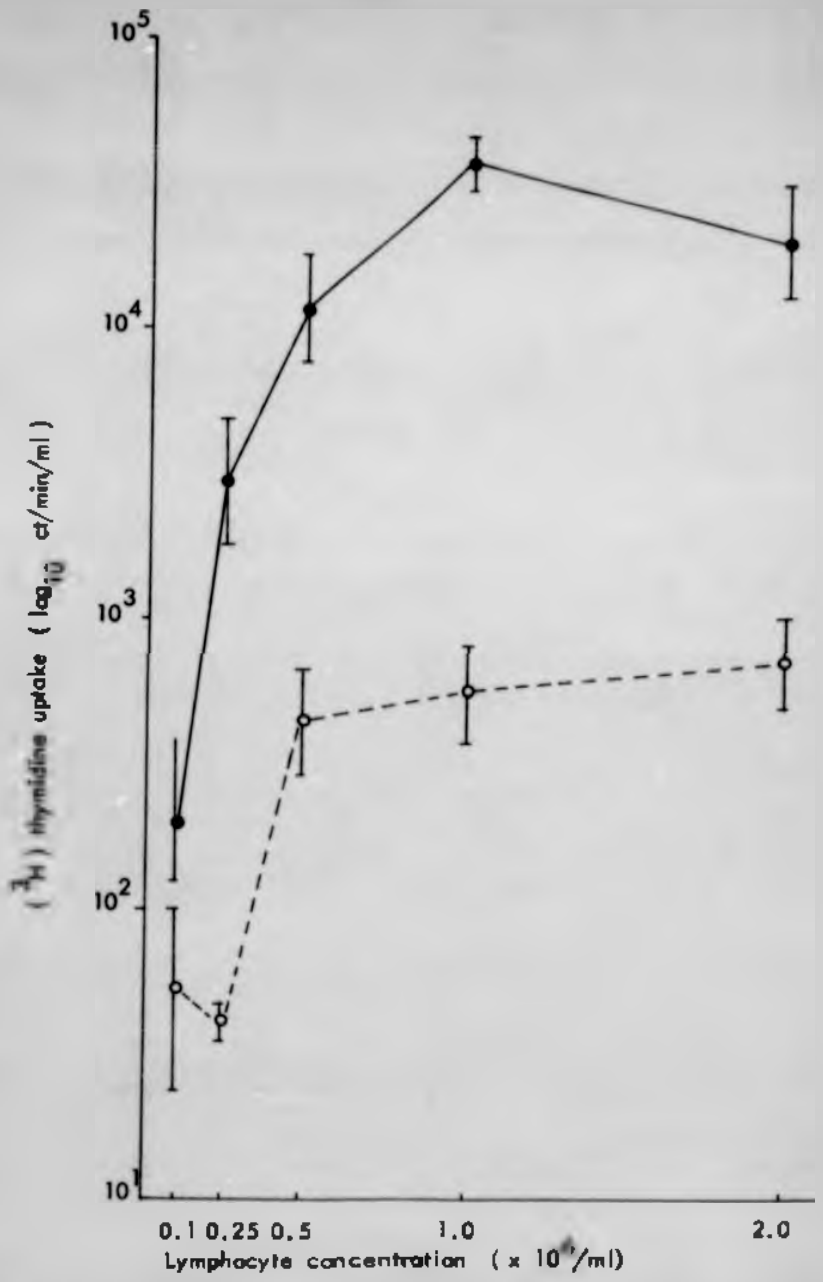


Fig.16 Effect of lymphocyte concentration on the incorporation of ($^3$H) thymidine. (●——●) Mean ± s.e. mean of PHA-stimulated lymphocytes from four donors. (o---o) Mean ± s.e. mean of the corresponding cultures of unstimulated lymphocytes.

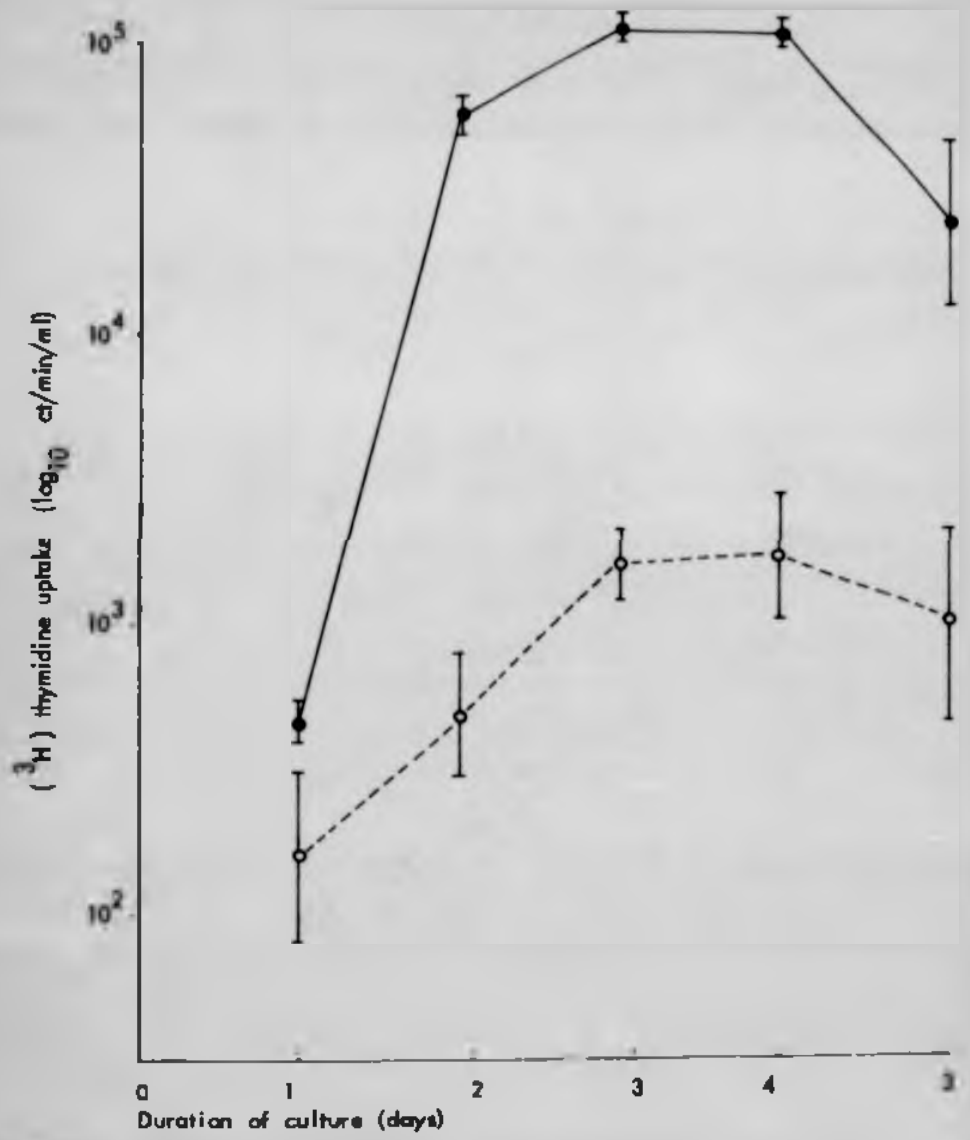


Fig.17 Effect of length of culture on the incorporation of ($^3$H) thymidine. (●——●) Mean ± s.e. mean of PHA-stimulated lymphocytes from eight donors. (○---○) Mean ± s.e. mean of the corresponding cultures of unstimulated lymphocytes.

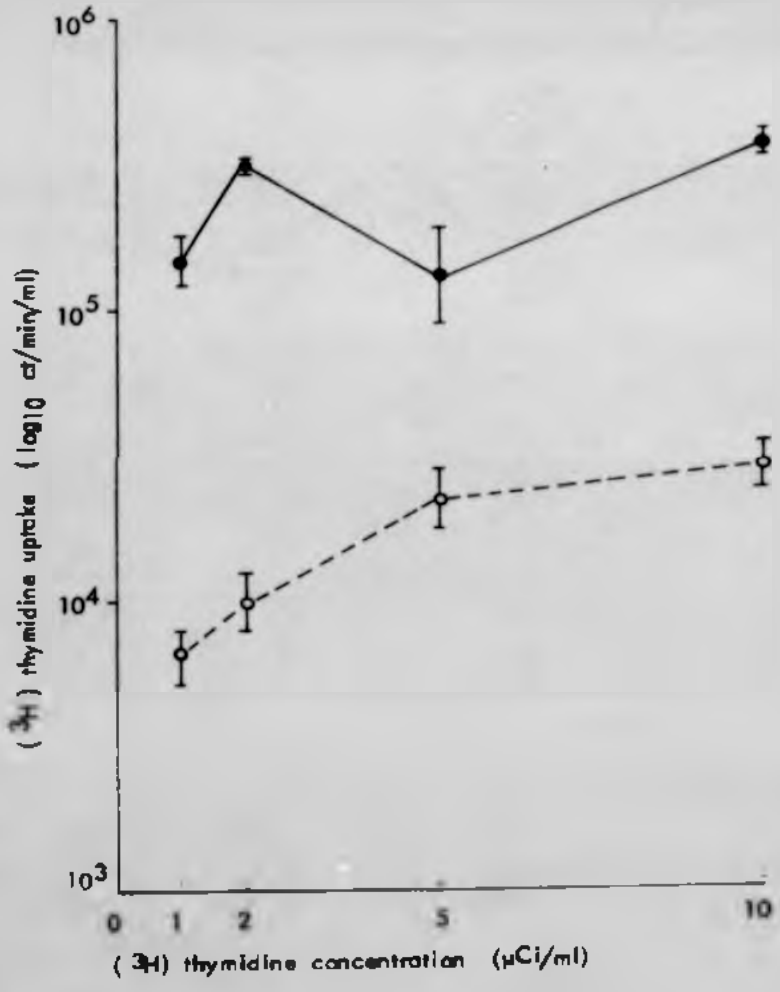


Fig.18 Effect of concentration of ($^3$H) thymidine on the incorporation of label. (●——●) Mean ± s. e. mean of PHA-stimulated lymphocytes from four donors. (○---○) Mean ± s.e. mean of the corresponding cultures of unstimulated lymphocytes.

Duration of the pulsing period. As indicated by the results depicted in figure 19, the length of the pulsing period does not exert much influence on thymidine uptake over a period of 2 to 24 hours. A period of six hours was considered to be optimal, although the differences are only marginal.

Optimal conditions for the response of lymphocytes to phytohaemagglutinin. Results of the experiments conducted on the effect of variables on the response of lymphocytes to PHA indicated that concentrations of 1 µg of PHA per ml and 1 x $10^6$ lymphocytes per ml, cultured for three days with 0.1 µCi per ml of ($^3$H)-thymidine added six hours before harvesting provides optimal or near-optimal conditions.

Comparison of the reactivity of lymphocytes to phytohaemagglutinin under optimal test conditions after storage for one hour and twenty-four hours in suspension medium.

Heparinized blood was mixed with an equal volume of S-MEM (Suspension) medium for preservation of the lymphocytes (Dr. Hilliard Festenstein, personal communication), and allowed to stand for twenty-four hours at room temperature (approx. 20°C). Blood for immediate processing from the same donors was also added to the same medium as a convenient means of obtaining a 1:2 dilution of the blood prior to separation of the lymphocytes. With the latter samples, however, procedures for the isolation of the lymphocytes and their cultivation in the presence of PHA were commenced after about one hour. Samples preserved for twenty-four hours were processed on the following day. As is obvious from the results shown in figure 20, differences between the corresponding pairs of results were not significant when tested statistically by the method for comparing two counts with Poisson distributions.

Determination of a dose response curve to tetanus toxoid in non-reactors.

In order to provide a dose response curve for tetanus toxoid as a guide for the selection of suitable test concentrations

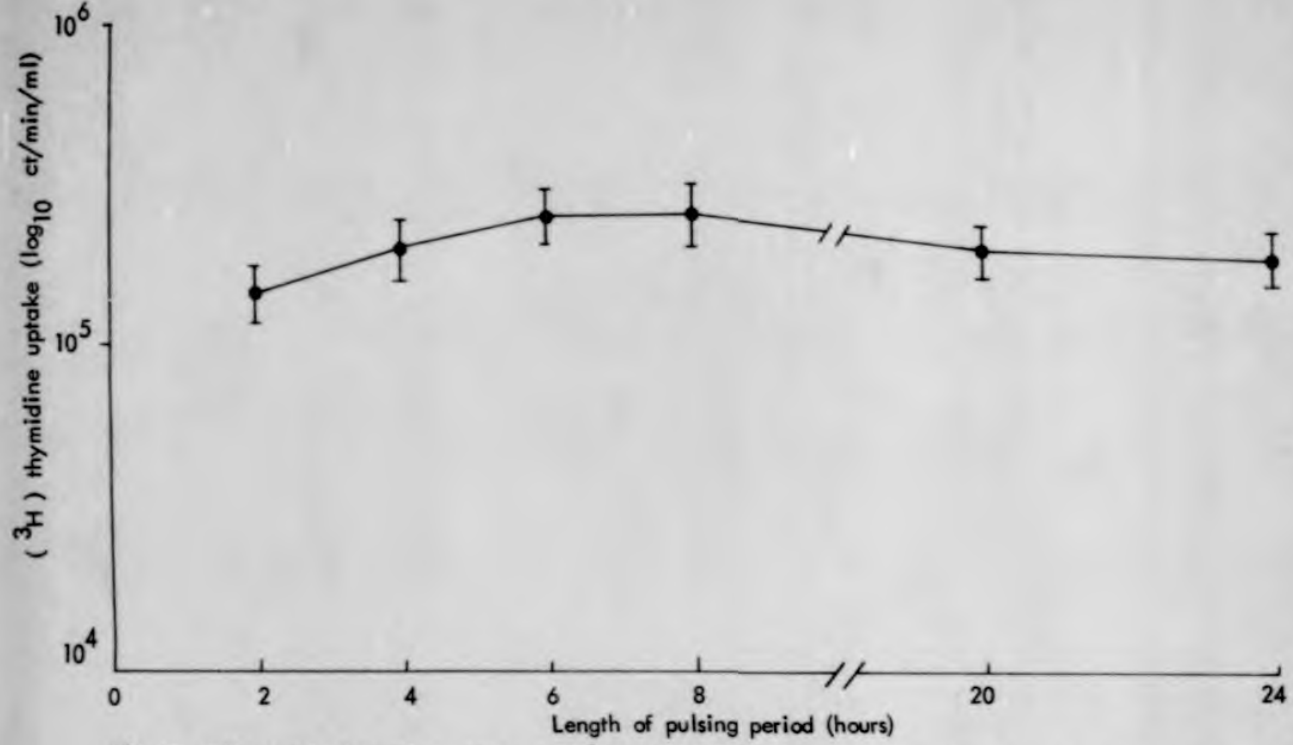


Fig.19 Effect of time of exposure to ($^3$H) thymidine on the incorporation of label by cultures of PHA-stimulated lymphocytes. Each point represents the mean response ± s.e. mean for lymphocytes from four donors.

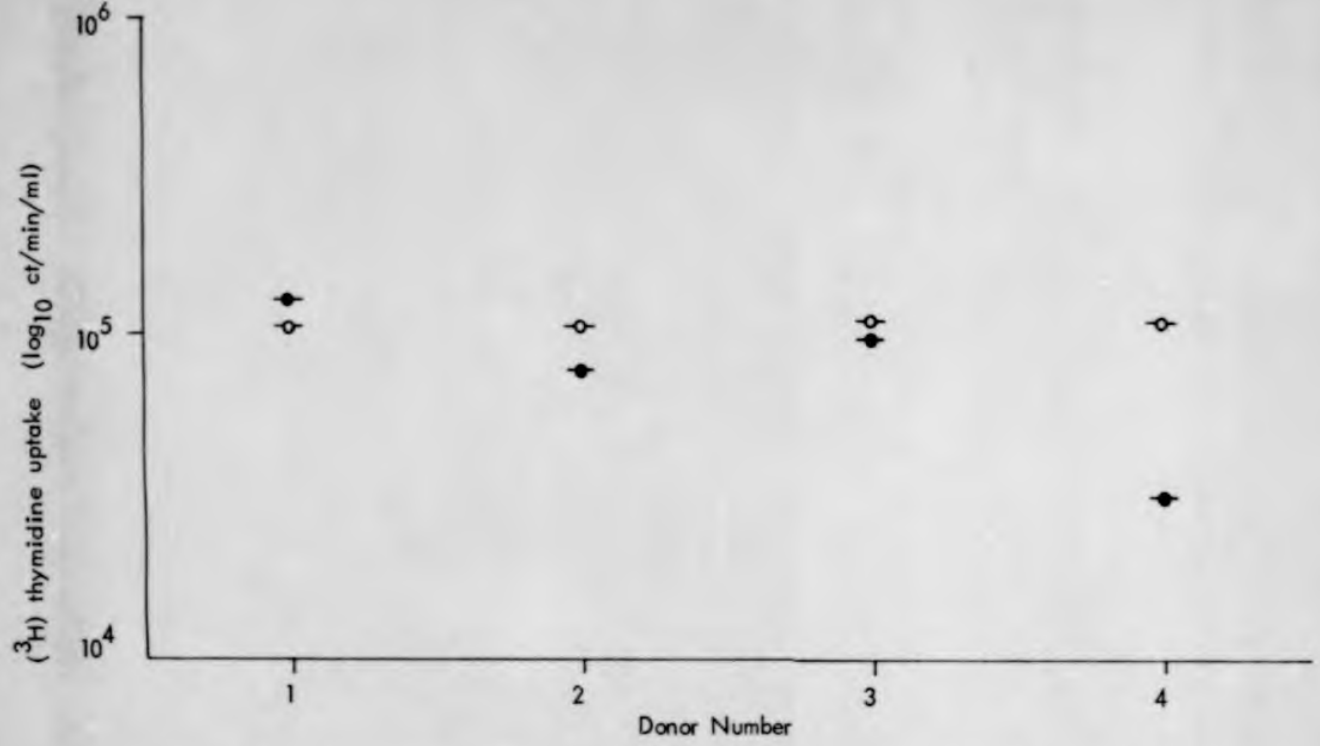


Fig.20 Comparison of the lymphocyte response of four donors to 1 µg PHA following processing after storage in S-MEM (Suspension) medium for approx. one hour ( ● ) and twenty-four hours ( o )

for the reactor group, the response of lymphocytes from non-reactors was investigated for four different concentrations of the antigen. Results of the investigations are shown in figure 21. Maximum uptake of thymidine occurred at 50 ug/ml of tetanus toxoid, but the differences in uptake at the four concentrations tested are minimal. However, differences in the response of non-reactors to tetanus toxoid according to age are apparent, as indicated in figure 22, despite the fact that there were only four subjects in the upper age group.

Comparison of the lymphocyte response of reactors and non-reactors to tetanus toxoid and to PHA.

Since Fitzgerald (1971) has shown that the concentration of PHA producing maximal response is not the concentration that allows the most sensitive discrimination between normal and abnormal responses, lymphocytes of reactors and non-reactors were tested against two concentrations of tetanus toxoid, viz: 10 µg/ml and 50 µg/ml; and against two concentrations of PHA, viz: 0.1 µg/ml and 1 µg/ml.

Reactor test group. Blood was collected from thirty-six reactors - eight females and twenty males. All responded in vitro to 1 µg/ml of PHA. However, only twenty-eight responded to one or both test concentrations of tetanus toxoid. Results of transformations to tetanus toxoid in four of the reactors were invalidated by a failure in the carbon dioxide supply to the incubator with the consequent development of an alkaline pH in the lymphocyte cultures; this is known to reduce the thymidine uptake dramatically (Hughes & Caspary, 1970). Another four of the reactor group gave exceptionally high counts for the lymphocyte cultures without antigen, i.e. two or three times the usual counts for control cultures, and these exceeded the counts obtained in the presence of antigen. Of the twenty-eight remaining, twenty-seven reacted to tetanus toxoid at a concentration of 10 µg/ml and twenty-one reacted to 50 µg/ml. Only one reacted to 50 µg/ml of tetanus toxoid and not to 10 µg/ml.

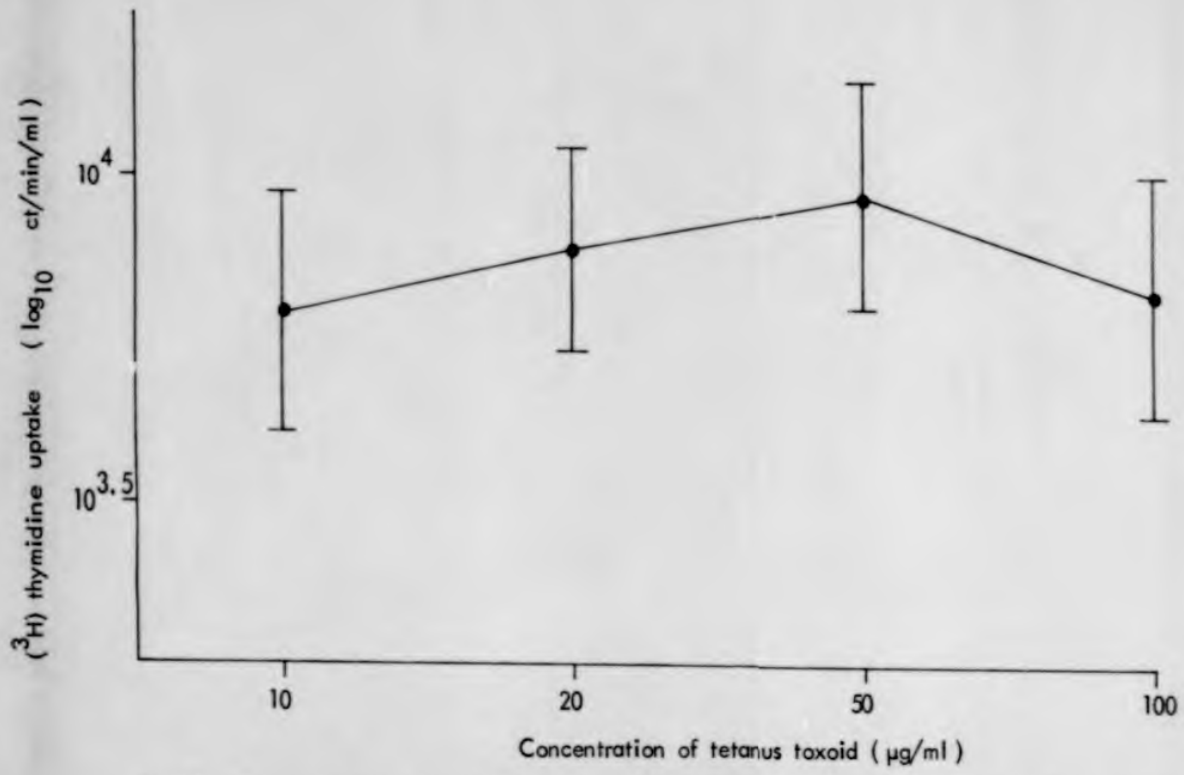


Fig.21 Dose response curve of lymphocytes to tetanus toxoid. Each point represents the mean response ± s.e. mean of lymphocytes from sixteen non-reactors.

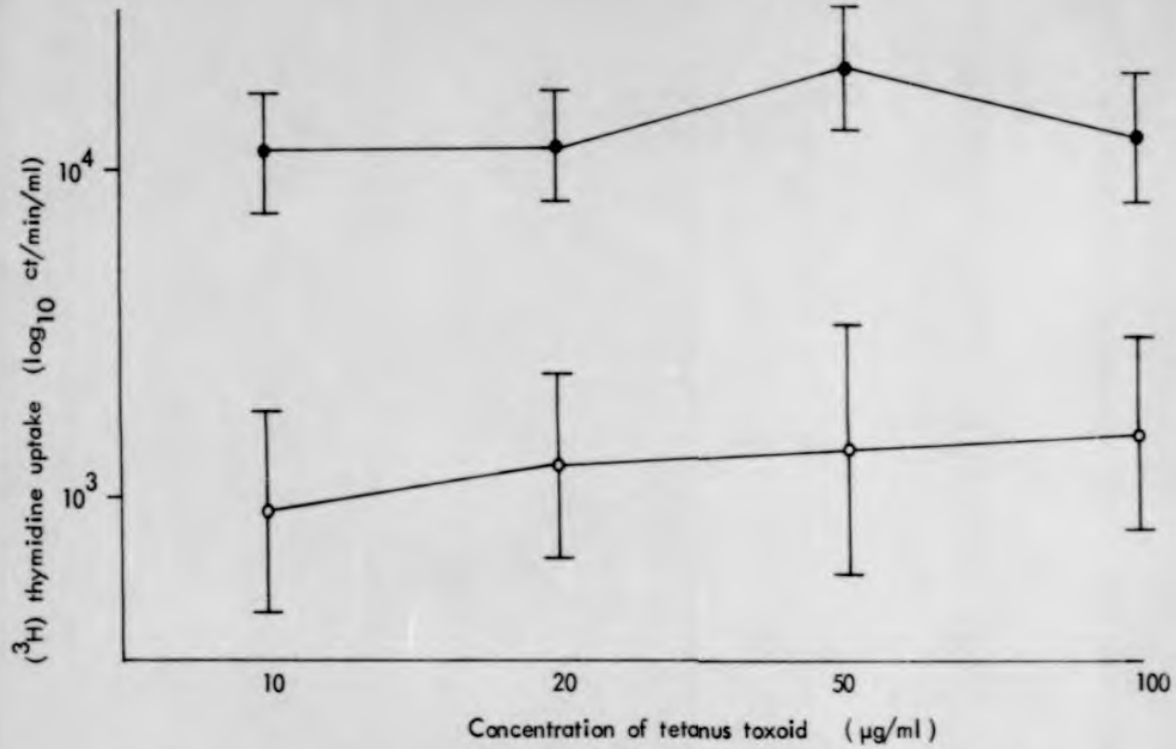


Fig.22 Relation between age and the lymphocyte response to four concentrations of tetanus toxoid. (●—●) Mean ± s.e. mean for lymphocytes from twelve subjects with ages ranging from 20-40 years approx. (o—o) Mean ± s.e. mean for lymphocytes from four subjects with ages ranging from 50-70 years approx.

The distribution of reactions in the twenty-eight reactors who showed some response to tetanus toxoid in vitro is given in table 39.

At the time of sampling, none of the reactors was taking oral contraceptives or any medication. Cigarette smoking and the occurrence of any recent viral infections were noted. One reactor had a history of penicillin allergy, but showed no apparent increase in lymphocyte activity in the control cultures containing medium alone.

Response to tetanus toxoid and PHA. Figure 23 illustrates the lymphocyte responses of the reactor and non-reactor groups to 10 µg/ml and 50 µg/ml of tetanus toxoid. There was no significant difference between the groups, when tested by t tests, for responses at either concentration.

Because of the influence of age on lymphocyte responses already indicated (figure 22), t tests were also conducted for the detection of significant differences in age of the groups. In the test of response to 10 µg/ml of tetanus toxoid, the mean age ± standard deviation was 41.37 ± 8.49 years for the reactor group, and 39.37 ± 14.82 years for the non-reactor group. In the test of response to 50 µg/ml, the mean age ± standard deviation was 40.80 ± 7.8 years for the reactor group, and 38.81 ± 15 years for the non-reactor group. There was no significant difference with respect to age of the reactor and non-reactor groups by t tests for either test concentration of tetanus toxoid.

Lymphocyte responses to PHA in the reactor and non-reactor groups also showed no significant differences by t tests. For a concentration of 0.1 ug/ml of PHA, the mean lymphocyte response ± standard error of the mean in terms of thymidine uptake expressed as cts/min/ml was 3.90 ± 0.13 for the reactor group, and 4.13 ± 0.20 for the non-reactor group. For a concentration of 1 ug/ml of PHA, the mean response ± standard error of the mean was 4.85 ± 0.06 for the reactor

TABLE 39

Distribution of reactions to tetanus toxoid according to type, severity and time of onset in the reactor group

| | LOCAL REACTIONS | | | | | GENERAL REACTIONS | | |
|---|---|---|---|---|---|---|---|---|
| Grade | No. | Onset, days after injection [c] | | | | Symptoms | No. | Onset, days after injection |
| | | <1 | 1 | 2 | 7 | | | |
| Moderate [a] | 23 | 2 | 5 | 12 | 2 | Nausea | 1 | 2 |
| Severe [b] | 5 | | 2 | 2 | 1 | Some malaise and "neck stiffness" | 1 | 1 |
| | | | | | | Malaise | 1 | 2 |

[a] Moderate - area of redness and/or oedema of between 2 and 12 cm diameter present at injection site (White et al., 1973).

[b] Severe - area of redness and/or oedema greater than 12 cm diameter present at injection site (White et al., 1973).

[c] Time of onset of reaction not recorded for two subjects.

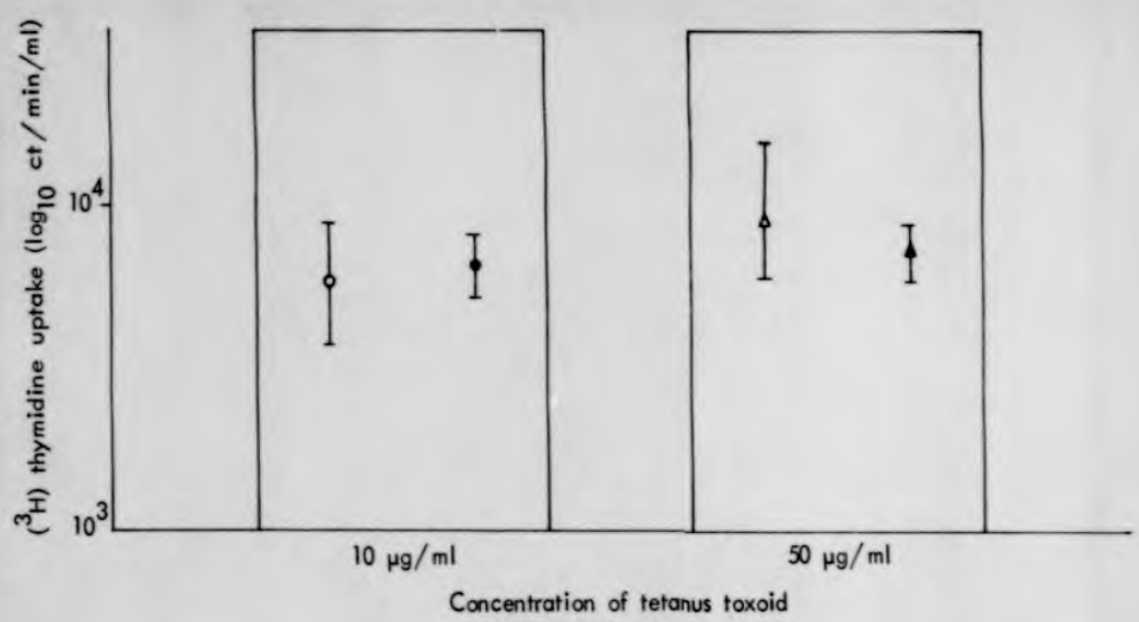


Fig.23 Comparison of the lymphocyte response of reactors and non-reactors to two concentrations of tetanus toxoid. ( o ) Mean response ± s.e. mean for sixteen non-reactors at 10 µg/ml. ( ● ) Mean response ± s.e. mean for twenty-seven reactors at 10 µg/ml. ( ▲ ) Mean response ± s.e. mean for sixteen non-reactors at 50 µg/ml. ( ▲ ) Mean response ± s.e. mean for twenty-one reactors at 50 µg/ml.

group, and 4.95 ± 0.05 for the non-reactor group.

Influence of age on the lymphocyte response to tetanus toxoid and PHA.

Figure 24 shows a decreasing lymphocyte response to 10 µg/ml of tetanus toxoid with increasing age of the cell donor. Since it has been shown that there was no significant difference in lymphocyte responses according to reactor status, the results obtained for reactors and non-reactors were combined to provide data for forty-three subjects. Analysis of these data showed that the regression equation is

$$Y = 5.1626 - 0.0342\ x$$

where Y is $\log_{10}$ cts/min/ml for thymidine uptake, and x is age in years. The correlation coefficient (r) has a value of −0.607 which is statistically highly significant ($p<0.001$).

Similarly, the regression equation relating the lymphocyte response to age of cell donor for stimulation by 50 µg/ml of tetanus toxoid was calculated as:

$$Y = 5.0125 - 0.0280\ x$$

where Y is $\log_{10}$ cts/min/ml for thymidine uptake, and x is age in years. The value of r is −0.5287 which is also highly significant ($p<0.001$). The scatter diagram with the regression line for these results is given in figure 25.

Figure 26 shows the decrease in lymphocyte response with advancing age for stimulation by 0.1 µg/ml of PHA. The regression equation is:

$$Y = 5.2169 - 0.0326\ x$$

where Y is $\log_{10}$ cts/min/ml for thymidine uptake, and x is age in years. The value of r is −0.428 which is significant ($p<0.01$).

Results obtained for lymphocyte responses to 1 µg/ml of PHA also indicated a general trend towards decreasing response with increasing age of cell donor, but the value of r was not significantly different from zero.

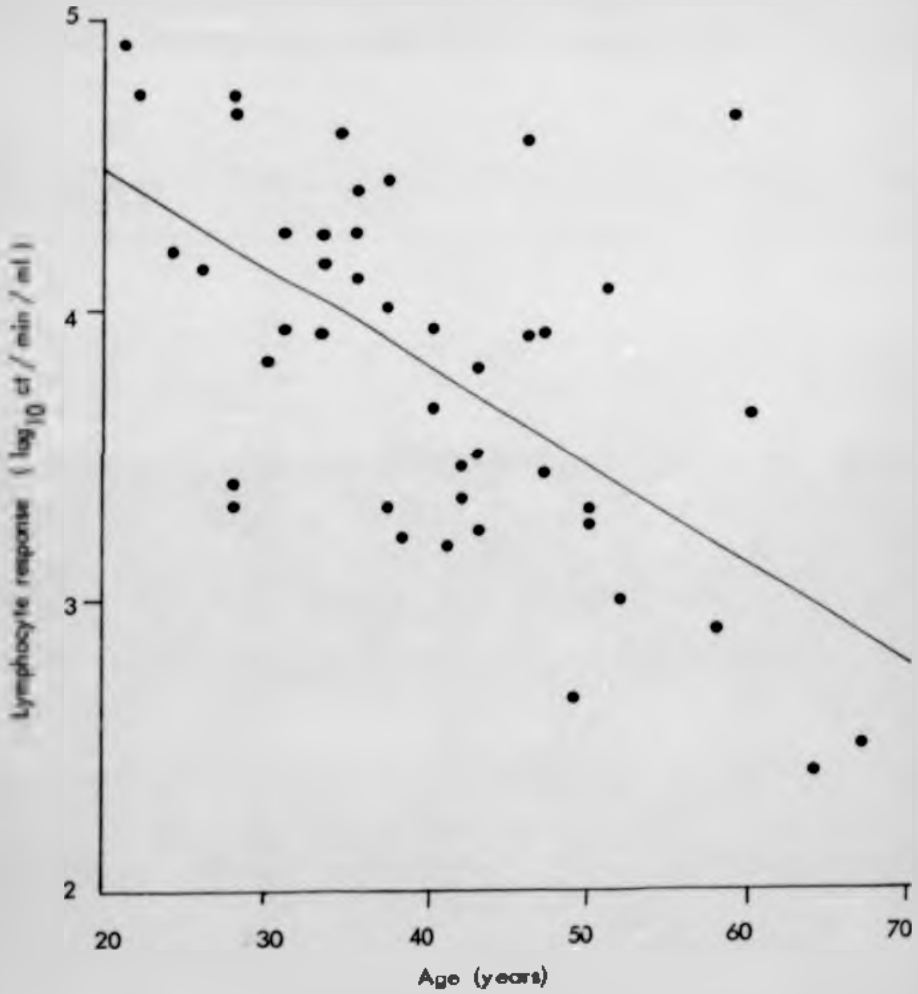


Fig.24 Decreasing lymphocyte response to stimulation by 10 ug/ml of tetanus toxoid with increasing age for forty-three subjects.

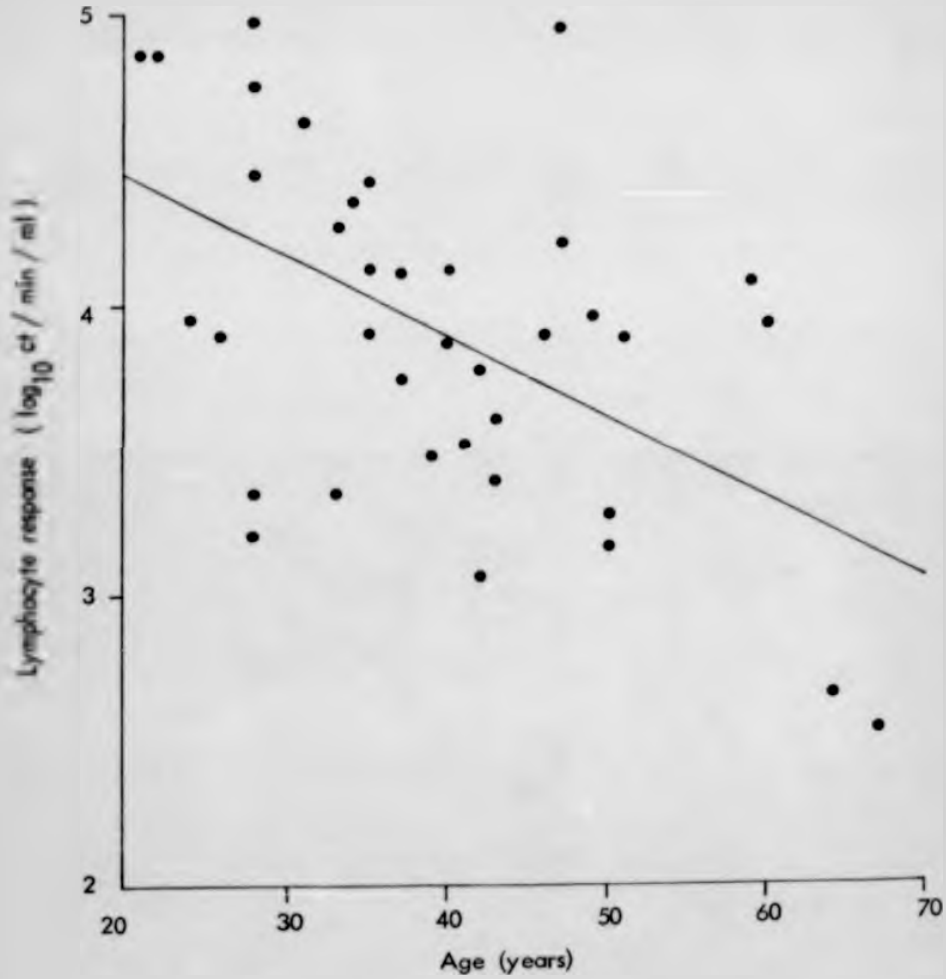


Fig.25 Decreasing lymphocyte response to stimulation by 50 µg/ml of tetanus toxoid with increasing age for thirty-seven subjects.

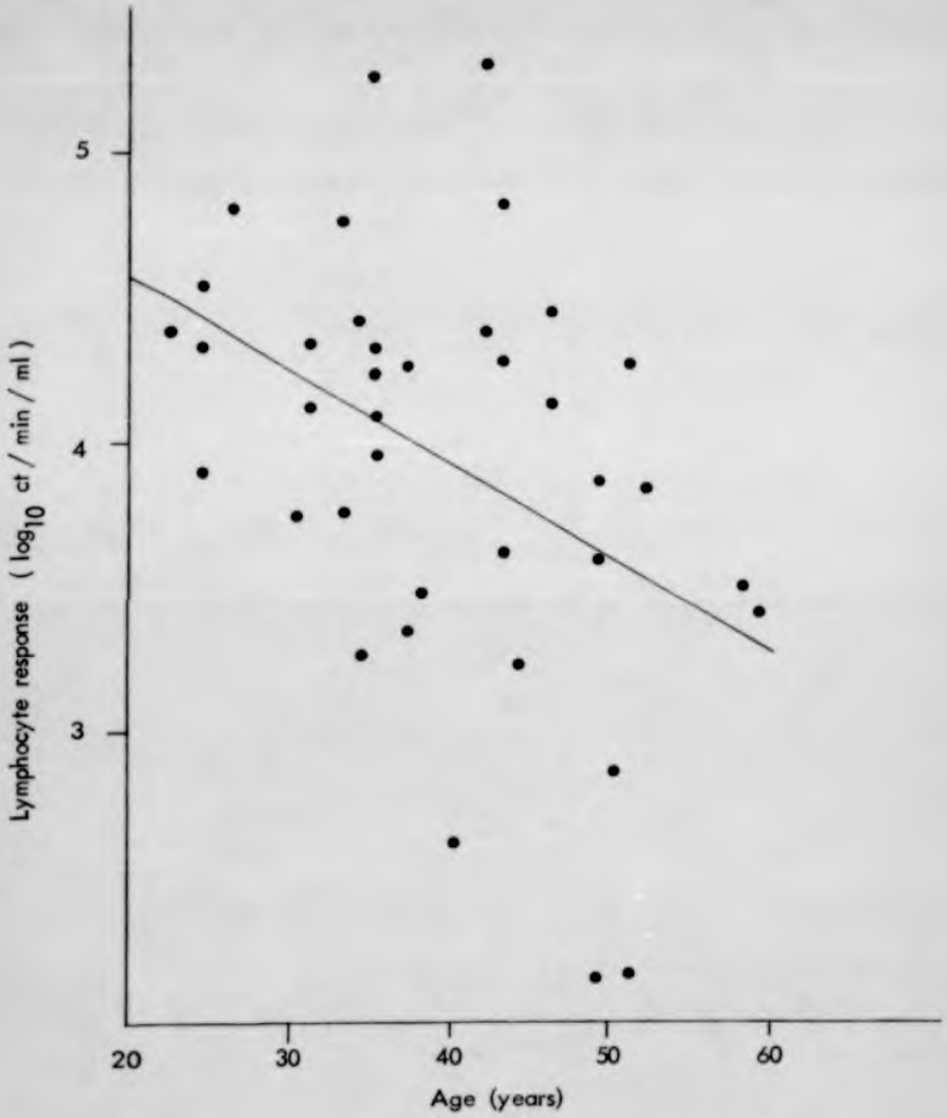


Fig.26 Decreasing lymphocyte response to stimulation by 0.1 µg/ml of PHA with increasing age for thirty-nine subjects.

Inspection of the scatter diagrams relating lymphocyte response to age in years indicated the presence of a few high responders in the upper age groups. An examination of the reactor status of these particular subjects revealed an interesting association between the severity of their reactions to tetanus toxoid vaccination and the lymphocyte response - despite the fact that the reactor group as a whole had shown no significant difference from the non-reactor group for lymphocyte response to tetanus toxoid. In figure 24, for example, the subject with co-ordinates (x, y) of 59 and 4.56 had shown the most severe local reaction of all those recorded for the reactor group tested, viz: a grade 3 reaction with erythema and pain involving the whole upper right arm. The subject with co-ordinates of 46 and 4.57 in the same figure was also one of the few tested with a grade 3 local reaction. The "outlier" in figure 25 with co-ordinates of 47 and 4.95 had produced a large nodule at the site of injection. However, the two probable reactors to primary injections of tetanus toxoid did not show a lymphocyte response which was significantly higher than those of others in their age group.

Comparison of lymphocyte response and antitoxin titres in subjects according to age.

Table 40 gives the distribution of the results of antitoxin titrations performed on blood which had been collected at the same time as blood for lymphocyte activity in the reactor group, and within about a month of the collection of blood for lymphocyte activity in the non-reactor group. Since both reactors and non-reactors varied greatly in their immunization histories with respect to the number and timing of their injections, no correlation between age and titres under these circumstances was expected. Nevertheless, formal t tests were performed for lymphocyte activity in the presence of 10 µg/ml of tetanus toxoid and for antitoxin titres for the age groups 20 to 39 years and 40 to 69 years. For the purpose of calculation, the antitoxin titres were taken to be the geometric mean titres of the range of titres quoted.

TABLE 40

Distribution of the tetanus antitoxin titres[a] of thirty-seven subjects by age groups.

| TITRE | AGE GROUPS | |
|---|---|---|
| | 20–39 years | 40–69 years |
| u/ml | | |
| 0.02–0.05 | 1 | - |
| 0.05–0.1 | - | - |
| 0.1 –0.2 | 2 | 4 |
| 0.2 - 0.5 | 1 | 4 |
| 0.5 - 1 | 2 | 5 |
| 1 - 2 | 4 | 3 |
| 2 - 5 | 3 | 3 |
| 5 - 10 | 1 | - |
| 10 - 20 | 2 | - |
| 20 - 50 | - | 1 |
| Total | 16 | 21 |
| Arithmetic mean titre | 3.28 | 3.92 |
| Geometric mean titre | 1.198 | 0.876 |
| Mean age (years) | 31.06 | 47.81 |

[a] Most of these antitoxin titrations were performed by Mr. Peter Knight of Wellcome Laboratories.

TABLE 41

Mean antitoxin titres for reactors and non-reactors

| GROUP | NUMBER | GEOMETRIC MEAN TITRE |
|---|---|---|
| | | u/ml |
| Non-reactors (representative sample) | 12 | 0.521 |
| Grade 2 reactors (representative sample) | 17 | 0.966 |
| Grade 3 reactors (total number) | 5 | 0.912 |
| Grade 3 reactors + reactors with general symptoms (total number) | 8 | 1.205 |

As expected, the lymphocyte responses of the two groups differed significantly ($p<0.001$) while the antitoxin titres for the two groups showed no significant difference. A comparison of the geometric mean titres of various groups of reactors with that of the non-reactor group showed that the former were about twice the latter (table 41).

## DISCUSSION

Lymphocyte reactivity to tetanus toxoid in vitro is the outcome of the interplay of many factors, and its correlation with reactions observed in vivo is influenced by many biological and technical variables. In the study reported here, no significant difference was found for the in vitro lymphocyte reactivity to tetanus toxoid between the group who developed reactions to tetanus toxoid vaccination and the control group, but one association did emerge, viz: an inverse relation between the age of the lymphocyte donor and the degree of lymphocyte transformation induced by tetanus toxoid. Although a decline in the responsiveness of peripheral lymphocytes to PHA in the older age groups has been reported repeatedly (Pisciotta et al., 1967 ; Del Pozo Perez et al., 1973; Hallgren et al., 1973; Roberts-Thomson et al., 1974; Poad et al., 1974), an age-related decline in the in vitro activity of sensitized peripheral lymphocytes to the sensitizing antigen has not apparently been reported before.

Despite the lack of specific lymphocyte reactivity to tetanus toxoid that was demonstrated by the reactor group as a whole, however, three of the five subjects giving severe (grade 3) local reactions did show a higher than normal response for their age. Thus it is likely that only in severe reactors is the increased lymphocyte reactivity of sufficient intensity to express itself against the background of the apparent determining influence of age in the detection system used. Furthermore, immediate and Arthus type reactions probably contributed significantly to the large

proportion of moderate (grade 2) reactors in the reactor group. In this context, and in line with the observations of Edsall (1959); Levine, Ipsen & McComb (1961); Edsall et al. (1967); and Relihan (1969), the geometric mean antitoxin titre for reactors was found to be about twice that for non-reactors.

Obviously, one important variable is the assessment of the type and extent of the in vivo reactions. Immediate, Arthus type and delayed type hypersensitivity reactions are difficult to differentiate and evaluate particularly as the reactions observed are often of a mixed type (Facktor, Bernstein & Fireman, 1972; White et al., 1973).

Other variables that determine or modify the development of a reaction relate to the conditions and circumstances of the tetanus toxoid immunization. While effects of a variable such as sex on reactions to vaccination might be expected to be reflected in the in vitro situation, the effect of the route of injection would not be reproduced in vitro. Yet, the route of injection appears to be a most important factor in determining the incidence of reactions; and one which, in the opinion of Relihan (1969), has not received the attention it deserves. Subcutaneous injection produces more local reactions than does intramuscular injection (Edsall, 1959; Relihan, 1969); and, in fact, the intracutaneous skin test with diluted tetanus toxoid produces reactions in most people irrespective of their tendency to give reactions on tetanus toxoid immunization. (Kittler et al., 1966; Facktor et al., 1972). Indeed, this latter finding invalidates the use of such skin tests as a means of detecting possible reactors prior to vaccination. White et al., (1973) even suggest that the greater incidence of reactions observed in older females may be due in part to the difficulty of giving them a true intramuscular injection because of the deeper layers of subcutaneous fat and smaller muscle mass that typically characterizes this group. No difference in lymphocyte reactivity according to sex was observed in the investigation reported here, but the number of females in the reactor group was comparatively small.

Lymphocyte reactivity to PHA is known to be depressed by oral contraceptives (Hagen & Frøland, 1972; Fitzgerald et al., 1973; Morishma & Henrich, 1974; Barnes et al., 1974), and by the intake of such substances as phenobarbital (Park & Brody, 1971) and aspirin (Crout, Hepburn & Ritts, 1975). Smoking has also been implicated as a cause of depressed immune responsiveness (Thomas, Holt & Kaset, 1974; Nymand, 1974), but no difference in reactivity of smokers and non-smokers was observed in the study. The absence of any obvious increased reactivity in the control cultures of the one subject with penicillin allergy was not surprising in view of the finding of Vischer (1966) that the lymphocyte cultures of only two subjects out of thirteen with penicillin allergy showed a significant proliferative response, as measured by lymphocyte transformation, in the presence of penicillin.

The extent to which lymphocyte transformation to specific antigens correlates with delayed type hypersensitivity reactions to those antigens is itself controversial. Some investigators, e.g. Mills (1966), Oppenheim (1968), and Rice et al. (1974) have reported a high degree of correlation or have attributed occasional discrepancies to a presumed higher sensitivity in the in vitro test; whereas others, e.g. Parkhouse (1967), Loewi, Temple & Vischer (1968), and Benezra, Gary & Davies (1969) have produced evidence which disproves the existence of a close correlation. The explanation for these conflicting findings may be that delayed type hypersensitivity reactions to various antigens differ in their susceptibility to external factors, such as the injection procedure, with a consequent variation in their correlation with in vitro test systems; but technical and biological variables affecting the in vitro tests must also be partly responsible.

In the lymphocyte transformation test used here, steps were taken to minimise known variability due to major factors at least. Thus, granulocytes were removed from the test system by isolation of the lymphocytes on a Ficoll-Triosil gradient since Hinz & Chickosky (1972) demonstrated a marked inhibitory effect on thymidine uptake with the addition of granulocytes to lymphocyte cultures stimulated by PPD; and Moore, Hayworth & Brown (1974) found a significant inverse correlation between the percentage of

granulocytes in their leucocyte cultures and response to 0.1 µg of PHA. After isolation, the lymphocytes were washed to remove tetanus antitoxin in order to avoid the stimulation which is produced in lymphocytes by antigen-antibody complexes (Bloch-Shtacher, Hirschhorn & Uhr, 1968; Möller, 1969). The foetal calf serum which was added to the culture medium was also vetted for the presence of tetanus antitoxin for the same reason. The lymphocyte concentration, duration of culture, and length of pulsing period for the tetanus toxoid system were consonant with conditions used by others for lymphocyte transformation to specific antigens (Hinz & Chickosky, 1972) including tetanus toxoid (Dukes, Parsons & Stephens, 1969).

Although the logistics of the study necessitated the storage of the blood samples for 24 hours in a suspension medium prior to isolation of the lymphocytes, this did not affect the reactivity of the lymphocytes to PHA when tested under optimal conditions for reproducible results and the quantitative measurement of PHA stimulation. As Tennant (1964) has shown, the testing of the activity of cells is a more sensitive means of assessing any cellular damage than is the dye exclusion test so it may be inferred that storage of the lymphocytes does not interfere with their ability to react to tetanus toxoid either.

In magnitude, consistency, and reproducibility, the lymphocyte responses obtained with PHA stimulation resembled those reported by Fitzgerald (1972) and Yamamura (1973) who also explored the effect of technical and other variables on a quantitation of the response. The general agreement between the results obtained with the technique and those found by others vindicates the use of the simplified harvesting procedure employed and endorses the general reliability of the methodology.

The harvesting procedure was a modified version of the culture extraction method of Penhale et al. (1974). In their technique, cultures from micro plates were transferred, after resuspension, to numbered discs on a pin-board for drying. When dried, the discs were removed from the pin-board, and placed in a large flask in which they were subjected to a series of communal rinses in processing solutions. They were subsequently removed

from the flask, and re-dried on sheets of absorbent paper before being placed in scintillation fluid for counting. Penhale et al. (1974) compared the reproducibility of lymphocyte transformation tests using this method of harvesting with tests using the popular method of manifold filtration, and showed that the coefficient of variation in the final counts was considerably lower for their own method. To facilitate the handling of a large number of specimens by a single operator for the study reported here, the "dry and communal rinse" system was further simplified by leaving the discs on the pin-board throughout the procedure. For this purpose, the pin-board was constructed of plastic material resistant to the action of the processing solutions, and the plastic pin-boards were accommodated in photographic developing dishes for exposure of the discs to the solutions.

Another probable advantage of the simplified sampling and harvesting procedure, as compared to the manifold filtration technique, is that the former method results in a much smaller amount of precipitated protein and DNA on each disc. This is of importance because of the self absorption problem associated with the disc method for counting precipitates containing tritiated thymidine. The deposited samples must form an "extremely thin layer" (Price, 1973) if excessive self absorption is to be avoided.

A disturbing aspect of the results obtained for lymphocyte transformation to tetanus toxoid, however, was the high background counts that sometimes occurred. "Failures" to react to one or both test concentrations of tetanus toxoid were almost invariably due to a high count for the control cultures containing medium only rather than to a low count for the test cultures containing tetanus toxoid. Foetal calf serum has been implicated as the constituent of the medium most likely to cause such high background counts. Johnson & Russell (1965) reported that the reaction of human lymphocytes to foetal calf serum showed an upsurge at seven to eight days in culture, whereas the lymphocyte response to specific antigen was maximal at five to six days;

but Wilson (1966) found that some human lymphocytes responded to the presence of foetal calf serum with a marked proliferation on the fifth day. Wilson (1966) suggested that the propensity of the lymphocytes from certain individuals to respond to calf serum proteins may reflect atopic sensitivities on the part of these donors, but this is not the only explanation, nor the most probable one. As pointed out by Bloom et al. (1973), both human and calf serum supplements may contain certain substances such as ABO or HLA antibodies which activate lymphocytes directly, as well as naturally occurring and cross reacting antibodies that may form antigen-antibody complexes which can stimulate non-sensitized lymphocytes. Despite these problems, the consensus of opinion and experience - as also enunciated by Bloom et al. (1973) - is that stimulation of lymphocytes by specific antigen usually does seem to require the presence of serum supplements in the medium.

The in vitro lymphocyte transformation test, although a conventional test for cell mediated immunity associated with T cell activity, also measures some B cell activity. This has been clearly demonstrated for the tetanus toxoid system by Geha et al. (1973) who used the method of density gradient centrifugation in bovine serum albumin of August et al. (1970) for the separation of human B and T cells of donors immunized with tetanus toxoid. They showed that culture fluid supernatants from tetanus toxoid-stimulated T cells contained a soluble product which, in the presence of antigen (Geha & Merler, 1974), caused blast transformation of a proportion of the B cells with the concomitant production of antibody. Antigen alone was incapable of triggering the B cells into blast transformation and the secretion of antibody. What the in vitro transformation test measures then is not only the lymphocyte transformation of sensitized T cells to the sensitizing antigen, but also the lymphocyte transformation of those B cells which respond to the

mitogenic product of the stimulated T cells. It is this combined activity of the lymphocytes from tetanus toxoid-immunized donors which shows a steady decrease with advancing age on tetanus toxoid stimulation. An increased lymphocyte reactivity in severe reactors only may be detected superimposed on the age effect.

## DISCUSSION AND CONCLUSIONS

Findings in the study of the relation between timing of maternal tetanus toxoid injections and the presence of protective antitoxin titres in their babies at delivery re-emphasize the variability in immune responsiveness that characterizes a population. Predictions based on the results of such studies can only be made in terms of probabilities, therefore. In spite of this limitation, it may be concluded that the administration of the first of two standard doses of adsorbed tetanus toxoid at least 70 days before delivery provides a reasonably acceptable rate of protection which increases significantly as the period between first injection and delivery is increased beyond 70 days. Any first injection given less than 70 days from delivery has only a 50% chance of resulting in a protective level of antitoxin in the baby. A single dose of standard tetanus toxoid given less than 60 days before delivery is not effective.

Precisely one-third of the total number of paired maternal and cord sera assayed for the determination of cord/maternal ratios gave a value of 2 for that ratio. Ratios greater than 1 are indicative of a materno-foetal passage of antibody against a concentration gradient i.e. are evidence for an active transport mechanism. The mean number of days from second injection to delivery for this group was about 60 as compared to 36 days for the group in which maternal and cord antitoxin titres were the same. This suggests that the active transport mechanism either develops slowly with time from the second injection, or is a slow mechanism which only becomes evident with time from the second injection. A relatively slow placental transfer mechanism that is favoured by Longo (1972) for antibody transport is pinocytosis, a common but poorly understood cellular process in which solutes and fluids are invaginated by portions of the cellular membranes and transported across the cell. Some support for the operation of this mechanism of transport in the placenta has come from the demonstration by electron microscopy of the presence of micropinocytotic vesicles containing high concentrations of protein in the placenta of bats (Stephens, 1969). Similar studies

on human placenta combined with histochemical and immunochemical techniques for identifying the nature of the contents of any vesicles found may provide information on the possible importance of this mechanism in the transport of antibody from mother to foetus.

Results of investigations into the in vitro reactivity of lymphocytes from reactors to tetanus toxoid showed that only in a few of the severe reactors was there any evidence for an increased response to tetanus toxoid antigen as measured by thymidine uptake with lymphocyte transformation. However, the results did reveal a clearcut decreasing lymphocyte reactivity to tetanus toxoid with increasing age of the lymphocyte donors. An inverse relation between age and reactivity to a specific antigen in vitro has not apparently been reported previously, although an age-related decline in PHA responsiveness is well established, and a reduction in the actual number of circulating T cells with age, as estimated by their rosette-forming capabilities, has also been demonstrated (Smith, Evans & Steel, 1974). The finding is relevant to the immunological theories of ageing as expounded by Burnet (1970) and Walford (1974); and, in particular, supports the importance of a gradual decline in thymus-related immune functions in the ageing process. Further, a declining lymphocyte reactivity to tetanus toxoid with age is not incompatible with the observed age-related increase in incidence of general autoimmune phenomena, or even in specific reactions to tetanus toxoid immunization, since many clinical and experimental studies have shown that primary immunodeficiency diseases in man as well as immunodeficiency disease produced by neonatal thymectomy and that developing during ageing in experimental animals are all accompanied by several autoimmune phenomena and diseases in high frequency (Good & Yunis, 1974). Any immunological theory for ageing must take into account this special feature of the age-related functional decline of the thymus viz: that as the normal immune response declines, autoimmunity becomes increasingly evident. In this connection, Walford (1974)

postulates that failures in the homeostatic control of tolerance and suppressor mechanisms are involved in the emergence of potentially self-reactive cells arising by somatic mutations, while Burnet (1970) emphasizes the importance of the loss in thymus-dependent functional capacity for immunological surveillance in dealing with emerging cellular anomalies. Further studies are indicated on the age-related response of pure populations of T cells to various antigens. The possibility of a difference in responses to antigens depending on whether they are "extrinsic" i.e. foreign or "intrinsic", i.e. self, as has been shown to occur in antibody production with advancing age (Rowley, Buchanan & Mackay, 1968), should be borne in mind in the interpretation of such studies.

In addition to the findings discussed above, studies on the immune response to tetanus toxoid have resulted in:
(a) *a method for the titration of tetanus antitoxin by toxin* neutralization in steps of twofold dilutions from 0.001 unit per ml with small volumes of serum. Titration from the lower limit of the range, which varies according to the toxin test dose, requires 0.6 ml of serum. For most antisera, however, the toxin dose level and the range of serum dilutions for testing can be chosen so that an initial serum dilution of 1:2 or greater will suffice. This means that only 0.3 ml of serum is then required. The method is simple and economical, and the results obtained are accurate and reproducible provided that no significant variation occurs in the body weight of the mice used.
(b) the determination of the variables affecting the titration of tetanus antitoxin by the indirect haemagglutination technique with glutaraldehyde-fixed sheep cells. When the technique is conducted under optimal conditions, its high sensitivity provides a reliable method for detecting the presence of antitoxin in sera. In addition, sensitization of the cells under optimal conditions promotes their reactivity with equine standard antitoxin so improving the correlation between haemagglutinin titres, as estimated by a comparison with the haemagglutinating activity of the standard, and titres obtained by toxin neutralization. The use of a diluent

containing 2-mercaptoethanol for inactivation of the IgM in sera also contributes to an improved correlation between the titrations. (c) a means of determining the presence of tetanus-specific IgM by an indirect immunofluorescence technique with tetanus toxoid-coated Sepharose beads as antigen. Prior absorption of the sera with protein A before testing removes most of the IgG so reducing the competition for antigenic sites available to IgM. The successful adaptation of microtitre techniques to the test system significantly reduces the manipulations and materials required. The use of this technique did not provide evidence for the trans-placental passage of tetanus toxoid by the demonstration of tetanus-specific IgM in cord blood. However, its application to the detection of specific IgM in maternal sera showed that production of tetanus-specific IgM occurred frequently after the second dose of tetanus toxoid. The finding is in agreement with a previous report on a high frequency in the production of tetanus-specific IgM after re-vaccinations with tetanus toxoid; and emphasizes that, in this regard, the immune response to tetanus toxoid does not appear to be typical of immune responses in general.

The use of tetanus toxoid as antigen for studies on the human immune response has provided information on immunological phenomena that concern man before birth and in old age.

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# APPENDICES

## Appendix (i)
## Tetanus Toxins

Toxins from the Netherlands, designated No.954 and No.956, were produced by ammonium sulphate precipitation (100% saturation) of a culture filtrate of Clostridium tetani strain CN655 obtained originally from the Wellcome Research Laboratories. The precipitates were dried over phosphorus pentoxide, distributed in ampoules, and freeze-dried. The toxins were supplied by:

Dr. J. Nagel,
Rijks Instituut Voor de Volksgezondheid,
Antonie van Leeuwenhoeklaan 9,
Postbus 1,
Bilthoven,
The Netherlands.

The British toxin, No.682-C2, had been used previously for calibration of the British Standard Antitoxin against the International Standard. It is unusual in that the Lf value is the same as the L+ value. A sample was obtained from:

Wellcome Research Laboratories,
Beckenham,
Kent, BR3 3BS.

L+ and MLD values of the toxins:

| Toxin | L+/100 | L+/1000 | L+/4000 | Lo/10000 | MLD |
|---|---|---|---|---|---|
| Dutch No.954: | 44 µg | 4.3 µg | 1.1 µg | 0.12 µg | 0.3 g |
| Dutch No.956: | 52 µg | 5.2 µg | 1.3 µg | 0.1 µg | 0.35 g |
| British No.682-C2: | $24 \times 10^{-4}$ ml | $2.4 \times 10^{-4}$ ml | $0.6 \times 10^{-4}$ ml | - | $0.2 \times 10^{-4}$ ml |

## Appendix (ii)
## Standard Tetanus Antitoxins

The Third British Standard for Tetanus Antitoxin, established in 1963, is available from the Medical Research Council.

One unit is the activity contained in 0.3304 mg of the preparation. When all the contents of an ampoule are dissolved, the solution contains 230 international units of tetanus antitoxin. It is stored in the dark below 0°C. A sample (No.60/13) was obtained from:

Division of Immunological Products Control,
National Institute for Medical Research,
Hampstead Laboratories,
Holly Hill,
Hampstead,
London, N.W.3.

The U.S. Standard Tetanus Antitoxin, lot No.E114, contained 5 international units per ml as supplied. It was obtained from:

Division of Biologics Standards,
N.I.H.,
Bethesda,
Maryland, 20014,
U.S.A.

This standard was stored in liquid form at about 4°C as directed.

## Appendix (iii)

## Phosphate Buffered Saline (Oxoid)

(Dulbecco & Vogt, 1954)

| | |
|---|---|
| Na Cl | 8 g |
| KCl | 0.2 g |
| $Na_2 HPO_4$ | 1.15 g |
| $KH_2 PO_4$ | 0.2 g |
| Dist. water | 1000 ml |

pH = 7.3/7.4

## Appendix (iv)

## Human Tetanus Immunoglobulin

"Tetagam", a preparation of human tetanus immunoglobulin, is manufactured according to a modification of Cohn's cold ethanol fractionation process for use in the prophylaxis and therapy of

tetanus. When reconstituted, each 1 ml of the solution contains approximately 250 I.U. Samples were supplied by:

Behringwerke Aktiengesellschaft,
355, Marburg (Lahn) 1,
Postfach 1130,
Germany.

## Appendix (v)

## Scheme for the preparation of dilutions at close intervals within a twofold dilution step.

Antilog of the dilution interval = $\frac{\alpha}{\alpha - \beta}$

where $\alpha$ is the total starting volume and $\beta$ is the volume of dilution required.

For a dilution interval of log 0.05, $\frac{\alpha}{\alpha-\beta}$ = antilog 0.05 = 1.122.

Hence $\alpha = 1.122\ (\alpha-\beta) = 1.122 - 1.122\alpha - 1.122\beta$.

If $\beta$ = 1 ml, then $\alpha = \frac{1.122}{0.122} = 9.2$ ml.

Thus the starting volume is 9.2 ml of a 1:2 dilution and the replacement volume is 1 ml.

| Resulting dilutions | Log of dilutions |
|---|---|
| 1:2 | $\bar{1}.70$ |
| 1:2.24 | $\bar{1}.65$ |
| 1:2.51 | $\bar{1}.60$ |
| 1:2.81 | $\bar{1}.55$ |
| 1:3.15 | $\bar{1}.50$ |
| 1:3.53 | $\bar{1}.45$ |
| 1:3.95 | $\bar{1}.40$ |
| 1:4.43 | $\bar{1}.35$ |

Experiments were conducted using eight mice per dilution.

## Appendix (vi)
## ACD anticoagulant
(Nelson, 1973)

| | |
|---|---|
| Glucose | 0.3 g |
| Sodium citrate | 0.3 g |
| Dist. water | 10 ml |

Solution autoclaved at 115°C for 15 min.
10 ml of blood, collected aseptically, is added to 1.5 ml of this solution.

## Appendix (vii)
## Dutch Tetanus Toxoid

A batch of purified tetanus toxoid, V.G.T. No.6, was obtained from:

Dr. J. Nagel,
Rijks Instituut Voor de Volksgezondheid,
Antonie van Leeuwenhoeklaan 9,
Postbus 1,
Bilthoven,
Netherlands.

It contained 1000 Lf/ml, and 1666 Lf/mg protein N. The tetanus toxoid was purified by ammonium sulphate precipitation between 15% and 23% (w/v). The precipitate was dissolved in saline; dialysed to remove the ammonium sulphate; sterilized by filtration; and distributed in quantities of 5 ml per bottle.

## Appendix (viii)

## Barbitone buffer for immunoelectrophoresis

(Campbell et al., 1970)

| | |
|---|---|
| Sodium barbitone | 15.87 g |
| 0.1N HCl | 230 ml |
| Dist. water | 2 litres |

pH = 8.2; Ionic strength (I) = 0.05

## Appendix (ix)

## Culture medium for Staphylococcus aureus

(Arvidson, Holme & Wadström, 1971)

1. Casamino acids (Difco) 40 g
   Yeast extract (Difco) 10 g
   Sodium B-glycerophosphate (Sigma Chemical Co.) 20 g
   Sodium lactate (50% v/v) 10 ml
   $Na_2HPO_4 . 2H_2O$ 1 g
   $KH_2PO_4$ 0.4 g
   $(NH_4)_2SO_4$ 1 g
   dl-tryptophane 80 mg
   l-cystine 100 mg
   Agar (Davis) 1.5 g
   Dist. water 1000 ml

2. Vitamin stock solution:
   Nicotinic acid (B.D.H. - "microbiologically tested") 20 mg
   Thiamine hydrochloride solution, 100 mg/ 1 (Wellcome) 100 ml

3. Trace elements stock solution:

| | |
|---|---|
| $MgSO_4 . 7H_2O$ | 0.2 g |
| $MnSO_4 . 4H_2O$ | 0.1 g |
| $FeSO_4 1 7H_2O$ | 0.06 g |
| Citric acid | 0.06 g |
| Dist. water | 100 ml |

The bulk of the medium is sterilized at 120°C for 20 minutes. The vitamin stock solution (No.2) and the trace elements stock solution (No.3) are sterilized separately through a sterilizing type GA-6 triacetate membrane filter (Gelman Hawksley Ltd.). Two ml of solution No.2 and one ml of solution No.3 are added to each 100 ml of melted, cooled bulk medium. The medium is distributed in large sterile bottles with flat sides in sufficient volume to provide a layer on the 150 $cm^2$ area of the flat sides.

## Appendix (x)

## Coon's buffered barbitone saline for immunofluorescence

(Holborow, personal communication)

| | |
|---|---|
| Sodium barbitone | 10.3 g |
| NaCl | 42.5 g |
| 1N HCl | 40.3 ml |
| Dist. water | 5 litres |

pH = 7.2

## Appendix (xi)

## Mountant for immunofluorescence preparations

(Heimer & Taylor, 1974)

| | |
|---|---|
| Glycerol (AR grade) | 3 g |
| PVA (Polyvinyl alcohol grade 51-50 Elvanol) | 1.2 g |
| Dist. water | 3 ml |
| Tris buffer (0.0995 M, pH 8.5) | 6 ml |

Preparation of Tris buffer (0.0995 M, pH 8.5):

| | |
|---|---|
| Tris (hydroxymethyl) methylamine (B.D.H.) | 2.42 g |
| N-HCl | 1 ml |
| Dist. water to 100 ml | |

The Tris is dissolved in 95 ml of distilled water; N-HCl is added dropwise until the solution is pH 8.5; and the final volume is made up to 100 ml with distilled water.

Preparation of the mountant:

The glycerol is weighed in a Sterilin universal container with a conical bottom (Sterilin Ltd., Richmond, Surrey, England). PVA (Du Pont Co., U.K. Ltd., Du Pont House, 18 Breams Buildings, London, E.C.4) is added to the glycerol in the bottom of the container and stirred well. Spreading of the PVA onto the side of the container is avoided. When the glycerol and PVA are completely mixed, the water is added. Tris buffer is then added, and the universal container is placed in a water bath at 50°C for about 10 minutes. The mixture is agitated occasionally to dissolve the PVA. Finally, the mixture is cleared by centrifugation. The mountant has a good shelf life, remains clear, and does not thicken on storage.

## Appendix (xii)

## Assay of tetanus antitoxin in maternal and cord sera: a working-week protocol

Monday: Test cord sera for antitoxin levels by the indirect haemagglutination technique (IHA). Prior absorption of the serum samples is not necessary because non-specific agglutinins for sheep cells are absent from cord sera.

Read the results of the toxin neutralization test performed during the previous week.

Tuesday: Determine the IHA titres. Group sera according to the toxin test dose level required for their assay on the basis of these titres.

Perform more IHA tests.

Read the results of TN tests performed during the previous week.

Wednesday: Determine the IHA titres and group sera accordingly.

Perform TN tests at the Lp/10000 level. Since the results of Lp/10000 assays are assessed after five days, they will be read on the following Monday.

Thursday & Friday: Perform TN tests at the L+/4000 levels. Results of these tests will be read after four days i.e. on the following Monday and Tuesday.

The maternal sera are subsequently titrated by the TN test without initial determination of their antitoxin titres by the IHA test since it has been found that the TN titres for paired maternal and cord sera usually differ by no more than one twofold dilution interval. This procedure avoids the need for any serum absorptions prior to IHA testing.

## Appendix (xiii)

## Complete S-MEM (Suspension) medium

| | |
|---|---|
| S-MEM (Suspension) medium with Earles salts but without l-glutamine | 100 ml |
| HEPES (1M) buffer, pH 7.3 | 2 ml |
| Penicillin-Streptomycin solution (10000 U/ml & 10000 ug/ml resp.) | 1 ml |
| Kanamycin solution (10000 ug/ml) | 1 ml |

Final pH = 7.2/7.3

## Appendix (xiv)
## Minimum Essential Medium (MEM) with Earles salts

| | |
|---|---|
| 10 x concentration of MEM with Earles salts | 10 ml |
| Dist. water | 90 ml |
| $Na_2HCO_3$ solution (7.5%) | 1.5 ml |
| Penicillin-Streptomycin solution (10000 U/ml & 10000 µg/ml resp.) | 1 ml |
| Kanamycin solution (10000 µg/ml) | 1 ml |

pH before addition of sodium bicarbonate = 2.5 approx.
Final pH = 7.4 approx. (after aeration).

## Appendix (xv)
## Complete RPMI-1640 medium

| | |
|---|---|
| RPMI-1640 medium with HEPES buffer (20 mM) | 80 ml |
| Heat-inactivated foetal calf serum | 20 ml |
| l-glutamine | 1 ml |
| Penicillin-Streptomycin solution (10000 U/ml & 10000 µg/ml resp.) | 1 ml |
| Kanamycin solution (10000 µg/ml) | 1 ml |

Final pH = 7.2/7.3

## Appendix (xvi)
## Eosin solution for testing the viability of lymphocytes
(Terasaki, Vredevoe & Mickey, 1967)

| | |
|---|---|
| Eosin yellowish, water and alcohol soluble (C.I. No.45380 from Raymond A. Lamb, 6 Sunbeam Road, North Acton, London, N.W.10). | 0.5 g |
| Dist. water | 50 ml |

pH adjusted to 7.8

0.05 ml of cell uspension in 0.5 ml of tissue culture fluid (e.g. complete RPMI-1640) is added to 0.5 ml of the eosin solution to give a 1:20 dilution suitable for counting on a Haemocytometer. Non-viable

cells appear as swollen, red-coloured cells as a result of uptake of the dye.