

CHARACTERIZATION AND EPIDEMIOLOGY OF VIRUSES  
BELONGING TO THE AFRICAN HAEMORRHAGIC FEVER  
GROUP, WITH PARTICULAR REFERENCE TO  
MARBURG ~~TYPE~~ VIRUSES

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### Abstract

Between July and September 1976, widespread interest on an international scale was directed towards outbreaks of viral haemorrhagic fever which occurred in the southern Sudan and northern Zaire. This thesis is an account of the work carried out by the author with the assistance of three members of the staff of the Special Pathogens Unit at the Microbiological Research Establishment, Porton Down, Salisbury. It describes the successful isolation and identification of the aetiologic agent, and also describes the successful transmission of the disease to guinea pigs and monkeys. Characteristic pathological lesions were found in all the experimental animals. The agent produced characteristic intracytoplasmic inclusion bodies in tissue culture which could be detected by both conventional staining and by immunofluorescent techniques. Studies have shown that the agent although morphologically identical to Marburg virus, was antigenically distinct. In concurrence with other principle investigators, the name Ebola virus was proposed.

The disease induced in monkeys by infection with Ebola virus was very similar to that which occurs in man. Rhesus and Vervet monkeys therefore seem to be suitable experimental animals in which to study the pathogenesis of the disease and also to evaluate the various aspects of therapy.

Infection with the Sudanese strain of the virus appeared to be less virulent in both guinea pigs and monkeys compared with that of the Zaire strain.

Rhesus monkeys were treated with human leucocyte interferon prophylactically and after experimental infection with the Zaire strain of Ebola virus. Viracmia was delayed and clinically survival appeared to be enhanced. There was no consistent difference in the pathological changes or the outcome of the infection between the

treated and the untreated animals.

Epidemiological studies carried out on material from the Sudan, showed antibodies to Ebola virus which were detected by immunofluorescence in 42 of 48 patients in Maridi who had been diagnosed clinically, but only in 6 of 31 patients in Nzara. The possibility of the indirect immunofluorescent test not being sufficiently sensitive is discussed. Of Maridi case contacts in hospital and in the local community only 19% had antibodies. Very few of them gave any history of illness indicating that Ebola virus can cause a mild disease or even sub-clinical infection.

Detailed virological investigations were carried out on a patient infected with Ebola virus.

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INTRODUCTION

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### Introduction

Haemorrhagic fevers of viral origin have been recognised for centuries with Smallpox and Yellow fever causing untold numbers of cases and countless deaths. Many of the other viruses which produce haemorrhagic illnesses have only come to light in the last 30 years. Although often regarded as "new" viruses it is much more probable that they have existed in nature for years as "silent" enzootic foci. The means by which all these viruses are transmitted to man varies considerably; smallpox, for example, is transmitted directly from man to man generally through aerosols, Yellow fever is transmitted through the bite of a mosquito, Kyasanur Forest disease through tick-bite, while arenavirus infections are acquired through contact with rodents and their excreta. Casals et al. (1970) drew up a classification of the viruses causing haemorrhagic fevers based on their route of transmission. Since then other viruses have been either newly recognised or shown to cause bleeding complications. A more up-to-date classification of these viruses is shown in Table 1. Although this classification takes into account the usual route of transmission, some human infections have occurred by other means such as contact with sick animals (eg. Rift Valley Fever) or sick patients (eg. Lassa, Marburg).

Not all the viruses listed in Table 1 occur in Africa but the list of those which do (Table 2) is still extensive. Smallpox (Variola) has been excluded since the disease has been almost totally eliminated from the world as a result of the World Health Organisation's extensive and highly successful eradication programme. The only possible remaining pockets of activity are in the Horn of Africa particularly in Somalia and south-east Ethiopia (WHO, 1977, 1978) but the prevailing civil unrest in these areas may delay final verification that the disease has been completely eliminated. Fortunately the illnesses which have been reported appear to be due to the less virulent variola



Table 1. Epidemic viral haemorrhagic fevers of man classified by their routes of transmission

Person-to-person:

Smallpox (Variola)

Mosquito-borne:

Chikungunya haemorrhagic fever

Dengue haemorrhagic fever

Rift Valley fever

Yellow fever

Tick-borne:

Crimean haemorrhagic fever (includes viruses of the  
Haemorrhagic fever/Congo/Hazara complex)

Kyasanur Forest Disease

Omsk haemorrhagic fever

Zoonotic:

Argentinean haemorrhagic fever (Junin virus)

Bolivian haemorrhagic fever (Machupo virus)

Lassa fever

Haemorrhagic fever with renal syndrome

Unknown route of transmission:

Ebola fever

Marburg fever



Table 2. Viral haemorrhagic fevers of man which are known to occur in Africa

Mosquito-borne:	Rift Valley fever
	Yellow fever
Tick-borne:	Congo/Crimean Haemorrhagic fever
Zoonotic:	Lassa fever
Unknown route of transmission:	Ebola fever
	Marburg fever

minor form where haemorrhagic symptoms are uncommon.

Chikungunya virus was first isolated in Africa from mosquitoes and patients, by Ross (1956) during an epidemic in the Newala district of Tanzania in 1952-53. The illness was characterised by high fever, rash and crippling joint pains (Robinson 1955, Lumsden, 1955). The native name is derived from the main symptom, being "doubled-up" as a result of the excruciating pains in the joints. Since then chikungunya virus has been frequently isolated from man and mosquitoes during epidemics in India and south-east Asia as well as in eastern, western, central and southern Africa. In India and south-east Asia, chikungunya virus has often been implicated in outbreaks of haemorrhagic fever. During an epidemic in Madras Thiruvengadam et al. (1965) reviewed 242 cases of chikungunya fever and found 11% with haemorrhagic complications and Sarkar et. al. (1967) reported 11 patients in Calcutta with severe haemorrhagic fever including 2 deaths from whom chikungunya virus was isolated. Some doubt however surrounds the view that chikungunya virus can cause haemorrhagic disease particularly as most outbreaks have occurred when all four dengue virus serotypes have also been active. In Thailand Nimmannitya et al. (1969) suggested that chikungunya never caused the severe shock syndrome displayed in haemorrhagic fever caused by the dengue viruses. Bleeding complications have never been reported in outbreaks of chikungunya infection in Africa and because of this, the virus is excluded from the list shown in Table 2. Similarly, although dengue viruses have been isolated in West Africa (Carey et al. 1971), no conclusive evidence that they cause haemorrhagic illness there has been presented and so they too are excluded from Table 2.

Rift Valley fever until recently has been regarded as a severe disease of sheep and cattle occurring particularly in South Africa where the virus is transmitted by Aedes caballus and Culex theileri.

Human involvement was generally fairly sporadic and the illness was most usually acquired through direct contact with sick or dead animals. The virus was first isolated in Kenya in 1930 by Daubney et al. (1931) and large epizootics have been regularly reported there since. South Africa has had the largest epizootics, 100,000 sheep and cattle dying in 1951. During the summer and autumn of 1974-75 several hundred human infections were reported during another huge epizootic (Van Velven, et al. (1977) and B.M. McIntosh, personal communication). Most of the patients were farm workers and veterinary surgeons who acquired their infection while cutting open carcasses and handling tissues of animals which had died from the disease. Seven patients died following the development of purpura, epistaxis, haematemesis and melaena accompanied by profuse gastrointestinal bleeding associated with extensive necrosis of the parenchymal cells in the liver. Thirty other patients developed central blindness associated with retinitis and a cotton wool exudate on the macula and several had permanent damage. At least one further patient developed encephalitis. The largest outbreak of human infection occurred in the last quarter of 1977 in lower Egypt (WHO, 1978). Unofficial estimates put the number of human cases as high as 20,000 with at least 90 deaths. (D.I.H. Simpson, personal communication). The outbreak involved the Governates of Sharquiyah, Qualyubiya and Giza between September and November, 1977. Retrospective investigations have shown that the human outbreak was preceded by an even more devastating epizootic in domestic cattle and sheep and also involved camels and water buffaloes. There is evidence, too, of extensive involvement of cattle and sheep in upper Egypt as early as July, 1977 but the disease was not recognised at first as Rift Valley fever and the disease has never been recorded there previously. It is thought that Culex fatigans acted as the mosquito host but many human infections were undoubtedly acquired through

direct contact with sick animals. There is as yet no information about the original source of the outbreak.

Yellow fever remains today as the most important cause of haemorrhagic disease in Africa. The exact extent of the enormous Ethiopian epidemic between 1960 and 1962 will never be known but Serie et al. (1964, 1968) estimated that there were between 15,000 and 30,000 deaths. There were several thousand cases and several hundred deaths in the Senegal epidemic of 1965 (Chambon et al. 1967) and smaller outbreaks have occurred more recently in Angola, Nigeria and several West African countries (WHO, 1976).

The first reported outbreak of what was later to be known as Crimean haemorrhagic fever took place among Russian troops helping with the harvest in the steppe region of western Crimea in 1944 (Chumakov, 1945). As well as isolations from human acute blood, strains were isolated from Hyalomma marginatum marginatum (Grobov, 1946). The first African isolate, known as Congo virus was made by Courtois in 1956 in what was then the Belgian Congo from the blood of a febrile child. Further isolations have shown the virus to be widespread in East and West Africa (Simpson et al. 1967; Causey et al. 1970). It was not until 1969 that Casals showed that the Crimean haemorrhagic fever and Congo viruses were identical and were partially related to another virus, Hazara, isolated in Pakistan (Begum et al. 1970). A syndrome similar to Crimean haemorrhagic fever has been described more recently from other areas of the Soviet Union and from Bulgaria and Yugoslavia. Cases generally occur in rural areas with peak incidence in June and July. Illness is usually severe with bleeding a common feature. The mortality rate is often as high as 30-50 per cent. This severe illness is in sharp contrast to the pattern of disease in Africa where haemorrhagic phenomena and deaths have only rarely been reported (Simpson et al. 1967).



Lassa fever is perhaps the most publicised of all the viral haemorrhagic fevers with a case fatality of 36-67 per cent among hospitalised patients. Twenty one medical workers are known to have been infected, of whom 10 had died. Lassa, like Marburg and Ebola viruses, once it has been successfully transmitted from its natural reservoir host to man, is capable of adaptation to produce man-to-man transmission. Thus it is theoretically possible for such an infection to be introduced from its country of origin to countries where the natural host does not exist and still be capable of successful transmission. Hence the considerable world-wide public health concern. Lassa virus was first isolated from an American missionary nurse following the sequential infection of two other nurses in Nigeria (Frame et al. 1970). The virologist who made the isolation in turn became infected. A seasonal outbreak of Lassa fever with a high mortality of 53 per cent among 23 patients admitted to hospital was reported in Jos, Nigeria in 1970 (White, 1972). In March, 1972 further cases of Lassa fever occurred among 4 patients and 7 members of staff in a hospital in the Zorzor district of Liberia, and 4 patients died (Monath, 1973). The fourth major outbreak was not hospital associated and took place between October, 1972 and October 1974 in and around Panguma township, Sierra Leone. There were 64 cases most of whom acquired their infection in the community. Several family outbreaks occurred. It was following this outbreak that investigators showed that the multimammate rat Mastomys natalensis is the possible reservoir host of Lassa virus (Monath et al. 1974). Lassa fever has continued to occur in West Africa, but usually as sporadic cases. Quite often medical personnel are those who manifest the disease presumably being infected through contact with a febrile patient. Lassa virus infections appear to be much commoner than was hitherto supposed and evidence for this has been supplied by Dr J.B. McCormick (personal communication) working in Sierra Leone. In an endemic area of Lassa virus activity he has found that almost

half of patients presenting with febrile illness have Lassa infection but very few develop severe disease.

The mode of transmission of Lassa virus from rodent to man or from man-to-man is not yet known. Similarly, the pathogenesis of Lassa virus infection in its natural host is still not understood. It is, however, reasonable to expect that Lassa, like some other members of the arenaviruses group, induces a chronic carrier state in its natural host and a persistent infection develops leading to both horizontal and vertical transmission. Rodents, such as Mastomys natalensis, may excrete the virus in urine and saliva and may thus contaminate food and water. Monath et al. (1974) suggested that the low level of sanitation, the storage of grain and food within houses and the ease with which rodents infect mud and thatch houses enhance the contact between rodents and man. The means by which the virus is spread from person-to-person is still not clear. Medical attendants or relatives providing direct personal care are most likely to contract the infection. Accidental inoculation with a sharp instrument or contact with blood has accounted for a few cases. Lassa virus has been isolated from the blood, pharynx and urine of patients (Buckley and Casals, 1970) so that indirect airborne spread of the virus as well as mechanical transmission are most likely. Virus has been isolated from patients' urine during convalescence indicating that the virus can persist after the acute illness has receded.

Lassa fever, like two other severe arenavirus infections (Junin and Machupo) often presents with a slow insidious onset and the non-specific early symptoms makes clinical diagnosis difficult. Later severe prostration, pyrexia, pharyngitis and tonsillitis with whitish exudative lesions and small vesicles and ulcerations, conjunctival infection, and occasionally a faint maculo papular rash become evident. In severe cases haemorrhages also occur. Although secondary cases are common, tertiary cases are rare.

Marburg virus disease (Martini and Seigert, 1971) is a severe distinctive haemorrhagic febrile illness first described in 1967 when 31 cases of illness with 7 deaths in Germany and Yugoslavia were traced to direct contact with blood, organs or tissue cell cultures from a batch of African green monkeys (Cercopithecus aethiops) which had been trapped in Uganda. Several secondary cases occurred in hospital personnel by contact with patients' blood. One further case was apparently transmitted by sexual intercourse 83 days after the initial illness, and virus was isolated from the semen. The case fatality rate was 29% for the primary cases but there were no deaths in the 6 secondary cases. The virus isolated from patients during the outbreak was quite distinct from any other known animal virus (Almeida et al. 1971; Peters et al. 1969).

The first recognised outbreak of Marburg disease in Africa and the first since the original 1967 outbreak occurred in south Africa in February 1975 (Gear et al. 1975). The primary case was a young Australian man who had hitch-hiked through Rhodesia. He died in a Johannesburg hospital and shortly afterwards his girl travelling companion and a nurse who had cared for him fell ill with the same disease. Both girls recovered. Virological studies showed that this outbreak was caused by Marburg virus. Again there was evidence of virus persistence in the body when virus was cultured from fluid aspirated from the anterior chamber of the eye 80 days after the onset of illness.

Between August and November, 1976 outbreaks of severe and frequently fatal viral haemorrhagic fever occurred in the equatorial provinces of Sudan and Zaire causing widespread international concern (WHO, 1976). In Nzara, Sudan there were 70 cases of which 33 died and in Maridi, also in the Sudan, the epidemic caused 229 cases, 117 of which were fatal. 76 members of the staff of 230 in Maridi hospital were infected and 41 died. In Zaire, the number of cases was 237

including 211 deaths.

The illnesses recorded with both Marburg and Ebola viruses are virtually indistinguishable. Both infections have always had an abrupt onset with severe frontal and temporal headache, followed by high fever, generalised pains particularly in the back. The patients rapidly became prostrated and soon developed severe watery diarrhoea which led to a rapid weight loss and dehydration. Diarrhoea, abdominal pain and cramping, nausea and vomiting often persisted for a week (Simpson, 1977). In the Sudanese outbreak knife-like chest and pleuritic pain was an early symptom and many patients complained of a very dry, rather than sore throat, accompanied by cough. On white skins a characteristic maculopapular rash (Figure 1) appeared between days 5 and 7 (Martini, G.A. 1969; Gear et al. 1975; Edmond et al. 1977). Lasting 3-4 days it was followed by a fine desquamation. On black skins the rash, often described as being "like-measles" was not so obvious and was often only recognised later with the appearance of skin desquamation (WHO, 1978). Conjunctivitis was a regular feature in all the outbreaks. An exanthem of the palate was reported from Germany but was not seen in South Africa. In Sudan a pharyngitis was commonly noted and the throat was found to be dry and accompanied by fissuring and open sores on the tongue and lips. Patients were generally admitted to hospital on the 5th day of illness and their general appearance was described as "ghost-like" with drawn anxious features, expressionless faces, deep-set eyes, a greyish pallor and extreme lethargy.

A large number of cases in both Marburg and Ebola outbreaks developed severe bleeding between days 5 and 7. The gastro-intestinal tract and lungs were most frequently involved with haematemesis, melaena and sometimes the passage of fresh blood in the stools. There was also bleeding from the nose, gums and vagina and subconjunctival haemorrhages were common. Petechiae and bleeding from needle puncture

Figure 1. Petechial skin rash on the buttocks of an Ebola patient  
(Courtesy of Dr R.T.D. Emond)

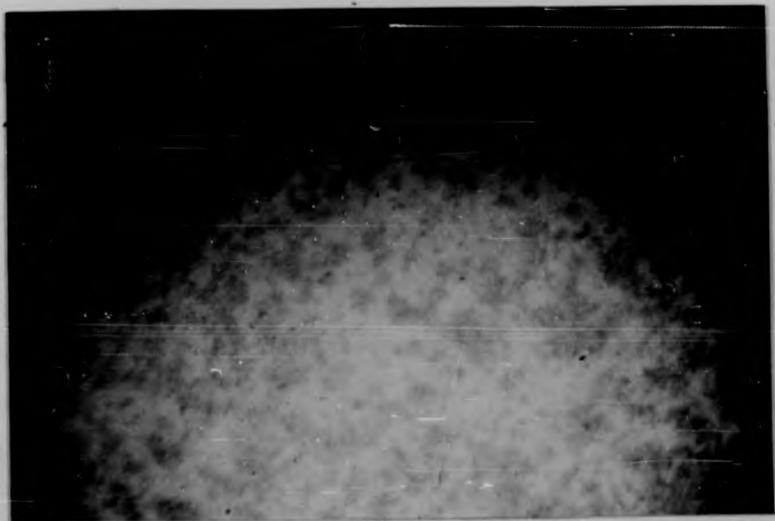


Figure 1. Petechial skin rash on the buttocks of an Ebola patient  
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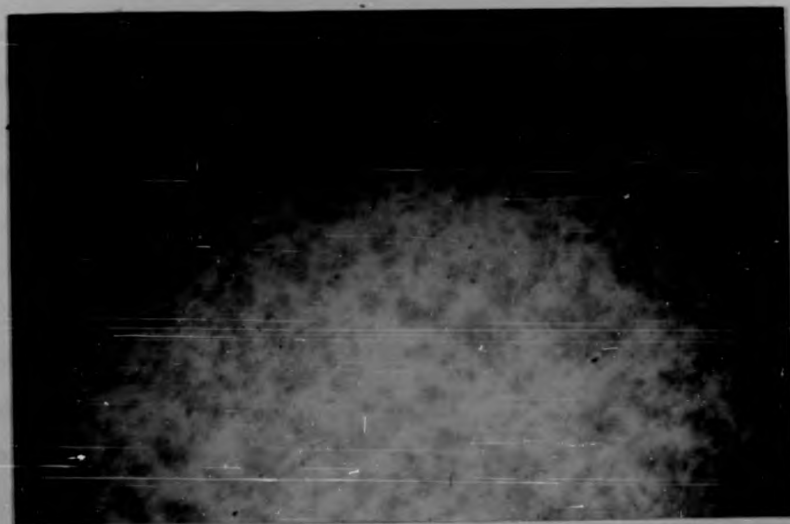




Figure 1. Petechial skin rash on the buttocks of an Ebola patient  
(Courtesy of Dr R.T.D. Emond)



sites were very common. Death generally occurred between days 7 and 16 usually preceded by severe blood loss and shock.

The virus strains isolated from both Zaire and Sudanese patients (Bowen et al. 1977; Pattyn et al. 1977; Johnson et al. 1977) were found to be morphologically identical to Marburg but antigenically distinct (Johnson et al. 1977). The name Ebola was given to the new strain of virus.

The Ebola virus outbreak in the Sudan was centred on the township of Nzara (20,000 inhabitants) and Maridi (15,000 inhabitants) in the Western Equatorial Province. In Nzara the first probable case fell sick on 27th June, 1976 and died on 6th July, 1976. He worked as a cloth storekeeper in a cotton factory in the town. Two weeks later a second storekeeper became ill and this was followed in a further two weeks by a third factory employee becoming ill. One of the contacts of the third case fell ill and was admitted to Maridi hospital where he died on the 17th August, 1976. Contacts with this patient in Maridi led to an enormous epidemic there.

During August, September and early October cases continued to occur among employees of the factory in Nzara. They in turn infected their families and friends. The last recorded case of infection in Nzara had an onset of illness on 10th October, 1976 by which time there had been 70 cases and 33 fatalities in the town. Maridi hospital meanwhile had become the centre of a large outbreak, the hospital acting as an amplifying factor of the disease. During the first two weeks of September several cases occurred in Maridi with 9 deaths. In the second half of September the epidemic became severe with 76 cases of infection and 42 deaths. There was a fall in the number of cases in early October, but later in the month the outbreak increased in severity. Stringent control measures brought into effect by the WHO/Sudanese team in late October brought the outbreak under control, the

last recorded case of infection being admitted to hospital on 25th November, 1976. The infection spread swiftly but only through close and prolonged, and usually nursing, contact with an active case. Health personnel in particular were involved through contact with patients' blood. When good nursing techniques, supplemented by the use of protective clothing, were introduced the number of contact infections fell dramatically.

The cotton factory in Nzara received particular attention from the WHO team as it appeared to be a common source of several Ebola virus infections during the July-October period. A large number of arthropods (mosquitoes and ticks), rodents, bats and other small mammals were collected during the investigations and blood and tissues removed for virological studies.

In Zaire the first recognised case occurred during the first week of September 1976 and is thought to have originated at a small mission hospital in Yambuku north of Yandonge Equateur Province. Parenteral injections may have played a role in transmission in this area. Patients infected in the hospital environment probably carried the infection back to their home villages, setting up new pockets of infection in their homes. The source of the infection in Zaire remains unknown but it may have been introduced to Yambuku by a patient presenting at the outpatient clinic with a non-specific febrile illness.

As the author had particular responsibilities at the Microbiological Research Establishment for the handling and containment of dangerous viral pathogens, it was originally conceived that work for this thesis would be concerned with certain aspects of research on Lassa virus or other viruses requiring this type of containment. Studies began on some of these viruses in 1975 but were suddenly interrupted by the enormous Ebola virus outbreak in the Sudan and Zaire. Such was the extent of international concern that the Special Pathogens Unit at the Microbiological Research Establishment was requested by the World

Health Organization to handle all the specimens emanating from Zaire and the Sudan in collaboration with the Centre for Disease Control, Atlanta, U.S.A. and the Prince Leopold Institute for Tropical Medicine, Antwerp. As a result the Unit's activities were directed entirely to studies on Ebola virus. The author's involvement was compounded further when, during laboratory investigations on Ebola virus, a member of the laboratory staff accidentally infected himself and contracted the disease (Emond et al. 1977). This left the author with no alternative but to devote his entire effort to work on Ebola virus, and as a result this thesis is necessarily almost entirely devoted to studies on Ebola virus.



Isolation attempts and human serological studies

INOCULA. Specimens from the northern Zaire outbreak were referred to the Microbiological Research Establishment, Porton, by Professor S.R. Pattyn of the Institute of Tropical Medicine, Antwerp. They were an acute-phase serum (No. 718), cell-culture material and brains from suckling mice which had already been inoculated with the serum. Later we received a specimen of liver from the same patient and also 5 acute-phase blood specimens from Zaire via Professor Pattyn. Specimens from the southern Sudan were collected by Dr D.I.H. Simpson, Dr Babiker el Tahir, Dr D.H. Smith, Dr K. Jones, Dr M. Cornet and Dr D.P. Francis who were there to investigate. They consisted of throat swabs, urine specimens, acute-phase blood specimens and convalescent serum specimens. These specimens were sent on dry ice or in liquid nitrogen.

Animals:

- a) Guinea pigs of the Dunkin-Hartley strain weighing approximately 250-300 grams.
- b) Vervet (*CERCOPITHECUS AETHIOPS*) monkeys and rhesus (*MACACA MULATTA*) monkeys of either sex.
- c) Porton white mice aged 2 days and adults aged 3-4 weeks.

Tissue culture: African green monkey cell line (Vero), baby hamster kidney cell line (BHK 21), rhesus monkey kidney cell line (LLC-MK2) and a primary human liver cell line. These were used as stationary monolayer cultures in either Corning 25 sq cm flasks or in Nunc plastic tubes 100 x 14 mm and maintained in either the Pirbright modification of Eagles medium (Capstick et al. 1962), Leibovitz medium (L15) or 199 with various concentrations of foetal calf serum.

Complement fixation test using the microtitre method of Sever (1962)



with a final erythrocyte concentration of one per cent and two complete units of complement in the test. The antigens were either:-

- a) infected guinea pig serum collected during the acute-phase of the infection (days 5 or 6).
- b) centrifuged suspension of infected guinea pig liver or spleen in complement fixation test diluent then inactivated with either 1/1000 betapropiolactone or 0.25 per cent formal saline and
- c) infected tissue culture medium inactivated with either 1/1000 betapropiolactone or 0.25 per cent formal saline.

These antigens were tested against specific Lassa, Marburg and Congo virus antisera.

Haemagglutination-inhibition tests were carried out by the microtitre technique described by Sever (1962) using sucrose-acetone extracted antigens prepared as described by Clarke and Casals (1958) from the following viruses:- (Chikungunya, O'nyong-Nyong, Yellow fever (17D), West Nile, Dengue 1 and 2, Bunyamwera, Germiston and Tick-borne encephalitis). The human sera tested were from early convalescent cases from the Sudan. All the sera were first inactivated at 56°C for 1 hour and then extracted with Kaolin and goose cell absorbed as described by Clarke and Casals (1958). Sucrose-acetone extracted antigens were also prepared from acute-phase human and guinea pig sera and from infected guinea pig liver and tested against the following specific virus antisera:- (O'nyong-Nyong, Chikungunya, Bunyamwera, Germiston, Pongola, Ntaya, West Nile, Spondweni, Witwatersrand, Wesselsbron, Yellow fever (17D), Nyando, Bwanba, Tick-borne Encephalitis, Banzi, Zika, Dengue 1 and 2, Uganda S., Rift Valley fever).

Virological methods. Suckling and adult mice were inoculated a) intracerebrally and b) intraperitoneally with 0.02 ml and 0.03 ml

Figure 2 to 7 show the cabinet layout in the maximum containment laboratory at the Microbiological Research Establishment.



Figure 2



Figure 3

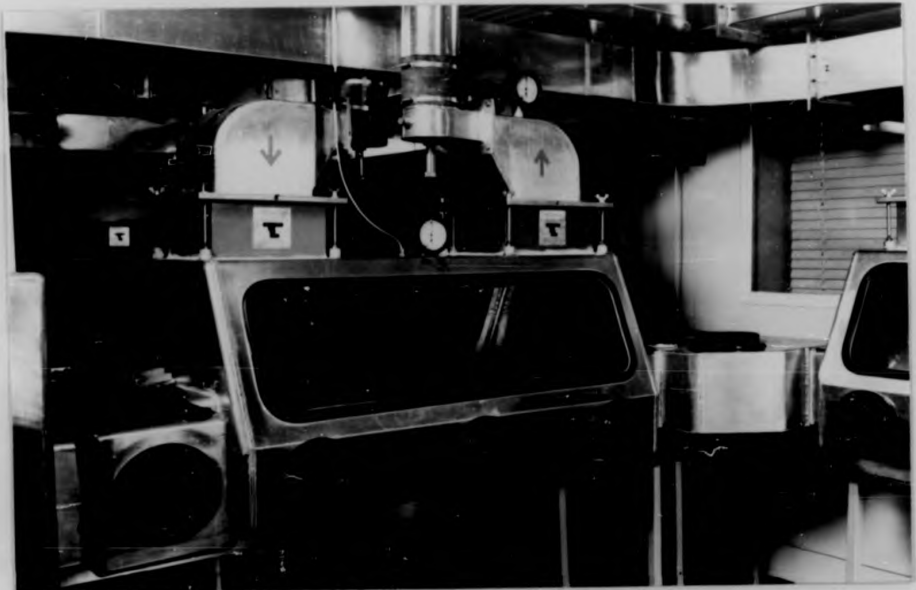


Figure 4

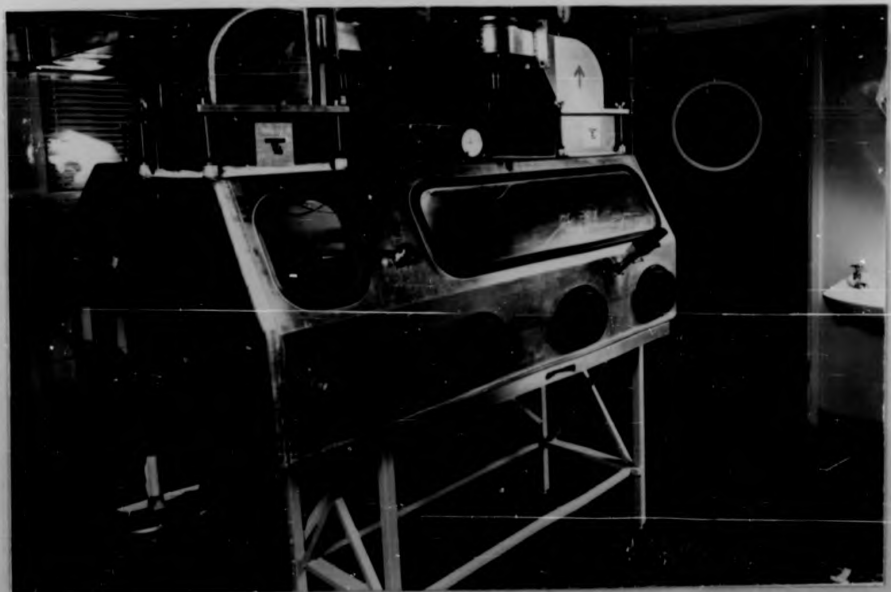


Figure 5

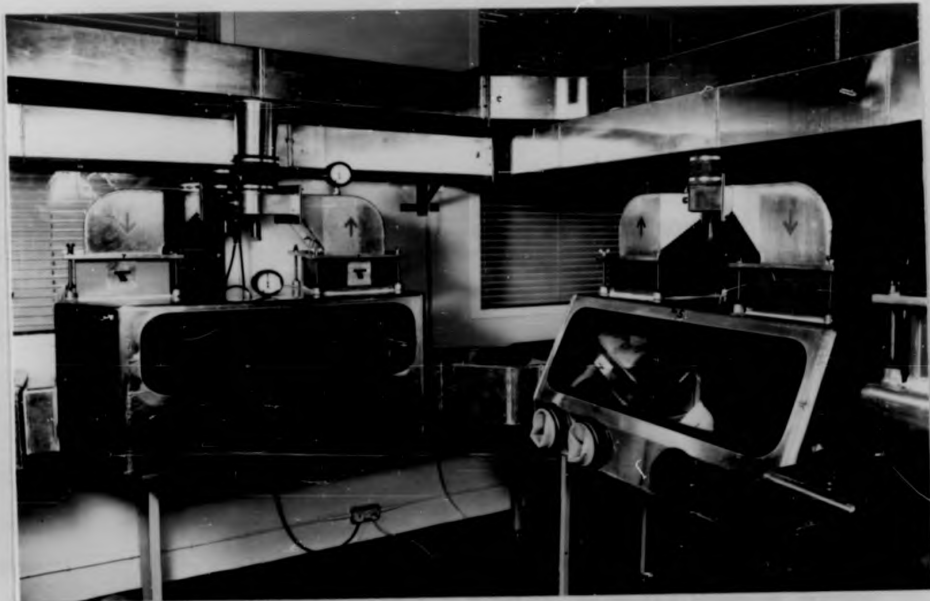


Figure 6



Figure 7

at least 24 hours.

Air is filtered into and out of each of the cabinets and is again filtered before it leaves the trunking at the end of the system. Material is passed into the system through a double-doored autoclave and is passed along from cabinet to cabinet through inter-connecting portholes.

Within the cabinet system are housed a small centrifuge, +4°C refrigerator, 2 water baths, one operating at 37°C and the other at 60°C, 2 microscopes and 2 incubators both operating at 36.5°C. Specimens are brought out of the system for storage purposes through a dunk tank containing 5% hypochlorite solution. The air pressure in the room itself is at a negative pressure gradient, through the changing rooms to the outside corridor. The air is filtered before it leaves the room and there is also a battery operated back-up system for the room in case of a power failure.

Animal experiments were carried out in a suite of rooms with entry and exit through an airlock and shower. Personnel wore protective clothing, gloves, rubber boots and full-face biological respirators and hoods.

All effluent from this suite of rooms was sterilised by boiling and extracted air was filtered. Before leaving infected animal rooms personnel swabbed down their respirators with hypochlorite solution and discarded their gowns and hoods into a metal bin. Boots were thoroughly washed down in hypochlorite solution and the rubber gloves were removed and left to soak in the hypochlorite solution. Having left the infected rooms the remaining clothing was removed in the airlock and placed in a metal bin. Boots and respirators were stored in lockers and the personnel then showered completely before returning to the clean changing area.

#### Studies in guinea pigs

Apart from the original inocula guinea pigs were inoculated with

the following:-

- a) Whole heparinised guinea pig blood taken during the febrile stages of disease at various passage levels.
- b) Whole heparinised monkey blood taken during the febrile stages of illness.
- c) Ten-fold dilutions of suspensions of tissues removed from infected guinea pigs, monkeys and mice.
- d) Monkey urine and rectal swab material.
- e) Arthropods, blood and tissues from various small mammals collected in the Sudan.
- f) Cells and medium from various tissue culture systems.

Guinea pigs were bled under ether anaesthesia by intracardiac puncture. They were inoculated intraperitoneally with 0.1 ml amounts.

#### Studies in monkeys

The animals used were young adult vervet (Cercopithecus aethiops) and rhesus (Macaca mulatta). The monkeys were held in individual metal cages, measuring 26" x 40" x 18".

Handling procedures. Monkeys were handled only under sedation with intramuscular injection of ketamine hydrochloride (Vetalar: Parke, Davis) 60 mg. They were bled from the femoral vein before inoculation with infective material. Daily rectal temperatures were recorded and 2 ml of heparinised blood samples were collected daily by femoral venepuncture and inoculated intraperitoneally into guinea pigs to detect circulating virus. Urine samples were collected aseptically at autopsy by bladder aspiration with syringe and needle and similarly tested for virus.

Necropsy was carried out on all monkeys shortly after death and various tissues removed for virological studies, histopathology and electron microscope studies. Suspension of heart, lung, liver, spleen, kidney, adrenals, pancreas, testes, bile, urine and faeces were prepared in phosphate buffered saline (pH 7.2) containing 0.75% Bovine



plasma albumin (Armour Fraction V), penicillin and streptomycin and inoculated into guinea pigs.

#### Inocula

Monkeys were inoculated with 3rd and 4th passage of infected guinea pig liver suspensions. The actual dose of the inoculum was calculated in each case by parallel intraperitoneal titrations in guinea pigs and expressed as guinea pig infectious units (G.P.I.U.)/ml.

#### Studies in mice

Porton white mice were used a) as 2 to 3 days old and b) adult mice aged 21 to 28 days.

#### Inocula

- a) Human acute phase serum.
- b) 2nd passage guinea pig liver suspension.
- c) Infected tissue culture medium.

#### Histological examination

Guinea pigs were killed with ether, monkeys with intravenous nembutal. Organs were removed immediately after death and portions of the following tissues were removed:- tongue, trachea, lung, liver, spleen, kidneys, adrenal glands, testes, myocardium, skeletal muscle, stomach, duodenum, ileum, caecum, colon, pancreas, salivary glands and lymph nodes. Since this disease is readily transmitted to man via skin wounds with very serious consequences the brain and spinal cord were not removed because of the risk of piercing a glove on a sharp piece of bone. Clinical neurological signs were absent in the monkeys, and therefore the risk did not seem justified. Tissues were fixed in 10 per cent buffered neutral formalin and embedded in paraffin wax. Sections were cut as 5  $\mu$ m stained by haematoxylin and eosin (H & E) and selected sections stained with either the Verhoeff-van Giesb method, Machiavello's technique for inclusion bodies, by the periodic acid-Schiff (PAS) and Gordon and Sweet's method, and with Mallory's phosphotungstic acid-haematoxylin (PTAH).



#### Stock seed

3rd, 4th, 5th and 6th passage guinea pig liver suspensions prepared by emulsifying in a Ten Broeck grinder as a 20 per cent suspension in phosphate buffered saline pH 7.2 containing 0.75% bovine albumin (Armour Fraction V).

#### Immunofluorescent studies

In tissue culture:- The indirect immunofluorescent test used was similar to that described by Wulff and Lange (1975) for Lassa fever. Vero cells were prepared in 25 cm<sup>2</sup> plastic flasks (Nunc) and maintained in modified Eagles medium or Leibovitz medium (L15) containing 2% foetal calf serum at pH 7.2-7.4. Each flask was inoculated with 1 ml of Ebola virus as a 10% guinea pig liver and spleen suspension. The medium was changed on the second day to reduce toxicity caused by using guinea pig tissue. As indicated by daily immunofluorescence antibody (IFA) testing of replicate preparations, flasks were incubated at 37°C until 30-40% of the cells had become infected, usually at 6-7 days.

For slide preparation media was first decanted off the infected cells and the cell sheet washed three times in a pH 7.2-7.4 phosphate buffered saline (PBS). The cell sheet was detached with 2 ml of 0.2% trypsin in versene; the cell suspension was removed and diluted in an equal volume of PBS containing 3% calf serum to inactivate the trypsin/versene activity. The suspension was centrifuged at 1000 rpm for five minutes, the supernatant discarded, and the cells resuspended in PBS. This procedure was repeated twice and the cells were finally suspended in PBS containing 0.2% of bovine serum albumin to a concentration of approximately  $5 \times 10^5$  cells/ml. The suspension was seeded into the wells of polytetrafluoroethylene coated glass slides each having 12 x 6 mm wells. Each well received one drop of cell suspension. The slides were allowed to dry under an ultraviolet lamp for 20 minutes followed by 5 minutes fixation in chilled acetone.

Sera were examined for the presence of Ebola virus antibodies by an indirect immunofluorescent method (Wulff and Lange, 1975). Duplicate dilutions of all test sera were made in PBS and sera were screened at dilutions of 1:4 and 1:8. Known human positive and negative sera were included as controls and PBS controls similarly used. All test and control sera were screened on both infected and uninfected slides. Diluted sera were placed in slide wells and held in a moist chamber at 37°C for 30 minutes and then washed in PBS for 5-10 minutes. Slides were then dried in air and a drop of a) Fluorocein-labelled rabbit anti-human IgG conjugate (Wellcome) at dilutions of 1:12-1:20 or b) Fluorocein-labelled rabbit anti-guinea pig IgG conjugate at dilution of 1:12-1:20 applied to all wells. The slides were again held at 37°C in a moist chamber for 30 minutes and then washed three times in PBS for 2-4 minutes on each occasion and finally rinsed in distilled water for 30 seconds. Slides were then dried in air and examined under a Reichert Fluorvar microscope using an HBO 50 w mercury vapour burner. Sera were only accepted as positive if clear fluorescence was observed at a dilution of 1:8. Sera which were positive only at a dilution of 1:4 were regarded as equivocal and graded as negative.

#### Electron microscope studies

##### Negative staining

Studies in infective guinea pig and monkey blood. Blood appeared to be one of the richest sources of the virus in body fluids with titres of approximately  $10^{6.5}$  G.P.I.U./ml at the height of the viraemia (Day 5-6). Six days after infection blood was obtained from guinea pigs and monkeys by cardiac puncture using heparin as an anticoagulant. The blood was immediately centrifuged at 2000 rpm for 5 minutes. Plasma was pipetted off and mixed with an equal volume of 4% formalin in distilled water and held at room temperature for 24 hrs.

Small drops of the fixed plasma were placed on specimen grids coated with formvar: left for 2-3 minutes then the excess removed with filter paper. Drops of 2% ammonium molybdate at pH 7.0 were then placed on the grids, the excess being removed with filter paper. The grids were then stabilised with a film of evaporated carbon and examined in a Philips EM 300 electron microscope using double condenser illumination and an accelerating voltage of 80 KV.

Negative staining of tissue culture derived material. Monolayers of Vero cells in Corning 25 sq cm flask were infected with human acute phase blood (E 718). Seven days after infection cell-culture fluid was mixed with an equal volume of 4% formalin in distilled water and held at room temperature for 24 hrs. This was then treated as for fixed plasma and examined in a Philips EM electron microscope.

Ultrathin section material

- a) Livers from guinea pigs infected with the 1st guinea pig passage material of the Zaire strain of Ebola virus E 718 were removed on the 5th post inoculation day. 1-2 mm cubes of the liver were fixed in 1% osmium tetroxide. After subsequent dehydration the fixed liver pieces were embedded in "epikote". Ultrathin sections were prepared on a Reichert U2 ultramicrotome and stained with uranyl acetate and lead citrate. Preparations were examined in Phillips EM 300 electron microscope.
- b) Livers from rhesus monkeys infected with 3rd guinea pig passage material of the Zaire strain of Ebola virus E 718.
- c) Spleens from rhesus monkeys infected with 3rd guinea pig passage material of the Zaire strain of Ebola virus E 718.
- d) Lungs from rhesus monkeys infected with 3rd guinea pig passage material of the Zaire strain of Ebola virus E 718.

Cubes of 2-3 mm were cut from all four organs and placed in phosphate-buffered glutaraldehyde in sealed containers. Forty eight

hours later 1 mm cubes were cut from these and placed in fresh phosphate-buffered glutaraldehyde. These were later washed in repeated changes of Palade/sucrose buffer pH 7.4 for 18 hours. The washed specimens were then part-fixed in 1% osmium tetroxide in the same buffer for two hours and washed for a similar time in distilled water. Dehydration was done in graded methanols, specimens spending 30 minutes in 3% uranyl acetate in 30% methanol. Embedding was in araldite, via graded propylene oxide mixtures. After polymeration for 48 hours at 60°C, sections were cut on a Cambridge Huxley, ultramicrotome and stained with lead nitrate before viewing on either a Zeiss E.M. 9A or AEI EM 801 (for the higher magnifications).

#### Epidemiological studies

Acute Samples. Blood, throat swabs, and urine samples were collected within five days of the onset of illness from eight acutely ill patients in Maridi Hospital isolation wards. All eight patients were febrile, grossly dehydrated and displayed many of the clinical features described (WHO, 1978 in press).

Blood samples were collected using disposable syringes and needles and immediately injected into vacutainer tubes. Throat swabs were placed in sterile saline in screw-capped polypropylene serum tubes (Nunc); urine samples were collected from bed-pan samples in similar polypropylene tubes.

After clotting, blood was centrifuged in sealed vacutainer tubes in the field laboratory and serum aspirated off into polypropylene tubes. All acute samples were stored in liquid nitrogen prior to dispatch to the Microbiological Research Establishment, Porton.

Post-mortem samples. Small pieces of liver, spleen, lung, kidney and bone marrow were collected in polypropylene tubes and stored in liquid nitrogen.

Convalescent plasma. Aliquots of immune plasma were collected using a modified plasmaphoresis technique. As no centrifuge was available plasma and cells were separated by gravity only over a 2-3 hours period.

Sera from contacts. Blood samples were collected using disposable syringes and needles and stored before centrifugation in sealed vacutainer tubes. In Nzara samples were collected in the cotton factory and in the homesteads around the township; in Maridi samples were collected in the hospital grounds, schools and surrounding homesteads. After centrifugation sera were stored in polypropylene tubes and stored at  $-5^{\circ}\text{C}$ . For despatch they were placed in liquid nitrogen and stored at the Microbiological Research Establishment at  $-20^{\circ}\text{C}$  until tested.

Arthropods and small mammal tissues: A large number of arthropods (mosquitoes and ticks), rodents, bats and other small mammals were collected during the investigation, blood and tissues were removed for virological studies.

Virological investigation on a case of Ebola virus infection: The first specimen of blood was collected 14 hours after the patient became feverish. This was examined by electron microscopy and also inoculated into guinea pigs. Virological investigations were carried out on daily samples of blood collected during the acute-phase of the illness and on blood, faeces, throat swabs, urine and seminal fluid collected during the convalescent phase of the illness.

Fixation and staining of tissue culture cells. Monolayer cultures of baby hamster kidney (BHK 21) and Vero cells maintained in Pirbright modification of Eagle's medium (Capstick *et al.* 1962) were grown on coverslips in Leighton tubes. Cultures were infected with infected guinea pig plasma and maintained at  $37^{\circ}\text{C}$ , medium being changed after 18 hours. Cultures were fixed for 2 hours in 5% picric acid in methyl alcohol and stained with a modification of Picro-Mallory (Zlotnik, 1952).

Cross challenge studies in guinea pigs. Guinea pigs were immunized with low guinea pig passage material of the following viruses, Marburg 67

(Pass 1), Zaire prototype E 718 (Pass 2) and the Sudan strain Boneface (Pass 2). Most guinea pigs survived the initial infection, these were re-challenged with the same virus after a period of three weeks. After a further period of three weeks, specific antibody levels were estimated on each guinea pig and the following cross challenge test carried out.

Immunising Virus	Challenging Virus		
	Marburg 67	E 718	Boneface
Marburg 67			
E 718			
Boneface			

#### Ebola virus inactivation studies

Ether inactivation: Equal volumes of infected guinea pig liver suspension and diethyl ether were mixed together and held at +4°C for 1 hour. The mixing was repeated at 15 minute intervals.

Thermal inactivation: A 2 ml volume of infected guinea pig liver suspension was sealed in a glass ampoule and immersed in a 60°C water bath. A sample was removed at 60 minutes, immediately chilled in an ice bath and then titrated in guinea pigs.

#### Interferon studies

Animals: These were young adult rhesus monkeys (*Macacca mulatta*) of either sex weighing between 3 and 4 kgms. The monkeys were anaesthetised by intramuscular injection of ketamine hydrochloride for sampling of blood and for inoculation. Rectal temperatures were recorded daily.

Virus inoculum: The virus was the prototype strain E 718 isolated from the patient who died in Zaire from Ebola haemorrhagic fever during the epidemic in 1976. The virus was passaged by several intraperitoneal inoculations in guinea pigs. The inoculum was prepared as a 10% suspension in phosphate-buffered saline pH 7.2 containing 0.75% bovine albumin, penicillin and streptomycin from a guinea pig liver in the

third guinea pig passage. The suspension was diluted and the dose of virus calculated by parallel titration in guinea pigs and expressed as guinea pig infective units. The monkeys received  $10^4$  guinea pig infective units in 0.4 ml amounts by intraperitoneal inoculation.

Human leucocyte interferon: Interferon was kindly supplied by Professor K. Cantell from the Central Public Health Laboratory, Helsinki. This was produced by induction with Sendai virus of leucocytes in suspension cultures obtained from buffy coats of human blood donation at the Central Public Health Laboratory, Helsinki. The specific activity of the final preparation was  $2 \times 10^6$  units/mg protein. The monkeys received  $3 \times 10^6$  units of interferon intramuscularly once daily.

Treatment schedule:

- a) Two of the rhesus monkeys received interferon 2 days before infection and the treatment was continued daily thereafter.
- b) Two monkeys were inoculated with the virus suspension and interferon was administered 1 hour later and daily thereafter.
- c) The third group of monkeys was given interferon when fever developed on the third day after inoculation with the virus and
- d) A group of three rhesus monkeys were inoculated with the virus only served as controls.

Sampling of blood and tissues

Samples of blood were obtained daily by femoral venepuncture. Biopsies were not carried out during life in view of the risk of uncontrolled haemorrhages. Autopsy was carried out on all monkeys shortly after death. Portions of the following organs were removed for virological and histological examinations:- lungs, heart, liver, spleen, adrenals, kidneys, intestinal tract, mesenteric lymph nodes and testes. Bile and faeces were also collected for virus titration.



Lassa virus inactivation studies

Ether inactivation: Equal volumes of virus containing tissue culture fluid and diethyl ether were mixed together and held at room temperature for 1 hour. The mixing was repeated every 15 minutes.

Ultraviolet light inactivation: 4 ml amounts of tissue culture medium containing  $10^{5.5}$  TCID<sub>50</sub>/ml of Lassa virus were dispensed into plastic petri dishes having a diameter of 50 mm. The depth of this fluid was approximately 3-3.5 mm. These were placed on a rocking shelf with a rocking cycle of 20 complete cycles per minute and at a distance of 6 inches from a 15 watt Philips germicidal tube giving an intensity of 600 microwatts/cm<sup>2</sup>. Exposure times of 15, 30 seconds, 1, 2 and 4 minutes were used.

Betapropiolactone (BPL) inactivation: Equal volumes of infected tissue culture medium and Betapropiolactone were mixed together to give the following final concentration of BPL, 1:500, 1:1000, 1:2000, 1:4000 and 1:8000. These were held at +4°C for 18 hours.

Thermal inactivation: Lassa virus was added to tissue culture fluid containing 10 per cent calf serum, this was then subjected to the following heat treatment, 56°C for 30 minutes, 60°C for 30 minutes and 60°C for 60 minutes.

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RESULTS

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Preliminary studies on the characterisation of Lassa virus

Tissue culture studies: Cytopathic changes in Lassa virus infected Vero cell cultures did not appear until the 4th day. These cytopathic changes began as single necrotic cells throughout the monolayer, these developed into focal areas involving groups of 10-15 cells that became rounded and granular. By days 5-6 these groups of cells became detached and were readily seen floating in the medium, leaving empty spaces in the cell monolayer. By the 8th day approximately 30-40 per cent of the cells became detached with the remaining cells showing rounding and granulation.

Following the infection of Vero cell cultures with approximately a 100 TCID 50 of Lassa virus, there was an initial drop in the infectivity titre. The virus concentration then gradually increased to a peak infectivity titre of between  $10^7$ - $10^8$  TCID 50/ml by the 4th or 5th day. This infectivity titre tended to fall to a level of  $10^6$  TCID 50/ml on days 7 and 8. At this stage the medium was fairly acid.

An interference phenomenon could readily be demonstrated in Vero cell cultures inoculated with high multiplicities of infection. When titrating a virus suspension containing approximately  $10^8$  TCID 50/ml in flasks containing  $3 \times 10^6$  cells, no cytopathic effect (CPE) developed in the flasks inoculated with the undiluted or  $10^{-1}$  dilutions. Invariably flasks inoculated with the  $10^{-3}$  dilution were the first to show signs of C.P.E.

Studies on the isolation and identification of Lassa virus

Studies by Wulff and Lange (1975) showed that the indirect fluorescent antibody technique could be used for the detection of Lassa virus antigen in infected Vero cell cultures. This provided a technique for the early identification of Lassa virus antigen and antibody and a means of making a relatively early diagnosis of Lassa virus infection in man.

Our results of daily testing of infected Vero cell cultures showed

that under the right experimental conditions (the right amount of virus in the inoculum) the isolation and identification of Lassa virus could be made from human specimens and a diagnosis confirmed within 48 hours. This was later confirmed in 1977 in this laboratory with an actual case of Lassa fever sent to Porton from Zaria in northern Nigeria.

Detection of Lassa virus antibody: Antibodies were detected by the indirect fluorescent antibody technique (Figure 8) using the procedure described in materials and methods for Ebola virus. A comparison between the indirect fluorescent antibody technique and the complement fixation test (Table 3) showed that the indirect fluorescent antibody test was the more sensitive of the two tests.

Table 3

Human Sera	Antibody Titre	
	CFT <sup>a</sup>	IIFA <sup>b</sup>
1	16	128
2	16	128
3	32	64
4	4	16

a) complement fixation test

b) indirect immunofluorescent antibody

Lassa virus inactivation studies

Ether sensitivity: Ether reduced the infectivity titre from  $10^{5.5}$  to  $10^{1.5}$  TCID 50/ml. Lassa virus was therefore considered to be ether sensitive but not completely inactivated under the condition of test.

Ultraviolet light inactivation: Ultraviolet light reduced the infectivity titre from  $10^{5.5}$  to  $10^{1.0}$  TCID 50/ml in 15 seconds, this reduced further after 30 seconds to a level where only the undiluted tissue culture fluid was positive; complete inactivation being

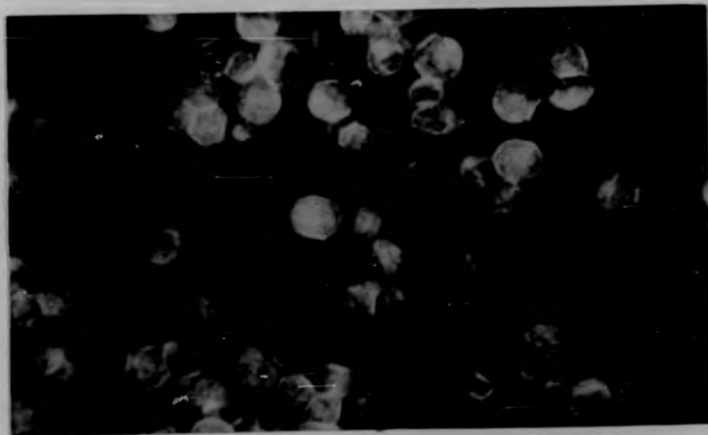
Figure 8. Vero cells showing Lassa virus inclusion bodies stained by the indirect fluorescent antibody technique.



Figure 8. Vero cells showing Lassa virus inclusion bodies stained by the indirect fluorescent antibody technique.



Figure 8. Vero cells showing Lassa virus inclusion bodies stained by the indirect fluorescent antibody technique.





achieved by 1 minute.

Beta-propiolactone (BPL) inactivation: The results of two experiments are reported in Table 4. In both experiments Lassa virus could not be isolated from any of the samples which were treated with a 1:2000 concentration of BPL and held at +4°C for 18-20 hours.

Table 4

Final conc. of EPL	TCID 50/0.5 ml Experiment	
	A	B
1: 500	Toxic for Cell	
1: 1000	Negative	Negative
1: 2000	Negative	Negative
1: 4000	$10^{2.5}$	$10^2$
1: 8000	$10^4$	$10^{4.5}$
1: 16000	$10^{5.5}$	$10^{5.5}$
Untreated control	$10^{5.5}$	$10^6$

Heat inactivation: The results of two heat inactivation experiments are reported in Table 5. Although the majority of the virus is inactivated at 56°C for 30 minutes, there is however a residual fraction that is relatively heat resistant. Heating at 60°C for 1 hour completely destroyed all infectivity in two separate experiments.

Table 5

Treatment	TCID 50/0.5 ml Experiment	
	A	B
None	$10^{6.5}$	$10^6$
56°C for 30 mins	$10^{2.5}$	$10^{1.5}$
60°C for 30 mins	$10^{1.5}$	$10^{0.5}$
60°C for 60 mins	Neg	Neg

Results of studies on Marburg type viruses

Isolation attempts and serological studies on human sera

The information received from various people in the field, suggested the possibility of Yellow fever, Congo fever, Lassa fever or Marburg disease. The approach therefore to the virus isolation studies took this information into consideration and the isolation attempts included:-

- 1) Inoculation of cultured preparations of Vero cells for possible Lassa fever; and also BHK 21 cells.
- 2) Inoculation of suckling and adult mice, intraperitoneally and intracerebrally for possible arthropod-borne viruses and
- 3) With Marburg in mind young guinea pigs weighing approximately 200-250 grams were inoculated intraperitoneally.

Tissue culture: No cytopathic changes were seen in low-power microscopic examination of either of the tissue culture systems used. Acidification of three cultures was noted after 6-7 days incubation at 37°C and fluid from these cultures produced a febrile illness in guinea pigs inoculated intraperitoneally. Electron microscope examination of this fluid on day 6, revealed virus particles which were morphologically indistinguishable from Marburg virus. (See section on tissue culture studies).

Details of electron microscope studies on these tissue culture fluids and of further studies in tissue culture are set out in a later section.

Mice: No detailed studies were carried out in mice apart from the original inoculation of acute phase sera and of suckling mouse brain material sent to us by Professor Pattyn of the Institute of Tropical Medicine, Antwerp. Suckling mice inoculated with this material began dying on the 5th day and were all dead by the 9th day. Further passage from sick mice was not attempted. No signs of illness were observed in any of the adult mice inoculated.

Guinea pigs: A clear positive result was obtained in guinea pigs.

Guinea pigs inoculated with acute-phase serum or whole blood

became febrile (40.5-41°C) after an incubation period ranging from 4-7 days and remained febrile for about 3-4 days during which time they were obviously ill. Whole heparinised blood taken during the febrile stage of the illness was successfully passaged in guinea pigs. A total of seven isolations were made in guinea pigs, 4 from specimens from northern Zaire and 3 from specimens from southern Sudan. Details of the successful transmission to guinea pigs is set out in a later section.

Serology: Apart from one serum having a low antibody titre (1/10) to Yellow fever, no antibodies were detected in any of the other patients' sera to any of the arbovirus antigens used. Neither were there any antibodies to Marburg or Lassa fever virus antigens.

The sucrose acetone extracted antigens which were prepared from acute-phase human serum, acute-phase guinea pig serum or liver, when tested against a broad range of arbovirus antisera in haemagglutination-inhibition tests also gave a negative result. Serum from guinea pigs bled 21 days after inoculation with E 718 acute phase serum when tested by the indirect fluorescent antibody technique in slide preparation of Vero cells infected with the isolate had a fluorescent antibody titre of 1/128. A comparison of the indirect fluorescent antibody titres of guinea pigs immunised against Marburg (67) and E 718 virus isolate (76) was carried out at C.D.C. Atlanta and at Porton with the following result:-

Immunising agent	Days after inoculation	Porton	
		I.F.A. titres with antigens of	
		Marburg 67	Isolate E 718
Marburg 67	23	128	<4
Isolate E 718	21	<4	128-256

C.D.C. Atlanta

Immunising agent	Days after inoculation	I.F.A. titres with antigens of	
		Marburg 67	Isolate E 718
Marburg 67	12	640	5
Isolate E 718	10	2	256

Cross Protection studies in guinea pigs: The results of the cross protection studies are reported in Table 6. These show that there was no cross protection in guinea pigs between Marburg virus and the new isolate from the Sudan and Zaire. At the time of challenge, these guinea pigs had fluorescent antibody titres ranging from 1/128-1/512. It was concluded that although morphologically the two viruses were identical, they were antigenically distinct. With the concurrence of the principle investigators involved, Professor S.R. Pattyn, Institute of Tropical Medicine, Antwerp, Dr Karl Johnson, C.D.C. Atlanta, U.S.A. and myself, the name Ebola virus was proposed for the new agent.

Table 6

Cross protection studies of Marburg and Ebola virus strains in guinea pigs

Immunising virus	Challenging Virus		
	Marburg 67	Ebola Zaire (E 718)	Ebola Sudan (Boneface)
Marburg 67	3/3 <sup>a</sup>	0/3	0/3
Ebola Zaire (E 718)	0/3	3/3	3/3
Ebola Sudan (Boneface)	0/3	3/3	3/3

a)  $\frac{\text{No. of guinea pigs protected}}{\text{No. of guinea pigs challenged}}$

Ebola virus inactivation studies

Ether sensitivity: Diethyl ether completely destroyed the infectivity of Ebola virus (Table 7).

Table 7

	<u>Log 10</u> <u>G.P.I.U./0.1 ml</u>
Control suspension	6.5
After ether treatment	0

Thermal inactivation: Heating at 60°C for 1 hour completely destroyed all infectivity for guinea pigs in three separate experiments.

Tissue culture studies: Virus isolation from the original serum and blood specimens was first attempted in preparation of cultured Vero cells. A partial cytopathic effect was seen under low-power microscopical examination. This effect did not progress to complete destruction of the cell sheet and was first thought to be attributed to the toxic nature of the specimen inoculated. There was, however, a distinct colour change in the medium of three of these cultures. By the 6th or 7th day after inoculation they became noticeably more acid compared to the control cultures. When young guinea pigs were inoculated with these three cultures a febrile illness developed after 4-6 days. The supernatant fluid from one of these infected cultures (E 718) was used for electron microscope studies.

Further passage of the infected tissue culture was attempted in fresh stationary cultures of Vero cells, BHK 21 cells, LLC MK2 cells and a primary human liver cell line without success. There was no obvious development of cytopathogenic effect in these cultures. When, at various intervals after infection, some of these cell cultures were frozen and thawed and the cells stripped from the surface of the flasks, then inoculated into guinea pigs, in all cases only the neat,  $10^{-1}$  and sometimes  $10^{-2}$  dilution of the tissue culture material produced a characteristic febrile illness in guinea pigs.

During the first passage of the isolate in Vero and BHK 21 cells, a number of the cells were found to contain pleomorphic basophilic intracytoplasmic inclusion bodies by the 5th-7th day (Figure 9). Apart from the presence of these inclusions there was no evidence to suggest that multiplication of Ebola virus was taking place in any of the four tissue culture systems examined. This contrasted with the tissue culture passage of Marburg virus where serial passage of tissue culture material resulted in the number and size of the inclusion increasing with the development of a regular cytopathic effect, (Zlotnik et al., 1968). Serial passage of Ebola virus tissue culture material resulted in the disappearance of these inclusion bodies. Further studies using infected guinea pig and monkey tissue as the inoculum showed that when high multiplicities of infection were used, (greater than one infected particle per cell) then a high proportion of the cells in the culture contained intracytoplasmic inclusions. When low multiplicities of infection were used, (approximately one infected particle per 10 cells) only a few cells were found to contain these inclusions. The specificity of these inclusions was illustrated by the indirect fluorescent antibody technique using specific Ebola guinea pig and human antisera as follows:-

- a) Uninfected monolayers treated with specific Ebola antisera followed by staining with the appropriate conjugate showed only low level non-specific fluorescence.
- b) Infected monolayers treated with normal guinea pig or human serum followed by staining with the appropriate conjugates showed no fluorescence.
- c) Infected monolayers treated with diluent (Phosphate buffered saline) followed by staining with conjugates showed no fluorescence.
- d) Infected monolayers treated with specific Ebola antisera followed by staining with appropriate conjugates showed specific fluorescence.

Figure 9. BHK 21 cell culture showing reddish-salmon coloured intracytoplasmic inclusion bodies. Picro-Mallory, x 900.





Figure 9. BHK 21 cell culture showing reddish-salmon coloured intracytoplasmic inclusion bodies. Picro-Mallory, x 900.



Figure 9. BHK 21 cell culture showing reddish-salmon coloured intracytoplasmic inclusion bodies. Picro-Mallory, x 900.



### Electron Microscope studies

Tissue culture material: The commonest form of Ebola virus seen by electron microscopy of negatively stained preparations of virus grown in Vero cells was as a sinuous cylindrical particle ranging from about 1-11 microns in length (mean 3-4 microns). Almost invariably the stain penetrated the particle to reveal a central core (Figure 10) approximately 20 nm in diameter. The envelope displayed projections about 10 nm long, so that the overall particle diameter was approximately 105 nm. The presence of an additional lipid coat exterior to the presumably lipoprotein envelope (Figure 19) was shown by the exclusion of negative stain and the absence of projections. When a prepared specimen grid was rinsed in ether before staining, the lipid was removed and projections became visible. The nature of the central core only became clear when on a few occasions the helix containing it was completely lost from the particle. The helix diameter was 33 nm with an 8-9 nm pitch (Figure 11). When the particle was not penetrated by stain a capsomere layer became apparent (Figure 12). This was not very well defined but the capsomeres appeared to have a mean diameter of 10 nm and a suggestion of a helical arrangement in parts of some particles. Figure 13 shows a particle with a portion of the nucleocapsid missing.

The sinuous particles of Ebola virus showed various forms of branching (Figures 14 and 15). What is believed to be an initial stage in branching is shown in Figure 16. The possibility that budding occurs is shown in Figure 17 where the end of the looped branch is partially rounded and partially in integral contact with the main particle. Looped ends to the particles were very common. Figure 18 shows the open loop where fusion of the end of the particle to the main shaft has occurred and Figure 19 shows a loop which appears to have been formed by several whorls of the particle which have ultimately fused together. Occasionally the ring type structure of Figure 20 which is 330 nm across was seen.

Figure 10. Sinuous particle (penetrated by stain) showing nucleocapsid core.

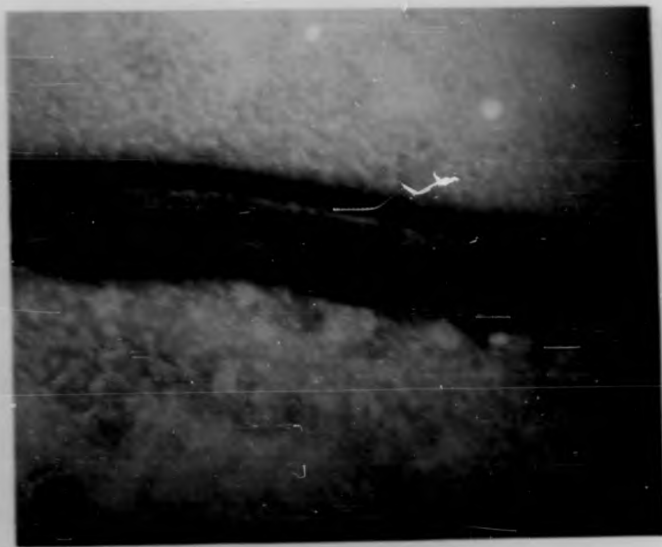


Figure 11. Nucleocapsid helix

Figure 10. Sinuous particle (penetrated by stain) showing nucleocapsid core.



Figure 11. Nucleocapsid helix

Figure 10. Sinuous particle (penetrated by stain) showing nucleocapsid core.

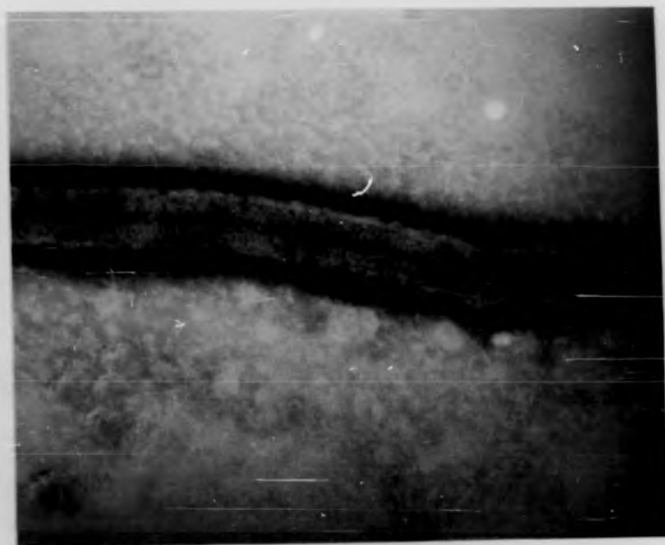


Figure 11. Nucleocapsid helix



Figure 12. Sinuous particle (not penetrated by stain) showing capsomere layer.

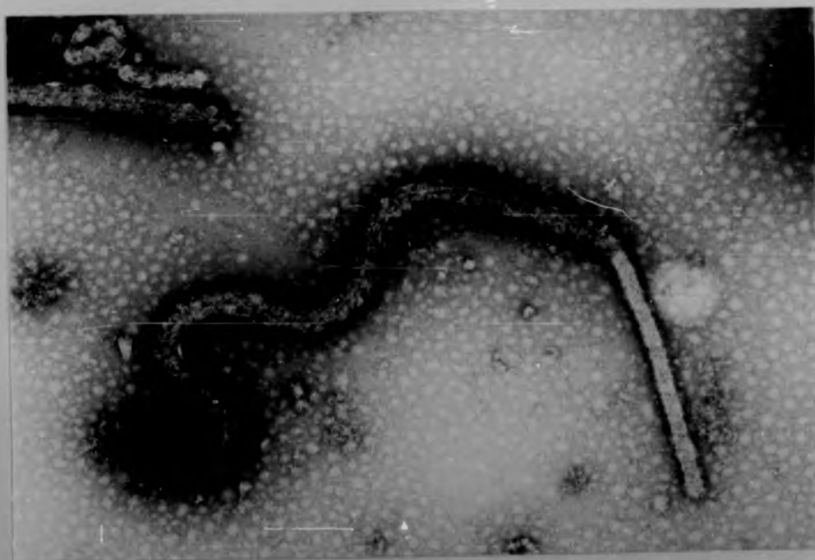


Figure 13. Particle showing a portion which has no nucleocapsid.



Figure 12. Sinuous particle (not penetrated by stain) showing capsomere layer.

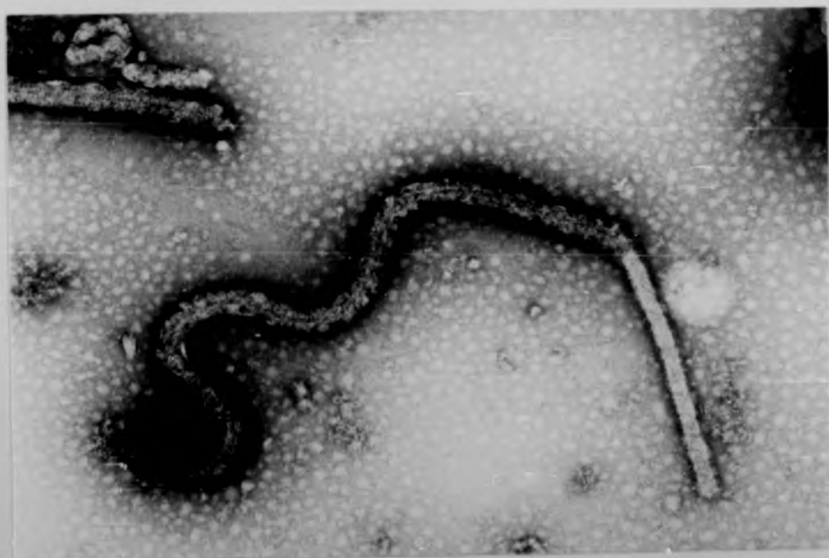


Figure 13. Particle showing a portion which has no nucleocapsid.

Figure 12. Sinuous particle (not penetrated by stain) showing capsomere layer.

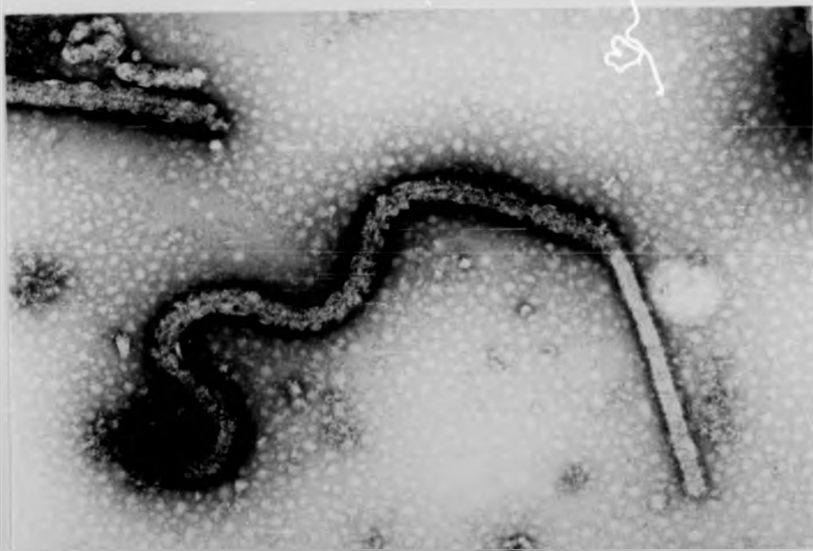
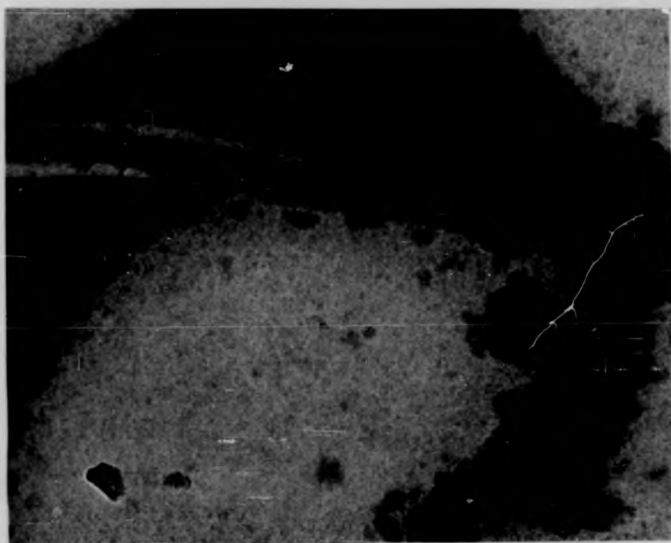


Figure 13. Particle showing a portion which has no nucleocapsid.

Figures 14 and 15. Two examples of branching.

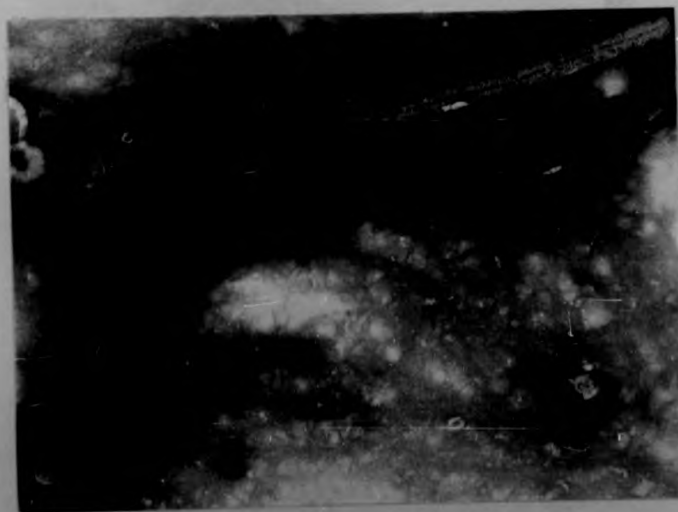
x 119000



x 43000

Figures 14 and 15. Two examples of branching.

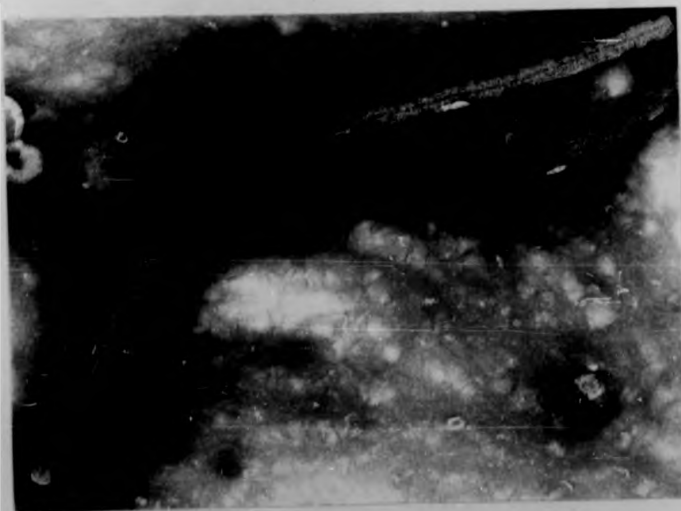
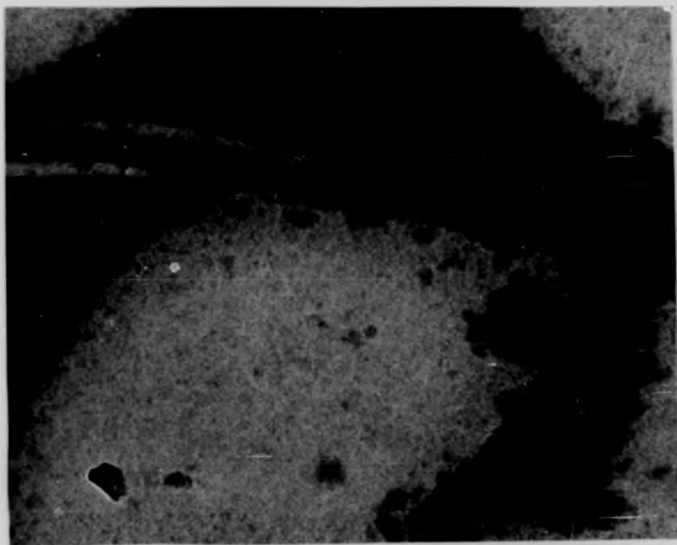
x 119000



x 43000

Figures 14 and 15. Two examples of branching.

x 119000



x 43000

Figure 16. Early stage of branching.

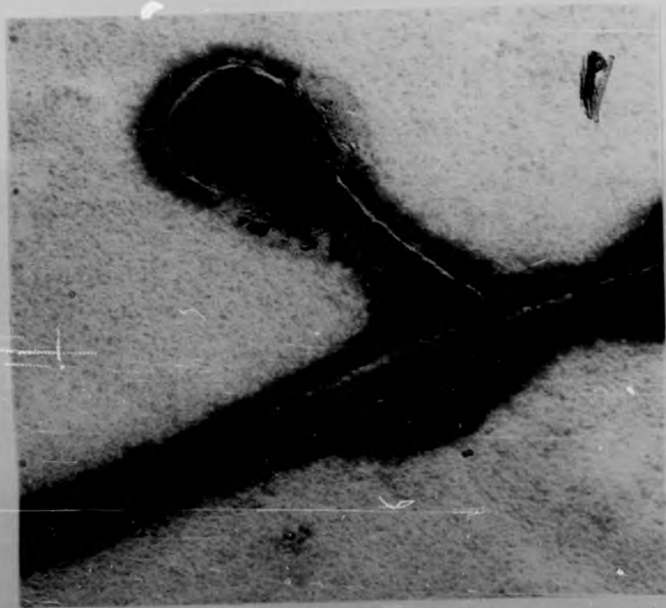
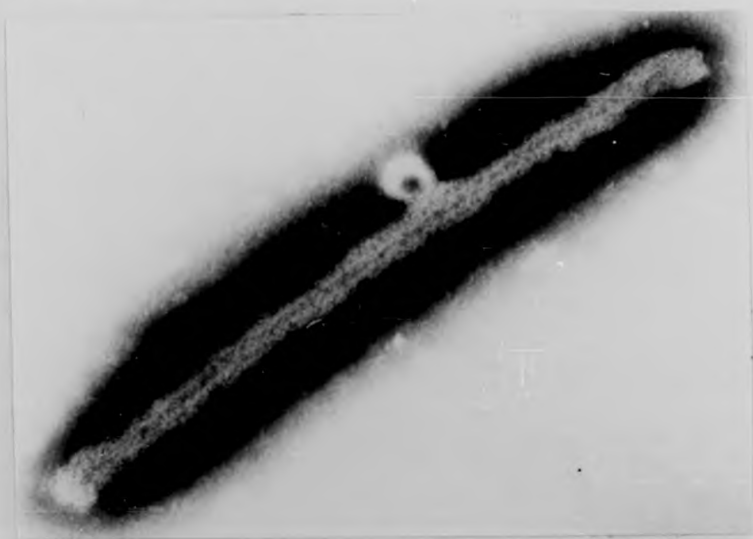


Figure 17. Example of possible budding where looped branch appears about to break away. x 94,500.



Figure 16. Early stage of branching.



Figure 17. Example of possible budding where looped branch appears about to break away. x 94,500.



Figure 16. Early stage of branching.

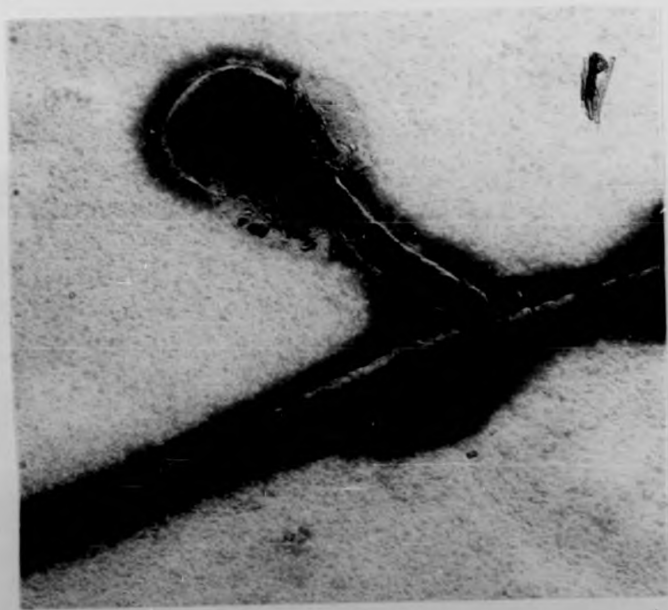


Figure 17. Example of possible budding where looped branch appears about to break away. x 94,500.

Figure 18. Open loop.

x 71000



Figure 19. Loop filled with whorls of particles.

x 94500



Figure 18. Open loop.

x 71000

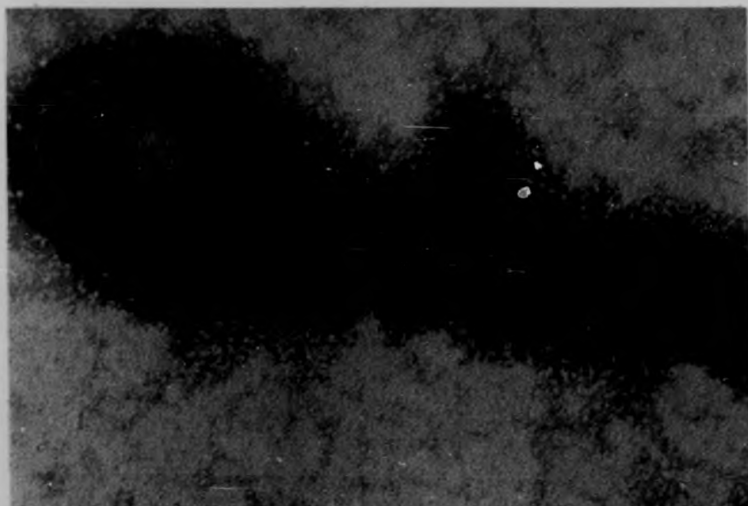


Figure 19. Loop filled with whorls of particles.

x 94500



Figure 18. Open loop.

x 71000



Figure 19. Loop filled with whorls of particles.

x 94500

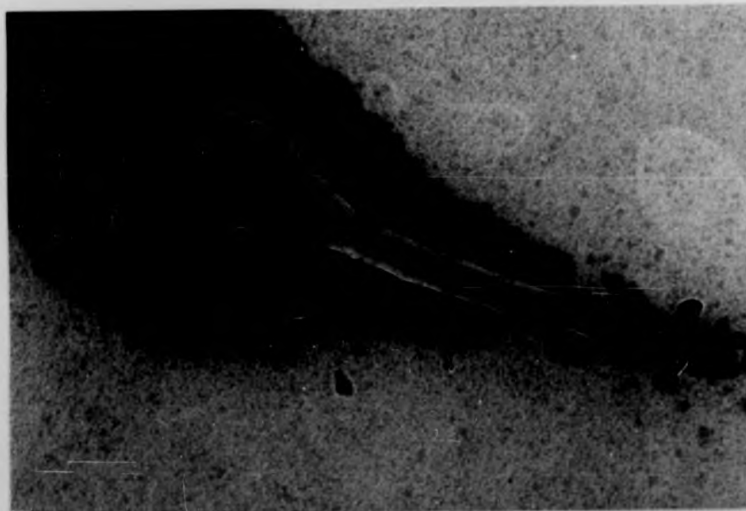


Figure 20. Ring shaped particle possibly formed by the pinching off of a loop similar to Figure 18.

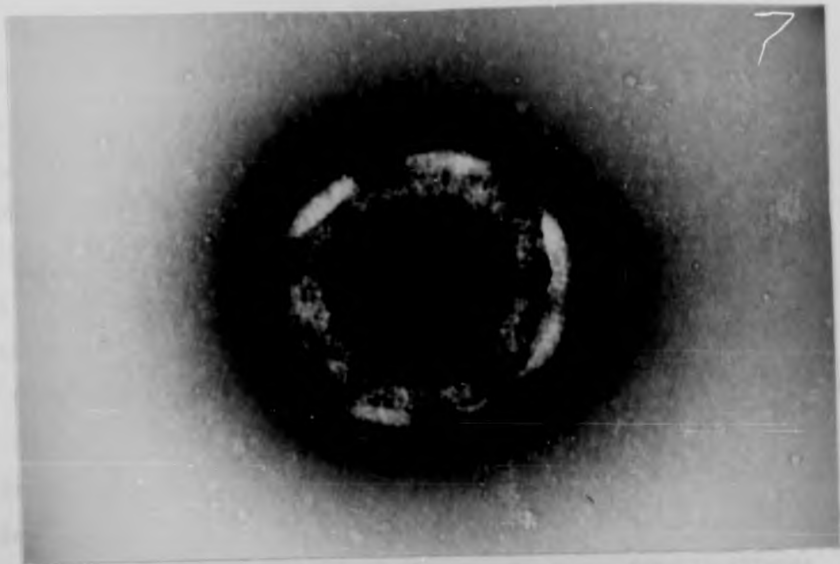


Figure 20. Ring shaped particle possibly formed by the pinching off of a loop similar to Figure 18.

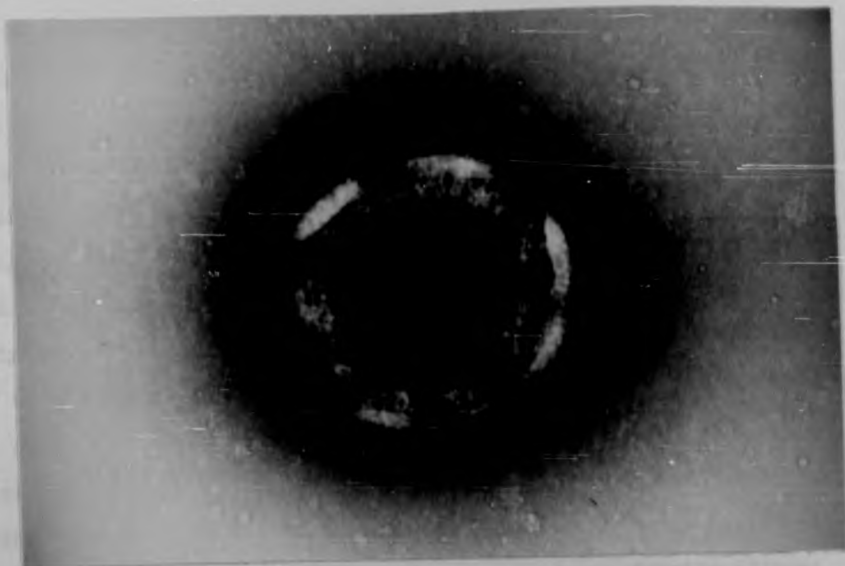
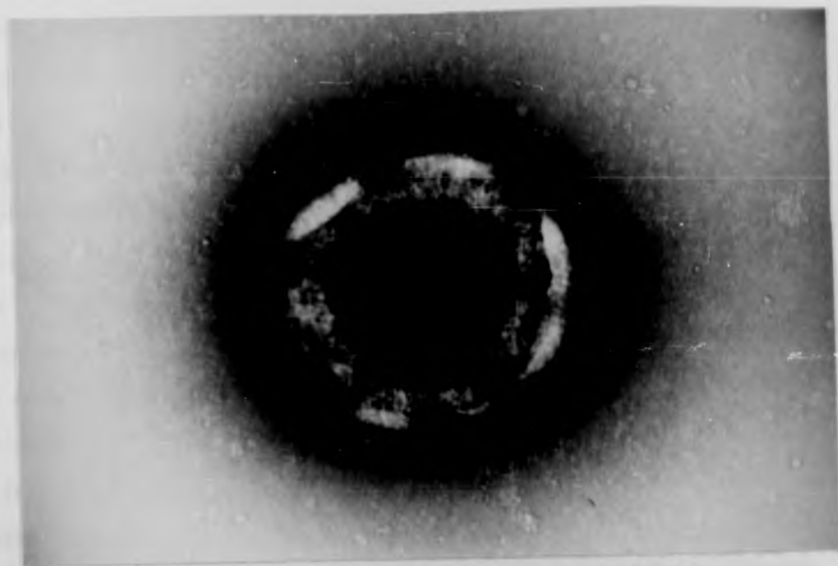




Figure 20. Ring shaped particle possibly formed by the pinching off of a loop similar to Figure 18.





#### Guinea pig virus isolation studies

Guinea pigs inoculated with acute phase blood from a Zaire patient, (A 42 year old woman, ME 718), became febrile ( $40.5^{\circ}\text{C}$ - $41^{\circ}\text{C}$ ) after an incubation period ranging from 4-7 days. The febrile illness lasted for about 3-4 days during which time the guinea pigs failed to thrive and generally looked ill. When heparinised blood was taken from these guinea pigs and re-inoculated into fresh guinea pigs, it produced a similar febrile illness.

During the first passage of this isolate in guinea pigs two of the guinea pigs were killed in the febrile period, one on day 5 and the other on day 8, and tissues were taken for histopathology, electron microscope examination and virological studies. Of the remaining ten guinea pigs that developed febrile illnesses only one died; the remaining nine pigs slowly recovered and appeared to be quite healthy within a period of about three weeks. These guinea pigs were subsequently shown to have antibodies detectable by fluorescent antibody test of titres ranging from 1/64-1/128. During the 2nd, 3rd and 4th passage of the aetiologic agent in guinea pigs the incubation period became fairly regular at 4-6 days with the number of infected guinea pigs dying increasing to a 100% by the 4th guinea pig passage. Tables 8 and 9 compares the number of guinea pigs developing a febrile illness that died respectively in the 3rd and 4th passage of the virus in guinea pigs.

Virus level in the blood and tissues: The concentration of the virus in various tissues removed from guinea pigs killed on days 5 and 6 are shown in Table 10. High levels of virus were found in all the tissues examined. It was not possible to determine whether this was a reflection of the high levels of virus in the blood.

#### Histopathological findings

Liver: There were numerous foci of necrosis which had no consistent lobular distribution and consisted of liver cells undergoing

hyaline degeneration and necrosis. (Figures 21-23). In some of the degenerating cells small pleomorphic eosinophilic bodies were present in the cytoplasm which were periodic acid/Schiff positive and stained bright red with the Machiavello technique but did not stain metachromatically with Giemsa. Kupffer cells were enlarged, some sinusoids contained lymphocytes, and the periportal areas were heavily infiltrated by lymphoreticular cells.

Spleen and lymph nodes: There was widespread depletion of the lymphoid tissue of the follicles, which contained small zones of necrosis. (Figures 24-26). Large numbers of macrophages were accumulated in the sinuses.

Lungs: Changes in the lungs were slight: localised thickening and infiltration of interalveolar septa by lymphoreticular cells.

Other organs: No lesions were detected in the brain, kidneys or adrenal glands.

Electron Microscope studies of guinea pigs: Ultrathin sections of liver obtained from a guinea pig killed on the 5th day post infection showed various forms of Ebola virus particles (Figure 27). In Figures 28 and 29 these virus particles can be seen actually budding from the cell membrane.

Serial passage of the Sudan strains of Ebola virus (Boneface) in guinea pigs: Serial passage of the Zaire strain of Ebola virus in guinea pigs, resulted in the number of animals dying increasing with passage so that by the 4th passage of the virus in guinea pigs all the guinea pigs died. In contrast, serial passage of the Sudan strain of Ebola virus showed that by the 4th guinea pig passage, none of the guinea pigs that showed evidence of infection died. (Table 11). By the 6th passage of the Sudan virus in guinea pigs, only 3 out of the 7 guinea pigs that became infected died. (Table 12).

Table 3  
 Titration of Ebola virus in guinea pigs  
 (3rd guinea pig passage)  
 Rectal temperatures (°C)

DAY	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
10 <sup>-1</sup>	( 38.5	38.9	39.6	39.6	39.5	40.6	41.1	40.6	39.7	37.3	36.5	DEAD				
	( 38.3	38.8	39	38.9	39.3	40.2	40.7	40.7	39.4	38.6	39.5	38.7	38.7	38		
10 <sup>-2</sup>	( 38.8	38.6	38.9	40.6	40.9	40.5	40	DEAD								
	( 38.2	38.3	38.5	40.1	40.6	40.3	40	DEAD								
10 <sup>-3</sup>	( 38.9	39.1	38.9	39.5	39.1	39	39.6	41	41.3	40.8	39.6	DEAD				
	( 38.5	38.8	38.8	39	39	39	39.4	39.7	40.7	40.5	39.8	39.2	39.2	38.3	38.7	
10 <sup>-4</sup>	( 38.5	38.9	38.8	38.8	38.9	40	40.2	40.8	41.2	40.6	41.1	40.3	39.5	39.2	39.5	39.2
	( 38.9	39	38.7	38.3	39.7	40.2	40.6	41.1	DEAD							
10 <sup>-5</sup>	( 39.3	39.1	39.3	39.5	39.1	39	40.6	42.1	40.6	DEAD						
	( 39.2	29.2	28.9	39.2	38.9	38.8	39.1	39.1	39	40.5	41.7	41.2	41.1	39.1	DEAD	
10 <sup>-6</sup>	( 39.1	39.5	39	39.1	38.7	38.5	39.2	39.1	39.2	38.9	39.3	38.7	39.8	39	38.8	38.5
	( 38.9	39.1	38.8	38.9	38.8	38.5	38.6	39.2	38.9	39.1	39.9	40.3	41.0	41.2	40.9	40.5

Table 9  
Titration of Ebola virus in guinea pigs  
 (4th guinea pig passage)  
 Rectal temperatures (°C).

DAY	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
10 <sup>-1</sup>	( 39	39.5	38.8	39.5	40.5	40.3	40.3	41.3	40	39.7	40.1	39.2	DEAD			
	( 39.5	39.8	38.7	40	41	40	DEAD									
10 <sup>-2</sup>	( 39.1	39.7	39.2	39.7	40.3	40.5	40.5	40.5	DEAD							
	( 39.5	39.4	38.9	39.8	40.5	40.5	40.1	40.5	40.2	39.7	40.5	40.1	38	DEAD		
10 <sup>-3</sup>	( 38.9	39.4	38.6	39	39.1	40.2	41.3	40.8	DEAD							
	( 38.9	39.1	38.4	38.4	38.4	40	40.5	40.5	39.4	DEAD						
10 <sup>-4</sup>	( 38.9	38.9	38.3	38.4	38.5	40	40	DEAD								
	( 38.3	39.5	38.5	38.8	39.1	38.3	38.7	41.1	40.8	40	35.6	DEAD				
10 <sup>-5</sup>	( 39	39	38.4	39	38.5	40	40.5	40.5	40	37.3	DEAD					
	( 39.4	39.7	38.9	39	38.8	40.9	40.9	40.5	39.5	DEAD						
10 <sup>-6</sup>	( 39.1	38.9	39.5	39.1	39	39.9	41.1	41.3	39.7	37.6	DEAD					
	( 39.1	39.5	39.1	39	38.9	39.1	38.9	39.5	39	39	38.9	39.2	39.2	38.9	38.6	38.8

Table 10

Titres of Ebola virus in guinea pig tissues expressed as  
Log 10 guinea pig infectious units/ml (or gram) of tissue

TISSUE	GUINEA PIG KILLED DAY 5	GUINEA PIG KILLED DAY 6
BLOOD	7.0	7.0
HEART	5.0	5.0
LUNG	5.0	5.0
LIVER	5.0	7.0
SPLEEN	6.0	7.0
ADRENALS	4.0	4.0
KIDNEYS	3.0	3.0
PANCREAS	7.0	7.0
MES LYMPH NODES	5.0	5.0

Figure 21. Guinea pig liver showing typical foci of hepatocellular necrosis. Haematoxylin and eosin x 208.

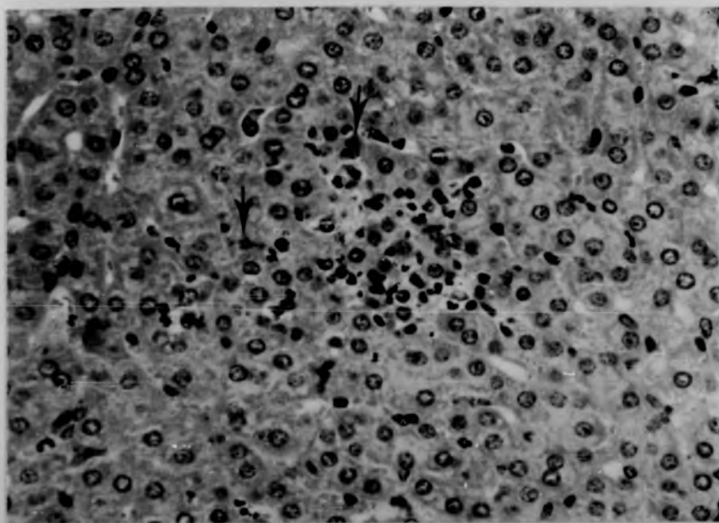
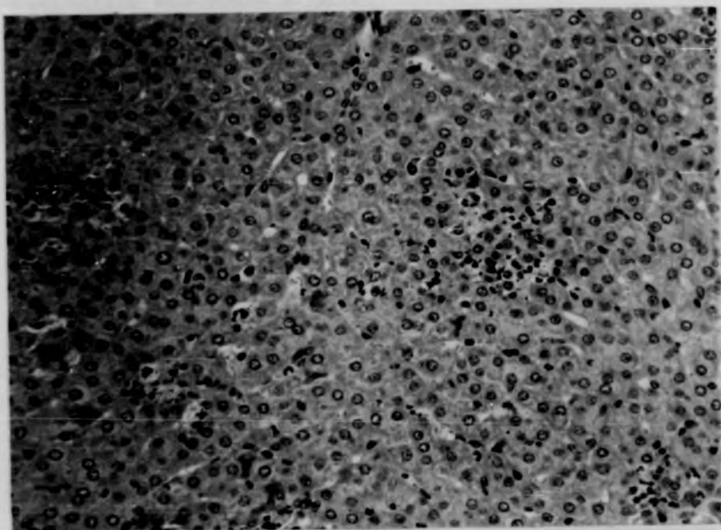


Figure 22. Focus of necrosis in guinea pig liver. Some hepatocytes contain eosinophilic intracytoplasmic inclusion bodies (arrows). Haematoxylin and eosin x 325.



Figure 21. Guinea pig liver showing typical foci of hepatocellular necrosis. Haematoxylin and eosin x 208.

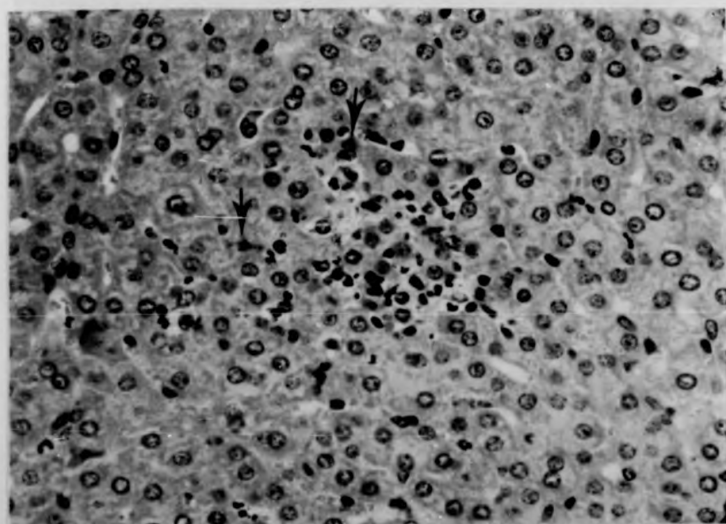
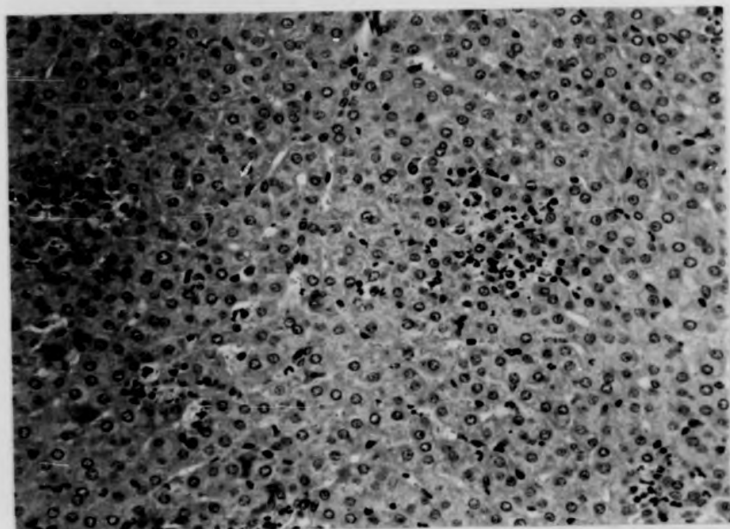


Figure 22. Focus of necrosis in guinea pig liver. Some hepatocytes contain eosinophilic intracytoplasmic inclusion bodies (arrows). Haematoxylin and eosin x 325.



Figure 21. Guinea pig liver showing typical foci of hepatocellular necrosis. Haematoxylin and eosin x 208.

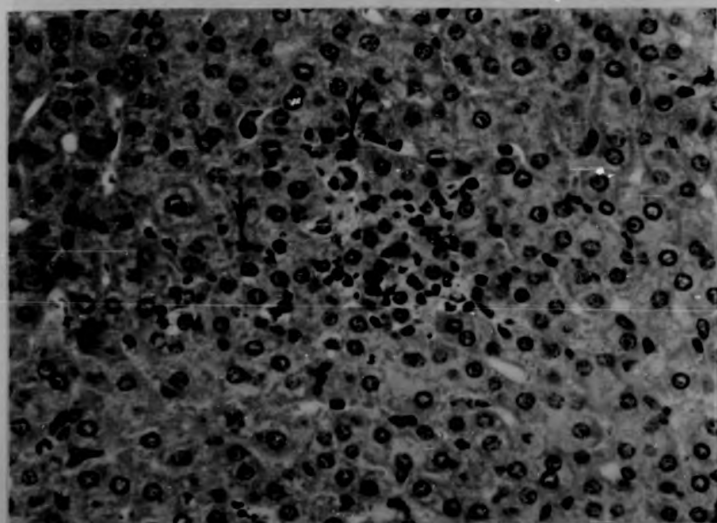
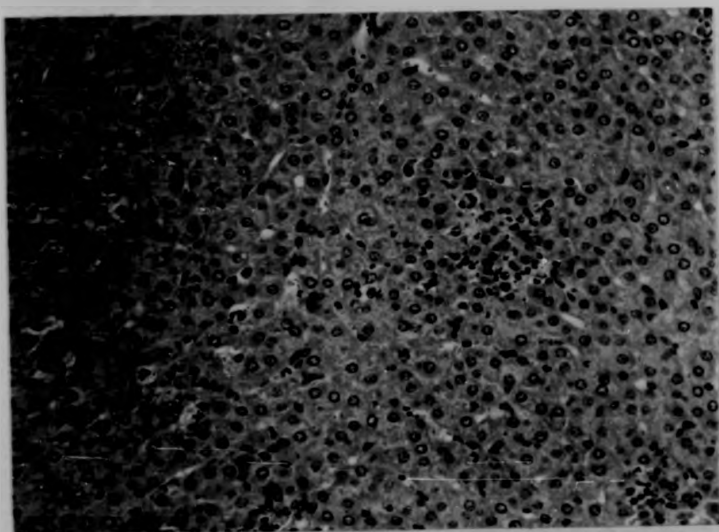


Figure 22. Focus of necrosis in guinea pig liver. Some hepatocytes contain eosinophilic intracytoplasmic inclusion bodies (arrows). Haematoxylin and eosin x 325.

Figure 23. Focus of necrosis in guinea pig liver. There is activation of a few Kupffer cells. Amorphous eosinophilic inclusion bodies are present in a few hepatic cells (arrows). Haematoxylin and eosin x 325.

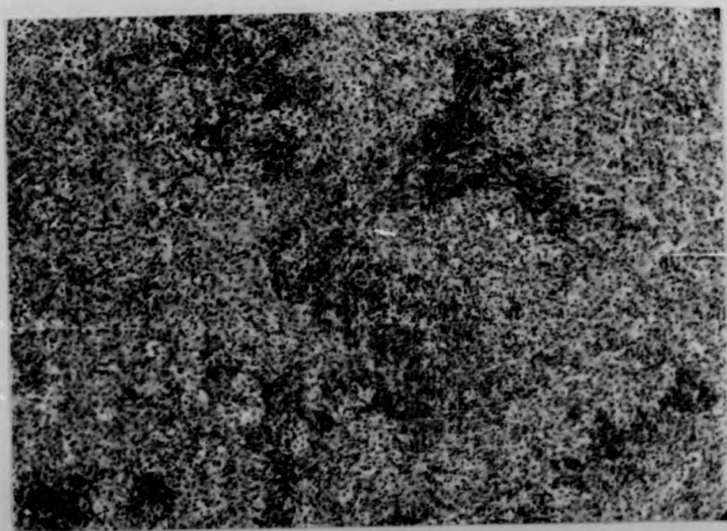
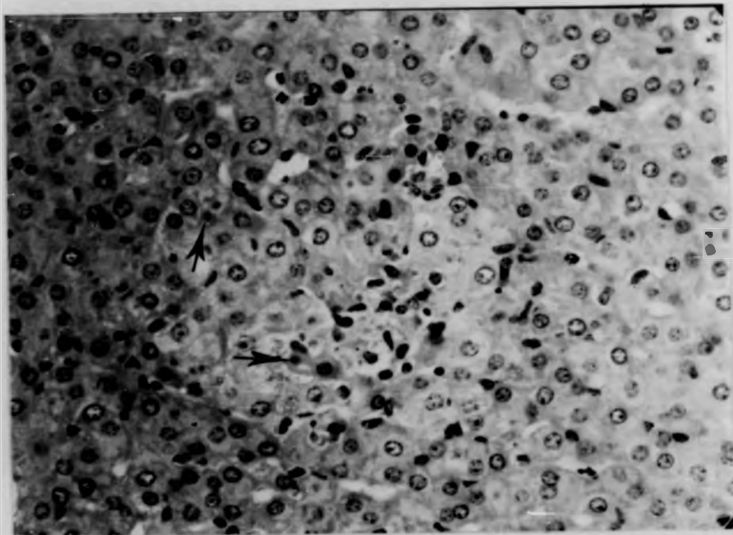


Figure 24. Necrosis of red and white pulp and depletion of lymphoid elements in guinea pig spleen. Haematoxylin and eosin x 82.

Figure 23. Focus of necrosis in guinea pig liver. There is activation of a few Kupffer cells. Amorphous eosinophilic inclusion bodies are present in a few hepatic cells (arrows). Haematoxylin and eosin x 325.

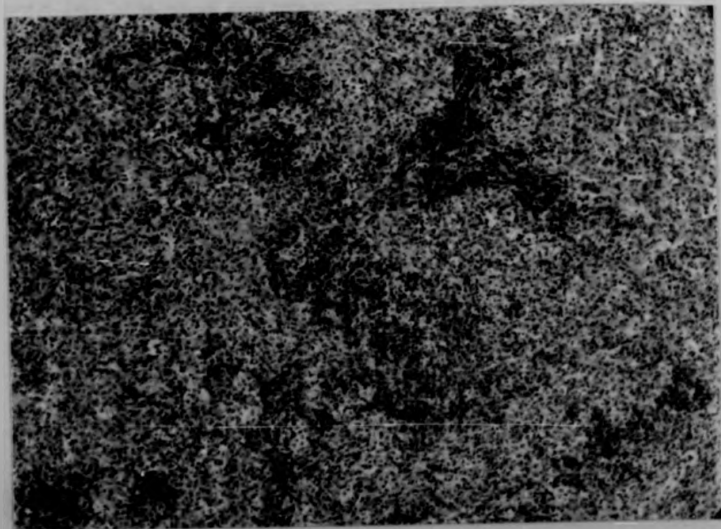
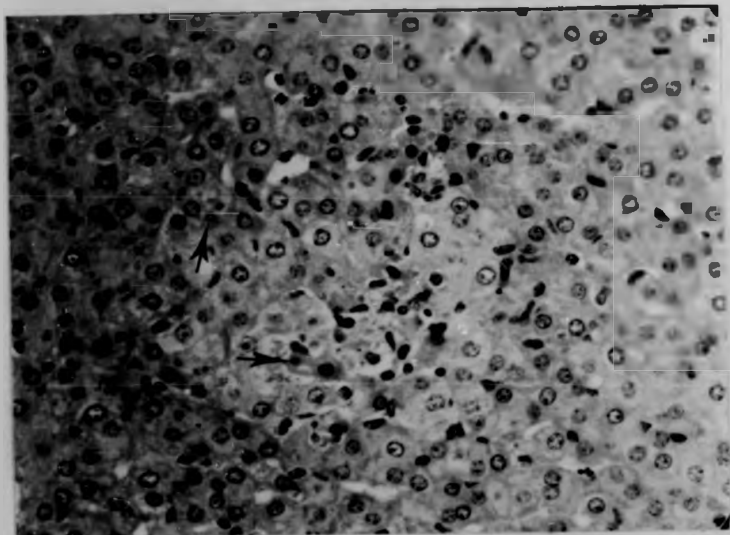


Figure 24. Necrosis of red and white pulp and depletion of lymphoid elements in guinea pig spleen. Haematoxylin and eosin x 82.

Figure 23. Focus of necrosis in guinea pig liver. There is activation of a few Kupffer cells. Amorphous eosinophilic inclusion bodies are present in a few hepatic cells (arrows). Haematoxylin and eosin x 325.

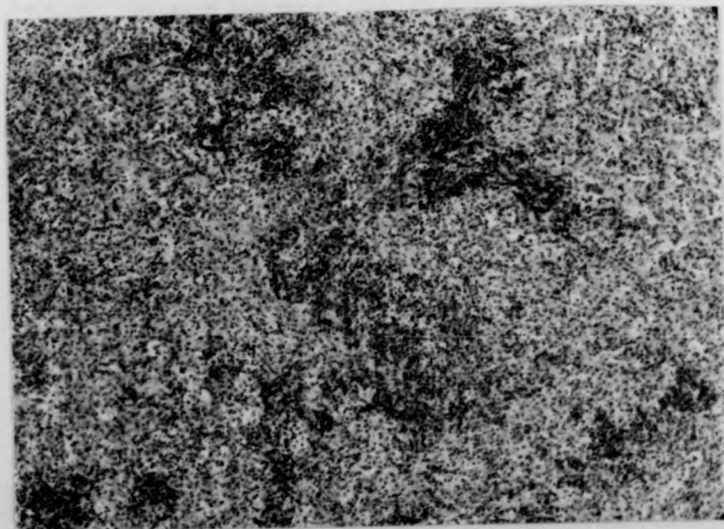
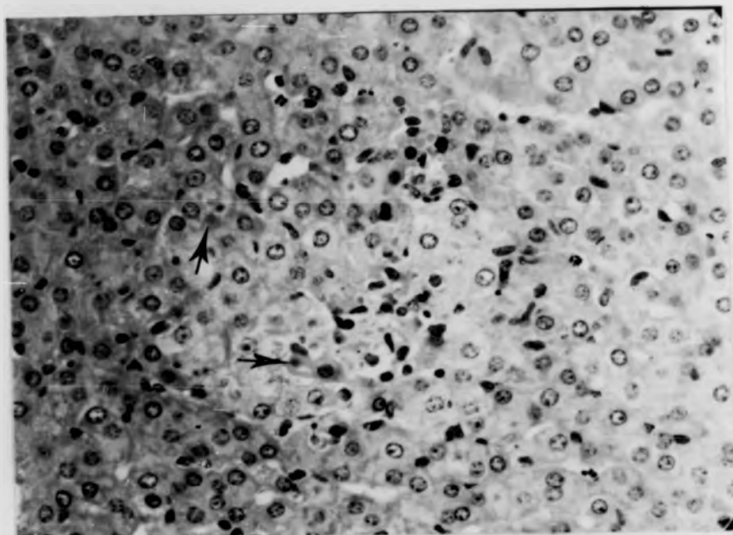


Figure 24. Necrosis of red and white pulp and depletion of lymphoid elements in guinea pig spleen. Haematoxylin and eosin x 82.

Figure 25. Guinea pig spleen showing loss of lymphoid tissue.  
Haematoxylin and eosin x 208.

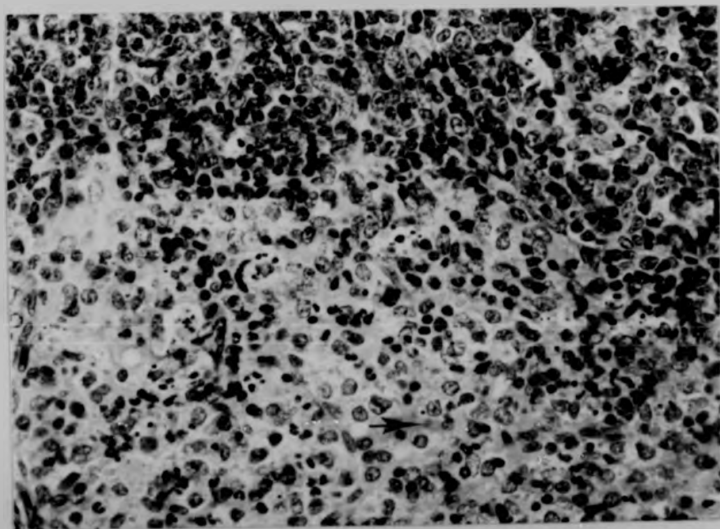
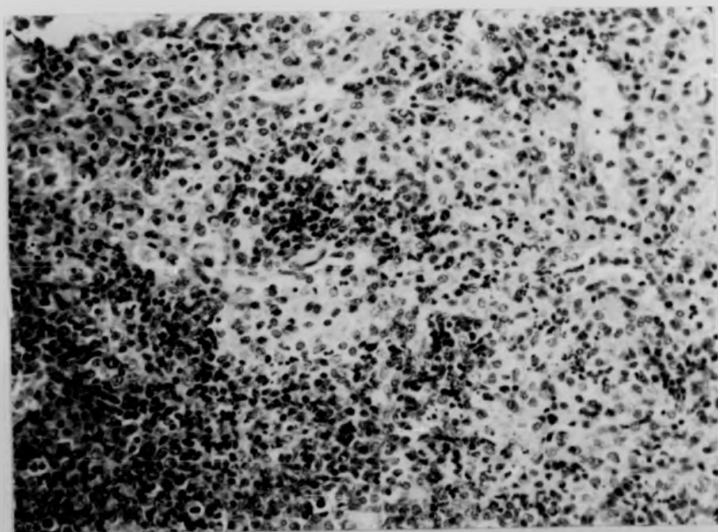


Figure 26. Area of necrosis of splenic white pulp, occasionally contains  
eosinophilic intracytoplasmic inclusion bodies (arrows).  
Haematoxylin and eosin x 325.



Figure 25. Guinea pig spleen showing loss of lymphoid tissue.  
Haematoxylin and eosin x 208.

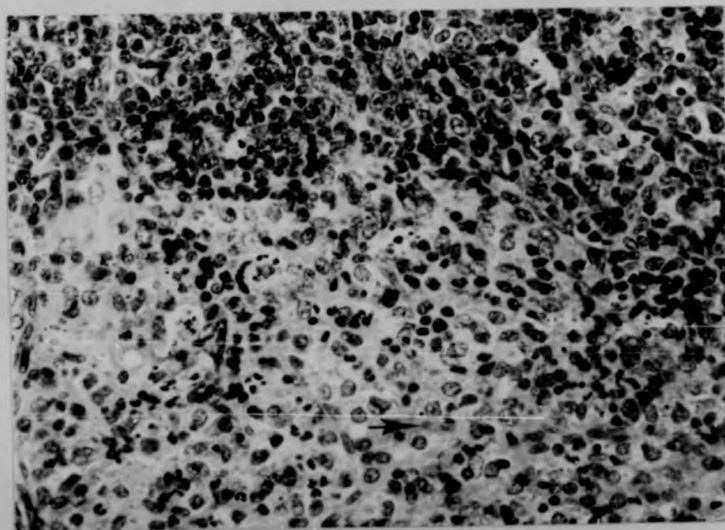
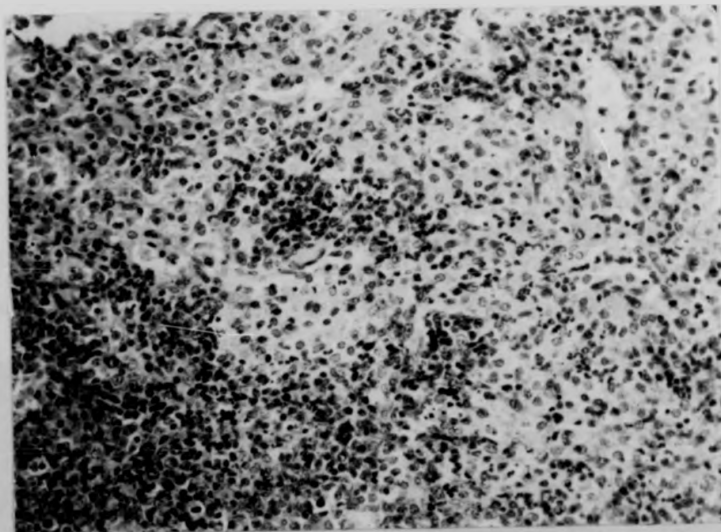


Figure 26. Area of necrosis of splenic white pulp, occasionally contains  
eosinophilic intracytoplasmic inclusion bodies (arrows).  
Haematoxylin and eosin x 325.



Figure 25. Guinea pig spleen showing loss of lymphoid tissue.  
Haematoxylin and eosin x 208.

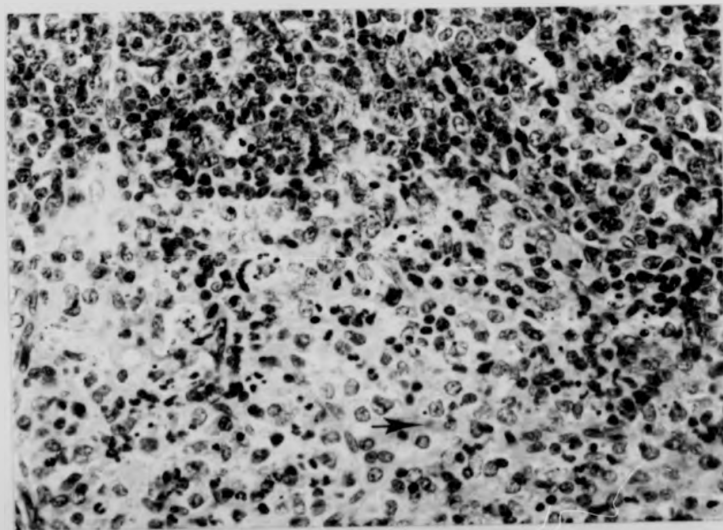
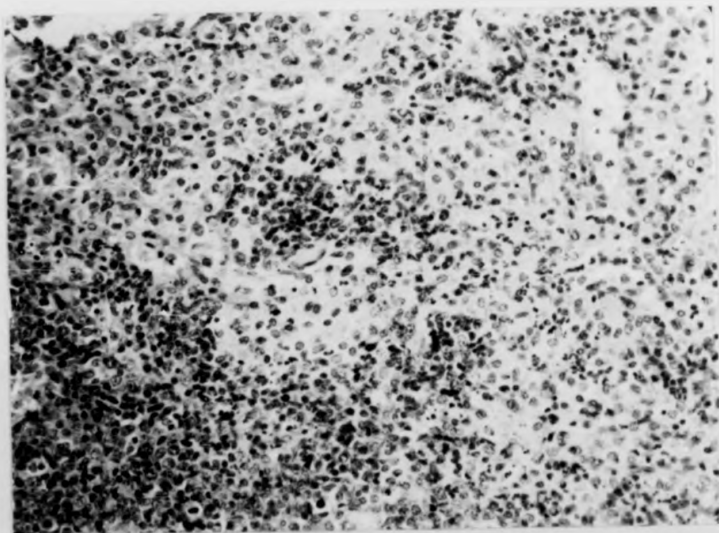


Figure 26. Area of necrosis of splenic white pulp, occasionally contains  
eosinophilic intracytoplasmic inclusion bodies (arrows).  
Haematoxylin and eosin x 325.

Figure 27. Electron micrograph of guinea pig liver showing a variety of forms of Ebola virus x 34000.

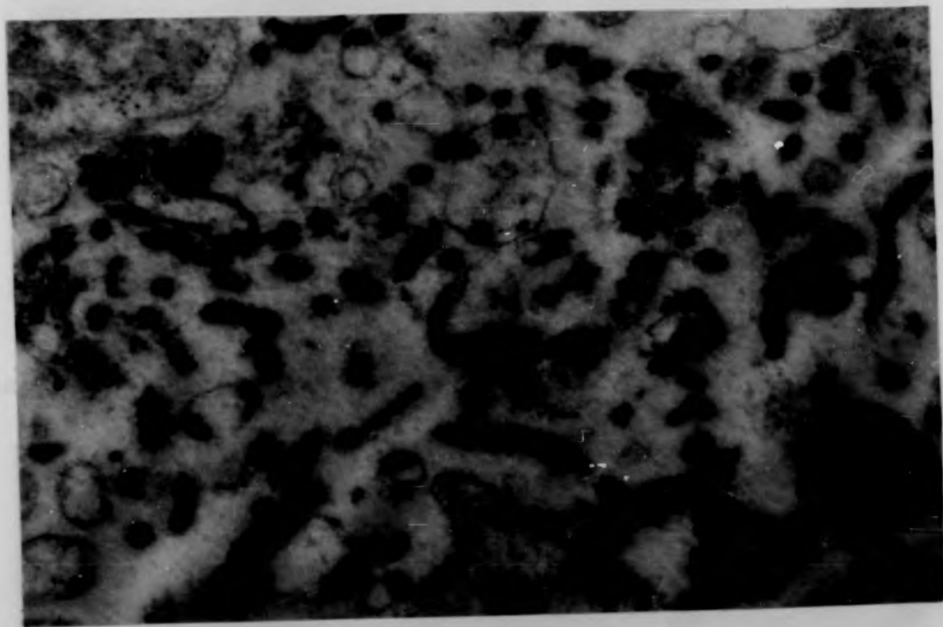


Figure 27. Electron micrograph of guinea pig liver showing a variety of forms of Ebola virus x 34000.

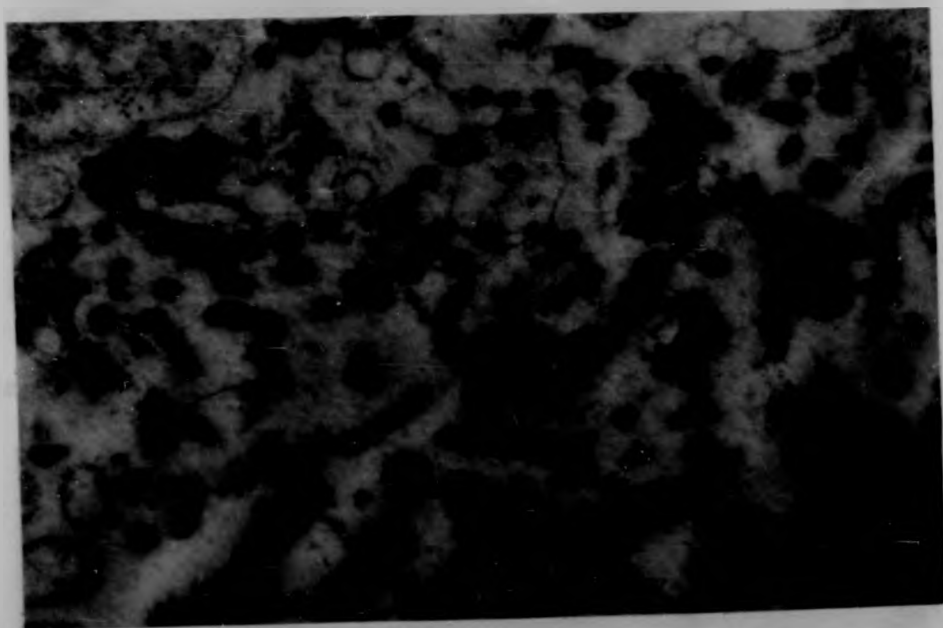


Figure 27. Electron micrograph of guinea pig liver showing a variety of forms of Ebola virus x 34000.

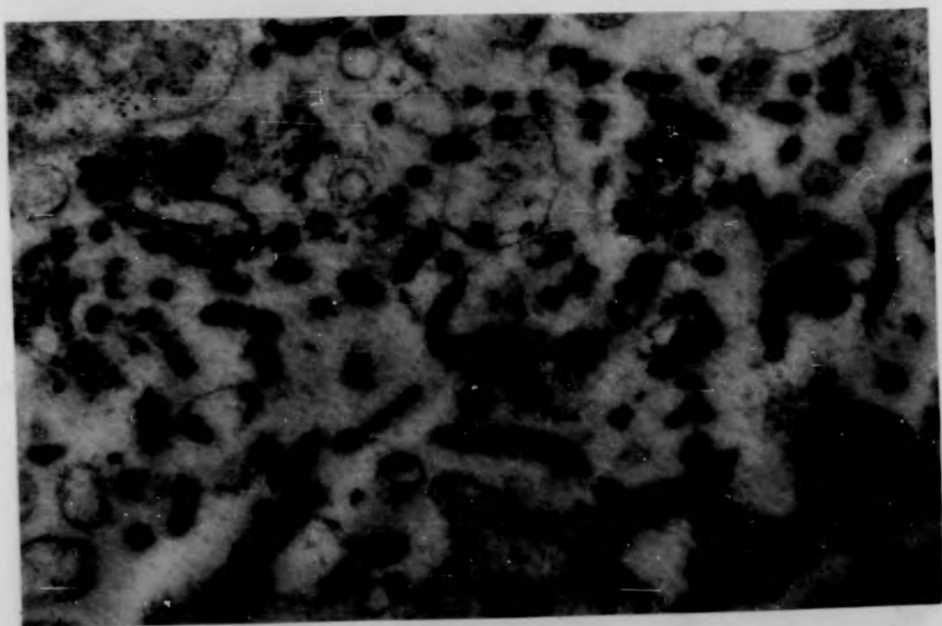


Figure 28. Infected guinea pig liver bile canaliculi showing virus particles budding from the cell membrane x 33000.

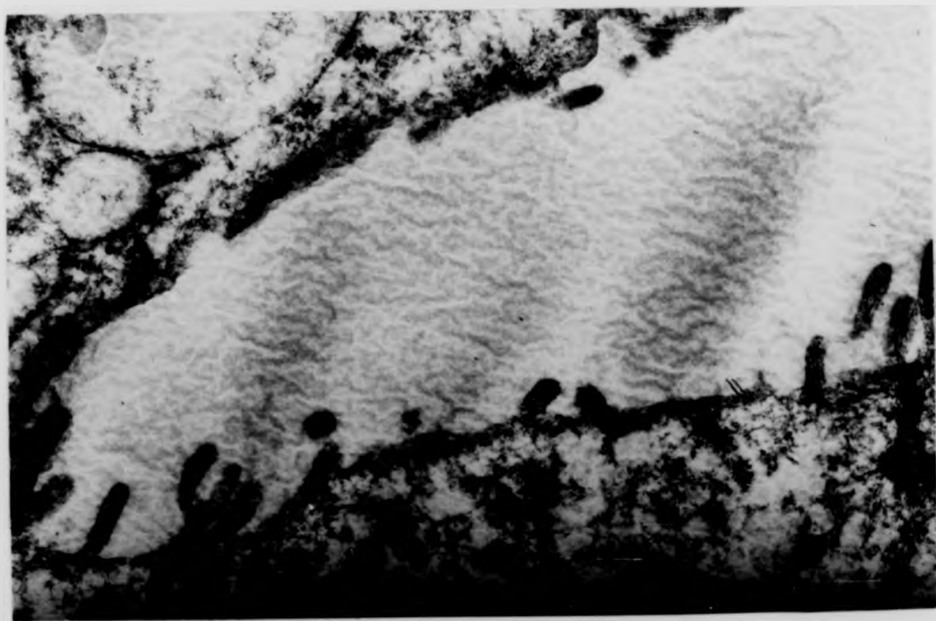


Figure 29. Infected guinea pig liver bile canaliculi showing virus particles budding from the cell membrane x 46000.



Figure 28. Infected guinea pig liver bile canaliculi showing virus particles budding from the cell membrane x 33000.

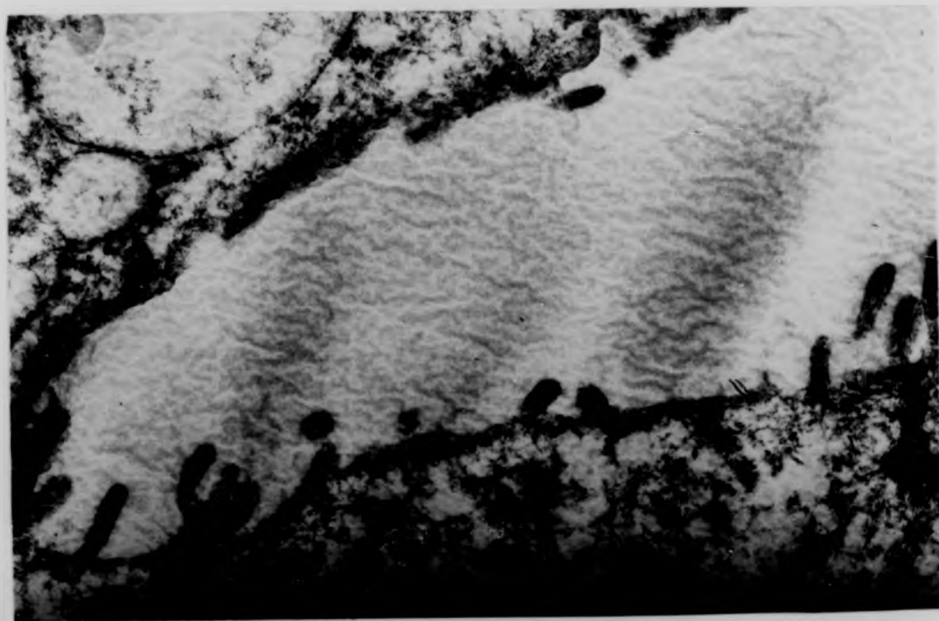


Figure 29. Infected guinea pig liver bile canaliculi showing virus particles budding from the cell membrane x 46000.





Figure 28. Infected guinea pig liver bile canaliculi showing virus particles budding from the cell membrane x 33000.

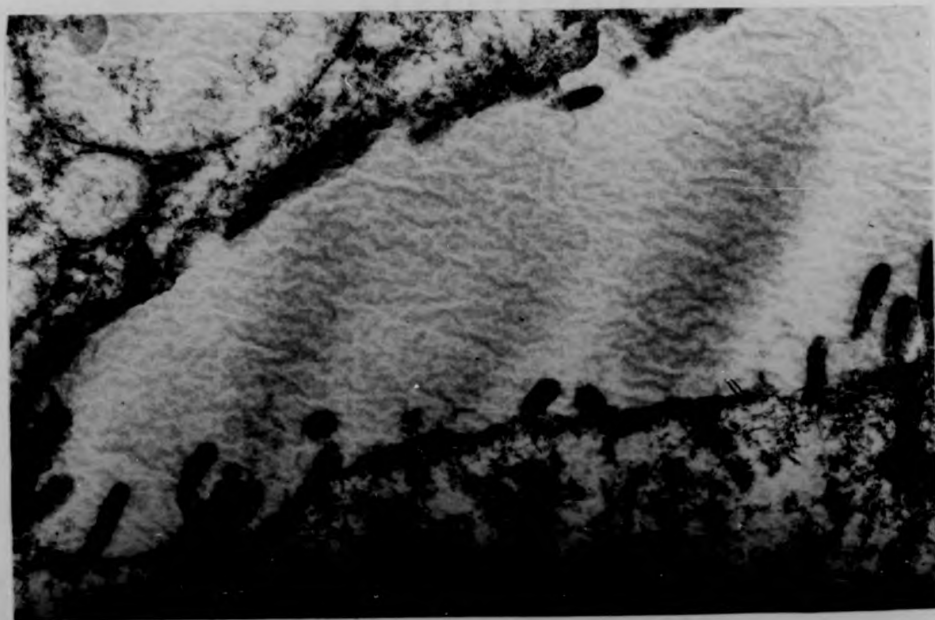


Figure 29. Infected guinea pig liver bile canaliculi showing virus particles budding from the cell membrane x 46000.

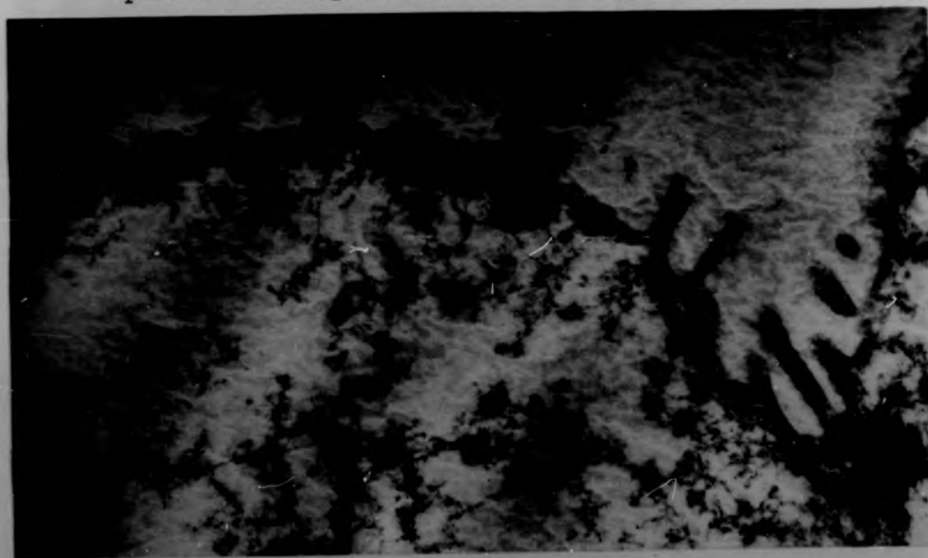


Table 11  
Serial passage of Ebola virus, Sudan strain (Boneface)  
in guinea pigs  
Rectal temperatures (°C)

Day	1	2	3	4	5	6	7	8	9	10	11	12	21	
Guinea pig Pass 1	39.2	39.0	39.1	39.5	40.5	40.6	39.8	39.3	39.1	36.7	ND	38.9	Survived	
Guinea pig a Pass 2	38.9	39.0	39.0	39.2	39.4	40.0	39.8	40	40.8	39.4	39.1	39.2	Survived	
Guinea pig b	38.5	38.6	38.9	38.8	39.1	40.1	40.4	40.6	40.6	39.5	38.9	38.9	Survived	
Guinea pig Pass 3	39.2	39.1	39.4	40.2	40.9	40.9	40.8	39.5	39.4	39.0	39.1	39.0	Survived	
Titration of guinea pig Pass 4	10 <sup>-1</sup>	38.8	39.4	38.4	39.7	40.2	40.7	40.6	41.1	40.1	39.8	39.4	38.8	Survived
		39.2	39.7	38.4	39	39.4	40.1	40.4	40.1	40.5	40.3	40.0	39.8	Survived
	10 <sup>-2</sup>	38.8	39.0	38.7	38.9	38.9	38.9	39.8	40.3	40.6	40.7	39.9	38.9	Survived
		38.6	39.7	38.4	39.2	39.4	39.7	40.4	40.7	40.6	40.6	40.0	38.9	Survived
	10 <sup>-3</sup>	39.0	39.2	38.9	39	39	38.9	40	40.6	40.6	40.6	40.8	39.2	Survived
		39.4	38.9	39.4	39.5	39.8	39.4	40.7	40.8	40.6	40.8	39.4	39.2	Survived
	10 <sup>-4</sup>	38.8	38.3	39.7	39.5	38.8	39.2	39	39.4	39.4	39.4	39.5	39	Survived
		39	38.9	38.9	39.4	38.8	38.9	39	39	39.3	38.9	39.7	39.2	Survived

N.D. Not done.

Table 12  
Titration of Ebola virus, Sudan strain (Boniface) Pass 6  
in guinea pigs  
Rectal temperatures (°C)

Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	21
$10^{-1}$	38.9	39.4	39.4	39.4	38.9	40.5	40.3	39.4	38.3	DEAD						
	39.0	39.6	39.6	40.0	40.4	40.2	39.1	39.4	39.4	39.0	38.8	38.3	38.9	39.3	38.8	Survived
$10^{-2}$	38.9	38.9	38.3	39.1	39.4	39.0	40.5	40.5	40.3	38.9	38.8	DEAD				
	39.0	39.3	38.8	40.1	40.4	40.3	39.6	40.1	39.4	38.9	38.7	38.5	39.0	38.7	38.7	Survived
$10^{-3}$	38.9	39.1	39.1	38.9	40.1	39.8	40.2	40.3	40.5	40.0	40.0	39.4	40.2	39.3	38.8	Survived
	38.9	38.8	38.9	39.0	39.1	39.7	39.6	40.2	40.5	40.3	39.5	38.9	39.2	38.7	38.7	Survived
$10^{-4}$	38.6	38.4	38.3	38.3	38.6	39.0	38.9	39.1	39.2	39.0	38.9	39.4	38.7	38.5	38.4	Survived
	38.9	39.1	39.1	39.1	39.0	39.2	39.1	40.2	40.5	40.5	40.3	40.7	40.1	DEAD		
$10^{-5}$	39.0	39.5	39.2	39.2	39.0	39.2	39.0	39.4	39.2	38.9	39.0	39.1	39.1	38.9	38.8	N.I.
	38.9	39.2	38.9	39.5	38.9	38.6	38.6	38.9	38.9	38.8	38.8	38.8	38.9	38.6	38.5	N.I.

N.I. = Not infected

#### Transmission to monkeys

Although monkeys had not been implicated in the transmission of the disease, it was thought important to infect monkeys experimentally with Ebola virus to determine whether they were susceptible to infection, to define the pathogenesis of this infection and to determine whether this would provide a useful experimental model for evaluating methods of therapy for use in human Ebola virus infections.

#### Monkeys infected with the Zaire strain E 718, Ebola virus

Clinical observations. Following a short incubation period monkeys became febrile on, or about, the third day after infection, with temperatures ranging from 40.2-40.6°C (Table 13). The pyrexia persisted in almost all cases until the terminal stage of the infection when the temperature became subnormal. By the fourth day the monkeys were quiet; they were not eating or drinking and normally sat huddled in their cages responding very slowly to provocation (Figures 30 and 31). Maculopopular skin rashes involving the forehead and face, the medical aspect of the fore and hind limbs and the chest developed in all rhesus monkeys between the 4th and 5th day (Figure 32) fading slightly before death which occurred between the 5th and 12th post inoculation day. The rash also tended to become confluent in patches mainly above the eyes and under the arms. No animals survived the infection, but neither of the two vervet monkeys infected developed skin rashes. Two of the monkeys had diarrhoea and all monkeys lost about 10 per cent body weight.

Virus levels in the blood. Virus was first detected in the blood on the second day reaching maximum virus titres of  $10^{5.5}$ - $10^{6.5}$ /ml on the 4th and 5th day (Table 14).

Virus levels in the tissues. Virus infectivity titrations showed high concentrations of Ebola virus in most of the organs examined (Table 15). It was not possible to determine whether some of these organs contained Ebola virus because of the high concentration of virus present in the

Table 13

Rhesus and Vervet monkeys inoculated intraperitoneally  
with 0.4 ml of 3rd passage guinea pig liver suspension

Rectal temperature (°C)

MONKEY	VIRUS <sup>1</sup> DOSE	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	TOTAL WEIGHT LOSS
Rhesus 6 Wt 3.686 kg	4	38.6	38.9	40.4	40.0	34.5	MORI- BUND			255g
Rhesus 7 Wt 3.629 kg	4	38.1	38.7	40.2	39.8	34.5	MORI- BUND			340g
Rhesus 12 Wt 3.289 kg	3	38.8	38.8	39.6	40.6	39.8	39.6	39.8	FOUND DEAD	396g
Vervet 1	4	38.6	38.9	39.6	40.2	39.7	34.5	MORI- BUND		N.D.*
Vervet 3	4	38.2	38.9	40.1	40.6	39.7	36.1	KIL- LED		N.D.

\*N.D. Not Done.

1) Log 10 guinea pig infectious units.

Figure 30. Rhesus monkey on the fourth day after infection. Sat  
huddled in the cage, obviously sick at this stage.





Figure 30. Rhesus monkey on the fourth day after infection. Sat huddled in the cage, obviously sick at this stage.

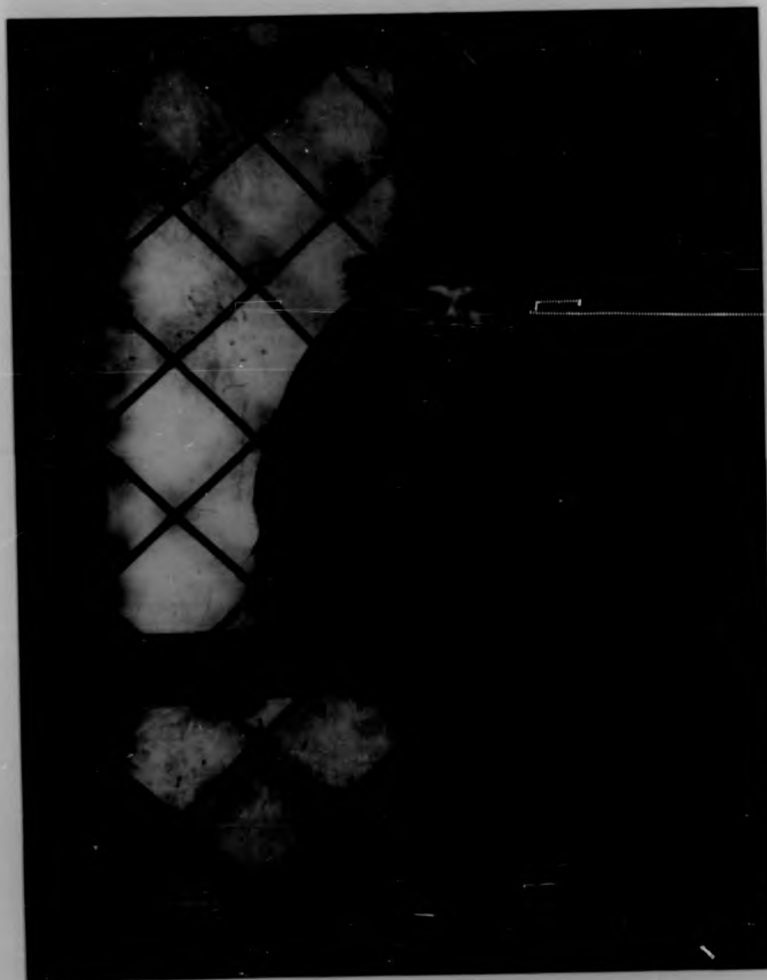


Figure 30. Rhesus monkey on the fourth day after infection. Sat huddled in the cage, obviously sick at this stage.



Figure 31. A close-up of the monkey as seen in Figure 30.

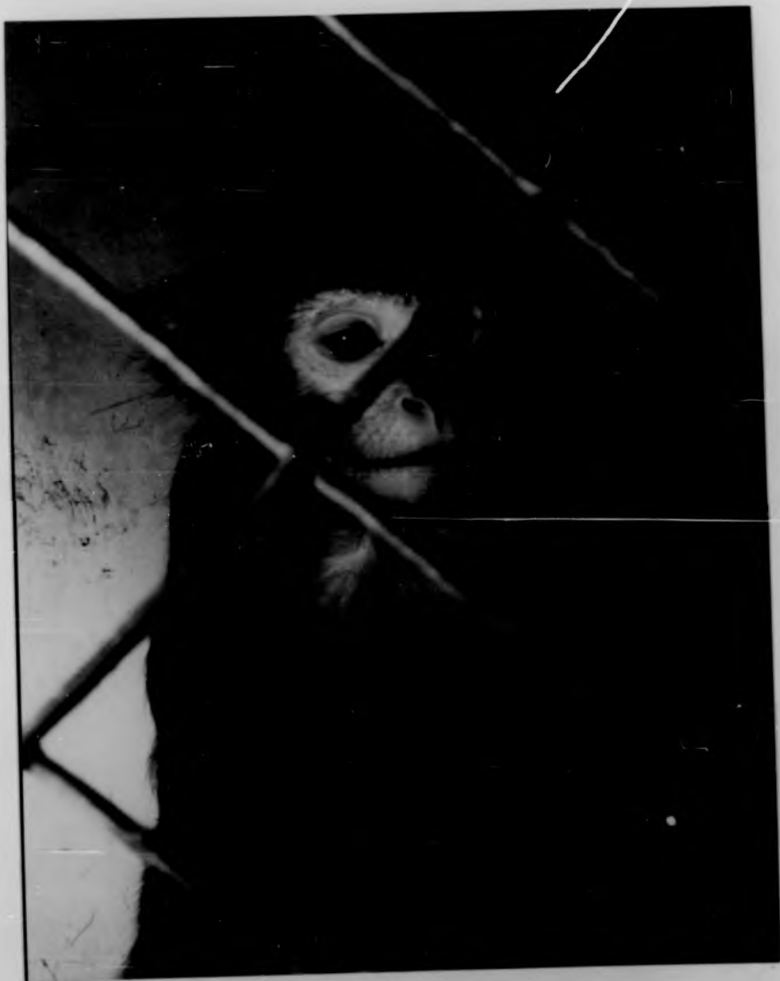


Figure 31. A close-up of the monkey as seen in Figure 30.



Figure 31. A close-up of the monkey as seen in Figure 30.

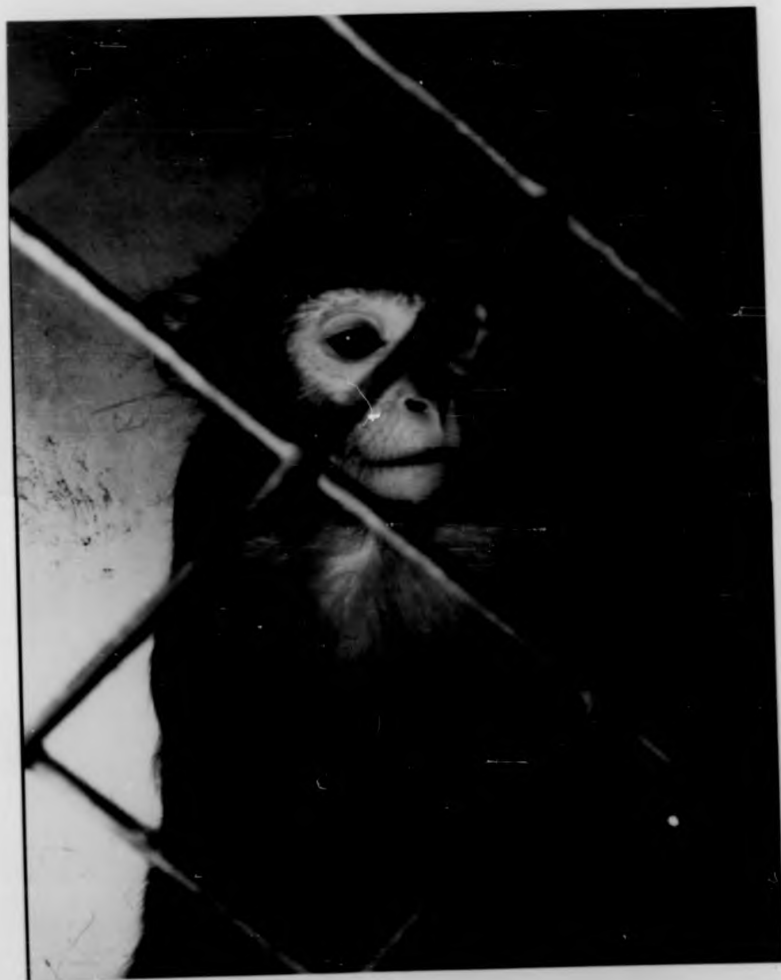


Figure 32. Rhesus monkey with maculo-papular skin rash. Particularly noticeable on the forehead, face and medial aspects of the fore limbs.





Figure 32. Rhesus monkey with maculo-papular skin rash. Particularly noticeable on the forehead, face and medial aspects of the fore limbs.



Figure 32. Rhesus monkey with maculo-papular skin rash. Particularly noticeable on the forehead, face and medial aspects of the fore limbs.



Table 14

Rhesus and vervet monkeys inoculated intraperitoneally  
with 0.4 ml of 3rd passage guinea pig liver suspension

Virus concentration in the blood

Expressed as Log 10 guinea pig infectious units/ml

MONKEY	VIRUS DOSE	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8
Rhesus 6	4.0	<0.5	2.5	4.5	5.5	MORI- BUND 4.5			
Rhesus 7	4.0	<0.5	2.5	4.5	6.5	5.5	MORI- BUND 5.5		
Rhesus 12	3.0	<0.5	2.5	4.0	5.0	5.0	6.5	6.0	FOUND DEAD
Vervet 1	4.0	<0.5	2.0	4.5	6.0	6.0	MORI- BUND 5.5		
Vervet 3	4.0	<0.5	1.5	4.0	6.5	6.5	KIL- LED 5.5		

Table 15

Titres of Ebola virus in monkey tissues expressed as Log<sub>10</sub>  
guinea pig infectious units/ml or grams of tissue

TISSUE	Rhesus 20 Killed Day 4	Rhesus 6 Killed Day 5	Rhesus 7 Killed Day 6	Vervet 3 Killed Day 6	Rhesus 12 Killed Day 8
BLOOD	6.0	5.5	6.5	6.5	6.5
HEART	4.5	7.5	5.0	6.0	6.5
LUNGS	7.5	8.0	6.5	6.0	7.5
LIVER	7.5	7.5	7.0	7.0	7.5
SPLEEN	7.5	6.5	8.0	7.5	7.5
ADRENALS	5.0	7.0	7.0	5.0	7.5
KIDNEYS	5.0	6.5	4.0	7.0	5.5
TESTES	6.5	6.5	6.0	N.D.	N.D.
MES LYMPH NODES	5.5	4.5	4.0	6.5	6.5
PANCREAS	N.D.	N.D.	N.D.	N.D.	7.5
BILE	NEG	NEG	NEG	N.D.	NEG
FAECES	NEG	NEG	NEG	N.D.	N.D.
URINE	N.D.	NEG	3.5	3.5	N.D.

\* N.D. Not Done

blood. However high concentrations of virus were consistently found in the liver, spleen and lungs as early as the fourth day after infection. Ebola virus appeared also to have some affinity for kidneys, adrenals, testes, lymph nodes and the pancreas. No virus was found in bile or faeces but low concentrations were found in urine of two out of three monkeys tested.

Gross necropsy findings: No gross lesions were observed in the monkeys killed on days 2 and 4 after infection. In the other rhesus monkeys dying between the 5th and 8th day, skin rashes were present to a variable extent. Visceral petechiae were also present in all animals, though their distribution was not constant. They occurred most frequently on the gums, oral mucosa, pleural and cut surfaces of the lungs, serosal surfaces of the intestine, caecum and colon, and in the tunica of the testes. In some animals there were large haemorrhages into the lumen of the intestine and colon, with ulceration of the mucosa. Massive haemorrhage had occurred from the left lung into the thoracic cavity of one monkey. There were only small quantities of ingesta in the gastrointestinal trace. Several monkeys had peritonitis with widespread early adhesions, and in some cases these involved the liver and spleen. The liver of most monkeys was pale, mottled and friable. Mesenteric lymph nodes were invariably enlarged and haemorrhagic, and the inguinal and cervical nodes of some animals were similarly affected.

#### Histopathology

Lesions were not found in the tissues of the monkey killed 2 days after infection.

Liver: Lesions were present in all monkeys but were much more severe and widespread in those given the higher dose of virus ( $10^4$  GPIU). They consisted of numerous small foci of necrosis of hepatic cells randomly distributed in the lobules. (Figure 33). In two rhesus monkeys given  $10^4$  GPIU and one vervet

Figure 33. Liver of vervet monkey 6 days after infection showing foci of hepatocellular necrosis (Haematoxylin and eosin) x 140.

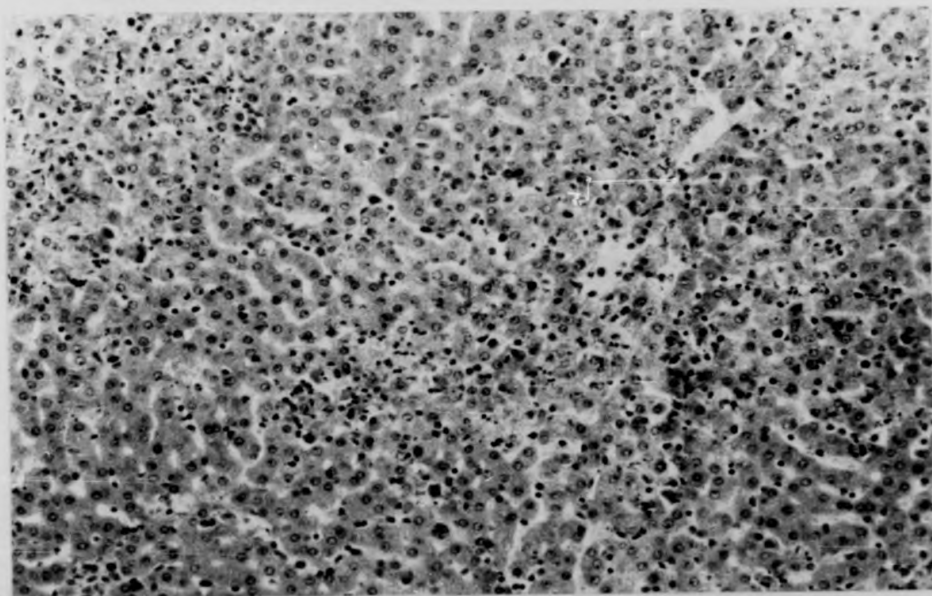




Figure 33. Liver of vervet monkey 6 days after infection showing foci of hepatocellular necrosis (Haematoxylin and eosin) x 140.

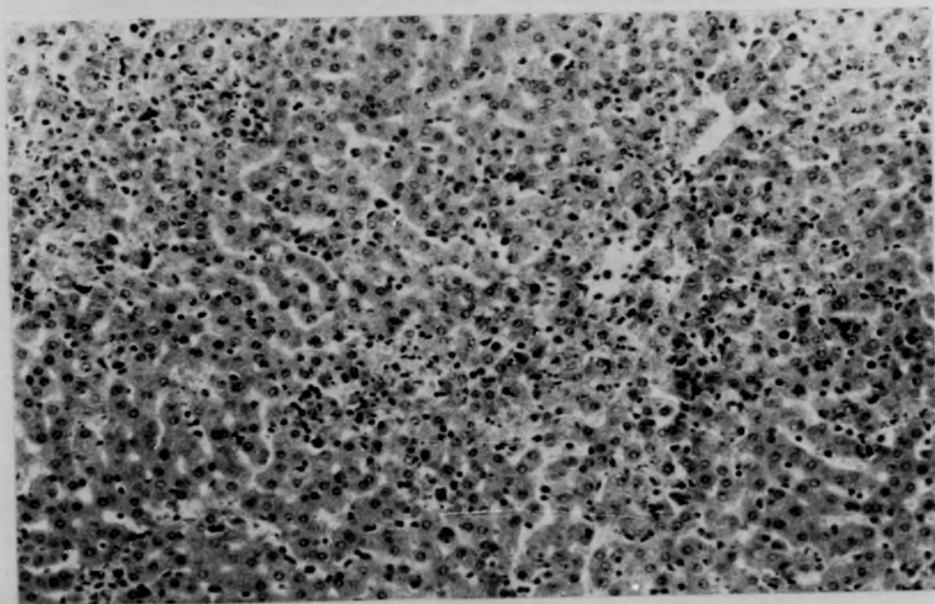
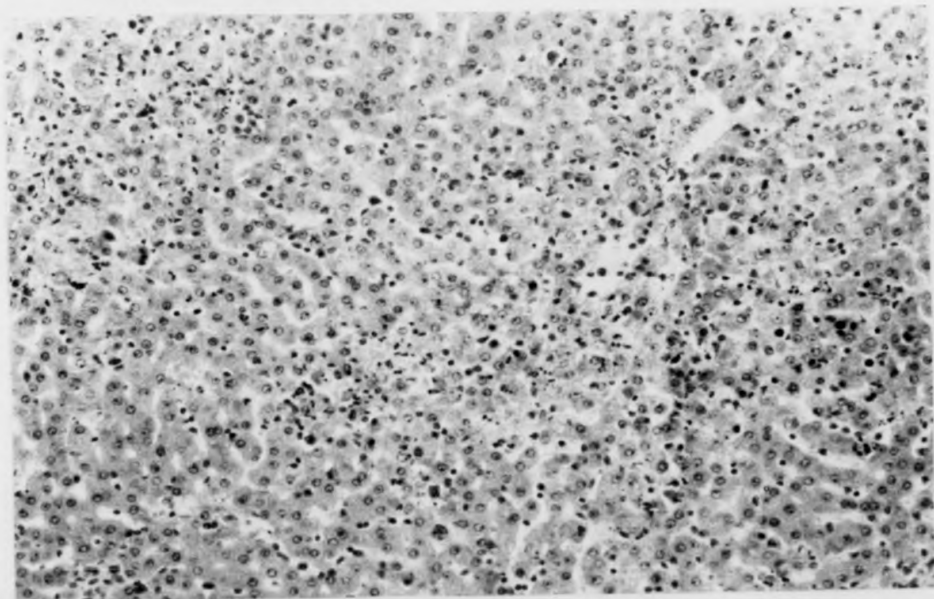


Figure 33. Liver of vervet monkey 6 days after infection showing foci of hepatocellular necrosis (Haematoxylin and eosin) x 140.



Kidney

There were also areas of coagulation necrosis up to 0.8 mm in diameter and haemorrhage onto the capsular surface. In a typical focus several hepatocytes were in various stages of degeneration and a number contained single or multiple intracytoplasmic eosinophilic inclusion bodies (Figures 34 and 35). The inclusions were extremely variable in shape, some were spherical, others rod-like or composed of large granular, amorphous masses. Their size ranged from 5-25  $\mu$ m. In the early stages of cellular damage the nucleus was not affected, but later nuclei were enlarged and pale and the cytoplasm was filled with granular eosinophilic inclusion material. The inclusion bodies stained a bright cherry-red in sections treated by the Machiavello method. Kupffer cells were enlarged and activated in many areas of damage and often contained cellular debris. Mitotic figures were observed in some hepatocytes and occasional multinucleate cells were seen. There was little infiltration of the lobule by leucocytes, and lymphoreticular infiltration of portal triads was slight or absent. In several of the more severely affected livers thrombosis and fibrin deposition had occurred in some central and portal veins.

Fibrin thrombi were present in the capillaries of a small number of glomeruli but the lesions predominantly involved tubules and larger blood vessels. There was intense congestion accompanied by small inter-tubular haemorrhages and occasionally fibrin deposition in the blood vessels of the cortex and outer medulla in most animals. Tubular necrosis with nuclear pyknosis and fragmentation of basement membranes was also present. This lesion affected loops of Henle and had an irregular focal distribution. Inclusion bodies could not be demonstrated. In collecting tubules and ducts there were commonly amorphous deposits, fibrin casts and plugs of cellular debris. Cellular

Figure 34. Vervet monkey liver 6 days after infection. There is necrosis of hepatic cells, mild infiltration by lymphocytes and activation of a few Kupffer cells (Haematoxylin and eosin) x 320.

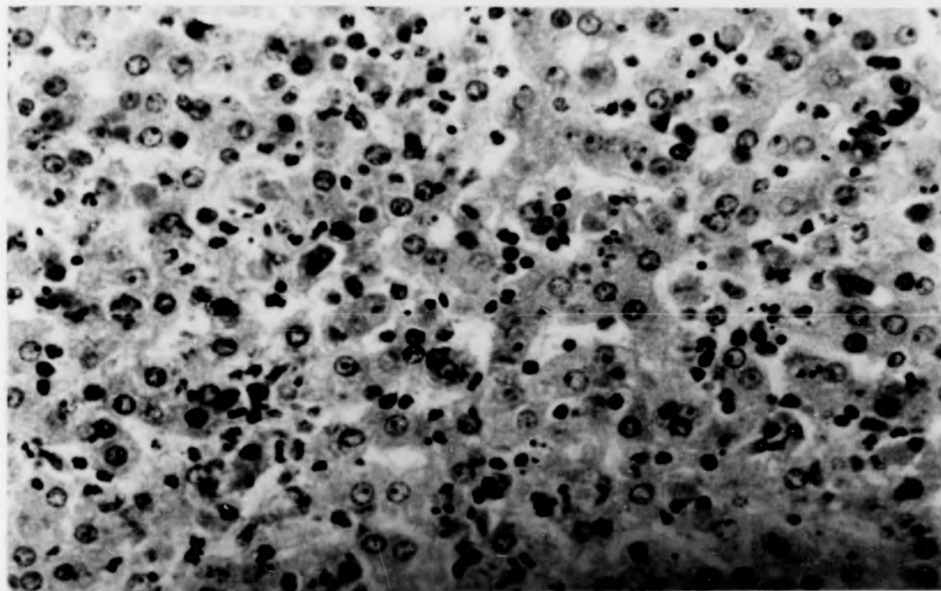


Figure 34. Vervet monkey liver 6 days after infection. There is necrosis of hepatic cells, mild infiltration by lymphocytes and activation of a few Kupffer cells (Haematoxylin and eosin) x 320.

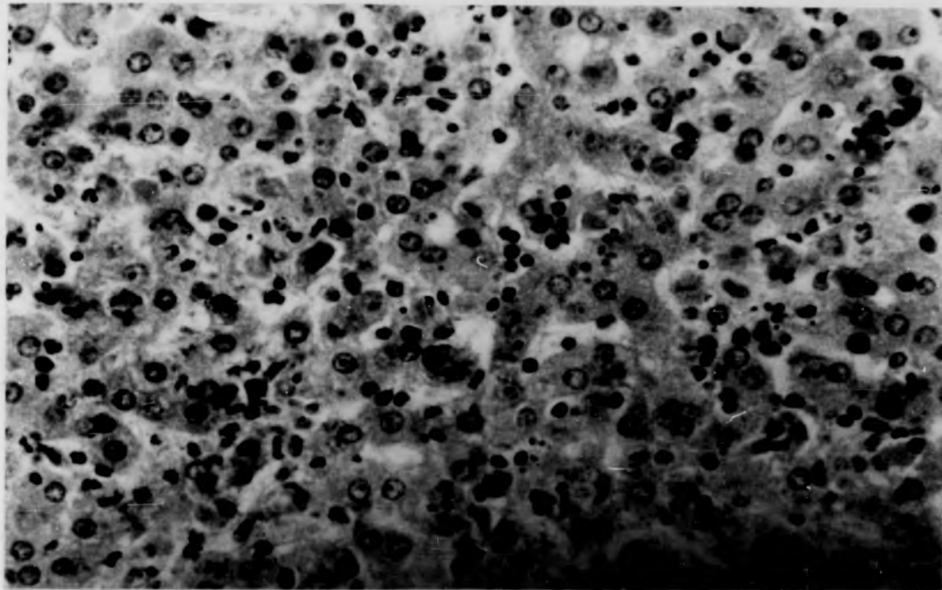
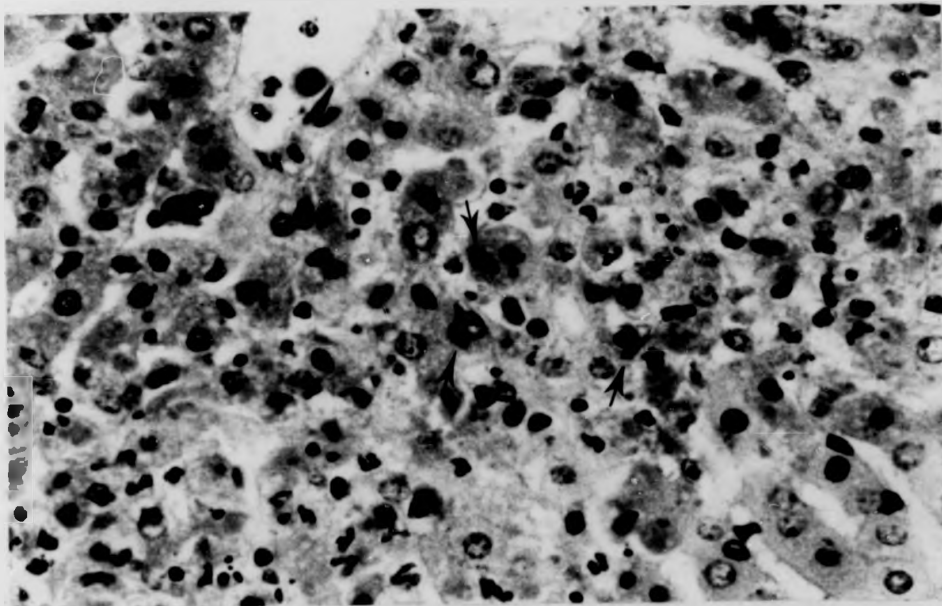




Figure 35. Typical area of liver damage in vervet monkey 6 days after infection. There are intracytoplasmic inclusion bodies in a number of hepatic cells (Haematoxylin and eosin) x 500.



infiltrations were absent.

Lungs: The degree of involvement of the lungs in different animals varied considerably. In some lungs there were only small isolated foci of necrosis of septa and intra-alveolar oedema formation, but in others there was widespread collapse and necrosis of inter-alveolar septa, oedema formation, fibrin deposition and haemorrhage into alveoli (Figure 36). Inclusion bodies were found in the cytoplasm of some alveolar epithelial cells in areas of damage. The epithelium of bronchi and bronchioli remained unaffected in all cases, the lesion apparently occurring at the alveolar and vascular level. Lesions of the type associated with infestation by the lung mite Pneumonyssus imocola were minimal and were readily distinguishable from the changes produced by the virus infection.

There was thrombosis and fibrin deposition in pulmonary venules in many lungs (Figure 37) and similar changes were also occasionally present in small branches of the pulmonary artery. There was no detectable damage to the walls of affected blood vessels.

Spleen: Necrosis of lymphoid tissue of the Malpighian corpuscles was present to some degree in all monkeys (Figure 38) and was accompanied by depletion of lymphocytes in most follicles and by congestion of the red pulp. In the most severely affected animals Malpighian corpuscles contained aggregates of eosinophilic amorphous material and there was also necrosis of the cells and connective tissue of the red pulp (Figure 39).

Lymph nodes: Generalised depletion of lymphoid tissue was evident and there was focal necrosis of many lymphoid follicles. Nodes most severely involved, such as the mesenteric, contained large areas of coagulation necrosis of cortical tissue and frequently

Figure 36. Lung of rhesus monkey on 12th day. Necrosis of some interalveolar septa has occurred and alveoli contain debris and oedema fluid (Haematoxylin and eosin) x 140.

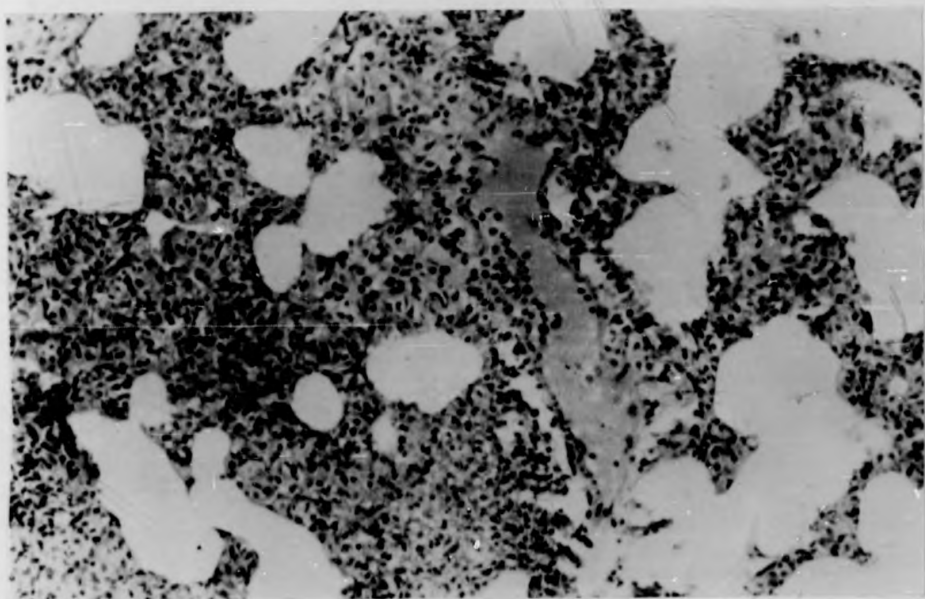


Figure 37. Lung of rhesus monkey 6 days after infection. A fibrin thrombus occludes a venule (Phosphotungstic acid haematoxylin) x 215.

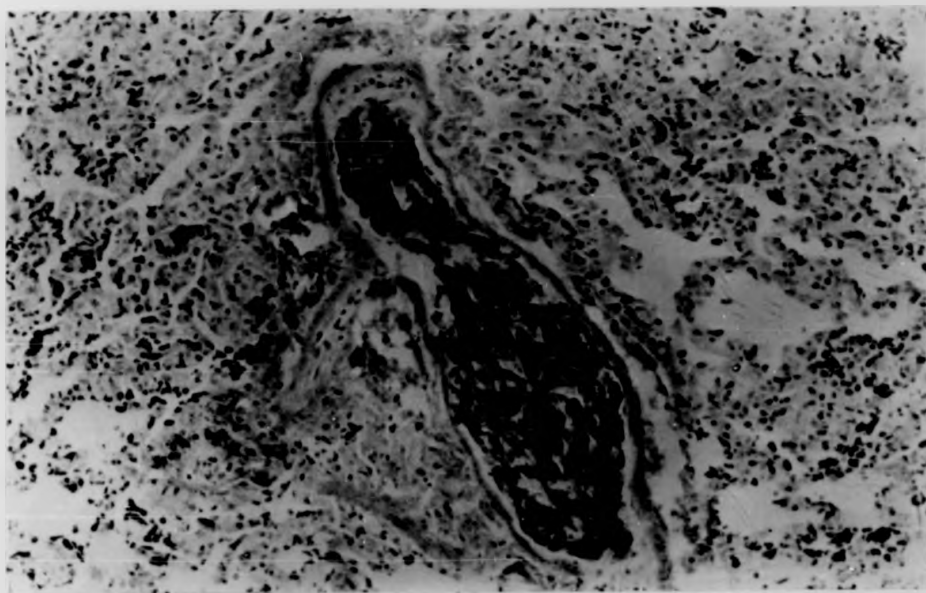


Figure 38. Rhesus monkey spleen, 6 days after infection. There is necrosis of the central white pulp and depletion of lymphoid tissue (Haematoxylin and eosin) x 140.

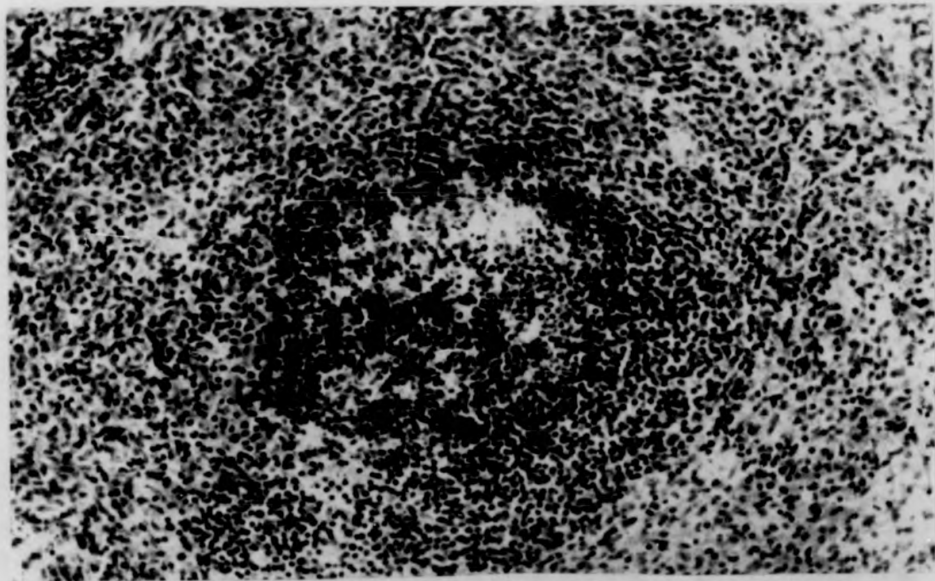
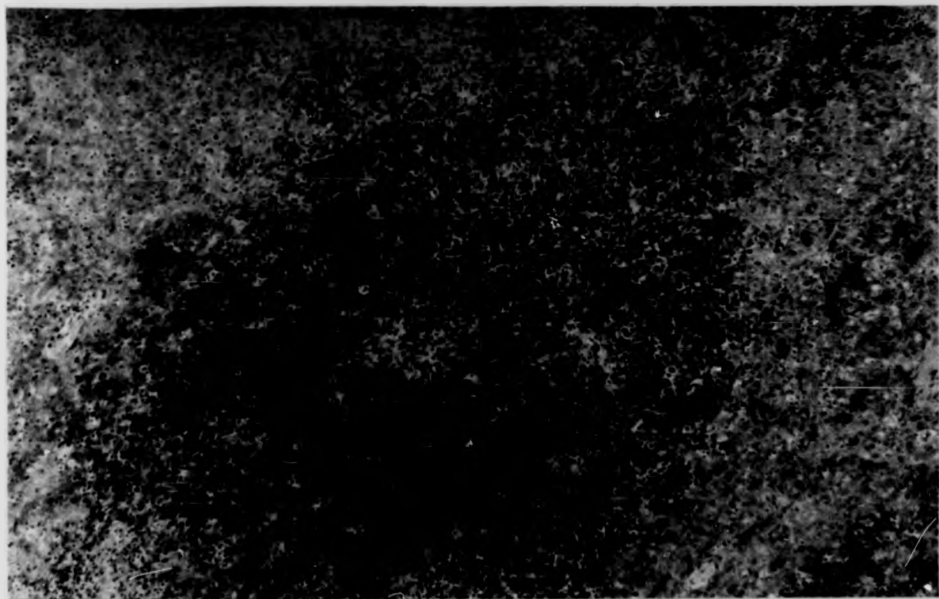


Figure 39. Spleen of rhesus monkey 6 days after infection showing necrosis of the central white pulp tissue and the surrounding red pulp (Haematoxylin and eosin) x 85.





also had haemorrhage into the subcapsular and medullary sinuses. In the longest surviving monkey, which lived until the 12th day after infection, many nodes exhibited sinus histiocytosis in addition to cortical necrosis.

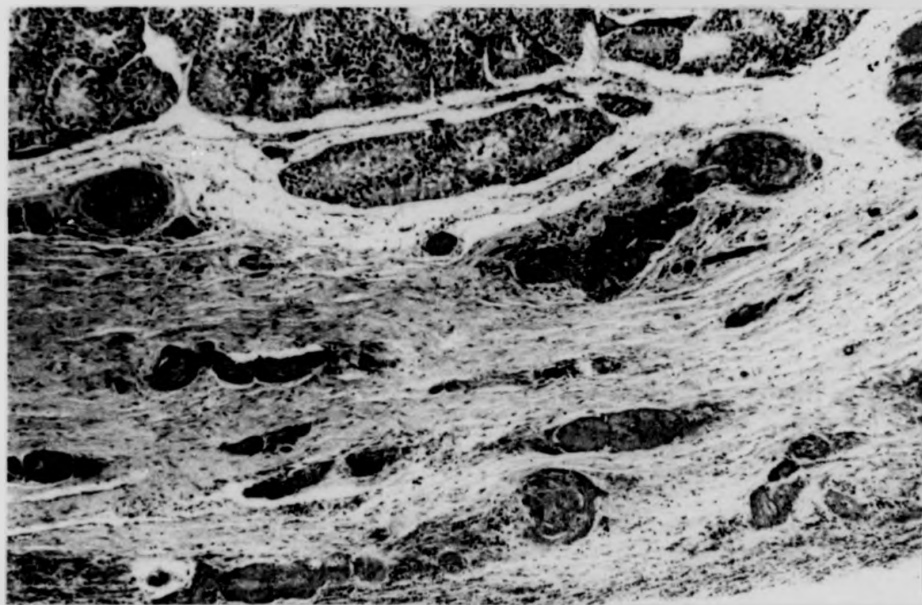
Adrenal glands: A consistent finding was intense congestion of the blood vessels of the cortex and medulla, with small haemorrhages, predominantly in the zona fasciculata. There was no damage to the parenchyma and no cellular infiltration of the glands.

Gastrointestinal tract: Lesions were not found in the stomach. Congestion and small haemorrhages from mucosal and submucosal blood vessels were present in the ileum and large intestine in all the animals. Necrosis of the surface epithelium, crypt tissue and lamina propia with ulceration occurred in five out of eight monkeys. Where there was necrosis of the mucosa it frequently extended into the underlying Peyer's patches. Villi in affected regions were shrunken and irregular in shape.

Testes: Five of the eight monkeys were males. Of these 3 had testes which histologically were sexually mature and the gonads of the remaining two were immature. There were no lesions in the testes of the monkeys killed 2 and 4 days after infection. In the fully established disease from the 5th day three animals (2 sexually mature, 1 sexually immature) had testicular lesions. These consisted of intense congestion and thrombosis of blood vessels of the tunica albuginea (Figure 40), small haemorrhages between seminiferous tubules, and cellular exudation, oedema and necrosis in the visceral layer of the tunica vaginalis and in the tunica albuginea. In addition the testes of the two sexually mature monkeys also showed focal necrosis of groups of seminiferous tubules.

Skin: Microscopical examination of the rashes showed congested capillaries and veins in the dermis and small haemorrhages. Separation and desquamation of superficial layers of the epidermis from the basal

Figure 40. Testes of rhesus monkey 6th day. Blood vessels of the tunica albuginea are occluded by thrombi (Haematoxylin and eosin) x 85.



layer occurred in some areas. There was no cellular inflammatory response.

Myocardium: Small haemorrhages were present between fibres in the ventricles of most monkeys. Occasional myocardial fibres showed hyaline degeneration in 4 animals. Cellular infiltrations were absent.

Other tissues: No changes were found in the tongue, trachea, bladder, pancreas, salivary glands or skeletal muscle.

Electron Microscope studies of monkey tissue: Ultrathin sections of liver, lung and spleen obtained from a rhesus monkey on the 6th day post infection showed large numbers of various forms of Ebola virus particles. The worst affected was the spleen where few cells remained recognisable and where rounded and elongated forms of the virus were fairly common (Figure 41). The lung also contained large numbers of these various particles usually in association with recent fibrosis (Figure 42). The liver apparently contained fewer virus particles than the spleen and still possessed areas of recognisable hepatocytes (Figures 43 and 44).

Figure 41. Electron micrograph of monkey spleen showing round and elongated forms of the Ebola virus x 32,000.

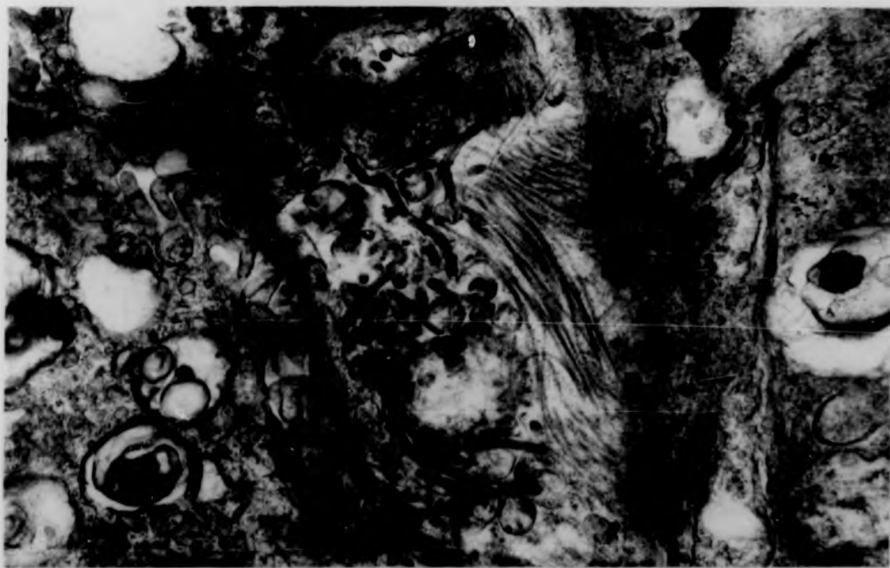
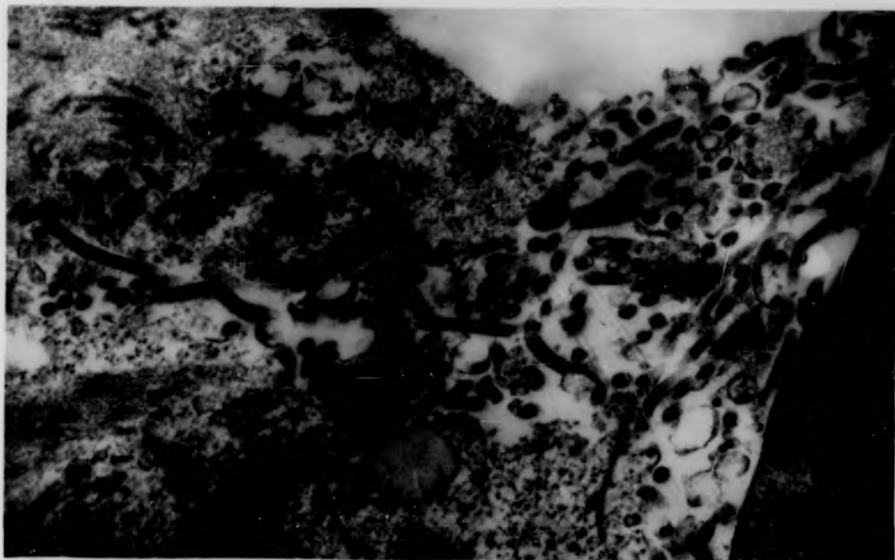
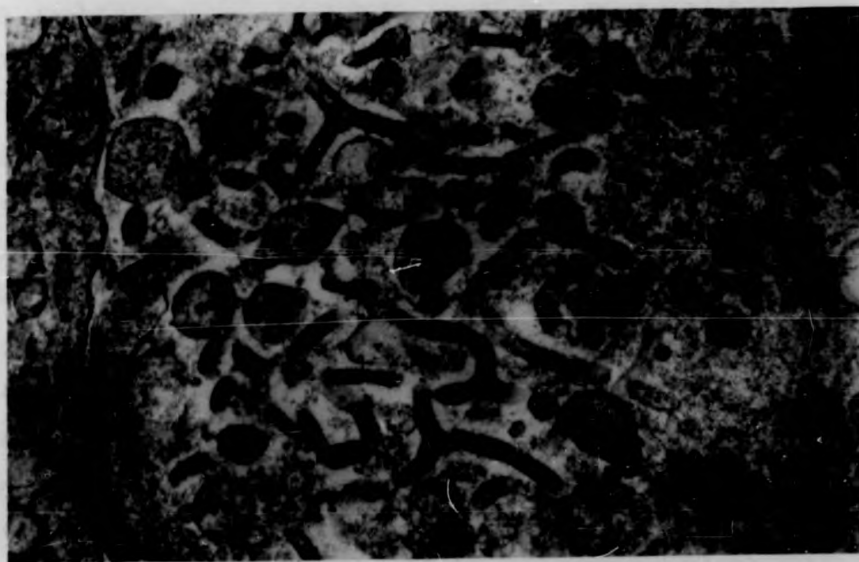
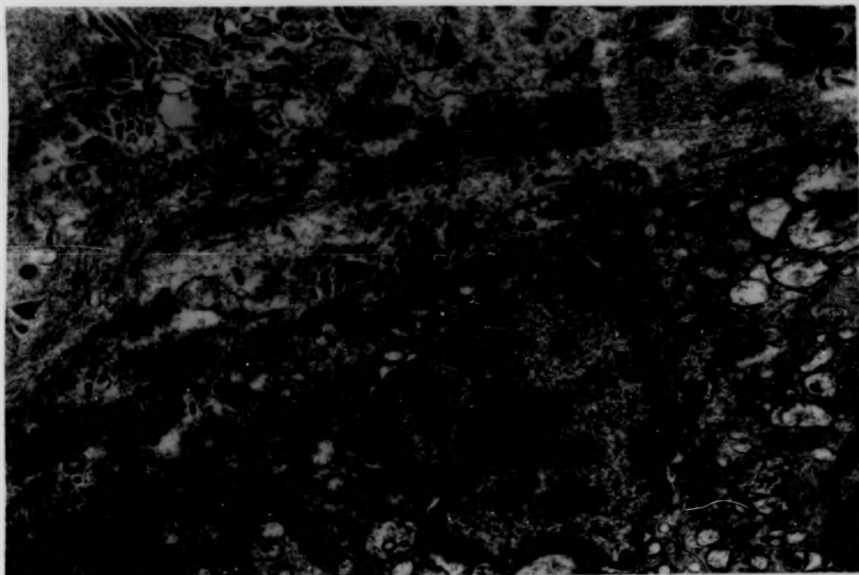


Figure 42. Electron micrograph of monkey lung showing various forms of Ebola virus x 20,000.

Figures 43 and 44. Electron micrographs of monkey liver again showing  
the various forms of Ebola virus x 12,600.



x 50,000

Monkeys infected with the Sudan strain of Ebola virus (Boneface)

Rhesus monkey (Rh 43)

Clinical observations: The clinical course of the disease in monkeys which were infected with the Sudan strain Boneface followed a similar pattern. Monkeys developed high fever, weight loss, anorexia, haemorrhages and a distinctive skin rash. However, although the course of the infection with the Sudan strain of Ebola virus appeared to be just as severe, the monkeys survived the infection. Figure 45 shows graphically the response of a rhesus monkey (Rh 43) to the infection. The monkey became febrile on the third day with a temperature of  $39.5^{\circ}\text{C}$ . The pyrexia reached a peak of  $40.6^{\circ}\text{C}$  on days 7 and 8 and persisted until day 14 when it dropped to  $38.6^{\circ}\text{C}$ . The temperatures spiked again on day 16 to  $39.5^{\circ}\text{C}$ , returning to normal on day 18 and remained normal thereafter.

Virus levels in the blood: The virus was first detected in the blood on the 4th day reaching maximum virus titres of  $10^{6.5}$  G.P.I.U./ml on day 6. This high level of virus in the blood was maintained on days 7, 8 and 9, dropping to  $10^{3.5}$  G.P.I.U./ml on day 10. The level of virus on day 12 was  $10^{1.5}$  G.P.I.U./ml but further specimens of blood collected on days 14, 16, 18 and 21 were all negative.

Fluorescent antibody levels in the blood: Fluorescent antibody was first detected in the blood at a titre of 1/4 on days 8 and 9, this increased to 1/8 on day 10 and to 1/16 on day 12. The antibody level then gradually increased to 1/1024 on day 21 and remained at this level up to day 32.

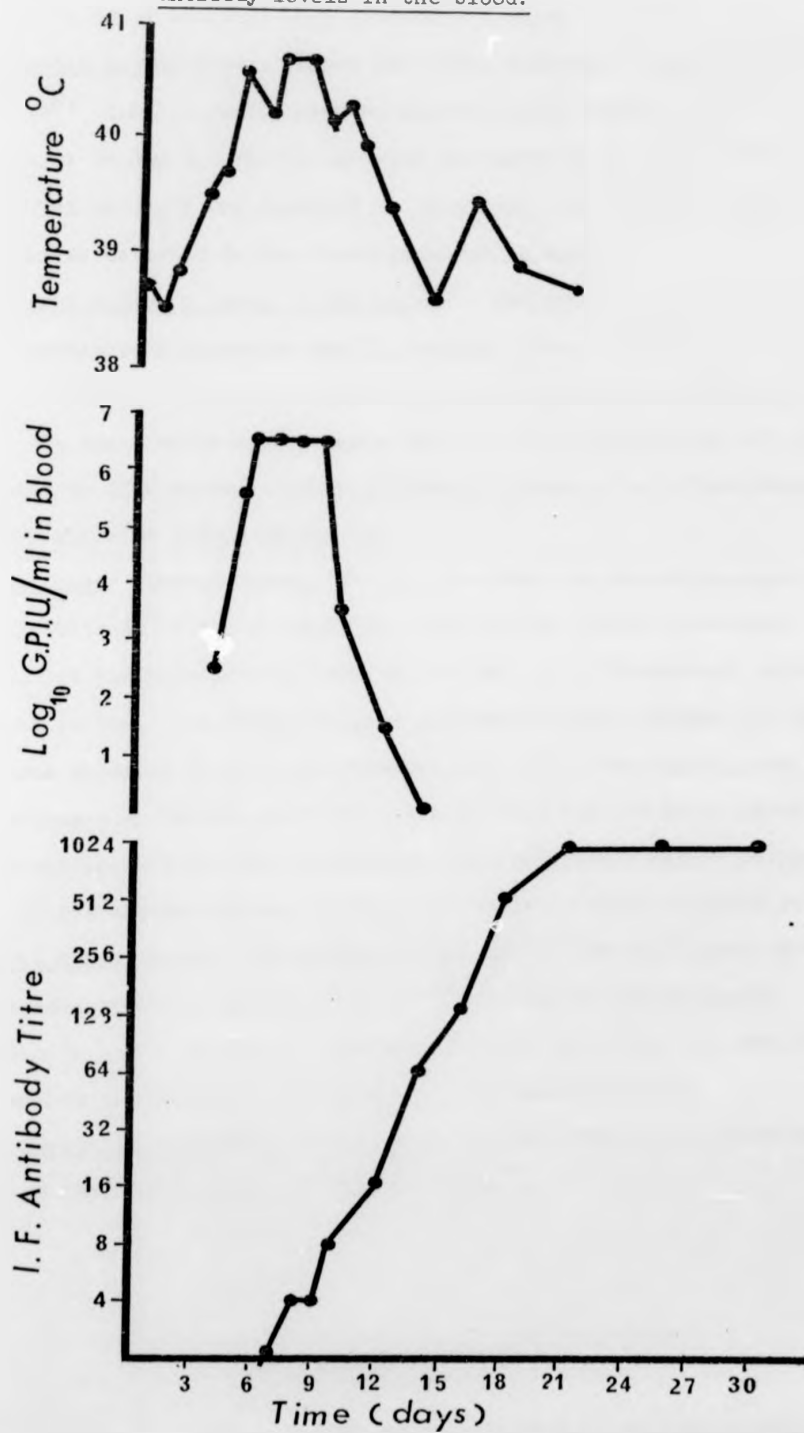
Rhesus monkey (Rh 11)

A second rhesus monkey (Rh 11) was infected with the Sudan strain Boneface. When this monkey became febrile, ( $40^{\circ}\text{C}$ ) on day 3, 20 ml of human convalescent plasma with a fluorescent antibody titre of 1/256 was infused intravenously over a period of 3-4 minutes. Figure 46 shows graphically the response of this rhesus monkey to the infection following the infusion of the convalescent plasma.

Clinical observations: The temperature continued rising to  $40.6^{\circ}\text{C}$  on



Figure 45. Rhesus monkey 43. Rectal temperature, virus and antibody levels in the blood.



days 4 and 5, then dropped to 40.1°C on day 6. The temperature rose to its peak on day 7 (40.8°C) then gradually dropped to normal by day 11.

Virus levels in the blood: Virus was first detected in the blood on day 3 ( $10^{2.5}$  G.P.I.U./ml), reaching maximum virus titre of  $10^{6.5}$  G.P.I.U./ml on day 6. The virus level gradually dropped to  $10^{2.5}$  G.P.I.U./ml by day 8 and remained at this level through days 9 and 10. No virus was detected in the blood from day 12 onwards.

Fluorescent antibody level in the blood: Following the infusion of 20 ml of convalescent plasma on day 3, antibody levels of 1/8 were recorded in the monkey's blood on days 4 and 5, this dropped to less than 1/4 on day 7. On day 8 antibody was again detected at a dilution of 1/4, this increased to 1/16 on day 12 then gradually increased to a fluorescent antibody titre of 1/1024 by day 45.

Re-challenged: Rhesus monkey (Rh 11) was later challenged intraperitoneally with  $10^4$  G.P.I.U. of Ebola virus (Sudan strain, Boneface) on day 56. At the time of challenge the monkey had a fluorescent antibody level of 1/1024. The monkey did not develop a fever, neither was there any virus detected in the blood between days 1-8. This monkey was again re-challenged on day 81, this time with  $10^4$  G.P.I.U. of Ebola virus (Zaire strain, E 718). The fluorescent antibody level before challenge was  $> 1/1024$  and the outcome of this challenge is shown in Table 16.

Clinical Observations: The monkey became febrile on day 4 with the temperature rising to a peak of 40.7 (°C) on day 5 then gradually dropping to normal on day 8. During this time the monkey was off its food and water from days 4-6 inclusive. No rash developed.

Virus levels in the blood: Ebola virus was detected in the blood at a level of ( $10^1$  G.P.I.U./ml) on days 5, 6 and 7.

Figure 46. Rhesus monkey 11. Rectal temperatures, virus and antibody levels in the blood.

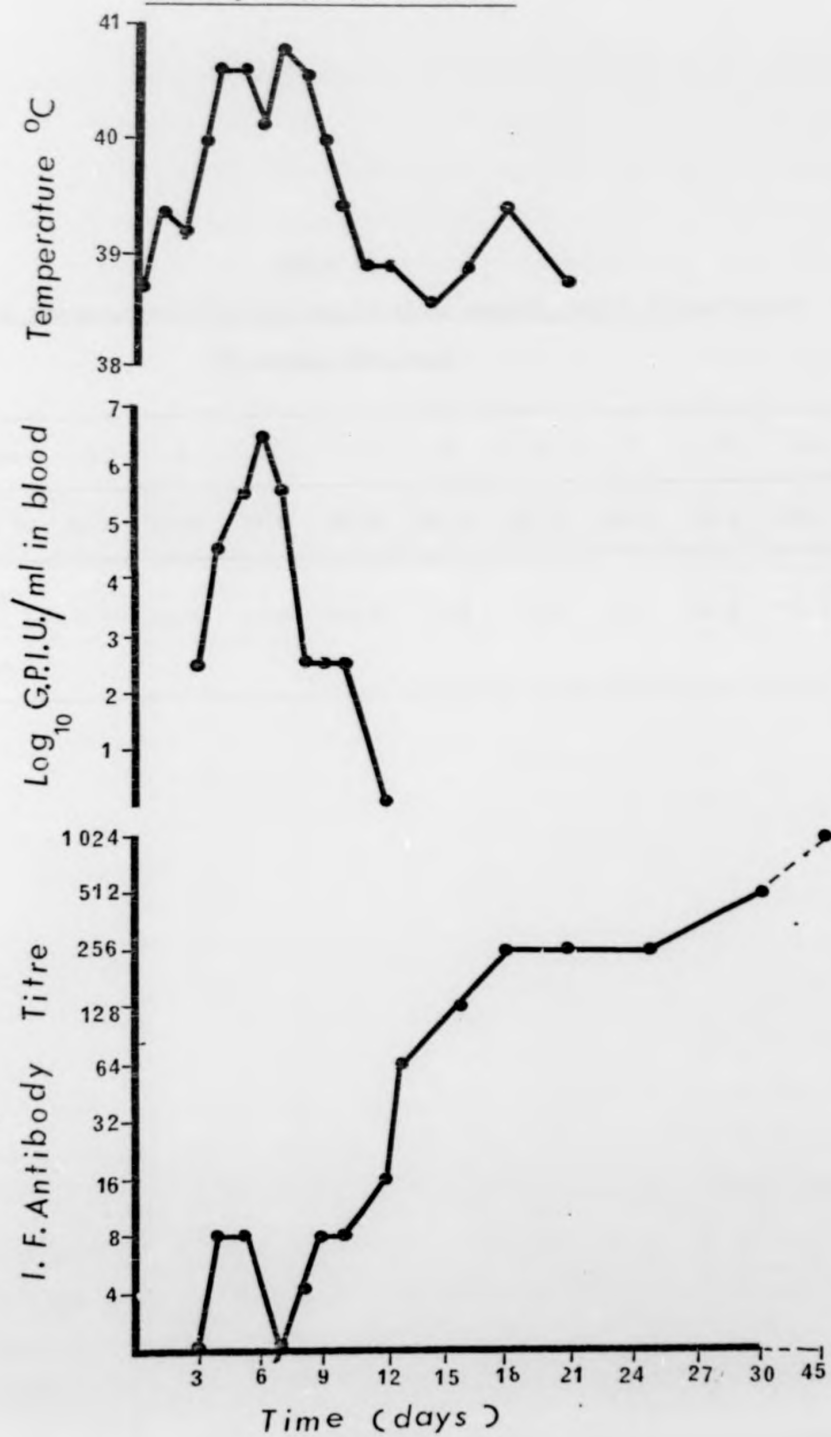


Table 16

Rectal temperature ( $^{\circ}\text{C}$ ) and Log 10 virus concentration in the blood  
following challenge

Days	1	2	3	4	5	6	7	8	9
Temp $^{\circ}\text{C}$	39.3	39.2	39.6	40.3	40.7	40.3	39.8	38.9	39.3
Virus level in the blood	<0.5	<0.5	<0.5	<0.5	1.0	1.0	1.0	<0.5	<0.5

The effect of interferon on experimental Ebola virus infection in Rhesus monkeys

Clinical observations: There were no marked differences in the clinical course of infection between the monkeys treated with interferon and the controls. Yet an impression was obtained that in the interferon-treated group the introduction of measures to combat disseminated intravascular coagulation, such as anticoagulant therapy and replacement of platelets and coagulation factors and fluid replacement may have prolonged survival. The monkeys became febrile on or about the third day after infection and the pyrexia persisted until death. Maculo-papular skin rashes with petechiae developed in all the animals on the 4th or 5th day and remained until death, although in 3 of the monkeys treated with interferon the rash was fading by the 6th or 7th day. The time of death of the monkeys in the different groups is shown in Table 17, and it is noted that survival may have been somewhat prolonged in 3 of the animals treated with interferon.

Table 17. Day on which death occurred

DAY	CONTROLS	INTERFERON		
		-2 DAYS	+1 HR	+3 DAYS
5	+			
6	+	+		
7	+			++
8		+	+	
9			+	

Viraemia: The titre of Ebola virus in the blood samples collected daily from each monkey was calculated by parallel titration in guinea pigs. The titres are shown in Table 18. Viraemia was not detected on the second day after infection in 3 out of 4 monkeys treated with interferon, whereas viraemia was evident in the 3 control monkeys and in the 2 monkeys treated with interferon 3 days after infection.

Findings at autopsy. Petechial and maculo-papular skin rashes of variable severity were present involving the forehead, face and cheeks

Table 18. Titre of Ebola virus in blood (Log 10/ml)

RHESUS MONKEYS	TIME IN DAYS AFTER INOCULATION							
	1	2	3	4	5	6	7	8
CONTROLS	< 0.5	2.5	4.5	5.5	4.5	-		
	< 0.5	2.5	4.5	6.5	5.5	5.5		
	< 0.5	2.5	4.0	5.0	5.0	6.5		
-2 DAYS	< 0.5	< 0.5	3.5	5.5	5.5	6.5	6.5	
	< 0.5	< 0.5	1.5	5.5	6.5	7.5	-	
INTERFERON +1 HR	< 0.5	< 0.5	1.5	3.5	2.5	3.5	6.5	6.5
	< 0.5	2.5	3.5	6.5	2.5	3.5	5.5	-
+3 DAYS	< 0.5	2.5	4.0	2.5	6.5	6.5		
	< 0.5	1.5	5.0	6.5	6.0	6.5		



the medial aspects of the limbs and the chest. Visceral petechiae were present in all the animals. Peritonitis was evident in most monkeys and intestinal haemorrhages and mucosal ulceration were found in many of the animals. The mesenteric lymph nodes were enlarged and haemorrhagic. The liver was pale and mottled. Petechiae and small haemorrhages were found on the pleural and cut surface of the lungs. Congestion and inflammatory changes were found in the tunica vaginalis and tunica albuginea of the testes.

There were no differences in the gross pathological findings between the treated and untreated animals, neither were there any consistent differences in the nature or severity of the lesions between the monkeys treated with interferon and the untreated animals.

#### Virus levels in the tissues

The distribution of virus in the tissues of two of the monkeys are shown in Table 19. These virus levels compared with those of the untreated monkeys (Table 15) are generally lower, particularly the virus levels in the spleen.

Table 19

Titres of Ebola virus in the tissues of monkeys which have been subjected to treatment with interferon. Expressed as Log<sub>10</sub> guinea pig infectious units/ml or grams of tissue.

Tissue	Rhesus 2 <sup>(1)</sup> Died Day 6	Rhesus 5 <sup>(2)</sup> Died Day 8
BLOOD	6.5	5.5
HEART	3.5	4.5
LUNGS	5.5	5.5
LIVER	6.5	6.5
SPLEEN	3.5	4.5
ADRENALS	4.5	4.5
KIDNEYS	3.5	1.5
TESTES	5.5	5.5
MES LYMPH NODES	5.5	4.5
FAECES	NEG	NEG
BILE	NEG	NEG

- 1) Monkey received interferon 2 days before infection and the treatment was continued daily thereafter.
- 2) Monkey was inoculated with the virus suspension and interferon administered 1 hour later and daily thereafter.

Virological and serological studies on a human case of Ebola virus infection

The first specimen of blood collected on the 11th November, 14 hours after the patient became feverish was examined by electron microscopy and virus particles were seen which looked suspiciously like those of Ebola virus. Guinea pigs which had been inoculated with this blood specimen developed a febrile illness and electron microscope examinations of their blood and tissues collected on the fifth and sixth day post inoculation showed particles which were again morphologically indistinguishable from those of Ebola virus.

Studies on blood collected during the acute-phase of the illness showed that the highest levels of virus in the blood ( $10^{4.5}$  guinea pig infectious units/ml) were recorded on the first and second days of the illness. Following treatment with interferon and convalescent serum the level dropped to  $10^{0.5}$  guinea pig infectious units/ml and remained at this level until the viraemia was undetectable on the ninth day from onset of illness (Table 20). No virus was isolated from throat swabs, faeces and urine collected between days 14 and 27. Ebola virus was, however, isolated from specimens of seminal fluid collected on days 39 and 61. Samples of seminal fluid collected on days 76, 92, 110 and 128, and two further specimens collected at three monthly intervals thereafter were negative.

Following the infusion of 450 ml of convalescent plasma (fluorescent antibody titre of 1/128-1/256) on the second day of illness antibody levels of 1/16 were recorded in the patient's blood from days three to nine. This increased to 1/32 on day 10 and gradually increased to a fluorescent antibody titre of 1/128 by day 34. The patient was then subjected to plasmaphoresis between 16th and 25th February, 1977. A total of seven units of plasma was taken which resulted in the fluorescent antibody level dropping from 1/128 to 1/32. A specimen of blood collected on 5th May 1977 had a fluorescent antibody titre of

Table 20

Day of sample and test from onset of illness	Activity of circulating antibody as F.A. titre	Recovery of infective virus as guinea pig I.P. infective units per ml or gram of sample tested	
		Positive	Negative
1	-	Blood, $10^{4.5}$	
2	1/2	Blood, $10^{4.5}$	
3	1/16	Blood, $10^{0.5}$	
4	1/16	Blood, $10^{0.5}$	
5	1/16	Blood, $10^{0.5}$	
6	1/16	Blood, $10^{0.5}$	
7	1/16	Blood, $10^{0.5}$	
8	1/8	Blood, $10^{0.5}$	
9	1/16		Blood
10, 11, 12, 13	1/32		Blood
14, 16, 20	1/64		Blood, Faeces, Urine and Throat Swab
23, 27	Not done		Blood, Faeces, Urine and Throat Swab
34	1/128		Blood
39	Not done	Seminal Fluid $10^{0.5}$	
61	1/128	Seminal Fluid $10^{0.5}$	Blood, Urine
76	1/128		Blood, Urine, Seminal Fluid
92, 110, 128, 219, 310	Not done		Urine, Seminal Fluid

1/16 while a specimen collected on the 9th November, almost one year from the onset of illness had a fluorescent antibody titre of 1/8.

Epidemiological studies on the Sudanese outbreak

Virus isolations: Blood, throat swabs and urine were obtained from 29 suspected cases of the disease in the Maridi isolation wards. Three strains of Ebola virus were isolated in guinea pigs, all from acute-phase blood from three patients - one from a 12 year-old schoolgirl (Malia Boneface) who died three days later, another from an 18 year-old male student (Moh'd Jeopard) who later recovered and the third was from a 25 year-old male (Philip Nimaya).

Serology: One hundred and four surviving Maridi patients were identified. All of them had been diagnosed as suffering from Ebola fever on clinical grounds alone. 35 of these patients were members of Maridi hospital staff and the remaining 69 patients were Maridi residents, some of whom were relatives of hospital staff. 30 of the 35 surviving hospital staff were bled for serological studies. 25 of them (83%) had detectable IF antibodies. Only 18 of the 69 patients who were not hospital staff were willing to be bled. 17 of these patients had detectable antibodies. Altogether 42 of the 48 patients diagnosed clinically as having been infected and who were bled had antibodies against Ebola virus (Table 21).

Among the probable and possible case contacts hospital staff were considered to be among those most at risk. 159 members of the staff had given no history of severe febrile illness during the epidemic. Sera from 64 of these staff members were tested for Ebola virus antibodies. The results are shown in Table 22. Seven staff members, including four nurses, a cleaner, a toilet cleaner and a water carrier had evidence of infection. None of the three doctors attending sick patients had antibodies.

One hundred and two members of the Maridi population who had been in contact with known cases of infection were tested. The majority of them were close family contacts and several had helped to nurse sick relatives during their illnesses. 22 case contacts had demonstrable antibodies

Table 21

Results of IFA tests on  
convalescent patients' sera

Hospital Staff

	<u>Number of</u> <u>survivors</u>	<u>Number</u> <u>tested</u>	<u>Number</u> <u>positive</u>
Medical Assistant	1	1	1
Nurses and midwives	9	8	7
Student nurses	17	14	11
Cleaners	4	4	3
Miscellaneous staff	<u>4</u>	<u>3</u>	<u>3</u>
	35	30	25

Non-hospital Staff

	<u>69</u>	<u>18</u>	<u>17</u>
Overall total	<u>104</u>	<u>48</u>	<u>42</u>



Table 22

Results of IFA tests on sera from  
Maridi Hospital staff contacts

	<u>Number bled</u>	<u>Number positive</u>
Doctors	3	0
Nursing staff (Including tutors, midwives, theatre staff)	35	4
Drivers	5	0
Cleaners	11	1
Toilet cleaner	1	1
Water carrier	1	1
Laboratory assistant	1	0
Messengers	3	0
Gardeners/carpenters	<u>4</u>	<u>0</u>
Totals	64	7

(Table 23). Close questioning revealed that nine of these close contacts had some evidence of febrile illness without manifesting severe disease. 29 members of a Maridi school were tested as a control group. None of them was thought to have had any contact with a known or suspected case of Ebola virus disease. However, three schoolboys had antibodies. None of them had any history of recent illness.

In Nzara 37 surviving patients diagnosed clinically as having had Ebola fever were identified and 31 of them were bled for antibody studies. Only six (19%) had demonstrable antibodies and none had antibody levels greater than 1:32 (Table 24). Among close family contacts, only one person out of the 78 who were tested had detectable antibodies although five of these contacts had an antibody titre of 1:4 which was not accepted as positive. Sera from 109 cotton factory workers were tested (Table 25). Seven members of the staff had antibodies with the largest number, three, in the cloth room and adjacent store. Three workers in the weaving section had antibodies while only one member of the 28 spinning section staff who were bled was positive. Antibody levels ranged from 1:16 to 1:64 and none of the seven workers gave a history of any recent illness.

#### Virus isolations from arthropods and small mammal tissues

Blood and tissues from 53 rats, 39 bats and 33 pools of arthropods (mosquitoes and ticks) were inoculated into guinea pigs for Ebola virus isolation attempts. No viruses were isolated.

Table 23

Results of IFA tests on sera from  
members of the Maridi population

	<u>Number</u> <u>bled</u>	<u>Number</u> <u>positive</u>
Case contacts	102	22
No known contact	29	3
	131	25

Table 24

Results of IFA tests on sera from Nzara  
convalescent patients and family contacts

<u>Number of survivors</u>	<u>Number</u> <u>bled</u>	<u>Number</u> <u>positive</u>
37	31	6
Number of contacts bled	78	1

Table 25

Results of IFA tests on sera from  
Nzara cotton factory staff

	<u>Number</u> <u>bled</u>	<u>Number</u> <u>positive</u>
Cloth room and store	17	3
Drawing-in section	9	1
Weaving section	55	2
Spinning section	28	1
Totals	<u>109</u>	<u>7</u>

Conclusion and Discussion

Many of the clinical features displayed in the infections caused by Lassa, Marburg and Ebola viruses are remarkably similar particularly in the early stages of the diseases which they cause. Fever, prostration, vomiting, chest pains, epigastric pain, rash and sometimes haemorrhages are common to all three, but the onset of Lassa fever is generally insidious (Mertens et al., 1973; Troup et al., 1970 and Frame et al., 1970) whilst Marburg and Ebola fevers have a very sudden onset. Pharyngitis is a very common feature in Lassa fever accompanied by pronounced conjunctivitis and periorbital oedema. Although conjunctivitis and pharyngitis have been reported with Marburg and Ebola fevers they are not such a regular feature.

Marburg and Ebola virus infections produce very similar features. The incubation periods have been variable. Martini et al., 1968; Martini, 1969 and Stille et al., 1968 reported incubation periods ranging from 3-9 days in the Marburg and Frankfurt outbreaks in West Germany in 1967, while the period of incubation during the 1975 South African incident was 7-8 days, (Gear et al., 1975). In the much larger Ebola virus epidemic that took place in Sudan and Zaire in 1976, the incubation period varied from 4-16 days with an average of 7 days (Simpson, 1977; WHO, in press). The single case which occurred in England in 1976 following a laboratory accident had an incubation time of 6 days (Emond et al., 1977). The illness caused by Marburg and Ebola viruses began abruptly with severe frontal and temporal headache, severe malaise, generalized aches most particularly in the lumbar region and sensitive and painful eyes, (Martini, 1969; Gear et al., 1975; Simpson, 1977). A high fever was obvious on the second day of illness and patients became progressively more debilitated over the next few days.

While the similarities of clinical features in Lassa, Marburg and Ebola virus infections may make diagnosis difficult, the isolation of virus readily distinguishes Lassa from Marburg and Ebola. Lassa virus grows readily in Vero cell culture producing an obvious cytopathic effect



(Buckley and Casals, 1970). In our studies a cytopathic effect appeared within 4 days of inoculation and was very obvious by day 5. Wulff and Lange (1975) showed that the indirect immunofluorescent antibody technique could be used for the detection of Lassa virus antigen in infected Vero cell cultures and we have successfully adopted the method for the detection of Lassa antibodies and for the identification of new isolates of Lassa virus. The most rapid identification of Lassa virus isolate was made 48 hours after the inoculation of Vero cell cultures with an acute-phase human serum sample sent to us from Nigeria. Wulff and Lange (1975) and more recently Wulff et al. (1977), Wulff and Conrad (in press) claim that the immunofluorescent test system is specific for the detection of Lassa virus antibodies and for distinguishing Lassa virus from other members of the arenavirus complex. Unfortunately we, like other workers, have been unable to develop a reliable neutralization test system for Lassa.

Ebola and Marburg viruses grow in a variety of cell culture systems. Marburg virus was successfully cultured in a wide variety of tissue culture systems both in primary and established cell lines (Smith et al., 1967; Siegert et al., 1968; Zlotnik et al., 1968; Malherbe and Strickland-Chalmley, 1968; Kissling et al., 1968; May and Knothe, 1968; Kunz et al., 1968; Slenczka et al., 1968, 1969 and Gear et al., 1975). The cell lines most studied were baby hamster kidney (BHK 21) and cultures derived from Ceropithecus aethiops kidney cells, GMK-AH1 and Vero cells. Ebola virus has been studied largely in Vero cells although a variety of different systems have been investigated by van der Groen et al. (1978). In early passage neither Marburg nor Ebola produced a specific cytopathic effect which is a sharp contrast to Lassa virus. A more obvious cytopathic effect may appear after several passages and Johnson et al. (1977) have claimed that cytopathic changes are discernible in Vero cells following inoculation of Ebola virus. Most workers however, have preferred to base their evidence of cell infection on the appearance of the characteristic intracytoplasmic inclusion bodies demonstrated by

conventional staining methods (Zlotnik et al., 1968; Mahoney and Strickland-Gibbsley, 1968; Sliemka et al., 1968) or by immunofluorescent staining techniques (Siegert et al., 1968; Carter et al., 1968; Gear et al., 1975; Johnson et al., 1977; Bowen et al., 1977). High virus titres have not been encountered in cell cultures, the maximum levels for Marburg virus being of the order of  $10^6$  G.P.I.U./ml even after several passages in such systems.

The structure of Marburg virus in thin section has been described by Peters and Muller (1968), Kissling et al., (1968), Zlotnik et al. (1968) and Siegert (1972), while negatively stained preparations have been widely examined (Siegert et al., 1967; Siegert, 1972; Peters and Muller, 1968, 1969; Kissling et al., 1968; Hoffman and Kunz, 1968; May and Knothe, 1968; Bowen et al., 1969; and Almeida et al., 1971). Ebola virus has been similarly studied (Johnson et al., 1977; Bowen et al., 1977, Pattyn et al., 1977, Knobloch et al., 1977; and Ellis et al., 1978). Two main structural forms of Marburg have been recognised (a) a filamentous or sinuous form and (b) a circular form resembling a doughnut or torus. Several intermediate forms have been described. There were 3 morphologically different types of the sinuous particle, the covered type being merely an uninterpretable form of one or other of the two genuine sinuous forms, one with an internal helix and one without. The form with the internal helix is presumably viable while that without is probably an empty or incomplete particle. Both forming and degraded particles showed that the naked helix is the internal component, both of the full sinuous form and of the mature circular torus. The one constant measurement linking the naked helix, the full sinuous form and the mature particle is the 28 nm diameter of the core of the internal helix (Peters and Muller, 1968; Kissling et al., 1968 and Zlotnik et al., 1968). Almeida et al., (1971) have suggested that the naked helix is the primary physical component of the virus and that this is surrounded by a sheath of a periodicity of 4.5 nm. The structure is completed by the addition of a lipid containing membrane bearing projections

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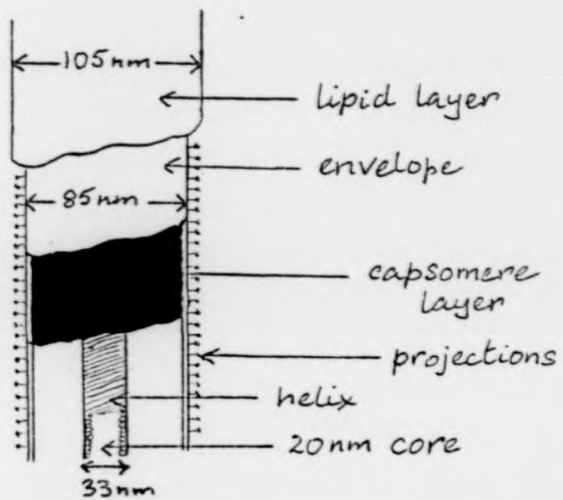
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on the surface. The first stage of virus maturity is reached when the full sinuous form develops constrictions along its length with subsequent breaking off of unit lengths which curve round to give the circular torus forms found in large aggregates. This type of morphogenesis, apart from the step giving rise to the torus, has been described for rhabdoviruses (Bradish and Kirkham, 1966; Nakai and Howatson, 1968). The similarity between Marburg virus and rhabdoviruses such as vesicular stomatitis virus and rabies was noted by Siebert et al., 1968 and Kissling et al., 1968. Like the rhabdoviruses, Marburg virus has been shown by Siebert et al., 1968; Kissling et al., 1968 and Bowen et al., 1969 to be ether sensitive and heat labile, to contain ribonucleic acid, and to be formed by budding through the cell membrane. However both the cross-sectional structure of the mature Marburg virus particle and the nature of the intracytoplasmic inclusions it causes differ significantly from corresponding rhabdovirus structures. The difference in core size must represent a fundamental difference in design. The size of Marburg virus alone makes it significantly different from members of the rhabdovirus group. Kunz et al., (1968) suggested the name "Rhabdovirus simiae" but Almeida et al., (1971) suggested that since the mature form of Marburg virus forms large aggregates of predominantly "doughnut-shaped" particles it should become a founder member of the group called "Toro-viruses".

Our electron microscope studies show that Ebola virus is structurally indistinguishable from Marburg virus. This similarity has been reported from several laboratories (Johnson et al., 1977; Bowen et al., 1977; Pattyn et al., 1977; Knobloch et al., 1977; Murphy et al., 1978 and Ellis et al., 1978). Peters et al. (1971) and Almeida et al., (1971) gave diagrammatic representations of the Marburg virus structure as the full (ie. complete) particle and empty (ie. incomplete) particle. We believe that Ebola virus has a very similar structure and the suggested structure of the "complete" sinusoidal particle is shown in Figure 47.

Figure 47

The suggested structure of the complete sinusoidal particle.



Although structurally identical, Marburg and Ebola viruses are quite distinct serologically. Johnson et al. (1977) showed that both viruses are antigenically different when studied by indirect immunofluorescence, with perhaps the exception of a weak reaction to Marburg antigen only at a dilution of 1/4 with a Zairean human convalescent serum. Ebola virus was distinct from Marburg virus strains isolated in Germany (1967) and in South Africa in 1975. The homologous Marburg titres with the 1967 and 1975 antigens and antisera were 1/128 and 1/64 respectively. We have confirmed the distinction between Marburg and Ebola virus by both cross-protection tests in guinea pigs and by immunofluorescence. We have also shown that there is a close similarity between the Zairean and Sudanese strains of Ebola viruses.

Very few physical and chemical studies have been carried out with Ebola virus. Like Marburg, Ebola is both heat and ether sensitive but no further investigations on inactivation have yet been carried out.

We had a unique opportunity to study the viraemic response in man following accidental laboratory infection with Ebola virus, clinical details of which have been described fully by Emond et al. (1977). Virus was detected early in the disease,  $10^{4.5}$  G.P.I.U./ml being detected on the first day of illness. No detectable change in the levels of circulating virus was evident on the day following initiation of interferon therapy. However within twelve hours of administering 450 ml of Ebola immune plasma the viraemia level had fallen to  $10^{0.5}$  G.P.I.U./ml. This much reduced level of circulating virus persisted throughout the acute stages of illness and virus became undetectable on the 9th day of illness.

Emond et al. (1977) were unable to draw any definite conclusions as to the value of either interferon or immunotherapy. As both were administered together their respective merits cannot be assessed. There is no doubt that viraemia levels were dramatically reduced soon after the administration of immune plasma, but the patient's clinical condition deteriorated despite the low virus levels in the blood.

The discovery of virus in the semen was not unexpected since Marburg virus is known to persist in seminal fluid for several weeks after infection (Martini and Schmitt, 1968; Martini, 1969). Marburg virus has also been recovered from the anterior chamber of the eye two months after the onset of illness (Gear et al., 1975).

The infection produced by Ebola virus in guinea pigs is indistinguishable from that caused by Marburg virus. All the isolations made by us from material sent from Sudan and Zaire were obtained in guinea pigs. The animals became febrile 4-7 days after intraperitoneal inoculation of acute-phase human serum samples. The febrile phase lasted for several days during which time the animals failed to thrive. Only 1 of 10 guinea pigs inoculated with original material died. The other guinea pigs slowly recovered and were subsequently shown to have developed specific antibodies detectable by the indirect fluorescent antibody technique. Passage of guinea pig blood taken during the febrile stages of illness produced a more uniformly fatal illness. Although morphologically and antigenically the Sudanese and Zaire strains of Ebola virus were shown to be very closely related if not identical, there was however a difference in the expression of virulence in the guinea pig. The suggested implications of this will be discussed later.

The pathological picture in guinea pigs was also remarkably similar to previous findings with Marburg virus. The liver, spleen and lymph nodes were the organs most consistently affected (Zlotnik et al., 1968; Zlotnik, 1969; Bowen et al., 1977). The liver showed numerous foci of necrosis with pleomorphic eosinophilic bodies seen in the cytoplasm of several degenerating cells. Kupffer cells were enlarged and periportal areas were heavily infiltrated by lymphoreticular cells. In the spleen and lymph nodes there was a widespread depletion of lymphoid tissue.

Ebola virus produced an illness in monkeys which closely resembled that found in man (Emond et al., 1977; WHO, 1976 and in press). High fever, severe weight loss, anorexia, haemorrhages and a distinctive



skin rash was most pronounced in the forehead and face where it was often confluent in patches. On the medial aspects of the fore and hind limbs the rash was more obviously maculo-papular. The rash in rhesus monkeys was always much more marked than in vervet monkeys which often had no rash at all. The illness produced in monkeys closely resembles that caused by Marburg virus (Simpson, et al., 1968; Simpson, 1969) and the infection in monkeys resulted in a uniformly fatal illness.

Both Ebola and Marburg virus predominantly affect the liver, spleen and lymph nodes and in addition Ebola virus has some affinity for the lungs, intestines and testes. Of particular interest is that the only pancreas examined had high concentrations of virus. This accords well with the clinical observation of pancreatitis in man following Marburg virus infection during the South African outbreak (Gear et al., 1975).

The pathological changes produced by Ebola virus in man (Johnson et al., 1977, Bull, WHO 1978) are very similar to those caused by Marburg virus (Gedigk et al., 1968; Gear et al., 1975). Similar pathological lesions were found in monkeys infected with Marburg (Zlotnik, 1969; Murphy et al., 1971) and Ebola virus (Baskerville et al., in press). Both viruses mainly affect the liver, spleen and lymph nodes, whilst Ebola has some affinity for the lungs, intestines and testes. Liver lesions are comparable in both diseases, although the intracytoplasmic inclusion bodies of Ebola infection in monkeys are much larger and more clearly defined. The inclusions in Marburg disease have been shown by electron microscopy to be aggregates of virus particles (Murphy et al., 1971) and it is likely that this will prove to be the case with the inclusions of Ebola. Changes in the livers of guinea pigs inoculated with the earliest passages of Ebola virus differed slightly from those in the monkey livers in having fewer inclusion bodies and a moderate lymphoreticular reaction in portal triads (Bowen et al., 1977). Also pulmonary lesions in guinea pigs were slight by comparison with those induced in the monkeys. At present it is not clear whether these

differences represent a genuine difference in species' response to the virus or whether they reflect changes in the behaviour of the virus with passage.

The widespread occurrence of thrombosis and fibrin deposition in the organs of monkeys infected with Ebola virus suggests that disseminated intravascular coagulation (DIC) may be a feature of the disease, as it is in some other viral haemorrhagic fevers (McKay and Margaretten, 1967). Gear et al., (1975) reported that 2 of the cases of Marburg virus infection occurring in South Africa had evidence of DIC although there was no evidence of this condition during the German outbreak in 1967 (Simpson, 1977). DIC cannot be conclusively diagnosed by microscopic criteria alone, and for confirmation clinical pathological parameters such as thrombocyte counts, prothrombin times and fibrinogen levels would be required. Because of the hazards to personnel from infected blood such a study in monkeys has not yet been possible. The distribution of the lesions of acute renal tubular necrosis may indicate a vascular origin for the damage, and is further evidence that DIC may be occurring, since these lesions are also found in patients with DIC. The DIC may be due to failure of the damaged liver to synthesise coagulation factors, or it may be due to a direct action of virus on blood components. For this critical levels of virus in the circulating blood may be necessary. This requirement was almost certainly fulfilled, since high titres of virus were demonstrated in the blood of all monkeys over a period of several days.

Ebola virus could be isolated from all affected organs and lymph nodes at various stages of the infection. Histopathological examination showed no evidence of direct damage to vascular endothelium, even in vessels exhibiting coagulation, but this does not preclude slight but significant effects which might be detectable only by electron microscopy. In the lungs there was no damage to airway epithelium at any level. This may be because the virus has no affinity for

ciliated columnar epithelium, but it may also be due to the parenteral route of infection. The subsequent viraemia ensured arrival of virus in the lung via the blood at the alveolar level, where lesions developed.

Our studies indicated that monkeys provided a useful experimental model for the study of Ebola virus infection. Consequently as immune plasma and interferon had been used to treat a human Ebola virus infection (Emond et al., 1977) we used human leucocyte interferon to treat experimentally infected monkeys. Our preliminary findings with a small number of rhesus monkeys treated prophylactically with human leucocyte interferon and at the time of infection are not discouraging. Clinically survival appeared to be enhanced and an impression was gained that life-support measures to combat disseminated intravascular coagulation and fluid replacement in the animals treated with interferon would have favoured recovery. Viraemia was delayed and there is obviously a need to establish the optimal dose and duration of treatment with interferon either prophylactically or early in the course of the infection.

At present, the availability of interferon is very limited. However, several chemical inducers of interferon have been investigated. Polyinosinic polycytidylic acid (Poly I. Poly C), a double-stranded RNA, is a potent inducer of interferon in mice and has been found to be effective against various virus infections both prophylactically and therapeutically. It is, however, a poor inducer of interferon in man and in non-human primates and it does not induce any detectable serum interferon. This may be related to the presence of an enzyme in the serum of primates which hydrolyses and inactivates Poly I. Poly C. Poly I. Poly C has been complexed with poly l-lysine and carboxymethyl cellulose (Poly ICLC) by Levy et al. (1975). This stabilised derivative is partially resistant to hydrolysis by primate serum and induces the formation of several thousand units of interferon per ml of serum in primates. This compound has been tested in non-human primates against several serious virus diseases. Thus, Poly ICLC was effective prophylactically in simian haemorrhagic fever. When this compound

was given to monkeys 6 hours after a large inoculum of yellow fever virus, about 75% of the treated animals survived and developed good serum antibody titres, in contrast to deaths of all the untreated monkeys (WHO, 1978). The use of Poly ICLC in experimental Ebola virus infection in non-human primates is being explored, since it can induce high levels of serum interferon with few major toxic side effects.

The administration of human immune plasma to infected monkeys had a less dramatic effect than was found in the single human infection treated in a similar manner. Two monkeys were inoculated with a Sudanese strain of Ebola virus which, as will be discussed later, appeared to be a less virulent strain than those emanating from Zaire. One monkey was given an intravenous infusion of human immune plasma at the onset of fever. Viraemia levels did not fall immediately as was found in man, but fell steadily over a period of several days. In the untreated monkey the level of virus in the blood remained high for several days before decreasing. Both monkeys survived infection in contrast to infection with Zaire strains where the outcome was always fatal.

Most of our epidemiological investigations have been carried out on material collected during the Sudanese outbreak. Details of this outbreak and its origin have been described (WHO in press; D.I.H. Simpson, personal communication). The first cases of haemorrhagic fever are thought to have originated in Nzara township in three employees of a cotton factory situated near the town centre. The factory, opened in 1952, forms part of a large agricultural cooperative with a total workforce of 2,000 people. 455 staff are employed in the factory which produces cotton cloth from raw cotton grown in small holdings throughout the region.

The first identifiable case was YG, a store-keeper in the factory, who became ill on 27th June, 1976 with a severe febrile illness, headache and chest pains. He developed haemorrhagic manifestations on the fifth day of illness with profuse bleeding from the nose and mouth and bloody diarrhoea. He was admitted to hospital in Nzara on 30 June and died on

6th July. During his illness at home he was nursed by his brother who in turn became ill on 13th July. His symptoms were similar but he recovered after a two-week illness and was able to give a description of both illnesses. A second store-keeper, BZ, who worked alongside YG, was admitted to Nzara hospital on 12th July and died on 14th July. Soon afterwards his wife also became ill and died at home on 19th July. Both had had severe febrile illnesses complicated by bleeding. The third man from the factory to fall sick was PG who was employed in the cloth room beside the store where YG and BZ worked. PG became sick on or around 18th July and was admitted to Nzara hospital on 24th July, dying on 27th July. Although all three men were employed in the weaving section of the factory their homes were widely separated geographically and their life styles were very different. There appeared to be no social contact between any of them. YG lived six miles south of Nzara in a remote rural homestead while BZ lived two miles east of the town. Both men lived quietly with their families and had few friends or contacts within their home environments. The third man, PG, was a batchelor who lived in the centre of Nzara close to a shop belonging to a general merchant, MA. PG was reportedly an embullient character, known to almost everyone in the area and he was closely associated with the merchant's family and employees. He was particularly friendly with two brothers, Samir S and Sallah S, staying in the merchant's household. All three young men helped in the shop and organised parties and dances in the area. During PG's illness he was visited and comforted by many people including two women, HW and CB, who nursed him before he was admitted to hospital.

Samir S became ill on 26th July and after a few days in Nzara he travelled with his brother Sallah to Maridi on 6th August intending to go to Juba. He became so ill in Maridi that he was admitted to Maridi hospital on 7th August and died there on 17th August. Sallah helped to care for Samir in Maridi hospital and then returned to Nzara on 18th August

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when he too had begun to feel ill. Meanwhile in Nzara four close contacts of PG - the two women HW and CB, together with SU, another cotton factory employee and close friend of PG, and RJ a nurse at Nzara hospital, had become ill and died from the disease. They in turn infected several others who had nursed them during their illnesses at home. Sallah S arrived in Nzara on 18th August and was so ill the following day that he was visited by a hospital nurse AI who administered chloroquine and antibiotic infections. Sallah and one of the merchant MA's sons died later in the same house from the disease. The nurse, AI, fell ill on 24th August and was eventually taken to Maridi hospital where he died on 3rd September. The merchant, MA, had become ill on 21st August and went for treatment to Omdurman having travelled by road to Juba and thence by plane to Khartoum. He died in Omdurman hospital on 30th August. Shortly after he left Nzara several of his family and employees also contracted the same illness. They, in turn, sequentially infected several others and small pockets of infection were set up around Nzara.

It was possible to relate 48 cases of infection and 27 deaths in Nzara to the original infection in PG acquired by direct close contact usually involving nursing and care of an infected individual. However, in July, September and October, further unrelated cases continued to occur in cotton factory employees for which no direct contact with previously sick persons could be established. They, in turn, infected members of their own families but as each family lived in relatively remote homesteads little extra-family contacts were involved and these pockets of infection were self-limiting. The last recorded infection in Nzara was reported to have had an onset on 27th October 1976.

The disease was introduced to Maridi, 80 miles away, by Samir S when he was admitted to Maridi hospital on 7th August. A close friend, a hospital nurse, a hospital cleaner and a hospital messenger, all of whom had had close contact with Samir during his illness, each developed the same disease and were admitted to various wards in the hospital.



Hospital contacts of these patients, including visitors to the hospital, who often helped to care for patients, seeded the infection around Maridi township. A further focus of infection was introduced to Maridi hospital on 29th August when AI, the nurse from Nzara, was admitted with the same disease. The hospital served as an efficient amplifier providing 'seed, sower and soil' from which the virus was disseminated throughout the town. The number of cases gradually increased until mid-September and at the end of the month there were a large number of cases, particularly in hospital staff. The number of cases declined in early October, possibly resulting from the use of protective clothing. A considerable increase in cases was observed in late October and early November which may have been partly due to lack of protective clothing when supplies ran out in mid-October. These later cases were more frequent in individuals who were not employed in the hospital but who had been in contact with patients within the hospital or in their homes.

No really satisfactory serological test has yet been devised to detect and quantify antibodies against either Marburg or Ebola viruses. Early workers (Smith *et al.*, 1967; Kissling *et al.*, 1968; Siegert and Slenczka, 1971) used complement fixation tests to detect antibodies to Marburg virus following the outbreak in 1967. They used crude antigens derived from guinea pig and monkey tissues but found them to be unreliable because they lacked specificity. Malherbe and Strickland-Cholmley (1971) compared antigens derived from animal tissues with those prepared in monkey kidney cell cultures and found both types to be unsatisfactory. Slenczka and Wulff (1971) and Slenczka *et al.* (1971) found that antigens derived from chronically infected Vero cells were much more satisfactory but still had doubts about their activity.

Neutralization tests have been carried out in guinea pigs and tissue culture systems but have proved costly in animals, cumbersome, time consuming and generally very unsatisfactory. Siegert and Slenczka (1971) and we ourselves have had some success with neutralization tests in Vero

and BHK 21 cells based on counts of intracytoplasmic inclusion bodies but the results were difficult to reproduce. The problem is that no really obvious cytopathic changes can be readily visualised in any of the cell culture systems used so far although Johnson et al. (1977) have claimed that some cytopathic changes are discernible in Vero cells. However these workers, like ourselves, have based their serological results on immunofluorescent tests. Immunofluorescence with Marburg virus was described in BHK 21 by Carter and Bright (1968) but recent work on Marburg and Ebola has been based on the indirect immunofluorescent technique described by Wulff and Lange (1975) for use with Lassa virus. The method is more sensitive than complement fixation and appears to be quite specific. It certainly distinguishes between Marburg and Ebola viruses (Johnson et al. 1977). Wulff and Conrad (1977) believe that the method could be used successfully for survey work since, at least with Marburg, antibodies can be detected by this method five years after infection.

The diagnosis of Ebola virus infection was confirmed by the indirect immunofluorescent test in 42 of 48 Maridi patients who had been diagnosed clinically. These results were confirmed in several patients by a parallel test kindly carried out at CDC, Atlanta by Dr Patricia Webb. There can be little doubt that the other six patients in whom no antibodies could be detected, had been infected with the same virus since they all had febrile illnesses accompanied by haemorrhages and symptoms as described (WHO, in press). The lack of detectable antibodies in 25 of 31 Nzara patients was surprising and disappointing especially as all of them had had illnesses clinically indistinguishable from proven cases of Ebola fever. The Nzara patients were bled at times ranging from six to seventeen weeks after the onset of their illnesses. It may be that their antibody levels may have fallen below those detectable by immunofluorescent testing. In view of earlier results with Marburg virus this possibility seems unlikely. However, antibody levels in proven cases of Ebola

infection in Maridi did fall quickly. 18 of 23 patients bled in November 1976 and again in January 1977 had distinct falls in antibody levels ranging from 2- to 5-fold. Later antibody levels were often detected at the lowest acceptable dilution of 1/8. Two patients actually became negative. These results could possibly be explained if the immunofluorescent test was directed towards detecting IgM antibodies alone. Unfortunately the test was specifically directed to detect IgG and therefore no satisfactory explanation for the lack of detectable antibodies can be advanced. Obviously further work needs to be carried out on developing more sensitive test systems.

The findings of Ebola virus antibodies in 25 of 131 (19%) Maridi case contacts and in 7 of 64 hospital staff contacts (19%) suggests that Ebola virus can cause milder illnesses and even subclinical infections. It also supports the view (WHO, in press) that Ebola virus is not readily transmissible from person to person except where there is close and prolonged contact with a patient suffering from severe disease. Clinical observation of cases occurring late in the epidemic showed that frank haemorrhagic manifestations were less apparent. There may have been some attenuation of Ebola virus resulting from continued passage in man.

The antibody results on sera collected from the Nzara cotton factory staff indicate that 9 of the 24 staff (37%) of the cloth room and adjacent store were infected. The levels of antibody, ranging from 1/16-1/256, indicate a fairly recent infection although the seven members of staff who were bled in November and were shown to have been infected gave no history of illness. These results point very strongly to the cotton factory having been a prime source of infection. Unfortunately virus isolation attempts and serological studies on rodents and bats collected in the factory have not yet indicated that these animals are involved in the natural cycle of infection with Ebola virus.

The WHO investigation team in the Sudan gained a clinical impression (D.I.H. Simpson, personal communication) that cases of infection seen in

later stages of the epidemic manifested fewer severe haemorrhagic complications and fatalities were fewer. By November 1976 the virus had undergone at least 10 generations in man. The fewer fatalities may have been due in some part to better clinical care, but the impression of a less severe disease remained. This impression was also noted when guinea pigs were used to isolate virus strains emanating from Sudan and Zaire. The virulence of Sudanese isolates for guinea pigs was found to be less intense than those isolates originating from Zairean patients. Similar findings were also found in monkeys. Infection with the Zairean strain of Ebola virus invariably resulted in a fatal infection with high fever, rash and marked viraemia, monkeys dying between days 5-8. On the other hand, monkeys infected with the Sudanese strain although developing fever, rash and viraemia, nearly always recovered after what appeared clinically to be a similar disease.

When one of these monkeys was challenged eight weeks later with the same Sudanese strain of Ebola virus, no clinical disease developed, there was no febrile response and no viraemia was detected. However, when this same monkey was later re-challenged with a Zairean strain of Ebola virus, it developed a low level viraemia and a febrile response despite the fact that high levels of fluorescent antibody to both strains of Ebola were present in the circulation. These findings are difficult to interpret, but suggest that cross-specificity as shown by serological tests may not be simply related to cross-protection as shown by tests in different hosts. All of these features may reflect different qualities of the virus, host, antibody interaction. Further investigations into this phenomenon are obviously required.

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