YELLOW FEVER VIRUS INFECTION
A CORRELATION OF COMPLEMENT-FIXING ANTIGEN
WITH HISTOPATHOLOGY

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SUMMARY.—A sequential and quantitative survey of brain and liver of suckling
mice for infective virus and complement-fixing antigen, after infection with
yellow fever virus, showed that while there was progressive increase of infective
virus content in both organs, only the brain showed a corresponding rise in
CF antigen. Histopathological examination revealed that the liver was not
significantly involved. The target organ was the brain, where the progressive
pathological changes culminated in an acute encephalitis by the 3rd day of
experiment. Organ destruction began with the molecular layer of the grey
matter. But by the 4th day after infection the entire cerebral cortex was in-
volved. At the initial stages the hippocampus was particularly affected.
Tissue damage did not appear to be entirely due to the differential quantitative
localization of infective virus. It was hypothesized that the CF antigen acting
singly or in conjunction with some hypothetical proteins may be principally
involved in the pathological outcome of the disease.

The pathogenesis and pathology of yellow fever virus infection are among the
most exhaustively studied viral infections. In 1890 Councilman described lesions
which later on came to be known as Councilman bodies; formed by the coalescence
of the contents of necrotic hepatic cells. It is also amply documented that the
pathological changes in the rhesus monkey are fundamentally similar to those in
man (Hudson, 1928a). In both cases the most distinguishing lesions are found
in the liver, where there is cloudy and fatty degeneration of the hepatic cells,
and a typical mid-zonal necrosis. The first definitive description of the liver
pathology in the rhesus monkey was by Stokes, Bauer and Hudson (1928). This
was later followed by a very detailed account of microscopic pathology by Hudson
(1928b). All these accounts were unanimous that the brain was without any signi-
ficant pathological involvement. Bearcroft (1957) established that the changes
in the liver parenchyma were initiated by single hepatic cells which later on involve
other cells of the organ by a radial spread. Cytochemical studies of the infected
liver cells were reported also by Bearcroft (1960). A detailed review of the path-
ological manifestations of the disease in different animals has been published
(Bugher, 1951).

The virus was first adapted to mice by Theiler (1930); who observed also that the
pathological picture in the mice was different from that in either man or monkey.
In mice the target organ was the brain instead of the liver. Theiler also observed

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that there was a greater localization of the virus in the brain than in the liver, where there was either very little virus or none at all. And death in mice seems to be as a result of encephalitis. However, it seems not yet settled whether the pathological changes in man, monkey or mice are due to the presence of infective virus or other virus-specific or virus-induced substances. Hughes (1933) observed that at the terminal stages of yellow fever infection in monkeys the concentration of precipitinogen, and therefore by inference also complement-fixing (CF) antigen, reflected the severity of the illness, and paralleled the extent of liver damage. A parallelism between infective virus content and precipitinogen concentration was, however, not demonstrated. Perlowagora and Lennette (1944) also reported a correlation between the destruction of hepatic cells in infected monkeys and CF activity. They also pointed to the dissociation of infective virus from CF antigen.

It was felt that perhaps a time course study of yellow fever infection in mice would throw some light on the question. This paper therefore reports the results of sequential and quantitative surveys of both brain and liver for the production of infective virus and CF antigen in Swiss white mice following infection with two strains of yellow fever virus. One of these is the classical 17D strain, and the other is a wild strain isolated from a recent human case. The results of the serological tests were correlated with the corresponding histopathology of the organs.

MATERIALS AND METHOD

Virus.—The 17D strain of yellow fever virus was originally obtained through the courtesy of the West African Council for Medical Research, Yaba, Lagos, Nigeria. The virus has been passaged here 7 more times by intracerebral (i.c.) inoculation of baby mice. The H43913 strain was isolated here from a fatal case during a recent yellow fever outbreak. It has undergone 4 mouse brain passages.

Complement-fixation test.—The method used was based on the microtitre technique of Weimbren (1958). Essentially the test consisted of dropping the reagents with cut No. 18 gauge hypodermic needles carried on oiling tubes. Each one of such drops delivered vertically is 0.025 ml. Plastic trays were used. A drop of the specific immune mouse ascitic fluid (IMAF) was followed by 2 drops of complement dilution (2 haemolytic units). A drop of antigen was finally added. After gentle tapping the trays were left at 4° overnight. Two drops of haemolytic system (3 per cent sensitized sheep cells) were thereafter added. The reagents were mixed again, and the trays were incubated at 37° for 30 min., with occasional shaking. The tests were read after the cells had been allowed to settle at 4°.

The antigens consisted of brain or liver suspensions of infected mice. The IMAF was prepared with the Asibi strain. Controls for normal antigens and normal ascitic fluids were included. Veronal buffer was used as diluent.

Haemagglutination test (HA).—This was carried out at room temperature with the infected organ suspensions (1 drop) and 1 per cent freshly collected goose RBC (2 drops). Before the addition of the RBC 1 drop of diluent was added. A stock suspension of RBC was initially prepared in saline. But immediately before use all the reagents were diluted in appropriate pH diluent so as to obtain a working pH of 6.3. The test was performed in microtitre plastic plates.

Histopathology.—Mice were sampled daily, anaesthetized with light chloroform, and the organs dissected out immediately. The brain was fixed in 10 per cent formalin, and the liver in Bouin's fluid. Sections were cut and stained with haematoxylin and eosin.

Experimental plan.—This was essentially as was reported previously (David-West, 1970) with only slight modifications. Briefly, the method was as follows: stocks of both strains of yellow fever virus were prepared in bovine albumin diluent (BA) which consisted of 0.75 per cent bovine albumin in 0.05 M phosphate buffered saline at pH 7.0. Penicillin and streptomycin in respective concentration of 2000 units and 600 mg./ml. were added. The stocks were titrated i.e. in 2-day old Swiss white mice; each mouse receiving 0.02 ml. of 10-fold
dilution. From the results of the titrations a dilution of each virus, shown to cause a uniform morbidity, was selected for the test.

Seven cages of mice of the same age as those used in the titration were used for each virus. Each cage contained 6 mice. One of the cages served as normal control on the 1st day of the experiment, while another provided normal controls on the day the last sampling was done. The remaining 5 cages were inoculated with virus and sampled each day for 5 days. From each day’s samples mice were kept in a Revco refrigerator (—60°C) for serology, while those for histology were processed immediately.

For serological tests 10 per cent (w/v) suspensions were prepared in BA from accurately weighed brains or livers dissected out at the time of testing. Infectivity titrations were done in 2-day old mice, and LD₅₀ titres calculated by Karber’s Method. The same suspensions were tested for both CF and HA activities. The reciprocal of the dilution of organ suspension giving at least a 2+ fixation or agglutination was taken as the end point.

The experiments were repeated 4 times.

RESULTS

Representative results of the experiments are reported.

Serological survey of brain and liver

Yellow fever virus strain 17D.—The results illustrated in Fig. 1 show that infective virus titre increased steadily in the brain from the 1st day after inoculation to the 4th day. The corresponding titres were 10⁴-⁰ LD₅₀ and 10⁶-⁵ LD₅₀. There was usually a slight drop in titre on the 5th and final day, when also the first signs of sickness were observed. The liver titre followed a similar pattern; 10²-² LD₅₀ on day 1 to 10⁴-³ on day 5. In most of the titrations it was observed that the

![Graph](image1)

![Graph](image2)

**Fig. 1.**—Multiplication of yellow fever virus (strain 17D) in baby Swiss white mice and the sequential production of complement-fixing antigen. Note haemagglutinin detection at the terminal stages of the infection.

**Fig. 2.**—Multiplication of yellow fever virus (strain H43913) in baby Swiss white mice and the sequential production of complement-fixing antigen. Note haemagglutinin detection at the terminal stages of the infection.
titre of infective virus in the liver was 1–2 logs lower than that in the brain. This difference was very seldom up to 3 logs. The concentration of CF antigen in the brain also increased progressively to a titre of 160 on day 5. No CF antigen was demonstrated in the liver under these conditions.

There was no plan to test for HA activity when the experiments were originally designed. However, when fresh goose RBC became available it was felt desirable to test for HA. The antigens used were the crude organ suspensions, which had been stored at $-60^\circ$ for about 2 months. Nevertheless, titres of at least 160 were attained with the brain suspensions on both the 4th and 5th days. The test was negative for the other days. A prozone effect was noticed. No HA activity was demonstrated in the liver.

Yellow fever virus strain H43913.—As was with the 17D strain, virus concentration in the brain increased after the 1st post-inoculation day (Fig. 2). The titre rose from $10^{4.2}$ LD$_{50}$ on day 1 to a titre of $10^{6.8}$ LD$_{50}$ on day 5. Infective virus titre in the liver also increased from $10^{2.8}$ LD$_{50}$ on day 1 to $10^{4.5}$ LD$_{50}$ on the 5th day. Although no drop in titre on the last day was observed in the results used here, however, in other experiments, a fall in titres of 1·0–1·5 logs was demonstrated. Complement-fixing antigen titre also increased from < 10 on day 1 to 80 on day 5. HA titres of 160 or greater were attained on day 4 and day 5, with the brain suspensions. Unlike strain 17D no prozone phenomenon was seen. And although the same titres were obtained with both strains the reaction with strain H43913 was much stronger (4 + as against 2 +). As with strain 17D the mice became sick on day 5.

Neither CF nor HA activity was detected in the corresponding liver suspensions.

Histopathology

Since the sequence of pathological changes was similar in both the 17D strain and the H43913 strain of the virus, only the results of the former will be described in detail, and any differences observed will be noted during the report. Furthermore, the patterns described here were consistent in all the experiments conducted.

Brain.—The control brains of day 1 and day 5 (Fig. 3 and 9) had thin un congested leptomeninges. The cerebral cortex had layers of polarized neurones and neuroglia. The basal ganglia were unremarkable. The ependymal lining of the ventricles was formed by 2 layers of columnar cells.

On the 1st day after i.c. inoculation (Fig. 4) the leptomeninges were slightly congested and oedematous. There was focal haemorrhage into the lateral and third ventricles; probably due to the trauma of injection. But this was rapidly resolved by the 3rd day. There was oedema of the white matter of the hippocampus. On the 2nd day (Fig. 5) the oedema of the leptomeninges increased with few mononuclear inflammatory infiltrates present. There was pavemencing of the leptomeningeal vessels by inflammatory cells. The molecular layer of the grey matter of the cerebral cortex was markedly oedematous. There was focal disruption of the cellular zone of the grey matter; most marked in the hippocampus. There was vacuolation of the nuclei of some neurones with large nucleoli. The cytoplasm was intensely eosinophilic in few cells. Inflammatory cells were few. The area of haemorrhage in the basal ganglia and temporal lobes had haemosiderin granules. On day 3 (Fig. 6) the mononuclear cells in the leptomeninges were fewer but the vessels were still slightly congested. The oedema of the molecular layer of the grey matter was significantly increased. There was
distribution of the polarized cellular lower zone of the grey matter. The neurones and some neuroglial cells had haloes around them. Mononuclear infiltrates were present around a few dead cells. This was maximal at the hippocampus. The intimate vasculature of the brain was slightly congested. There was no perivascular cuffing. At this stage, acute encephalitis was established. No inclusion bodies were, however, seen. On the 4th day (Fig. 7) the leptomeninges were virtually free of inflammatory cells. There was complete disruption of the zonal layers of the cerebral cortex with diffuse infiltration of acute inflammatory cells. There was marked vacuolar degeneration of neurones and neuroglial cells with satellitosis and neuronophagia in areas. There were focal intense subependymal inflammatory infiltrates. The intimate vasculature was congested, but perivascular cuffing was not prominent. There were a few questionable eosinophilic cytoplasmic inclusion bodies especially in the parietal lobe neurones. No nuclear inclusions were observed. On the 5th day (Fig. 8) the leptomeninges were congested. There was intense, generalized, acute encephalitis with reticular degeneration and markedly congested intimate vasculature.

Compared with strain H43913, the 17D strain caused a more severe and diffuse form of acute encephalitis. This may be due to its long adaptation to the mouse brain. There was greater oedema and greater diminution in the width of the molecular layer of the grey matter, and a more severe disruption of cerebral architecture. Skip areas were present in the brain infected with H43913, but not with 17D strain. There was the possibility of inclusion bodies being present in the lesions of 17D, but not in those of H43913. The progressive intensity of the lesions from day to day was very striking.

Liver.—The control liver of day 1 had trabeculae of hepatocytes in a syncytial pattern, unlike that of day 5, which had distinct cell margins. The foci of haematopoietic cells including megakaryocytes were more diffuse in the sinusoids of day 1

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EXPLANATION OF PLATES

Fig. 3.—Normal brain (day 1). Leptomeninges free of cellular infiltrates but widened artefact). Grey matter forms 2 distinct zones: upper molecular layer and a lower cellular zone. × 120.

Fig. 4.—Brain infected with yellow fever virus (strain 17D)—day 1. Leptomeninges unremarkable. Oedema of molecular layer of grey matter, with focal area of haemorrhage. White matter shows no appreciable lesion. × 120.

Fig. 5.—Brain infected with yellow fever virus (strain 17D)—day 2. Leptomeninges with scanty mononuclear infiltrates. Marked oedema of the molecular layer of grey matter. Vacuolation of cytoplasm of some neurones and neuroglial cells in the cellular zone of the grey matter, which still maintains its polarity. × 120.

Fig. 6.—Brain infected with yellow fever virus (strain 17D)—day 3. Congestion of leptomeningeal vessels. Persistent oedema of molecular layer of grey matter. More extensive vacuolation of neurones in the cellular zone of the grey matter. Halo around some blood vessels in the white matter. × 120.

Fig. 7.—Brain infected with yellow fever virus (strain 17D)—day 4. There is gross congestion of the leptomeningeal blood vessels and some mononuclear inflammatory infiltrates. The molecular layer is reduced in width but still oedematous in areas. There is complete loss of polarity of the cellular zone, with lytic necrosis of neurones and neuroglial cells involving the entire brain. There are several pyknotic and karyorhectic nuclei as well as infiltrates of acute inflammatory cells exhibiting satellitosis focally. × 120.

Fig. 8.—Brain infected with yellow fever virus (strain 17D)—day 5. Very similar to Fig. 7, except for slightly greater lytic necrosis and vacuolation of the grey matter cells. × 120.

Fig. 9.—Normal brain (day 5). The thin leptomeninges is lifted off the grey matter (artefact) but free of inflammatory infiltrates. The grey matter and the white matter show no oedema or destruction of cells. The cellular zone of the grey matter is well polarized. × 120.
control, than in day 5, where they formed discrete foci. Each hepatocyte had a vesicular nucleus with a prominent nucleolus and a granular eosinophilic cytoplasm. The radicals of the portal vein were patent. Bile ducts were scanty and inconspicuous. There was some congestion of the radicals of the portal vein on the first experimental day. Bile ducts were slightly more prominent. There was slight centrilobular ballooning, vacuolar and hydropic degeneration of some hepatocytes. On the 2nd day, scanty focal areas of feathery degeneration of hepatocytes were present subcapsularly and near bile ducts. On the 3rd day, many of the hepatocytes had clear cytoplasm, distinct cell margins and resembled plant cells. The portal veins and sinusoids were focally, but markedly congested. In areas the sinusoidal littoral cells were prominent. On day 4, the plant-cell like cells were still present, but a few were ballooned. On the final day of the experiment (day 5) there was focal but striking periportal ballooning of hepatocytes. Inflammatory cells were never seen at any stage in any area of the liver. Therefore, there was no hepatitis.

The livers of mice infected with H43913 showed remarkably few changes, and these were only on the 3rd and 4th days of the experiment.

DISCUSSION

The mechanism of the destruction of cells by viruses is an intriguing subject. And in view of the intimate association of viruses with the physiology of the host cells, it would be safe to state that such cell destructions are the ultimate outcome of a host of biochemical events triggered off in the host cells following infection. A number of possibilities have been considered in the literature. These include a virus directed switch-off of host cell metabolic activity (Baltimore, Eggers, Franklin and Tamm, 1963) or the synthesis of some virus-induced proteins which act either directly or indirectly. In support of the last hypothesis certain cytotoxins have been implicated (Pereira, 1961; Ginsberg, 1961); also Defendi (1962) suggested lytic lysosomal enzymes. These hydrolases may be released by the disruption of lysosome membranes by some virus-induced or virus-specific proteins. There is an accumulating wealth of evidence to support this proposal (Bablanian, Eggers and Tamm, 1965; Mallucci and Allison, 1965; Amako and Dales, 1967; Scholtissek, Becht and Drzeniek, 1967; Guskey, Smith and Wolff, Bablanian, 1970). It should be pointed out that all the above reports were based on \textit{in vitro} systems. However, they probably would also provide a valid basis for interpreting results of animal tests.

The progressive stages of tissue destruction after infection of mice with yellow fever virus were followed in the present studies. An attempt was made at correlating the sequential development of pathological changes with corresponding production and localization of infective virus and CF antigen. The patterns observed after infection with the 17D strain were remarkably similar to those obtained following infection with a wild strain, H43913, of the virus. However, tissue destruction was generally more severe with the former strain; probably as a result of longer adaptation in mice. The target organ of pathological involvement was the brain in the mouse whereas it is not in man or monkey. This is in complete accord with earlier reports. The brain lesions progressively developed into an acute encephalitis on the 3rd day of the experiment. At the early stages there was oedema of the leptomeninges and the molecular layer of the grey matter.
Later in the infection the cells of the cellular zone of the grey matter lost their polarity and become disorganized. The hippocampus was particularly involved. By the 4th day after infection the entire cerebral cortex was involved, and there was complete disruption of the zonal architecture of the cortex. There was diffuse infiltration of inflammatory cells, and marked degeneration of neurones and neuroglial cells. Satellitosis and neuronophagia were present in some areas. All the above pathological changes notwithstanding, the mice were not visibly sick until the 5th day after infection. However, sickness appears to be as a result of the encephalitis. There was some focally located haemorrhage on the 1st day after infection, which may have been due to injection trauma. But it was resolved early in the course of the infection.

There was no significant involvement of the liver; only a few focal areas of ballooning or feathery degeneration of hepatocytes were seen. But there was no inflammation at any stage of the infection, and no hepatitis.

The serological surveys established a consistent positive correlation between the concentration of CF antigen and the intensity of brain damage. No CF antigen was demonstrated in the liver, although there was a progressive increase of infective virus titre in this organ, as was with the brain. Several possible reasons may be advanced to explain these observations. One possibility is that the differential localization of the pathological lesions may be a reflection of the quantitative difference between infective virus titres of the brain and the liver. Secondly, it may be proposed that the CF antigen or other substances, possibly proteins, closely associated with the antigen are directly or indirectly responsible for the tissue damage. And the antigen and/or the hypothetical proteins may be acting in a way analogous to that suggested by the "lysosome hypothesis". The likelihood that infective virus particles as such may not fully provide the answer is considered on the following grounds. First, although in most of the titration the LD$_{50}$ titre in the liver was 1–2 logs lower than the corresponding titre in the brain, there were occasions when both titres were comparable. And even under these conditions no lesions were seen in the liver. Furthermore, unlike infectivity and HA activity (Smith and Holt, 1961) there seems not to be a strict relationship between LD$_{50}$ titre and CF activity. For instance, in the experiments reported by Perlowagora and Lennette (1944), while some virus titre of $10^4.9$ LD$_{50}$ showed no CF activity others with $10^4.8$ LD$_{50}$ were associated with a CF titre of 80. Also some with a titre of $10^5.5$ LD$_{50}$ had no CF activity while others with a titre of only $10^4.4$ LD$_{50}$ demonstrated CF titre of 20. Bergold and Weibel (1962) failed to observe virus particles with the electron microscope in the liver of a fatal human case; although the typical pathological manifestations of the disease were seen in light microscopy. Baruch, Carbonell and Weibel (1963) failed to demonstrate virus particles in Councilman bodies. Finally, the route of inoculation does not seem to influence the pathology of the organs (Hudson, 1928b). In mice even after i.p. inoculation of virus, it was only the brain that was affected pathologically (Theiler, 1930)

It would appear that the second proposal offers a more attractive explanation for tissue damage in yellow fever infection. In the report of Perlowagora and Lennette (1944) the survival of some of the monkeys seems to be a function of the absence of CF antigen, and not of infective virus; since "large amounts" of virus was demonstrated in these animals. It is also known that in some human infections the typical histopathology of the liver parenchyma was not observed. This may probably be due to a defect in the production of the CF antigen or the
associated hypothetical proteins. Councilman (1890) suggested toxaemia and not infective virus as the cause of the hepatic lesions. A relationship between CF antigen and pathological manifestations of infection has also been demonstrated for 2 other arboviruses (David-West, 1970).

A rigid host factor seems to operate also in yellow fever infection, and this may explain, at least in part, the inter-species transposition of the target organ in man and monkey on the one hand and mice on the other.

Although special stain for inclusion bodies was not used, with the haematoxylin and eosin stain some questionable intracytoplasmic eosinophilic inclusion bodies were seen especially in the parietal lobe neurones. No such bodies were observed in the nucleus. This contrasts with the intranuclear inclusion bodies (Torres bodies) found in man and monkeys (Cowdry and Kitchen, 1930). Theiler (1930) also reported intranuclear inclusions in mice. Some small acidophilic granules were also observed in the cytoplasm.

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