Memoranda

VIRAL HEPATITIS AND TESTS FOR THE AUSTRALIA (HEPATITIS-ASSOCIATED) ANTIGEN AND ANTIBODY*

"Australia" antigen has been shown to be closely associated with serum hepatitis. The presence of the antigen and its corresponding antiserum can be detected in human beings (and in certain primates) by a number of laboratory tests. This is of great potential importance to blood transfusion and similar services because detection and exclusion of blood donors carrying the antigen might significantly reduce the risk of hepatitis from transfusions and other procedures.

In this paper the present state of knowledge of "Australia" or "hepatitis-associated" antigen is reviewed. The currently employed tests are described in detail and their use, interpretation and limitations are discussed. Though it appears from early studies that the application of routine screening tests to blood donors would only reduce the risk to recipients by less than 25 %, the more sensitive tests becoming available may increase this percentage and it is recommended that where competent laboratory services are available steps should be taken to set up a scheme for testing donors—provided that the current limitations of such a scheme are clearly recognized.

I. INTRODUCTION

Three years ago the possible relation of "Australia antigen" (discovered earlier by Blumberg and his associates) to viral hepatitis became apparent and since then advances in knowledge have been reported almost weekly. The antigen has now been shown to be closely associated with serum hepatitis—a finding of great practical importance affecting procedures in which human blood and blood products are used. It was therefore thought useful and timely to review the present state of knowledge, to describe in detail the current laboratory tests for the detection of antigen and antibody, to discuss the use of these tests and the interpretation of results, and to indicate the lines of research which might be fruitfully pursued in the immediate future.

The nomenclature of viral hepatitis and of the probable agents associated with it has long been in a state of confusion and the current publications on Australia antigen reflect clearly the tendency for different workers to use different terms for the same phenomenon. In this paper the two commonest terms—Australia antigen (the name given originally by Blumberg and his colleagues) and hepatitis-associated antigen—have been employed interchangeably, at the discretion of the authors of the individual sections.

II. REVIEW OF VIRAL HEPATITIS [1]

Viral hepatitis is commonly defined as acute inflammation of the liver caused by either of two agents—virus A, the presumed etiological agent of infectious or epidemic hepatitis; and virus B, which is associated with serum hepatitis or homologous serum jaundice. This definition excludes, by common usage, infection in which hepatitis may occur and which is caused by well recognized viruses such as yellow fever virus, cytomegalovirus, herpes simplex virus and others (WHO Expert Committee on Hepatitis, 1964).

Epidemic jaundice has been recognized for many centuries, and is most commonly spread by the faecal–oral route, usually by person-to-person con-

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[1] Prepared by Dr A. J. Zuckerman.

tact. The faecal contamination of water and food has resulted in many large outbreaks. Infectious hepatitis can also be transmitted by blood and blood products since viraemia occurs during the period of incubation of the disease and may persist for some months after recovery. Viraemia and excretion of the virus in the faeces also occur in patients with the subclinical and anicteric forms of this infection.

Serum hepatitis has only a comparatively short history and its origin is speculative. Havens (1961-62) suggested that virus B represents the emergence of a new strain derived from virus A which had undergone some changes during prolonged survival in the host with viraemia and alimentary disease. Thus, by implication, the antigenic differences between the two agents may be only relatively minor. An alternative hypothesis is that the origin of the serum hepatitis variant is obscured by its antiquity and that it has been maintained at a low level in ancient populations by such procedures as tattooing, scarification and other skin penetrations with various implements (Zuckerman, 1970a), and by transmission by blood-sucking insects. Transmission of hepatitis by syringes, in the course of transfusion of blood and blood products, and by inadequately sterilized instruments is now well recognized.

Many laboratory tests, diagnostic procedures and screening-tests for viral hepatitis have been described over the last four decades, but all have been shown to be non-specific. Attempts to transmit human hepatitis to laboratory animals and exotic animal species have been unrewarding, with the possible exception of the work with marmosets (Deinhardt et al., 1967; Holmes, Walsh et al., 1969; Holmes, Wolfe et al., 1969; Deinhardt, 1970). The numerous attempts to isolate and cultivate the human hepatitis agents in tissue culture have so far failed and, although many "candidate viruses" have been isolated, a consistent etiological association between these and hepatitis has not yet been demonstrated.

Much valuable information on the epidemiology and clinical features of the disease and some data on the biological characteristics of the infectious agents of human hepatitis were obtained from transmission experiments to human volunteers in the early 1940s (Neefe, Stokes & Gellis, 1945; Havens, 1945; MacCallum, 1946; MacCallum et al., 1951) and also more recently by Krugman, Giles & Hammond (1967), Giles et al. (1969) and Krugman & Giles (1970). The work with human volunteers established the existence of two distinct epidemiological and immunological forms of viral hepatitis and the fact that serum-hepatitis-like illness could be transmitted to children by the oral route, although less readily than infectious hepatitis.

THE AUSTRALIA ANTIGEN

The discovery of the Australia antigen by Blumberg and his associates has been the most spectacular advance in the seemingly insoluble problem of human hepatitis. By using the two-dimensional micro-Ouchterlony immunodiffusion technique, Allison & Blumberg (1961), Blumberg, Dray & Robinson (1962) and Blumberg (1964) observed the development of precipitating antibodies against a complex system of serum β-lipoprotein antigens in some patients who had been transfused with blood. Such precipitating antibodies were found more frequently in patients who had received a large number of blood transfusions over a period of time, for example, patients with haemophilia or thalassaemia. During the search for additional precipitating lipoprotein antibodies an antibody was detected in the serum of two frequently transfused American sufferers from haemophilia which gave a precipitin line with only one of a panel of 24 test sera. The antigen in this single serum contained little or no lipid and clearly differed from the serum β-lipoproteins. Since the reacting serum was obtained from an Australian aborigine, the antigen was named "Australia antigen". Subsequent studies on the distribution of the antigen in normal populations in different geographical areas of the world revealed that this antigen was very rare or absent in normal North American and European communities but that it occurred frequently in the serum of apparently healthy people living in the tropics and South-East Asia (Blumberg, Alter & Visnich, 1965b). The antigen was detected more frequently in the serum of patients with some forms of leukaemia, in patients with nodular (lepromatous) leprosy, and in patients with thalassaemia and others who had received multiple transfusions. A particularly high frequency (30%) was found in patients with Down's syndrome who were in institutions and among whom the prevalence of viral hepatitis is notoriously high, and in the serum of patients with acute viral hepatitis (20%). The possible relationship of the Australia antigen with viral hepatitis thus became apparent (Blumberg et al., 1967; Blumberg, Sutnick & London, 1968). From then onwards the fascinating story of the Australia antigen has continued to unfold and

progress at an ever-increasing pace. Prince (1968), by using methods very similar to those employed by Blumberg and his associates, detected an antigen, which he termed the SH (serum hepatitis) antigen, in the sera of patients during the incubation period and early acute phase of post-transfusion hepatitis, in those of patients with serum hepatitis and in the sera of 8 out of 4844 volunteer blood donors. The SH antigen is believed to be closely similar to, if not identical with, the Australia antigen. An association between the Australia antigen and post-transfusion hepatitis was also noted in Japan (Okochi & Murakami, 1968) and in various centres in the United States of America (Gocke & Kavey, 1969; Gocke, Greenberg & Kavey, 1969; Holland et al., 1969; and many others). A number of terms describing similar if not identical antigen–antibody systems have been introduced, including the SH antigen, hepatitis-antigen, Australia-SH or Au/SH antigen and more recently the hepatitis-associated antigen (McCollum, 1969). All these terms are interchangeable. The antigen was not found in the serum of patients suffering from disorders of the liver other than hepatitis and its complications, thus indicating that the Australia antigen does not merely represent a non-specific product of hepatocellular damage. Indeed, the distribution of the antigen could be explained on the basis that it is an infectious agent.

The morphology of the Australia (hepatitis-associated) antigen

Bayer, Blumberg & Werner (1968) examined in the electron microscope serum fractions rich in Australia antigen after rate zonal separation of whole serum on a 10%–30% (w/w) sucrose density gradient and electrophoresis in cellulose acetate. Negative staining with sodium silicotungstate revealed spherical particles measuring 19 nm to 21 nm in diameter in these preparations. A definite substructure and central cores were noted in some of the particles. Aggregates of these particles, as well as a few elongated tubular structures with similar diameters and measuring up to 230 nm in length, were visualized in the electron microscope when specific human or animal antiserum was added. The serum specimens containing the Australia antigen were collected from patients with acute myelogenous leukaemia, chronic reticuloendotheliosis and Down's syndrome with hepatitis. No particles were found in control sera.

Almeida et al. (1969) described the morphological appearance of virus-like particles visualized by electron microscopy in immune aggregates of serum samples obtained from a known long-term asymptomatic carrier of serum hepatitis and from heroin addicts who contracted hepatitis after sharing syringes. The main antigenic constituent was a rather pleomorphic spherical particle approximately 20 nm in diameter, but with a range of 16 nm to 25 nm. The outstanding feature was the presence of many tubular forms, often several hundred nanometres in length with a more constant diameter close to 20 nm.

These tubular structures frequently displayed a periodicity of approximately 30 Å on the surface. This spacing is the only geometric arrangement which could be resolved and a subunit construction was not detected on the spherical particles. Empty particles and particles with cores were found. The particulate structures associated with the Australia antigen thus have several features in common with known virus morphology. Such particles were not detected in normal human serum specimens.

Similar findings have also been reported by Hirschman et al. (1969) and Barker et al. (1969), who also observed that partially purified Australia antigen treated with ether consisted mainly of spherical particles which were more uniform and smaller in size (average diameter 16 nm) than the untreated antigen preparations. Treatment with ether seemed to remove a 20-Å outer coat from the particles. The tubular forms were practically absent from ether-treated preparations.

Dane, Cameron & Briggs (1970) described virus-like particles measuring about 42 nm in diameter in serial serum specimens obtained from 3 patients with hepatitis in which pleomorphic spherical and tubular particles characteristic of the Australia antigen were also present. Human antiserum to Australia antigen was added to the serum under test before high-speed centrifugation and these larger particles were often seen in the immune aggregates with both small and tubular forms. In the larger particles which were penetrated by the phosphotungstic acid a definite substructure was visualized. These particles had an outer coat measuring about 70 Å in thickness surrounding an inner body about 28 nm in diameter. The inner body had a shell 20 Å in thickness. Several tadpole-like forms were found, with the tail resembling a typical tubular form in continuity with the outer coat of a large particle which was not penetrated by the stain. Many of the large particles appeared to have five or six sides rather than being completely spherical, and at times the inner bodies had a similar appear-

ance, implying icosahedral symmetry. The large particles aggregated with the small and tubular forms when mixed with two different human antisera to the Australia antigen, suggesting that they all shared a common surface antigen. Similar particles with identical morphology have been found independently in the sera of patients with hepatitis in which the Australia antigen was also present by Zuckerman, Taylor & Bird (1970)—Fig. 1 and 2—and by Field & Cossart (personal communication, 1970). Dane, Cameron & Briggs (1970) put forward the hypothesis that the 42-nm particles are the virus of serum hepatitis and that the small 19 nm–22 nm particles and the tubular forms of the Australia antigen are non-infectious surplus virus-coat material, which is produced at the site of replication of the virus, and further that it seems possible that the Australia antigen results from the degradation of the larger and more typical virus-like particles. However, Zuckerman, Taylor & Bird (1970) obtained evidence, which at present is in no way conclusive, suggesting that the larger 40-nm–45-nm particles may result from aberrant tubular forms and that these larger particles may merely represent tightly coiled elongated forms. The striking resemblance should also be noted between the various forms of the Australia antigen and the similar morphology of the structures which Bancroft, Hiebert & Bracker (1969) produced by the reassembly on polyanions of proteins extracted from several spherical plant viruses. Attention is also drawn to the work of Shoeman, White & Mannering (1969) on the formation of tubular aggregates from hepatic microsomes. The walls of the tubules were found to display a periodicity produced by parallel rows of globular subunits which were nearly perpendicular to the long axis of the tubule.

The nature of the Australia antigen

The Australia antigen is a protein associated with varying amounts of lipid. It is immunologically distinct from normal low-density serum lipoprotein and the amount of lipid, as shown by staining with Sudan black and azocarmine, is far less than that present in serum lipoproteins (Blumberg, Alter & Visnich, 1965b). It is eluted by 0.1–0.2 M phosphate buffer at pH 7.0 from a DEAE cellulose column chromatogram after IgG and together with β-lipoproteins. The immunoelectrophoretic mobility of the antigen is similar to α_1-globulin (Alter & Blumberg, 1966). The buoyant density as determined by isopycnic banding on density gradients was between 1.063 g/cm^3 and 1.30 g/cm^3 in potassium bromide (Alter & Blumberg, 1966), 1.20 g/cm^3 in caesium chloride, 1.16 g/cm^3 in sucrose and 1.15 g/cm^3 in potassium tartrate gradients (Gerin et al., 1969a). After treatment with Genetron, ether or sodium deoxycholate the density is increased somewhat (Barker et al., 1969; Gerin et al., 1969a). The sedimentation coefficient was 110 as determined by rate zonal centrifugation on linear sucrose gradients. The stability, as measured by complement-fixing reactivity, was not affected by repeated freezing and thawing, or by heating at 56°C overnight or at 60°C for 1 hour, but it was destroyed by 85°C–100°C. Reactivity was unaffected by exposure to ether at 4°C overnight, to 1% sodium deoxycholate or 1% Tween 80–ether for 1 hour, or by pretreatment for 3 hours at pH 2.7 at room temperature. Treatment with 1% sodium dodecyl sulfate destroyed its complement-fixing activity (Gerin et al., 1969a).

No carbohydrate component has been found in the antigen. No nucleic acid has so far been identified.

These biophysical and biochemical findings immediately raise the problem of the biological nature of the antigen. Although the morphology has a number of features in common with recognized virus structure, the other known characteristics of the particles resemble more closely the group of agents referred to as the " slow viruses " which are associated with scrapie in sheep, kuru in man, Aleutian disease of mink and mink encephalopathy (Almeida et al., 1969; Gerin et al., 1969a). Other possibilities are that the particulate structures found in the serum represent incomplete virus particles or virus-coat protein (Dane, Cameron & Briggs, 1970), or that their nucleic acid content is very low, or that the antigen may be a protein which is produced specifically by the liver in response to infection by the virus of hepatitis. However, the detection of the antigen in serum sometimes long before any evidence of hepatocellular damage may be considered as evidence against this last view. In this context it seems that the site of replication of the antigen has not been conclusively demonstrated although the results reported by Nowoslawski et al. (1970) do suggest the successful cellular localization in the liver. Millman et al. (1969) and Coyne et al. (1970) have previously localized the antigen in liver cell specimens, prepared from liver biopsy material obtained from patients with hepatitis, using the direct immunofluorescent antibody tech-

nique. The presence of fluorescent particles in such liver cell preparations was shown to be strongly correlated with the presence of the antigen in the sera of these patients. More recently, small clusters of particles measuring about 20 nm in diameter and frequently arranged in chain-like arrays were identified by electron microscopy by Nowoslawski et al. (1970) in the nuclei of hepatocytes of 6 patients from whom specimens of liver were obtained at necropsy. The Australia antigen was present in the sera of all 6 patients, who suffered from various malignant lymphoproliferative disorders, and furthermore the antigen was detected by specific immunofluorescence in the nuclei and/or cytoplasm of the hepatocytes. None of these patients had any clinical or biochemical evidence of hepatitis, but in two of them there were histological changes which were compatible with chronic persistent hepatitis. Specific immunofluorescence or small virus-like particles were not found in the liver of 6 other patients with lymphoproliferative disorders in whom the antigen could not be detected serologically.

Attempts to culture the antigen, using fractions purified from serum containing it, have not been successful so far in a variety of primary and continuous cell lines of both human and animal origin, and, although encouraging preliminary results have been obtained using cultures of differentiated human liver cells, considerable further work is still required (Zuckerman, 1969). It seems possible that the actual infectious agent, although it has not yet been finally identified, may also be present in fractions obtained from some sera containing the Australia antigen.

The relationship between Australia antigen and viral hepatitis

The association between the antigen and viral hepatitis is now firmly established and this has been the subject of many publications, extensive reviews and editorials (Blumberg, Sutnick & London, 1968, 1969; Prince, 1969; Zuckerman, 1969; *Lancet*, 1969; *Brit. med. J.*, 1970). It has been detected, using a variety of laboratory tests, in patients with post-transfusion hepatitis, in multiply-transfused patients, in asymptomatic carriers of serum hepatitis among blood donors, in narcotic addicts, in patients and staff of renal dialysis units, in sporadic cases of hepatitis in urban communities, in some patients with chronic hepatitis and a few patients with cirrhosis, in the long-incubation variety of hepatitis (MS-2 strain) experimentally transmitted to human volunteers, and in a variable proportion of apparently normal people living in the tropics.

Zuckerman & Taylor (1969) found it in high concentration in the serum of a well-documented former blood donor, who had been implicated in serum hepatitis in recipients of his blood and in transmission experiments in volunteers, 18 years after the original incidents. MacCallum (personal communication, 1969) detected it in the original serum specimen collected from this carrier and stored since 1950–51. Persistent carriage for a number of years has also been recorded in a number of patients by Blumberg, Sutnick & London (1968), Giles et al. (1969), Turner & White (1969) and others.

However, discrepancies and conflicting results have been obtained with regard to the specificity of the antigen. In a number of studies it was found to be associated with both the infectious and serum varieties of hepatitis (Blumberg, Sutnick & London, 1968; Gocke & Kavey, 1969; Hirschman et al., 1969; Cossart & Vahrman, 1970). Other investigators have demonstrated that it is associated more specifically with the serum hepatitis type of infection and not with epidemic hepatitis (Prince, 1968; Giles et al., 1969; Mosley et al., 1970; Krugman & Giles, 1970). The virtual impossibility of distinguishing between the two clinically and pathologically similar forms of hepatitis is the underlying problem. Other factors which make the differentiation between the two types of infection difficult are that patients in whom there is no definite history of parenteral inoculations are likely to be placed in the infectious type of illness, that serum hepatitis may be transmitted by the oral route (Krugman, Giles & Hammond, 1967), and that infectious hepatitis may be transmitted by the parenteral route. Characterization of the infectious type of hepatitis is only possible on meticulous epidemiological grounds such as those outlined by Mosley (1970)—namely, an average incubation period to onset of symptoms of approximately 30 days, clear evidence of transmission by person-to-person contact or common-source transmission and protection of individuals in the same epidemiological setting by human immune serum globulin in small doses. It would seem, therefore, that the absence of the antigen, especially when serial serum samples have been tested, distinguishes at least some groups with typical infectious hepatitis from patients with serum hepatitis. It should also be noted that there is substantial epidemiological and experimental evidence

that infectious and serum hepatitis are caused by immunologically distinct etiological agents.

GENETIC FACTORS IN VIRAL HEPATITIS

Inherited resistance and susceptibility to infections have been described in a number of animal species. Zuckerman & McDonald (1963), on the basis of blood group distribution among hepatitis patients, postulated that some genetic factors may play a part in susceptibility to infection with hepatitis. A statistically significant excess of blood group A and a corresponding deficiency of group O was found among 378 patients with acute viral hepatitis in the Royal Air Force when the ABO and Rh blood group distribution of these patients was compared with that for a sample of 47 108 RAF recruits, making due allowance for their region of birth. Lewkonia & Finn (1969) found a disproportionate excess of blood group O in a circumscribed outbreak of serum hepatitis among patients and staff of a renal haemodialysis unit. It was noted that the clinically more severe cases were also of group O, once more suggesting that host factors may be important in the genesis of this disease. The subject of non-specific immune mechanisms in viral hepatitis has recently been reviewed (Zuckerman, 1970b).

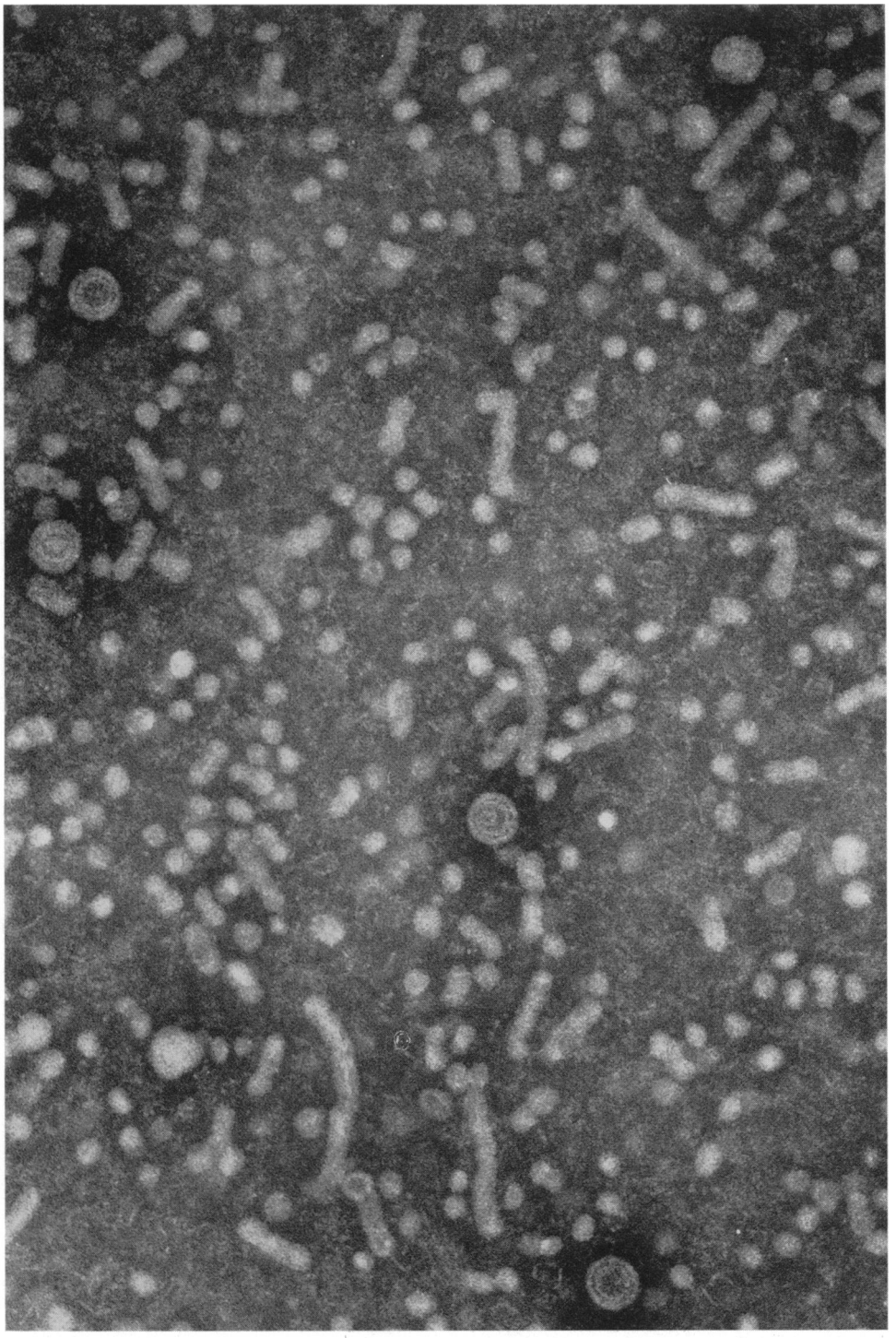
In extensive family studies carried out by Blumberg and his associates in the islands of Cebu, in the Philippines, and in Bougainville, New Guinea, clear evidence was obtained of family clustering. Further studies in Cebu and Bougainville (Blumberg et al., 1966; Blumberg et al., 1969) were consistent with the hypothesis that there is a host susceptibility to persistent carriage of the Australia antigen and that this susceptibility is controlled by an autosomal recessive gene (Au^1). Genetic factors may thus be of importance in determining whether or not an individual becomes infected with hepatitis virus and whether or not the illness becomes manifested clinically or remains subclinical, or whether complete adaptation leads to healthy carriage of the antigen. Genetic factors and defects in immunological mechanisms may also account partly for the susceptibility to chronic infection in patients with Down's syndrome, leukaemia and lepromatous leprosy (Blumberg, Sutnick & London, 1968).

SOME IMMUNOLOGICAL ASPECTS OF SERUM HEPATITIS

Some years ago an immunological basis for the pathogenesis of serum hepatitis was postulated by Hotchin (1962, 1966) and Hotchin & Collins (1964), who compared this type of hepatitis with lymphocytic choriomeningitis of mice and with kuru in man. It was suggested that asymptomatic infection with the etiological agents causing lymphocytic choriomeningitis and kuru may be associated with a state of immunological tolerance. In serum hepatitis breakdown of tolerance to the infectious agent leads to a type-IV hypersensitivity reaction resulting in damage of the hepatic cells containing the virus. An alternative possibility is that the cellular damage in hepatitis is mediated by antigen–antibody complexes, i.e., a type-III hypersensitivity reaction (Steiner et al., 1961). Popper, Paronetto & Schaffner (1965) considered this as a possible pathogenic mechanism in chronic active hepatitis. Enhanced toxicity of circulating antigen–antibody complexes resulting in hepatocellular damage might be the outcome of the union of these complexes with free antigen within the liver. Experimentally, however, circulating antigen–antibody complexes produce more severe lesions in the renal glomeruli than in the liver. Nevertheless, the observations of Conrad, Schwartz & Young (1964) of non-specific inflammatory changes in renal biopsies obtained from patients with viral hepatitis indicate that further work in this direction is required. Feizi & Gitlin (1969) also described a patient with immune complex disease of the kidney (acute glomerulonephritis) associated with chronic hepatitis and cryoglobulinaemia.

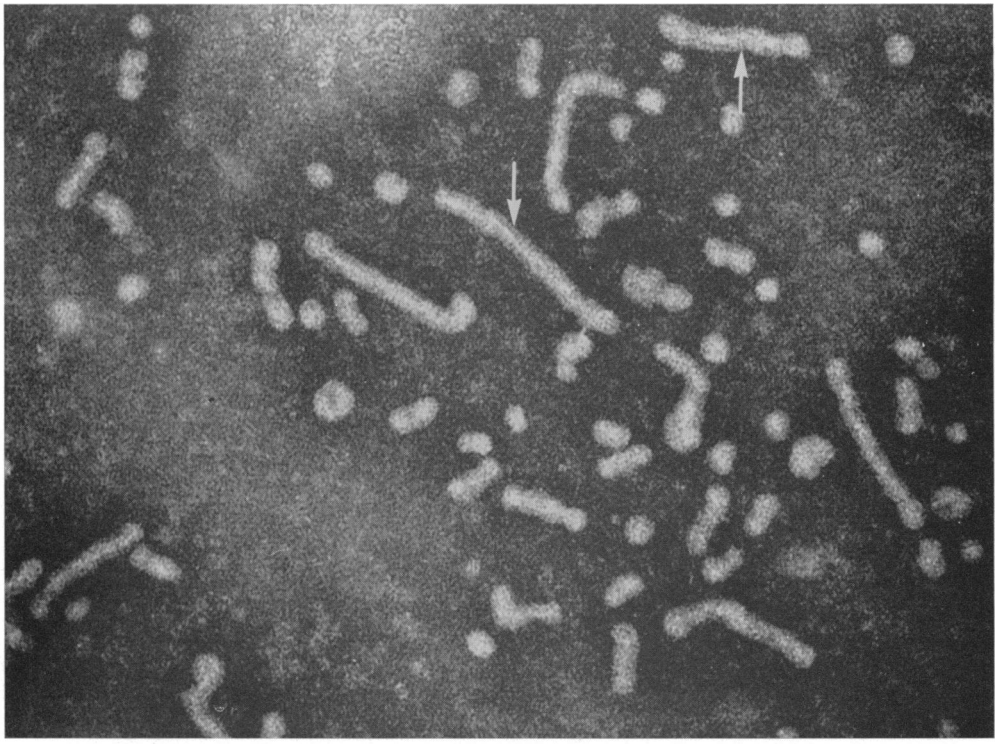
The presence of immune complexes circulating in the blood of some patients with acute and chronic hepatitis was noted by Barker et al. (1969), Shulman & Barker (1969) and Taylor, Zuckerman & Brighton (1969). These observations were confirmed by electron microscopy (Almeida & Waterson, 1969). The long-term asymptomatic carrier of the serum hepatitis virus described by Zuckerman & Taylor (1969) had a large number of antigen particles in his serum with little or no antibody to the antigen as judged by the electron microscopic picture. In a patient with chronic active hepatitis, in whom the Australia antigen was present, antigen–antibody complexes were found in the peripheral blood as well as unattached antigen particles, and this may be regarded as antigen excess. In another patient with fulminating hepatitis the electron microscope findings were those suggestive of antibody excess. These observations again support the contention that there are a number of similarities between Australia antigen (serum) hepatitis and type-III hypersensitivity reactions (serum sickness), in which

FIG. 1. SERUM CONTAINING AUSTRALIA ANTIGEN, SHOWING 3 DISTINCT MORPHOLOGICAL ENTITIES—SMALL PLEOMORPHIC SPHERICAL PARTICLES,[a] TUBULAR FORMS OF VARYING LENGTH,[b] AND LARGER SPHEROIDAL PARTICLES[c, d]



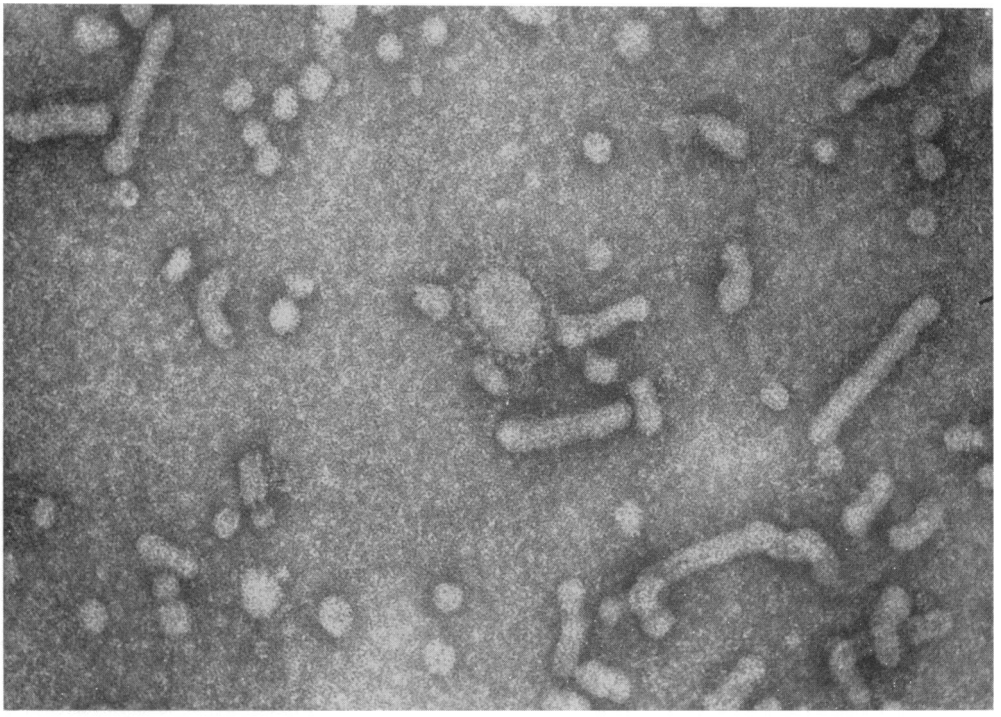
[a] Approx. 200 nm in diameter.
[b] Constant diameter of approx. 20 nm.
[c] Approx. 40 nm in diameter.
[d] Photomicrograph from a series by A. J. Zuckerman, Patricia E. Taylor and R. G. Bird. Magnification: ×164 000.

FIG. 2. CHARACTERISTIC TUBULES DISPLAYING A PERIODICITY OF APPROX. 30 Å ON THE SURFACE [a]



[a] Photomicrograph from a series by A. J. Zuckerman, Patricia E. Taylor and R. G. Bird. Magnification: ×145 000.

FIG. 3. SERUM CONTAINING AUSTRALIA ANTIGEN PARTICLES (SMALL AND LARGE SPHERICAL PARTICLES AND TUBULAR FORMS) AS WELL AS CORONAVIRUS-LIKE PARTICLE [a]



[a] Photomicrograph from a series by A. J. Zuckerman, Patricia E. Taylor and R. G. Bird. Magnification: ×165 650.

the severity of the illness also appears to depend on the balance between antigen and antibody.

Other observations also lend support to the hypothesis concerning the possible role of type-III reactions in the immunopathogenesis of serum hepatitis. The Australia antigen has been detected in patients some time before there was any laboratory or clinical evidence of hepatitis. Is the development of antibody associated with the genesis of liver damage and therefore with clinical symptoms in some patients? In a limited study the occurrence of hepatitis in patients with antibody to the Australia antigen implied either that such antibody does not protect completely against hepatitis or, alternatively, that in these patients the development of hepatitis following blood transfusion was caused by an agent serologically distinct from the Australia antigen (Holland et al., 1969).

Finally, both the early and more recent experimental transmission of hepatitis to human volunteers have demonstrated the presence of at least two epidemiologically and immunologically distinct forms of hepatitis. Homologous but no heterologous immunity after infection with classical infectious hepatitis (short-incubation form of hepatitis) was clearly shown. More recently Giles et al. (1969) and Krugman & Giles (1970) found the antigen in nearly all the volunteers inoculated with serum containing the MS-2 agent (long-incubation variety of hepatitis) but not in any of the volunteers inoculated with serum containing the MS-1 agent (short-incubation variety of hepatitis). In addition, the well recognized effect of pooled human immunoglobulin in attenuating infectious hepatitis and its almost consistent failure to influence the course of post-transfusion hepatitis also lend support to the view of lack of immunological identity between the Australia antigen and the infectious hepatitis virus. Further evidence has recently been provided by the observations of Krugman & Giles (1970), who found that human immunoglobulin consistently neutralized the infectivity of MS-1 serum, whereas in most cases human immunoglobulin failed to neutralize the infectivity of MS-2 serum.

CORONAVIRUS AND HUMAN VIRAL HEPATITIS

Anticomplementary activity has been noted by Taylor and by Zuckerman in a number of sera obtained from patients with chronic active hepatitis. When serum from one such patient was examined under the electron microscope the presence of distinct virus-like structures was observed and the morphology of these particles was typical of a member of the coronavirus group (Zuckerman, Taylor & Almeida, 1970). Of particular interest was the observation that all these particles were in association with antibody. The complement-fixing antibody titre to mouse hepatitis virus 3, a recognized coronavirus, was 1 : 640. This observation is analogous to that found in some patients with serum hepatitis, namely, antigen–antibody complexes in the circulation which may play a part in some forms of hepatitis. It is also of particular interest that a human serum should contain a coronavirus, seemingly associated with hepatitis, when a similar relationship has been shown in the mouse and it is possible that the coronavirus described by Zuckerman, Taylor & Almeida (1970) may be a human counterpart to the mouse hepatitis virus.

The completely independent observation of Deinhardt et al. (1970) of paramyxovirus-like particles which were morphologically similar to those described by Zuckerman, Taylor & Almeida (1970) in acute-phase sera of two patients with infectious hepatitis and in acute-phase sera of marmosets with experimentally induced hepatitis is of considerable interest. Deinhardt et al. (1970) examined acute-phase sera of patients with viral hepatitis and of marmosets with experimentally induced hepatitis by caesium chloride density-gradient centrifugation, electron microscopy and testing for infectivity in marmosets. Paramyxovirus-like particles were found in acute-phase marmoset sera and in acute-phase sera of two human patients; one of these human sera induced hepatitis in marmosets. The particles were in the fractions of about 1.21 g/cm^3 and these fractions were infectious for marmosets. The virus-like particles were not found in fractions of other densities and they were not infectious to marmosets. Paramyxovirus-like particles could not be demonstrated in preinoculation (normal) marmoset sera, two normal human sera or in the acute-phase serum of a patient with serum hepatitis in which the Australia antigen was found. The Australia-antigen-positive serum and its 1.20–1.25 g/cm^3 and 1.30–1.32 g/cm^3 caesium chloride density-gradient fractions were also not infectious for marmosets.

More recently, Zuckerman, Taylor & Bird (unpublished observations) noted the presence of coronavirus-like particles in two serum specimens obtained from different sources. These sera con-

tained at the same time the structures usually associated with the Australia antigen (Fig. 3). It is not possible, however, to interpret at this stage the significance of these latter observations.

Both Zuckerman and his associates and Deinhardt and his associates stress that these observations must be interpreted with considerable caution since these findings do not prove that the coronavirus-like particles are the causative agents of human infectious viral hepatitis, but further studies of their possible significance at least in some forms of human viral hepatitis are justified.

III. IMMUNODIFFUSION TEST [1]

The Australia antigen was discovered by Blumberg (1964) and Blumberg, Alter & Visnich (1965b) using the immunodiffusion method of Ouchterlony as modified by Blumberg & Riddell (1963); this is still the common method used for comparisons with new methods. The following is a description of the immunodiffusion method as used in the authors' laboratory, which includes the use of a reinforcement pattern of wells and staining of the precipitin bands, both of which increase the sensitivity of the test. The reinforcement pattern also permits the simultaneous testing for antigen and antibody.

The antisera and antigen used in this laboratory are described. They have now been distributed to more than 150 laboratories and have been the basis for comparison of most of the published studies on the Australia antigen.

METHOD FOR IMMUNODIFFUSION

Reagents

Phosphate-buffered saline, pH 7.4 (Sorensen's phosphate). Two phosphate–saline solutions are prepared : solution (*a*) 9.46 g Na_2HPO_4, 8.5 g NaCl in 1000 ml of distilled water; and solution (*b*) 9.07 g KH_2PO_4, 8.5 g NaCl made up in 1000 ml of distilled water. Solution (*a*) is titrated with solution (*b*) to give a pH of 7.41. A few grains of sodium azide are added to the buffer to prevent the growth of bacteria. The solutions should be stored at 4°C.

2.0% Agar for pre-coating slides. Dissolve 2 g of Nobel Agar (Difco Laboratories, Detroit, Michigan) in 100 ml of distilled water and heat, while stirring, to a gentle boil. The agar should be made up fresh for each new batch of plates.[2]

1.1% Agar for preparation of gel. Add 1.1 g Difco Nobel Agar to 100 ml of phosphate-buffered saline. Heat on a hot-plate, using a magnetic stirrer, until it is brought to a boil. A fresh batch of agar is made each time it is required. The flask in which the agar is being dissolved may be kept loosely covered to prevent evaporation. Protamine sulfate should not be added to the agar since it can lead to confusing results.

Agarose gel. Agarose (L'Industrie biologique française, Gennevilliers, France) may be substituted for Nobel Agar for some studies.[3] A fresh batch of the agar is made each time it is required. 1.1 g of agarose is dissolved in 40 ml of barbital buffer and 60 ml of distilled water. Large batches of barbital buffer are made by adding 36.84 g barbituric acid and 103.1 g sodium barbital to 14 litres of distilled water; this gives a pH of 8.2 (I/2 = 0.0375).

Protein staining. Dissolve 1.0 g of azocarmine (Matheson, Coleman and Bell, East Rutherford, N.J., USA) in 1 litre of 1 M acetic acid.

Preparation of slides

1. Wash lantern slides 3¼ in×4 in (8.25 cm× 10 cm)—or microscope slides.

2. Keep the 2.0% Nobel Agar (see "Reagents" above) liquefied by heating before use and use hot while pre-coating the lantern slides.

3. Pre-heat the clean slides for a few seconds on the hot-plate at approximately 70°C. Spread 0.3 ml to 0.5 ml of agar across the width of the plate using the side of another lantern slide. Place on the table top to cool. Mark the underside of each slide with a wax marking-pencil before storing between sheets of weighing-paper. Place the slides for 30 min in an oven at 40°C–50°C. The entire group of pre-coated slides may then be wrapped in aluminium foil and stored for several weeks.

[1] Prepared by Dr B. S. Blumberg, Dr W. T. London & Dr A. I. Sutnick.

[2] In some laboratories pre-coating of the slides has been found to be unnecessary. Some laboratories also use Petri dishes instead of slides; they are considered easier and safer to handle.

[3] Many laboratories have found agarose more sensitive than agar for routine testing.

4. Following pre-coating, the slides have to be coated with the agar. Heat the gel-agar (1.1% agar or agarose gel) until clear and then continue heating until it comes to the boil. Then distribute 15 ml of the agar (using a 25-ml pipette) on each pre-coated lantern slide, allowing the agar to flow evenly to all edges and corners of the slide. This must be done quickly and air bubbles should be avoided.

5. Cool the slide for a few minutes at room temperature; then store in a moist chamber in a refrigerator at 4°C. The plates should be stored in the cold for several hours before use. They may be stored in the covered moist chamber for 7 to 10 days.

Inoculation of slides

1. A 7-well pattern is cut in the agar gel using a template constructed with stainless steel cutters. The wells are 2 mm in diameter and are 3 mm apart from each other. The cutter is placed on the agar and pressed firmly. The agar plugs are removed by suction using a Pasteur pipette with vacuum. A total of 6 (or 9) patterns is placed on a lantern slide plate measuring 3¼ in × 4 in (8.25 cm × 10 cm).

2. A Pasteur pipette with an opening of about 1 mm in diameter is used for inoculating the sera. For convenience a numbering system is used in which the peripheral wells are numbered clockwise from 1 to 6. The wells are filled clockwise starting at position No. 1 (12 o'clock). After all the peripheral wells are filled, antiserum to the Australia antigen is added to the centre well. Between 5 μl and 10 μl of serum are required to fill each well.

3. The reinforcement pattern is made by placing a serum known to contain Australia antigen in wells 1 and 4 and the human or animal antiserum in the centre well. In this manner the test sera (in wells 2, 3, 5 and 6) are each adjacent to a known positive serum and a known antiserum.

4. The slide is placed in a moist chamber and incubated at 25°C. This temperature is slightly above the ambient temperature and is used to maintain a constant temperature during the period of incubation.

Scoring the precipitin lines

The slides are held for reading at 24 and at 48 hours or for 7 days. They are read in a darkened room using oblique illumination. Maintaining the slides for 7 days may reveal additional positive precipitin lines. The results are scored on a record sheet which matches the patterns used on the lantern slides. This may be carried out using an annular fluorescent lamp set in a black box with a square opening at the centre.

Staining

1. After the precipitin lines have been scored, the slides are placed in a container covered with phosphate-buffered saline. They are washed for 18 to 24 hours with frequent changes of saline. Care should be taken not to damage the gel when changing the saline washes.

2. The slides are placed on paper towels with the agar facing up and are overlaid with filter-paper cut to the size of the gel. The filter-paper is punctured with a 20-gauge needle over the well holes to accelerate drying. The air pockets are pressed out.

3. The slides are allowed to dry at room temperature. After several hours the agar appears as a flat transparent film.

4. The filter-paper is removed by gently washing the slides with distilled water.

5. The slides are placed in a container with stain (see " Reagents; protein staining " above) for 40 min. The slides are then washed twice, approximately 15 min for each wash, with 2.0% acetic acid, using a shaker. The second wash may be saved and used as the first wash for subsequent washing of other slides. The slides are air-dried and read by transillumination, i.e., not in the viewer used for observing the precipitin lines.

CHARACTERIZATION OF AUSTRALIA ANTISERA AND ANTIGENS BY THE IMMUNODIFFUSION TEST

Test antisera to the Australia antigen may be compared with a known antiserum in two ways.

Immunodiffusion using the Ouchterlony pattern

The antigen is placed in the centre well of a pattern and the antiserum to be tested is placed adjacent to the well containing the antiserum. If a line of identity is obtained, the antisera may be considered to have at least some specificities in common.

Panel studies

A panel of sera was selected from persons living in different parts of the world with and without liver disease and with and without the Australia antigen. The reactions of the known antiserum and the test

antiserum against the panel are compared. If the reactions are identical, the new antiserum is accepted as being suitable for use in screening other sera for Australia antigen. It should be obvious that the probability of finding differences between antisera depends on the composition and the size of the panel of sera against which they are tested. The " population " approach to specificity testing is given in detail elsewhere (Raunio et al., 1970); it permits identification not only of very similar antisera but also of categories of antisera and their degrees of difference from each other.

IV. COMPLEMENT-FIXATION TESTS

Two methods for complement fixation are described. The first method is the standard microtitre method and the second method is the technique as carried out at the Centre national de Transfusion sanguine, Paris, France.

COMPLEMENT-FIXATION TEST: STANDARD MICROTITRE TECHNIQUE [1]

The complement-fixation test is widely used in the serological diagnosis of viral, rickettsial and bacterial infections. Because of its relative simplicity and reproducibility under standard conditions it has proved to be one of the most valuable serological techniques available for virological investigations. Complement-fixation is a general reaction liable to occur when any antigen is allowed to react with its specific antibody in the presence of complement. The technique as it is used today is based on the early observations of Bordet, who in 1898 observed that red blood cells could be lysed when allowed to react with antiserum specific to the red blood cells. Lysis was found to depend on the interaction of two distinct substances, a specific thermostable substance developed as a result of immunization and known as antibody, and a thermolabile component present in the normal fresh serum of most vertebrates and now known as complement. When an antigen–antibody reaction takes place in the presence of complement, the complement takes part in the reaction and is " fixed " by the antigen–antibody complex. It is then not available for the haemolysis of subsequently added red blood cells sensitized by previous treatment with haemolytic serum. The failure of the sensitized red cells to lyse when added to the reaction mixture provides a method of demonstrating that an antigen–antibody reaction has taken place.

Although the immunodiffusion test provides a highly specific test for the hepatitis-associated (Australia) antigen, its sensitivity is not very great, and few attempts have been made to quantitate either the antigen or antibody to the antigen by this means. The microtitre complement-fixation test (Sever, 1962) provides a more sensitive method for the detection of the antigen (Shulman & Barker, 1969; Purcell et al., 1969; Taylor, Zuckerman & Brighton, 1969). It also allows the quantitation and serological comparison of different preparations of antigen and specific antibody. The basic technique used for the test is a modification of that described by Bradstreet & Taylor (1962).

Antibody for the test is usually obtained from persons who have received multiple transfusions of blood, some of which presumably contained the antigen (Blumberg, Alter & Visnich, 1965a). It may also be found in persons who have had frequent close contact with cases of acute viral hepatitis (Wright, McCollum & Klatskin, 1969). Detectable antibody is rarely found in convalescent sera from patients with acute viral hepatitis, and the complement-fixation test is therefore used diagnostically by the detection of the antigen rather than by the demonstration of a significant rise in antibody titre.

Equipment required

Besides the large equipment generally present in most laboratories (i.e., centrifuge, refrigerator, freezer, pH metre, flasks and cylinders) the following microtitre equipment for the actual test is required:

- 0.025-ml loops for diluting serum
- 0.025-ml calibrated dropper pipettes
- U-plates containing 96 wells (capacity 0.125 ml), Lucite or disposable
- 0.025-ml loop-delivery testers
- Transparent tape, 3 in (7.5 cm) wide, for sealing plates
- Incubator (37°C)

Because many of the specimens to be tested will be highly infectious, the use of disposable U-plates in preference to Lucite plates is recommended in

[1] Prepared by Dr Patricia E. Taylor.

order to eliminate the necessity for decontamination and rewashing. Disposable dropper pipettes also may be used but these require some care in handling in order to avoid over-wetting the tip of the pipette. This would result in faulty delivery. Non-disposable dropper pipettes are sterilized by boiling for 1 hour. Rubber suction bulbs, and not oral suction, should be used with pipettes and dropper pipettes.

Reagents

Diluent. Glass-distilled or deionized water of low conductivity is used for the preparation of reagents. Analytical grade reagents are used throughout. Barbital-buffered saline is prepared as follows:

NaCl	85.00 g
$MgCl_{2.6}H_2O$	1.68 g
$CaCl_2$	0.28 g
5,5-diethylbarbituric acid	5.75 g
Na 5,5 diethylbarbiturate	2.00 g

Dissolve the barbituric acid in 500 ml hot water. Add the other components and make up to 2000 ml. Autoclave at 15 lbf/in² (1.05 kgf/cm²) for 20 min and store tightly stoppered. Working solutions are prepared by making a 1/5 dilution with distilled water. The pH of the diluted stock must be 7.2, and the chloride concentration, which can be titrated with $AgNO_3$, should be 0.85 g per 100 ml.

Oxoid tablets (Oxoid Barbitone CFT Diluent, Code Br. 16; Oxoid Ltd, London) based on the above formula can be used for the preparation of the diluent. Add 10 tablets to 1000 ml distilled water in a flask. Place in a water-bath at 56°C for 30 min. Autoclave at 15 lbf/in² (1.05 kgf/cm²) for 20 min and store tightly stoppered. The pH should be 7.2 and the chloride concentration 0.85 g per 100 ml.

Sheep red cells. Defibrinated sheep red cells or cells preserved in Alsever's solution are suitable. These should be stored at 4°C. For use, sediment the cells at 1000 rev/min for 10 min in a 10-ml centrifuge tube. Carefully remove the supernatant and resuspend the cells in 7 ml or 8 ml 0.85% saline solution. Centrifuge at 1000 rev/min for 10 min and repeat the process for a total of 3 washings. Finally, pack the cells at 2000 rev/min for 10 min. Discard the supernatant and prepare a 4% red cell suspension by adding 1 volume of packed cells to 24 volumes of barbital-buffered saline. To ensure that the cell suspension is of the required concentration, place 1 ml of the suspension in a haematocrit and centrifuge at 2000 rev/min for 10 min. If the reading on the haematocrit does not indicate a red cell concentration of 4%, adjust the whole suspension carefully.

Haemolytic serum. Either glycerolated horse haemolytic serum or rabbit haemolytic serum may be used. Rabbit haemolytic serum generally gives a higher titre than horse haemolytic serum. Both are very stable at 4°C.

Complement. Normal guinea-pig serum preserved by Richardson's method (Richardson, 1941) is used as the source of complement. Small aliquots of this are stored frozen and remain stable for prolonged periods. However, periodic restandardization is advisable. Once thawed, this preparation should not be refrozen. As complement stored by Richardson's method is hypertonic, reconstitute by adding 1 volume of the reagent to 7 volumes distilled water (*not saline*). This results in a 1/10 dilution of the original complement-containing serum. Further dilutions of the complement are made with barbital-buffered saline. Since diluted complement is unstable, it should be kept cold and prepared immediately before use.

Titrations

Haemolytic serum and complement. In order to determine the optimal sensitizing concentration of the haemolytic serum and the titre of the complement, serial dilutions of the haemolytic serum are titrated against serial dilutions of complement in a chessboard titration as shown in Table 1. In order to minimize the degree of error in making dilutions and titrating reagents, the following precautions should be taken:

(*a*) Never blow out the final drop from a pipette.

(*b*) Never use the last tenth of a pipette.

(*c*) Use a clean pipette for each dilution of reagent unless the highest dilution is pipetted first, then work from that to the lowest dilution.

With a dropper pipette, add 0.05 ml barbital-buffered saline to each well of the plate (Table 1). This represents 0.025 ml antiserum and 0.025 ml antigen in the actual test. Add a further 0.025 ml barbital-buffered saline to the control column of wells to take the place of complement.

Prepare 2 ml 1/20 complement by adding 1 ml 1/10 complement to 1 ml cold barbital-buffered saline. Place 0.6 ml barbital-buffered saline in each of 6 test-tubes. Add 1 ml 1/20 complement to the first of these 6 tubes to give 1/32 dilution. Make serial dilutions by transferring 1 ml 1/32 complement

TABLE 1
CHESSBOARD TITRATION OF HAEMOLYTIC SERUM AND COMPLEMENT

Reciprocal dilutions of haemolytic serum	Reciprocal dilutions of complement							Control
	20	32	51	82	131	210	336	
50	0	0	0	2	3	3	4	4
100	0	0	0	3	4	4	4	4
200	0	0	0	2	4	4	4	4
400	0	0	0	tr	2	3	4	4
800	0	0	1	2	3	4	4	4
1 600	3	3	4	4	4	4	4	4
3 200	4	4	4	4	4	4	4	4
Control	4	4	4	4	4	4	4	4

0 = complete haemolysis
tr = approximately 10 % cells remaining
1 = approximately 25 % cells remaining
2 = approximately 50 % cells remaining (end-point reading)
3 = approximately 75 % cells remaining
4 = approximately 100 % cells remaining

Optimal sensitizing concentration of haemolytic serum = 1/400.

One unit of complement (HC_{50}), i.e., dilution giving 50% lysis = 1/131.

from the first tube to the second tube and so on to the end of the row. The dilutions of complement thus prepared are shown in Table 2.

Add 0.025 ml of the complement dilutions to the appropriate column of the plate with a dropper pipette starting with the highest dilution as shown in Table 1. Leave overnight at 4°C. Cover the plate in order to minimize evaporation during incubation.

TABLE 2
DILUTIONS OF COMPLEMENT

Reagent	Reciprocal dilutions of complement						
	20	32	51	82	131	210	336
Diluent (ml)	1.0	0.6	0.6	0.6	0.6	0.6	0.6
Diluted complement (ml)	1.0 of 1/10						
	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	

On the following morning, prepare doubling dilutions of haemolysin from 1/50 to 1/3200 in 0.5-ml amounts. Use barbital-buffered saline as diluent. Discard 0.5 ml from the last tube. Include a tube containing 0.5 ml barbital-buffered saline for the red cell control. Prepare 4% sheep cell suspension as described. Place 0.5 ml of this suspension in an adjacent row of tubes. Sensitize the cells by rapidly pouring the diluted haemolysin into the sheep cell suspension and mix by rapid swirling. Incubate both the sensitized and the unsensitized control cells in a 37°C water-bath for 30 min. Shake the cells gently for 15 min.

Transfer the plate containing the complement dilutions from 4°C to an incubator at 37°C. Leave for 30 min.

With a dropper pipette, add 0.025 ml unsensitized control cells to the bottom row of the plate (Table 1). Using the same pipette, add 0.025 ml sensitized cells to the appropriate row of the plate, beginning with the highest dilution. Tape, shake and place the plate in the 37°C incubator for 30 min. Shake once at 15 min and again at the end of the period of incubation.

Remove the plate from the incubator and leave it at 4°C for 1 hour or 2 hours to allow the cells to settle before reading. The plate is read as follows:

0 = complete haemolysis, i.e., no cells remaining
tr = approximately 10% cells remaining
1 = approximately 25% cells remaining
2 = approximately 50% cells remaining
3 = approximately 75% cells remaining
4 = approximately 100% cells remaining

The end-point is taken as the dilution giving 50% lysis, i.e., reading 2 above.

The optimal sensitizing concentration of haemolytic serum is that dilution which gives most lysis with the highest dilution of complement, i.e., 1/400 in Table 1. One unit of complement (HC_{50}) [1] is the dilution giving 50% lysis (reading 2) at the optimal sensitizing concentration of haemolytic serum, i.e., 1/131 in Table 1; 2½ units of complement are used in the test. If 1 unit (HC_{50}) is contained in 0.025 ml 1/131 complement, then 2½ units are contained in 0.025 ml of $5/2 \times 1/131$ dilution, i.e., 1/52.4 complement.

[1] In other laboratories, particularly in the USA, a unit of complement refers to the minimal quantity of complement which produces complete haemolysis.

Antiserum and positive control antigen. Doubling dilutions of antiserum are titrated against doubling dilutions of known positive antigen in a chessboard titration in order to obtain the optimal concentration of specific antibody, i.e., the highest dilution of antiserum that gives the highest antigen titre.

Add 0.025 ml of barbital-buffered saline to each well of the plate as in Table 3. Inactivate undiluted antiserum and undiluted antigen for 30 min in a 56°C water-bath to remove endogenous complement. After the sera have cooled to room temperature, add 0.025 ml undiluted antigen to each well of the first column of the plate (Table 3) with diluting loops. This gives a 1/2 starting dilution of antigen in the plates. Prepare further 2-fold dilutions with the loops up to 1/512.

TABLE 3
CHESSBOARD TITRATION OF ANTISERUM AND ANTIGEN

Reciprocal dilutions of antiserum	Reciprocal dilutions of antigen									Control
	2	4	8	16	32	64	128	256	512	
4	4	4	4	4	4	4	4	3	2	0
8	4	4	4	4	4	4	4	1	0	0
16	3	4	4	4	4	4	4	2	tr	0
32	4	4	4	4	3	3	2	1	0	0
64	2	2	tr	1	0	0	0	0	0	0
128	0	0	0	0	0	0	0	0	0	0
256	0	0	0	0	0	0	0	0	0	0
Control	0	0	0	0	0	0	0	0	0	0

Cell control 4

Complement control	2 ½ units	2 units	1 unit	½ unit
	0	tr-0	2	4

Optimal dilution of antiserum = 1/32.
Titre of antigen with optimal dilution of antiserum = 1/128.

Dilute equal volumes of antiserum with barbital-buffered saline in a series of tubes to give dilutions of 1/2 to 1/128. With a dropper pipette, add 0.025 ml of the antiserum dilutions to the appropriate rows of the plate as in Table 3, starting with the highest dilution. Add 0.025 ml barbital-buffered saline instead of antiserum to the control antigen row and 0.025 ml barbital-buffered saline instead of antigen to the control antiserum column. Prepare the complement dilution to contain 2½ units HC_{50} in 0.025 ml by adding complement to cold barbital-buffered saline and mixing gently. Add 0.025 ml to each well. Add 0.075 ml barbital-buffered saline to a well for the sensitized cell control. This represents 0.025 ml antigen, 0.025 ml antiserum and 0.025 ml complement. Include also complement controls to contain 2½ units, 2 units, 1 unit and ½ unit HC_{50}. For the 2-unit control add 0.4 ml of 2½-unit complement solution to 0.1 ml barbital-buffered saline. Make doubling dilutions of this to obtain the 1 unit and ½-unit solutions. Add 0.05 ml barbital-buffered saline in place of 0.025 ml antigen and 0.025 ml antiserum to 4 wells for the complement controls, then add 0.025 ml of the appropriate dilution of complement starting with the highest dilution. Cover the plate and incubate at 4°C overnight.

On the following morning, prepare sensitized cells by adding 1 volume of the optimal sensitizing haemolysin dilution to 1 volume of 4% cell suspension and mix with rapid swirling. Place in a 37°C water-bath for 30 min. Mix gently for 15 min. Transfer the plate from 4°C to an incubator at 37°C and leave for 30 min. With a dropper pipette, add 0.025 ml sensitized cells to all wells. Tape, shake and place the plate in the 37°C incubator for 30 min. Shake after 15 min and at the end of the period of incubation. Remove the plate from the incubator and place at 4°C for 1 hour or 2 hours to allow the cells to settle before reading.

The optimal dilution of antiserum is that which gives most fixation with the highest dilution of antigen and is selected only if all of the controls are acceptable. In Table 3, the optimal dilution of antiserum is 1/32 and the titre of the antigen with the optimal dilution of antiserum is 1/128. Four units of antibody are used for the detection of antigen and are contained in 0.025 ml 1/8 antiserum.

Four units of antigen are used for the detection of antibody and are contained in 0.025 ml 1/32 antigen.

The test

Detection of antigen. The protocol for the test is shown in Table 4. All sera, whether containing antigen or antibody, are inactivated undiluted for 30 min at 56°C to destroy endogenous complement. However, the sera still remain potentially infectious and must be handled with care.

Add 0.025 ml barbital-buffered saline to 2 rows of 8 wells in the plate. When the undiluted, inactivated

TABLE 4
COMPLEMENT-FIXATION TEST FOR HEPATITIS-ASSOCIATED ANTIGEN

Wells in plate	Serum [a] (ml)	Diluent (ml)	Antibody (ml)	Complement (ml)		Sensitized cells (ml)	
Serum under test	0.025	—	0.025	0.025		0.025	
Serum control	0.025	0.025	—	0.025	Overnight incubation at 4°C followed by 30 min at 37°C	0.025	30 min at 37°C followed by 4°C to allow cells to settle before reading
Positive antigen control	0.025	—	0.025	0.025		0.025	
Antigen control	0.025	0.025	—	0.025		0.025	
Specific antibody control	—	0.025	0.025	0.025		0.025	
Reagent controls;							
Complement 2 ½ units	—	0.05	—	0.025		0.025	
2 units	—	0.05	—	0.025		0.025	
1 unit	—	0.05	—	0.025		0.025	
½ unit	—	0.05	—	0.025		0.025	
Sensitized cells	—	0.075	—	—		0.025	

[a] 0.025 ml of each dilution.

test serum has cooled, add 0.025 ml to the first well of each row with a diluting loop. This gives a starting dilution of 1/2. Make doubling dilutions of serum up to 1/256 with the loops. Add 0.025 ml antiserum containing 4 units of antibody to the first row, and barbital-buffered saline instead of antiserum to the second row, which is equivalent to the serum control as shown in Table 4. Include all controls shown in Table 4. Add 0.025 ml complement containing 2½ units to all wells except the cell control well. Cover the plate and incubate at 4°C overnight. The subsequent procedure is the same as that described above under " Antiserum and positive control antigen " (preceding page) and is illustrated in Table 4.

The test is valid only if all the controls are satisfactory. The end-point is taken as the dilution at which approximately 50% of the cells are lysed, i.e., the dilution for which a reading of 2 is recorded.

For a number of reasons, the screening of test sera at a single dilution only (e.g., 1/4 or 1/8) for the presence of antigen is not practical. Antigen-containing sera often have very high titres of 1/512 or greater. Many of these exhibit a prozone effect which may extend up to a dilution of 1/16 or greater. Some sera contain antigen only at low titres of 1/2 to 1/4. Furthermore, sera from patients with acute viral hepatitis are frequently anticomplementary at titres of 1/2 to 1/16, i.e., they fix complement in the absence of added specific antibody. On occasions, both antigen and antibody have been detected in the same specimen of serum (Taylor, Zuckerman & Brighton, 1969), and much of the anticomplementary activity is probably due to the presence of antigen–antibody complexes (Shulman & Barker, 1969). However, in these sera, antigen may still be detected at titres beyond the anticomplementary titre. If screening is to be undertaken, initial testing of the following dilutions is recommended: 1/2, 1/8, 1/32, 1/64, 1/128.

Detection of antibody. Complement-fixing antibody may be detected in the same way as the antigen, using 4 units of specific antigen. Antibody titres are generally found in the range of 1/2 to 1/64.

Comments

When human serum is used as the laboratory standard antibody or antigen in the complement-fixation test for the detection of the hepatitis-associated antigen or antibody to the antigen in serum specimens, it should be borne in mind that other antigen–antibody reactions may give misleading results. The use of purified antigen preparations and the production of specific antibody in experimental animals will undoubtedly lessen this hazard.

Antisera containing precipitating antibodies which react well in immunodiffusion may often give very low titres for complement-fixing antibody.

The number of units (2½) of complement selected for use in the test was determined by carrying out a three-dimensional complement-fixation test, i.e., by performing a chessboard titration of antigen and antibody in the presence of varying concentrations of complement ranging from 1.7 to 3 units.

COMPLEMENT-FIXATION TEST: MICRO-TECHNIQUE USED AT THE CENTRE NATIONAL DE TRANSFUSION SANGUINE, PARIS [1]

A complement-fixation micro-technique is used for the detection of the Australia antigen. Sera are screened at three concentrations, undiluted, 1/16 and 1/256, in order to overcome the problem of the prozone phenomenon.

Antibody used to detect the antigen is selected from sera containing precipitating antibody to the Australia antigen. Some 30%–45% of these contain complement-fixing antibody. Titres of complement-fixing antibody up to 1/8 are usually found. Serum with a titre of at least 1/2 is used undiluted in the test. Various antisera with the same antibody titre may not detect the same proportion of sera containing the antigen. Two different antisera should therefore be used for screening. Antiserum from a healthy blood donor is preferred to antiserum from a patient who has had multiple transfusions in order to avoid other antigen–antibody reactions. Anti-A or anti-B and anti-Lewis agglutinins which are capable of fixing complement may be present in the blood of non-transfused blood donors. These should be removed from sera by absorption with group A and group B red cells.

Human serum from an AB blood group donor is used as the source of complement.

Tests are carried out in Falcon tissue typing plates. One microlitre of undiluted serum containing antibody is added to 1 μl of each concentration of the serum under test; to this, 3 μl of complement are added. 3.6 units per ml of complement are used in the test. One unit of complement is the dilution giving 50% haemolysis. The plate is incubated at 37°C for 1 hour before the addition of 1 μl of sensitized 3% sheep red cells. Both positive and negative controls are included in each test. The test is read after 30 min incubation at 37°C. Positive sera are further tested at dilutions to 1/4096 to give end-point titres. Anticomplementary activity up to a titre of 1/16 may be present. Testing such sera at higher dilutions often results in the disappearance of anticomplementary activity and the appearance of positive reactions for the antigen.

The technique used for the detection of antibody is similar to that used for the detection of the antigen. Serum or a pool of sera containing specific antigen is used to detect antibody. The optimal dilution of antigen is the middle dilution between the minimum and maximum dilution resulting in the fixation of complement. The antigen preparation should be devoid of anticomplementary activity and of immune agglutinins, and theoretically the antigen should be purified. In order to avoid anticomplementary activity the serum samples under test should be used fresh or frozen and thawed only once. Serum from an O blood group donor is preferable. Absorption of the serum with AB and O red cells may be used to remove immune agglutinins. The prozone phenomenon observed in antigen-positive sera is not encountered with antibody-positive sera and such sera may be tested undiluted.

The complement-fixation technique is more sensitive than immunodiffusion for the detection of the Australia antigen. However, immunodiffusion is more sensitive than complement fixation for the detection of antibody to this antigen.

Further details of the actual technique utilized are as follows.

Reagents

Levine–Meyer buffer is prepared as follows:

Solution A

NaCl	85 g
Na 5,5-diethylbarbiturate	3.75 g
Twice-distilled water	1400 ml
Then add:	
5,5-diethylbarbituric acid	5.75 g
Hot twice-distilled water	500 ml

Solution B

$MgCl_2$	2.03 g
$CaCl_2$	0.33 g
Dissolve in twice-distilled water	10 ml

Add 5 ml of solution B to solution A. Make up to 2000 ml with twice-distilled water. Check the pH, which should be 7.2–7.3. This concentrated stock solution is kept at 4°C for 3–4 weeks. For use, an isotonic solution is prepared by making a 20% dilution of the stock solution in distilled water.

Sheep red cells collected under sterile conditions in acid-citrate-dextrose solution.

[1] Prepared by Dr J. P. Soulier and Dr Doris Ménaché.

Anti-sheep red cell rabbit *haemolytic serum* (Institut Pasteur).

Human complement in the form of whole group AB serum (mixture of 3 sera).

Preparation of sheep red cells

Remove with sterile precautions 10 ml of sedimented sheep red cells. To the above 10 ml add 40 ml–45 ml of sterile physiological saline (0.85% NaCl solution). Centrifuge for 10 min at 1700 rev/min and 4°C, discard the supernatant and the buffy coat layer. Add 40 ml–50 ml of 20% Levine–Meyer buffer. Centrifuge for 10 min at 1700 rev/min and 4°C and discard the supernatant. Prepare a 3% red cell suspension by adding 1 ml of the packed red cells to 33 ml of 20% Levine–Meyer buffer and adjust to obtain 600 000 red cells per mm^3.

Sensitization of the red cells

Take 8 ml of 20% of Levine–Meyer buffer containing 10 000 haemolytic units of haemolysin and shake well. Add the haemolysin solution to 8 ml of 3% red cell suspension. Incubate for 30 min in a water-bath at 37°C. Store at 4°C.

Titration of complement

Human serum (AB) is used as source of complement. To determine the exact amount of complement that should be used in the assay, a preliminary titration is done for each batch of complement, using either a macromethod or a micromethod. In the micromethod, the human serum that is the source of complement is tested in Falcon tissue typing plates, at dilutions from 1/10 to 1/60, the conditions outlined in Table 6 below being followed exactly. However, the macromethod is to be preferred because it is the more sensitive. It is performed using 9 tubes, each of which contains a constant amount of red cells and varying proportions of complement and buffer; the total volume is kept constant at 2.8 ml (see Table 5).

The tubes are kept for 30 min in a water-bath at 37°C, and then centrifuged for 10 min at 4000 rev/min at 4°C. The degree of haemolysis of the supernatant is determined by using a photocolorimeter with the 540 nm green filter, with 20% isotonic Levine–Meyer buffer as a blank.

The 50% haemolytic unit of complement (1 H_{50} unit) is derived from a linear representation of haemolysis (see Fig. 4).

The H_{50} unit lies by definition in the middle of the sigmoid curve. In the example shown in Fig. 4: 3.8/720=1/180. For a 3% suspension, use 6 H_{50} units, i.e., a 1/30 dilution.

The amount of complement in the final incubation mixture per ml (see below) is:

$$\frac{3\times 6}{5} = 3.6\ H_{50}\ \text{units.}$$

The test

The test is carried out by the micromethod (see Table 6), using Falcon tissue typing plates with an overlay of liquid paraffin. All sera to be tested are inactivated undiluted at 56°C for 30 min to destroy complement.

Detection of the Australia antigen

Undiluted serum and serum diluted 1/16 and 1/256 are tested. Serum containing antibody to the Australia antigen is used undiluted.

Detection of antibody to the Australia antigen

The optimal dilution of the Australia antigen as outlined above is used and the serum under test is used undiluted. After incubation the test is read by using a frosted-glass plate lit from below, and the results are recorded as follows:

++++ = No haemolysis
+++ = 25% haemolysis
++ = 50% haemolysis
+ = 75% haemolysis
0 = 100% haemolysis

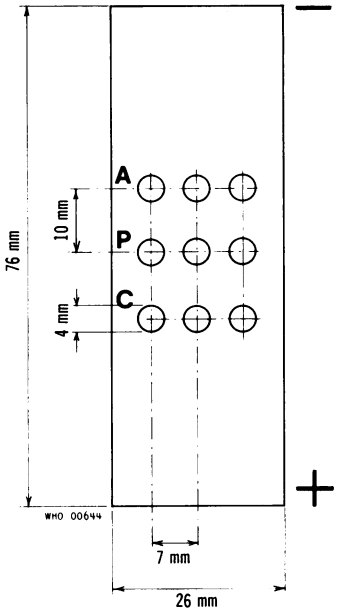
Only +++ or ++++ complement fixation is considered significant (++ or + being doubtful reactions).

TABLE 5
AMOUNTS (ml) OF EACH REAGENT FOR COMPLEMENT FIXATION BY MACROMETHOD

Reagent	No. of tubes								
	1	2	3	4	5	6	7	8	9
Complement (human serum, diluted 1/60)	0.2	0.4	0.6	0.8	1	1.2	1.4	1.6	1.8
Buffer (concentrated stock solution, diluted 1/5)	2.2	2.0	1.8	1.6	1.4	1.2	1.0	0.8	0.6
3% sensitized red cells	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4

FIG. 5

WELLS IN AGAR ON MICROSCOPE SLIDE [a]



[a] A = antigen; P = patient's serum; C = antibody.

FIG. 6

ARRANGEMENTS OF WELLS FOR SERIAL TESTS (A) FOR ANTIGEN AND ANTIBODY DETECTION AND (B) FOR ANTIGEN DETECTION ONLY

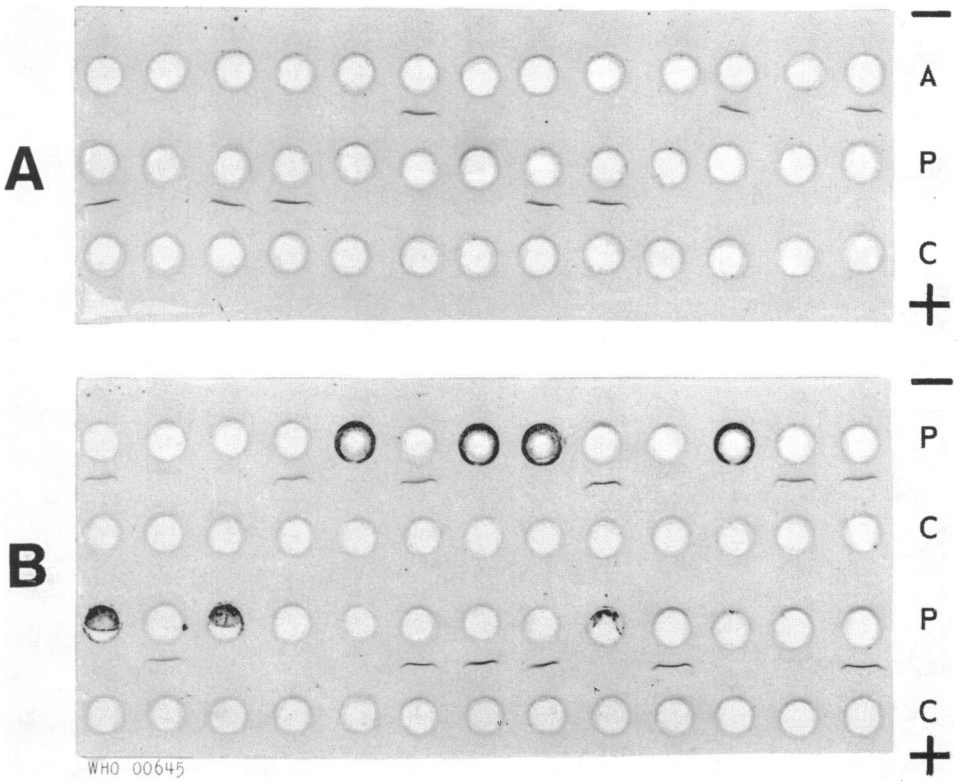
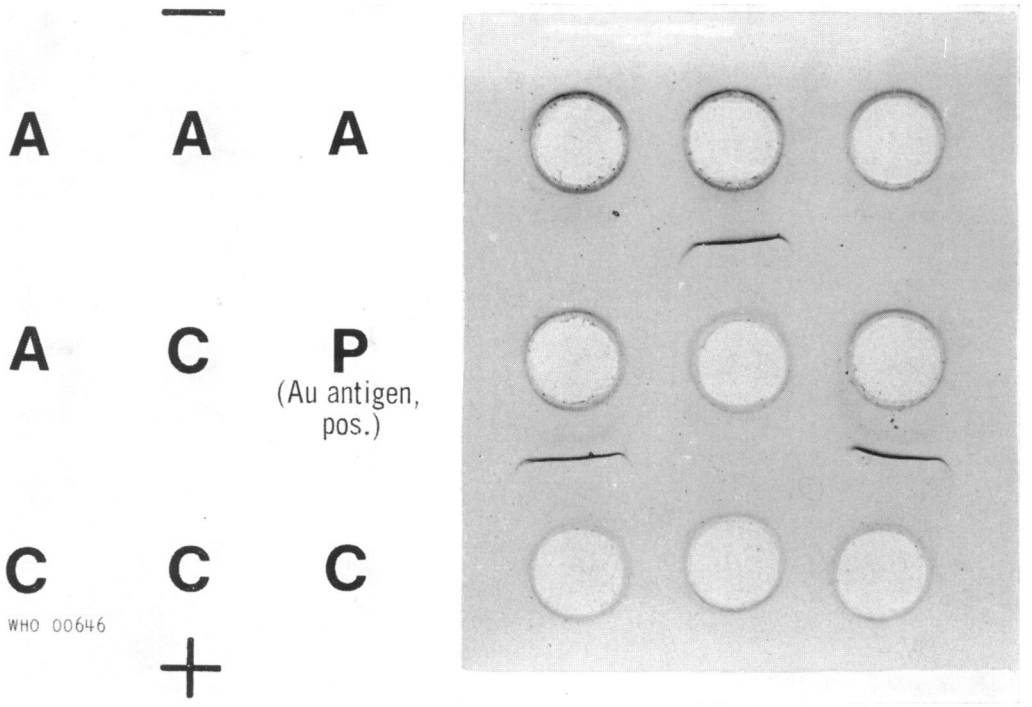


FIG. 7

IMMUNOPRECIPITATION AFTER ELECTROPHORETIC RUN [a]



[a] Left series, control with antigen. Middle, control with antibody. Right series, antigen-positive patient's serum.

FIG. 8

TESTS OF SERA WITH DIFFERENT ANTIGEN CONTENTS IN QUANTITATIVE ELECTROPHORETIC IMMUNODIFFUSION (LAURELL TECHNIQUE)

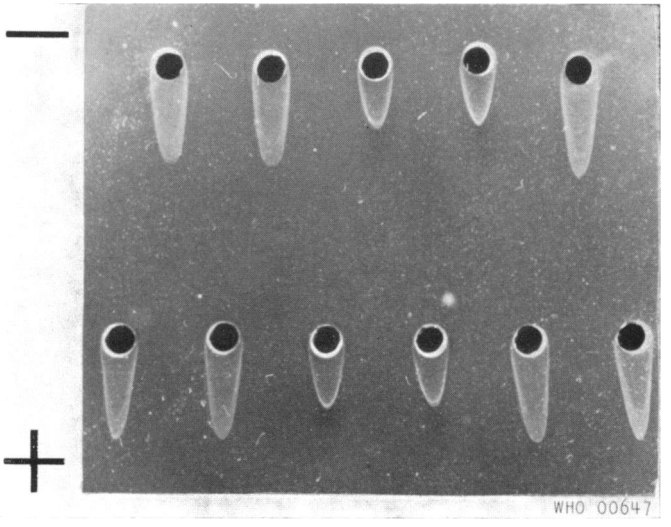


FIG. 4

DETERMINATION OF H_{50} UNIT FROM HAEMOLYSIS PLOTTED IN RELATION TO OPTICAL DENSITY

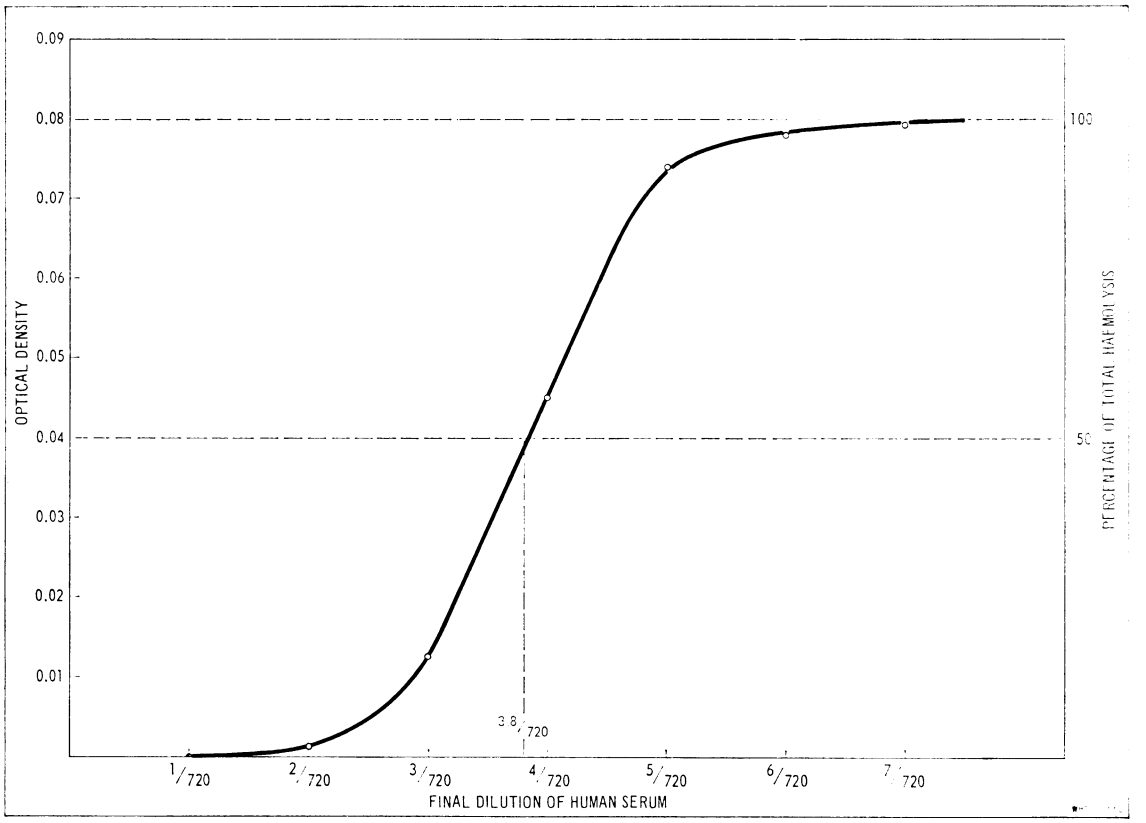


TABLE 6

AMOUNTS (μl) OF EACH REAGENT AND PROCEDURE FOR 2-STEP COMPLEMENT-FIXATION ASSAY

Reagent	Tube reaction	Controls	
		Serum alone	Antigen (or antibody alone)
Serum under test	1	1	0
Antigen or antibody	1	0	1
Complement (serum at the calculated dilution)	3	3	3
Buffer (concentrated solution, diluted 1/5)	0	1	1
Incubate for 1 hour at 37°C			
3 % sensitized red cells	1	1	1
Incubate for 30 min at 37°C			

V. IMMUNOELECTROPHORETIC METHODS[1]

The immunodiffusion method of Ouchterlony as modified by Blumberg & Riddell (1963) and by Prince (1968) has been widely used for the detection of both the Australia antigen and antiserum. A method of immune precipitation electrophoresis was recently devised by Pesendorfer, Krassnitzky & Wewalka (1970) in order to increase the sensitivity and speed of detection of the antigen and antibody.

IMMUNE PRECIPITATION ELECTROPHORESIS METHOD OF PESENDORFER, KRASSNITSKY & WEWALKA (1970)

Principles

Under certain electrophoretic conditions the Australia antigen moves like an α_2-globulin to the anode, and the antibody, a γ-globulin, moves to the cathode. When these two fractions meet, a line of precipitation is formed similar to the precipitin line obtained in the Ouchterlony test. Agar placed on a microscope slide is particularly suitable for this type of immunoelectrophoresis. A set of 3 wells is punched in the gel. The antiserum is placed in a well on the anodal side of the slide since the γ-globulin moves towards the cathode and the antigen is introduced into a well at the cathodal side since the mobility of the antigen is similar to that of an α_2-globulin. The conditions should be kept constant if clear-cut and reproducible results are to be obtained; this is especially important during the preparation of the agar and the buffer since some electro-osmosis is required for the migration of γ-globulin.

The method

Reinagar (Behring-Werke) in a concentration of 0.75% with sodium barbital acetate buffer at pH 8.4 (μ = 0.064) is used. No further purification of this agar is necessary. The composition of the 0.13 M buffer is as follows:

Sodium diethylbarbiturate	8.142 g
Sodium acetate	6.476 g
0.1 N Hydrochloric acid	90.0 ml
Distilled water	to 1000 ml

[1] Prepared by Dr F. Pesendorfer, Dr O. Krassnitsky & Dr F. G. Wewalka.

The agar (7.5 g) is made up in 1 litre of buffer, warmed in boiling water until it is dissolved; 2 ml of warm agar are poured on a microscope slide (26 mm × 76 mm). For a glass plate measuring 250 mm × 75 mm, 25 ml of agar in solution are required. Care must be exercised to ensure that no agar flows over the edge of the glass. The thickness of the agar will be a little more than 1 mm. These plates may be used immediately after cooling or they may be kept at room temperature in a moist chamber for up to 48 hours. No bacteriostatic agent is added if the plates are to be used within 18 hours but if the plates are to be kept for a longer period of time the addition of a bacteriostatic agent is recommended.

A cardboard template is placed under the glass, and wells of 4 mm in diameter are punched in the gel, using a cork-borer. Sets of 3 wells are arranged longitudinally as shown in Fig. 5, the distance between the centres of the wells being 10 mm. Three sets may be arranged on one microscope slide, the lateral distance between the centres being at least 7 mm. When 250 mm × 75 mm glass plates are used, up to 35 sets of wells may be punched (Fig. 6A). The patient's serum is added to the middle well (P), the antiserum is run into the well on the side of the anode (A) and serum containing the antigen on the side of the cathode (C). The wells should be filled completely (approximately 25 μl).

A control serum containing the antigen is included in the first set of each run (Fig. 7). In the middle well of the second set a serum containing the antibody is used. These two controls should be present in each electrophoretic chamber on each run and function as an indicator for immunoprecipitation. This arrangement allows for the testing for antigen and antibody at the same time. Electrophoresis is carried out at a constant current intensity of 8 mA per slide. If 250 mm × 75 mm glass plates are used, a constant current of 50 mA is necessary. In our laboratory the Boskamp Microphor System is used but any electrophoretic chamber which can be adapted for immunoelectrophoresis may be used.

The agar and buffer are connected by filter-paper strips (Schleicher Schüll 2043b). Under these conditions the results may be read within 3 hours. Oblique illumination is used for reading the plates. Staining is not necessary. The slides may be stored for a short time after the electrophoretic run in a

moist chamber before the results are recorded. The agar plates may subsequently be dried and kept in the dried state or stained after washing with normal physiological saline (0.85% solution) with Ponceau-red or Amido-black 10b. With the electrophoretic apparatus used in this laboratory three runs may be carried out in one day so that 99 sera may be tested. The results may be read after each run. This allows for the examination of sera from a large number of patients and blood donors.

If only the detection of the Australia antigen is sought the template is modified. Only 2 wells are required for each set, but 2 rows can be arranged on the same plate. In this case 69 sera may be tested in each run, amounting to 207 sera a day per electrophoretic chamber (Fig. 6B).

The immunoelectrophoretic method, which is similar to the electroprecipitin test (Watson & Whinfrey, 1958) or the overtaking electrophoresis method (Lang, 1955), cannot readily give reactions of identity like those obtained by the Ouchterlony technique. Consequently in positive cases a preliminary test for immunological identity is usually carried out.

This immunoelectrophoretic method has proved of value (Krassnitzky, Pesendorfer & Wewalka, 1970) and 30 000 tests have now been carried out in the First University Medical Clinic, Vienna.

THE ONE-DIMENSIONAL RADIAL IMMUNODIFFUSION METHOD OF MANCINI, CARBONARA & HEREMANS (1965)

This type of test is usually used for the quantitation of serum proteins but it may also be used for the detection of the Australia antigen. Antigen diffusing into agar incorporating the homologous antibody produces precipitation rings, and the more antigen present the larger the diameter of the ring. Agar containing 10% of undiluted antiserum is required for this test. Hydroxymethylaminomethanol (pH 7.6 at 25°C), 0.001 M tris-ethylenediaminotetraacetic acid buffer is used for making up the agar. Wells with 3 mm diameter are filled with the serum under test. The precipitation rings should be recorded after 48 hours. This method allows for a quantitative estimation but, on the other hand, a considerable amount of serum containing antibody is required.

LAURELL'S (1960) ELECTROPHORETIC IMMUNODIFFUSION TECHNIQUE

The Laurell technique gives a better quantitative measure of the antigen. Under these conditions the antigen diffuses in a 1.3% agarose gel incorporating the antiserum. The 250 mm × 75 mm glass plates are used. The agarose is made up with the barbital buffer referred to above in the description of the method of immune precipitation electrophoresis. To 25 ml agarose at 50°C are added 5 ml of antiserum; this is poured on the plate. Four rows of wells, each with a diameter of 3 mm, up to a total of 96 wells, are cut in the agarose after it has set. A current of 50 mA is applied for 4 hours. The areas of precipitation thus obtained are shown in Fig. 8. The maximum distance of the line of precipitation from the centre of the well should correlate with the amount of antigen present as with other proteins. An accurate estimation of the quantity of the Australia antigen may be obtained if this test is standardized. Sera containing antibody may also be standardized using reference antigen.

A comparison of these different methods together with the Ouchterlony method was carried out by Pesendorfer & Krassnitzky (1970). Of the four methods, immune precipitation electrophoresis was found to be the most sensitive for the detection of the Australia antigen, followed by the Laurell technique. The least sensitive method was the Ouchterlony technique as modified by Prince (1968).

VI. ELECTRON MICROSCOPY AND IMMUNE ELECTRON MICROSCOPY [1]

The method of negative staining in electron microscopy was introduced by Brenner & Horne (1959). The technique utilizes the principle of surrounding within a rigid electron-dense material particles such as viruses, isolated subcellular components and other biological structures. The technical details of this quite simple procedure have been the subject of an excellent recent review by Horne (1965). The method

[1] Prepared by Dr A. J. Zuckerman.

allows several freshly prepared specimens to be examined quickly in the electron microscope and a fairly large number of specimens may be examined in the course of a day. There is good and often very good preservation of the biological material under test with minimum distortion of structure. Negative staining results in high contrast, remembering, of course, that the effect is reversal of the final image when compared with positively stained preparations. Finally, only relatively small amounts of the material to be tested may be required. The main disadvantage of this technique is that a high concentration of particle suspension is usually required, often 10^9–10^{12} particles per ml (a condition which seems to be frequently satisfied in both patients and carriers of infective hepatitis). Furthermore, the addition of specific antiserum to the serum under examination leads to the formation of antigen–antibody aggregates, a procedure which would concentrate particles within the immune complex. The negative staining technique has been used successfully by Bayer, Blumberg & Werner (1968), Hirschman et al. (1969) and Almeida et al. (1969) for studying the morphology of the hepatitis-associated antigen in immune aggregates (see also Zuckerman, 1969). The presence of immune complexes circulating in the blood of some patients with acute and chronic hepatitis suggested that the addition of human or animal antiserum for hepatitis-associated antigen to the sample under examination by electron microscopy was not necessary (Shulman & Barker, 1969; Taylor, Zuckerman & Brighton, 1969; Almeida & Waterson, 1969).

Two techniques of preparation of serum specimens for examination in the electron microscope are currently practised at the London School of Hygiene and Tropical Medicine. The first method employs a serum–antiserum mixture; in the second method antibody is not used.

SUPPLIES AND REAGENTS

Apparatus

1. MSE Superspeed 40 centrifuge with a swing-out head.
2. Polypropylene centrifuge tubes with stainless steel caps.
3. Balzars Mikro BA 3 vacuum evaporator.
4. Copper support grids (New 200 mesh/in, Old 400 mesh/in or AEI-type grid).
5. AEI EM 801 electron microscope.

Reagents

1. *Cleaning fluids.* Quadralene liquid detergent, a mixture of ammonia, soaps, trisodium edetate and complexing agents. Inhibisol, a 95% mixture of 1,1,1-trichloroethane and 5% organic inhibitors.

2. *Sterile phosphate-buffered saline (PBS).*

3. *3% phosphotungstic acid (PTA), pH 6.0.* A stock solution of 3% phosphotungstic acid is made up in distilled water. The solution may be kept for a period of up to 4 weeks. The stain is prepared freshly each week from the stock solution. A quantity of 10 ml–15 ml of 3% phosphotungstic acid stock solution is adjusted to approximately pH 5.5 with 0.1 N potassium hydroxide. The solution is centrifuged in a bench centrifuge at about 1000 rev/min for 10 min and the supernatant decanted into a clean centrifuge tube. The pH of the supernatant solution is adjusted to exactly 6.0 with 0.1 N potassium hydroxide and the phosphotungstic acid is centrifuged again for 10 min. The resultant solution may be used for up to 1 week but it must be centrifuged for 10 min before every staining process.

Preparation of the grids

Standard 3-mm size copper grids (New 200 mesh/in, Old 400 mesh/in or AEI-type grids) are used. The plastic Formvar (polyvinyl formal) and a thin film of evaporated carbon are used as the specimen support film. A Formvar film, prepared by using a 0.3% w/v Formvar in chloroform solution, is floated on the surface of clean distilled water. The copper grids are placed matt-side downwards on the film and the grids are picked up on a piece of Whatman No. 1 filter-paper or stainless-steel gauze. The grids and plastic film are dried in an oven at 60°C and then coated with a thin film of evaporated carbon in a Balzars Micro BA 3 evaporator. The grids are stored in a glass desiccator containing silica gel.

Cleaning and preparation of the polypropylene centrifuge tubes

It is essential to use sterile centrifuge tubes which are free of dirt and it pays to be meticulous in preparing the tubes. New tubes are sonicated for 10 min in a mixture of 1 part Quadralene liquid detergent to 3 parts distilled water. The tubes are rinsed thoroughly in distilled water and then in methylated spirit to remove the water. Subsequently the centri-

fuge tubes are sonicated for 10 min in a solution of Inhibisol filtered through paper, rinsed first in methanol and then in absolute ethanol and finally dried in an oven at 60°C. The dry tubes are capped with aluminium foil and sterilized in steam at 121°C for 15 min.

PREPARATION OF THE SERUM UNDER TEST

Serum–antiserum mixture

A 1-ml quantity of serum is mixed thoroughly with 0.1 ml of antiserum. The mixture is maintained at 37°C in a water-bath for 1 hour and then stored at 4°C overnight. In the morning the serum–antiserum mixture is transferred with a sterile pipette to a new, clean, sterile 10-ml polypropylene centrifuge tube, sealed with a clean sterile stainless-steel cap and centrifuged at 4°C in an MSE Superspeed 40 centrifuge for 30 min at 2500 rev/min. This procedure removes any blood cells and cell debris which may be present. The supernatant is run into another new sterile polypropylene centrifuge tube and an equal quantity (i.e., 1 ml) of sterile phosphate-buffered saline added. The tube is closed with a stainless-steel cap and the diluted serum centrifuged for 30 min at 15 000 rev/min (25 000 *g*). The supernatant is discarded into a jar containing 10% hydrochloric acid and the pellet resuspended in 2 ml of phosphate-buffered saline; the tube is capped and recentrifuged for 30 min at 12 000 rev/min (15 500 *g*). The supernatant is discarded and the tube inverted in a beaker lined with dry filter-paper, thus allowing any remaining fluid to drain off. Each pellet is stained negatively as soon as possible.

Preparation of the serum without addition of antiserum

A 1-ml quantity of the serum under test is run into a new, clean, sterile 10-ml polypropylene centrifuge tube, sealed with a sterile stainless-steel cap and centrifuged at 4°C for 30 min at 2500 rev/min. The supernatant is transferred into a new sterile centrifuge tube and 1 ml of sterile phosphate-buffered saline is added. The diluted serum is centrifuged at 20 000 rev/min (45 000 *g*) for 1 hour. The supernatant is discarded and the pellet resuspended in 2 ml of phosphate-buffered saline and centrifuged at 20 000 rev/min (45 000 *g*) for 1 hour. The supernatant is discarded and the tube inverted in a beaker. Each pellet is stained negatively as soon as possible.

STAINING AND MOUNTING PROCEDURES

Each pellet is resuspended in 0.1 ml of sterile distilled water. A drop of this suspension is mixed, on a clean glass slide, with an equal volume of 3% phosphotungstic acid at pH 6.0. A carbon–Formvar-coated grid is picked up with straight watchmaker's forceps, with the carbon side of the grid uppermost. A Petri dish containing 10% hydrochloric acid is placed a little way under the specimen grid, into which excess suspension and stain may drop. A droplet of 1/1 solution of the suspension and staining reagent is transferred to the grid with a fine Pasteur pipette, and excess fluid removed by touching the torn edge of a piece of filter-paper to the grid (Fig. 9). The staining and mounting of the specimens is carried out in a safety cabinet.

EXAMINATION IN THE ELECTRON MICROSCOPE

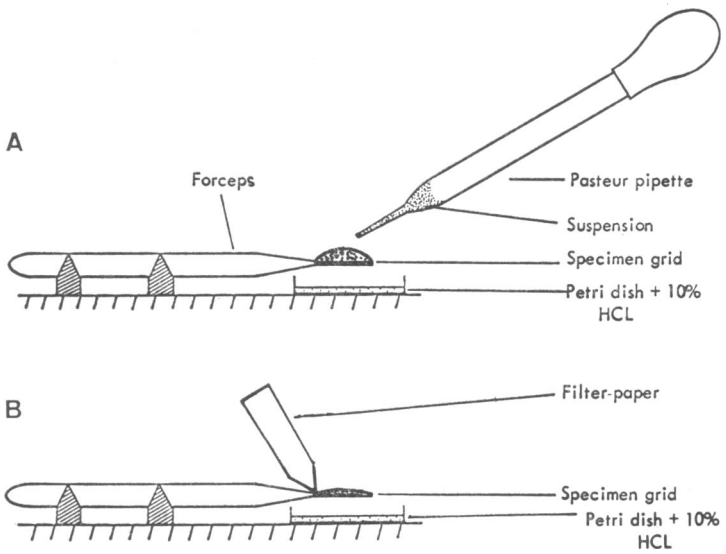
Negatively stained preparations should be examined in the electron microscope immediately. Harris & Westwood (1964) have shown that the morphological appearance of vaccinia virus was dependent on the time elapsing between the preparation of the grids and their examination in the microscope. Residual moisture on the grids that were left on the bench for some hours allowed the continued slow penetration of the stain into the virus particles, giving rise to altered appearance.

At least two grids are examined for each serum specimen at magnifications of × 63 000 and × 100 000 until the hepatitis-associated antigen particles or immune complexes are identified, or for 30 min before being classed as negative when no antigen particles or aggregates could be seen. A known positive serum, i.e., a serum specimen containing the hepatitis-associated antigen, should be included with every run of tests.

COMMENT

The methods described for electron microscopy may be applied for the examination of selected specimens of serum and, of course, as a research tool. If the equipment and personnel are available, negative staining procedures may be applied for diagnostic purposes. Electron microscopy has been found a most useful method for the examination of specimens giving equivocal results by other procedures. One such example is the examination of sera giving anticomplementary activity (Zuckerman, Taylor & Almeida, 1970).

FIG. 9
NEGATIVE STAINING FOR ELECTRON MICROSCOPY: DROPLET METHOD



VII. RADIOIMMUNOASSAY TECHNIQUES [1]

Walsh, Yalow & Berson (1970) recently described a radioimmunoassay technique for detecting and measuring the hepatitis-associated antigen and antibody. The technique is 20 to 100 times more sensitive than complement fixation for detecting both antigen and antibody and appears to measure a type of antibody to this particular antigen which is more persistent than the antibody measured by gel diffusion and complement fixation. The technique as described by Walsh, Yalow & Berson (1970) consists in the iodination of partially purified hepatitis-associated antigen with ^{125}I, additional purification of the antigen, and reaction of the labelled antigen (either alone or in combination with added unlabelled antigen) with antibody to this antigen. After an incubation interval of 4 days, antigen–antibody complexes (bound, B) are separated from unbound (free, F) antigen by physical means and the ratio of the bound to free antigen (B/F) is determined with a gamma-ray counter. The antibody is detected by its reaction with labelled hepatitis-associated antigen to form complexes. The hepatitis-associated antigen is detected by its ability to displace labelled antigen from a standard quantity of antibody. The antigen and antibody are quantitated by constructing standard curves of B/F for different concentrations of antigen or antibody and comparing B/F ratios of such known concentrations with those of unknowns.

Recently the radioimmunoassay technique was modified in the author's laboratory. These modifications included adaptation to the microtitre format (Sever, 1962) and separation of bound and free antigen by serological means (Gerloff, Hoyer & McLaren, 1962) to permit rapid testing of many specimens for antibody.

PREPARATION OF LABELLED ANTIGEN

The method used is that described by Walsh, Yalow & Berson (1970) as modified from Hunter & Greenwood (1962). The antigen is purified as described on page 986 by two cycles of isopycnic banding in caesium chloride. To a small glass or plastic tube the following are added rapidly, in order: 20 μl of 0.25 M phosphate buffer, pH 7.4; 5 μl–10 μl of partially purified antigen containing approximately 100 μg of protein; 700μCi–1000μCi of ^{125}I (carrier-free) in 1μl–4μl 0.1 N NaOH; 15 μl of chloramine T (3.5 mg per ml in 0.25 M phosphate buffer); 20 μl of sodium metabisulfite (4.8 mg per ml

[1] Prepared by Dr R. H. Purcell.

in 0.25 M phosphate buffer); and 20 μl of human plasma free of antibody to the hepatitis-associated antigen. Add 200 μl of 0.02 M barbital buffer, pH 8.5, containing 2.5 mg per ml of human serum albumin (RIA diluent) and apply to a 1 cm × 25 cm Sephadex G-200 column. Elute with RIA diluent and collect 1-ml fractions. Determine radioactivity and immunoreactivity on each fraction as described below.

RADIOIMMUNOASSAY (METHOD OF WALSH)

All dilutions are performed in RIA diluent. Mixtures are prepared in small glass or plastic test-tubes.

Detection of the antigen

To a series of tubes, add such amount of labelled antigen in 50 μl as gives 2000 counts/min to 5000 counts/min. Add 50 μl of a quantity of antiserum sufficient to bind approximately 50% of the labelled antigen. Add 100 μl of dropping dilutions of unlabelled antigen to the tubes. The dilutions should constitute a linear rather than a geometric progression. It may be necessary to experiment in order to find a suitable range of dilutions of unlabelled antigen. Incubate for 3 days at 4°C. Add 50 μl of human plasma stained with bromophenol blue,[1] mix and apply 200 μl of the mixture to moistened Whatman 3MC paper strips. Separate bound from free antigen by chromatoelectrophoresis in 0.1 M barbital buffer, pH 8.5, 24 V/cm for 90 min. Dry the strips in an oven (125°C). Cut the strips in two places with a pair of scissors, midway between the origin and the blue spot formed by the blue-stained plasma, and half an inch (or about 1¼ cm) distal to the blue spot. Roll up the two pieces of paper containing the origin and the blue spot and count in a well-type gamma-ray counter. Discard the paper distal to the blue spot (this contains free ^{125}I). Plot the ratio of bound (at origin) to free (at blue spot) antigen (B/F) as a function of the concentration of unlabelled antigen added to the mixture (standard curve). For convenience, the highest dilution of a standard antigen source which will cause a measurable reduction in the binding of labelled antigen to antibody is designated "1 hepatitis-associated antigen unit".

Sera to be tested for the antigen are diluted 1/5 and added to a mixture of labelled antigen (2000 counts/min–5000 counts/min) plus a quantity of antiserum sufficient to bind 50% of the labelled antigen. Appropriate controls from which a standard curve may be constructed are included in every test. The mixtures are incubated and separated as above and the concentration of the hepatitis-associated antigen in the unknown specimens is determined from the standard curve.

Detection of antibody

A 1/5 dilution (100 μl) of the serum to be tested is added to a tube containing 100 μl of labelled hepatitis-associated antigen (2000 counts/min–5000 counts/min). Incubate, add 50 μl of plasma and separate as described above. Sera positive for antibody are quantitated by preparing 10-fold dilutions and adding labelled antigen (2000 counts/min–5000 counts/min) to each tube. The tubes are incubated and the B/F ratio is determined as described above. The titre of the antibody to the hepatitis-associated antigen of a serum is defined as the highest dilution which will bind 15%–20% more labelled antigen than a negative control serum.

RADIOISOTOPE PRECIPITATION (RIP) TECHNIQUE FOR THE DETECTION OF ANTIBODY

Disposable 96-well, V-bottom microtitre plates of thin flexible plastic (Cooke Engineering Inc., Alexandria, Va., USA) are employed. Sera are screened for antibody by adding 0.025 ml of a 1/2 dilution of the serum to a well of the microtitre plate. Add to each well 0.025 ml of labelled antigen diluted in barbital-buffered saline, pH 7.5, with 1% bovine albumin (RIP diluent) to yield 500 counts/min–1000 counts/min. The plates are covered and incubated on a rocker platform at 37°C for 90 min in a humidified box, then incubated at 4°C for 1 day or more. After incubation, 0.15 ml of an appropriate dilution of anti-human serum or anti-human globulin [2] is added to each well. The mixture is reincubated on a rocker platform at 37°C for 60 min in a humidified box, then incubated overnight at 4°C. The following morning the microtitre plates are sealed in polyethylene bags and centrifuged at 2000 rev/min at room temperature for 10 min in an International

[1] Add approximately 10 mg of bromophenol blue per ml of plasma; allow to stand for 30-60 min; dialyse against RIA diluent.

[2] Anti-human serum or globulin is titrated in the presence of human antibody to the hepatitis-associated antigen and labelled antigen and the highest dilution resulting in the maximum percentage of precipitated antigen is used. Anti-human serum is usually sufficiently potent to use at a dilution of 1/4.

centrifuge, using special trunnions commercially available for this purpose (Cooke Engineering Inc.). After centrifugation, the plates are removed from the bags and 100 μl of supernatant fluid are transferred with an automatic pipette from each well to a gamma-ray counting tube. A dissecting needle, set in a wooden handle, is heated in a flame and used to cut the wells of the microtitre plate apart. Each well with its contents is transferred to a gamma-ray counting tube. Samples are counted in a well-type gamma-ray counter and the percentage of antigen bound to antibody is determined by the following formula:

$$\% \text{ bound antigen} = \frac{\text{counts/min in well} - \text{counts/min in 100 } \mu\text{l of supernatant}}{\text{counts/min in well} + \text{counts/min in 100 } \mu\text{l of supernatant}}$$

The percentage of bound antigen of a negative serum, even at a dilution of 1/2, should be approximately 10 or less. Sera containing the antibody are quantitated by preparing 2-fold dilutions in RIP diluent in a microtitre plate and tested as above. The antibody titre of a serum is defined as the highest dilution that will bind a given percentage of the labelled antigen (20%–50%, or, alternatively, twice that bound in a negative serum).

A similar technique for measuring the antigen and antibody has recently been described by Coller & Millman (1970).

These techniques are still being developed and are being used principally in research. Because of the special precautions required in handling radioactive isotopes these methods cannot as yet be recommended for general use.

VIII. THE PLATELET AGGREGATION REACTION [1]

Antigen–antibody complexes interact with the surface of blood platelets. This interaction produces changes in the platelets, which can be revealed either by the release of some intracellular substances or by platelet aggregation. A sedimentation pattern test on microplates is a sensitive method for recording the platelet aggregation induced by immune complexes (Penttinen & Myllylä, 1968). The system can be used as an immunological method analogous to the complement-fixation test. The platelet aggregation by immune complexes is not based on the serological " bridging " mechanism, but is due to the change in adhesive properties of the platelet surface. The alteration of the platelet surface triggered by the immune complex is dependent on the metabolic state of the platelets, and therefore platelets to be used for the aggregation test must be separated in a system which preserves their viability.

Blood platelets are aggregated by many agents in plasma. Apart from aggregation by immune complexes in the ionic medium used, platelet aggregation may also occur in response to aggregated gamma-globulin and other gamma-globulin-mediated processes. This system, however, is quite insensitive for adenosine diphosphate and some other platelet-aggregating agents. The investigator must be constantly aware of the possible nonimmunological factors that may operate in the test system.

[1] Prepared by Dr K. Penttinen and Dr G. Myllylä.

PREPARATION OF PLATELETS

Platelets are separated by the method of Aster & Jandle (1964). ACD (acid-citrate-dextrose) with extra citric acid, sufficient to bring down the pH to 6.5, is used as an anticoagulant and the separation is carried out at room temperature. Blood platelets can be obtained as a by-product from blood banks, the other blood components such as red cells and plasma being used for clinical and other purposes. The platelet pellet contains no more than 1 contaminating cell per 10 000 platelets. The platelets are washed twice with 0.145 M saline and once with the buffered saline used in the test, then resuspended carefully and adjusted to a platelet count of about 200 000/mm^3, according to the optical density of the suspension. Some 30 ml–150 ml of the platelet suspension can be obtained from 1 unit (400 ml of blood) representing an average of about 15% of the total original amount of the platelets. The platelets must be used on the same day, and for practical purposes a pool of platelets from three donors is recommended.

BUFFERS

Many kinds of buffer have been tested and most of them have been found unsuitable. The following buffers can be used.

1. *Modified Ringer's solution* (NaCl 68.3g, KCl 3.0g, $NaHCO_3$ 4.2g, Na_2SO_4 2.8g, distilled water 10000 ml).

The pH of the medium is adjusted to 6.7 with 1N HCl before use. This buffer has been found to be preferable for testing for the Australia antigen.

2. *Phosphate-buffered saline* free of magnesium and calcium (NaCl 80.0 g, KCl 2.0 g, $Na_2HPO_4 \cdot H_2O$ 14.0 g, KH_2PO_4 2.0 g, distilled water 10 000 ml). The pH of the medium is adjusted to 7.8 with 1 N NaOH before use.

3. *Unbuffered 0.145 M NaCl* (infusion grade) may also be used.

Glucose is added to all media to give a final concentration of 0.15 mg/ml.

THE PLATELET AGGREGATION TEST

It is advisable to carry out preliminary checkerboard titrations, which cover a range of antigen–antibody combinations, particularly when the Australia antigen is being examined, since prozone reactions may be marked and the row of positive combinations is short.

Microtitre pipettes, U-plates (preferably disposable) and the apparatus employed for the complement-fixation test may be used.

Test sera are inactivated at 56°C for 30 min. Starting with a dilution of 1/10, serial 2-fold dilutions are made. One drop (0.025 ml) of each dilution is mixed with one drop of double dilution of antigen. After incubation for 1 hour at room temperature, 0.05 ml of the platelet suspension is added. The plates are incubated at 5°C–8°C overnight in a temperature-controlled refrigerator. Temperatures below 3°C inhibit the reaction and higher temperatures give variable results. Reading of the sedimentation patterns is carried out using dark background illuminations; a smooth white button or a dark even pattern on the bottom of the well indicate negative or positive results respectively.

Controls

Sufficient numbers (10) of platelet controls with 0.05 ml of buffer and 0.05 ml of the platelet suspension are included in each test. In addition to positive and negative serum controls, daily investigation of the sensitivity of the platelet suspension is advisable. This is best carried out by a checkerboard titration of a known antigen with its specific antiserum. Monospecific synthetic antigen 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) and monospecific rabbit serum against the NIP hapten coupled to bovine serum albumin which is in use in the authors' laboratory gives good results (Penttinen et al., 1969). The number of positive antigen–antibody combinations, which is designated as the " score ", gives a better indication of the sensitivity of the platelet batch than serum and/or antigen titres alone. Serum titres of potent antisera are particularly poor controls. When the " score " required for satisfactory sensitivity of the platelets has been established, series with a variation of $\pm 15\%$ are acceptable.

The sensitivity of the method

With some virus antigens, such as cytomegalovirus, rubella virus or arbovirus group B antigens, the platelet aggregation test technique gives 3–10 times higher serum titres than the complement-fixation technique. The antigen requirements are correspondingly smaller. The advantages of the platelet aggregation technique relate to the study of small-sized antigen preparations (Myllylä et al., 1969; Penttinen, Kääriäinen & Myllylä, 1970; Penttinen et al., 1970).

COMMENTS

Screening tests for the Australia antigen and antibody by the platelet aggregation technique have not been compared with the complement-fixation test. However, Australia antigen titres of 1 : 80–1 : 1280 obtained with known positive sera by the platelet aggregation technique seem to correspond to the titres obtained by complement fixation. Comparison with the gel diffusion test suggests that the sensitivity of the platelet aggregation test may be somewhat greater; for example, 2 sera with titres of 1 : 80 and 1 : 160 from a series of 10 sera giving positive reactions by the platelet aggregation test were negative by the gel diffusion test. Finally, the starting dilution of serum to be used for the platelet aggregation test may be between 1/10th and 1/50th of that required for the gel diffusion test so that a substantial saving of reagents is possible.

The occasional reactivity of serum with platelets which occurs without added antigen may be considered to correspond to anticomplementary activity in the complement-fixation reaction. Many anticomplementary sera can be tested, however, by the platelet aggregation reaction without difficulty. The sera which show direct platelet aggregation can be analysed by sucrose gradient centrifugation followed by platelet aggregation titrations of the fractions so obtained. It is possible to characterize the nature of immune complexes in such fractions by the direct

platelet aggregation reaction and by changes caused by known antigens and antibodies.[1]

In conclusion, the platelet aggregation test is useful for the diagnosis and study of hepatitis. The advantages of this test are its sensitivity and applicability to the characterization of immune complexes. The disadvantage of the platelet aggregation reaction is the varying sensitivity of different batches of platelets, which make it essential to have meticulous controls. This method has so far been applied only to a limited number of serum specimens and considerable further experience is needed.

IX. FLUORESCENT ANTIBODY TECHNIQUES [2]

Application of the fluorescent antibody technique for the detection of Australia antigen is still at the experimental stage and it is described here in order to facilitate further studies to determine its usefulness.

THE DIRECT METHOD

Preparation of the antiserum and conjugation with fluorescein

Antiserum to the Australia antigen is prepared by hyperimmunization of rabbits with human serum containing this antigen followed by absorption of the rabbit serum with normal human serum (Melartin & Blumberg, 1966). Human antiserum to the Australia antigen has also been used as a source of antibody, but the titre may be much lower than that found in rabbits or other immunized animals.

The absorbed antiserum to the Australia antigen is passed through DEAE Sephadex in order to separate the immunoglobulin G fraction. The purity of this fraction is tested by immunoelectrophoresis to confirm the presence of IgG, and it is also tested by immunodiffusion against serum containing the Australia antigen to determine the presence of specific antibody to this antigen. When this is established the IgG fraction is conjugated to fluorescein isothiocyanate isomer I (Baltimore Biological Laboratories) by the method of Cherry, Goldman & Corski (1960). The conjugate is then purified by the method of Curtain (1961). With this method several bands of fluorescent material will come off the column and the bands which are chosen for use are those in which the ratio of absorption at 280 nm to that at 495 nm is greater than 1 as determined by spectrophotometric readings.

The conjugated antiserum is considered ready for use only if it produces a single precipitin line with serum containing the Australia antigen and does not react with serum in which the Australia antigen is not present and if the precipitin line produced by the conjugated antiserum produces a line of identity with that produced between Australia antigen and its unconjugated antiserum.

Absorption of the conjugated antiserum with mouse liver powder is useful for the removal of non-specific background fluorescence. This is achieved by mixing 100 mg of dried liver powder with each millilitre of fluorescent antiserum, incubating in a water-bath at 37°C for 1 hour, and cooling in the refrigerator overnight. The material is then centrifuged and the supernatant fluid is saved. The fluorescent antiserum is stored undiluted in 1 ml–2 ml amounts at −30°C. It is diluted before use and the degree of dilution varies with each antiserum. In the authors' laboratory the rabbit antiserum has been diluted 1/20 and the human antiserum 1/4. Phosphate-buffered saline at pH 7.4 is used as the diluent. The pH is important since fluorescence is lost when the pH falls below 7.0. The conjugated antiserum should always be centrifuged on each occasion prior to use to remove any debris which may have accumulated.

Further evaluation of the specificity of the conjugated antiserum must be determined by statistical comparisons between the results of fluorescent staining and the results obtained by immunodiffusion or complement-fixation tests on serum for the presence of the Australia antigen. A statistically significant correlation should be shown between staining with the fluorescent reagent and the presence of the Australia antigen in the serum and/or the clinical diagnosis of hepatitis.

Preparation of specimens

The tissues to be examined should be processed as soon as possible after they have been obtained from the patient. If the tissues cannot be processed immediately they should be stored in the cold

[1] Penttinen, K., Vaheri, A. & Myllylä, G.—paper in preparation.

[2] Prepared by Dr Veronica Coyne, Dr I. Millman and Dr B. S. Blumberg.

in tissue culture medium containing calf serum. Small pieces of tissue approximately 0.5 cm long are minced in a few drops of McCoy's 5a tissue culture medium (Grand Island Biological Co.) containing 10% newborn calf serum (Hyland Laboratories). McCoy's medium alone, Earl's or Hanks' balanced salt solutions, or phosphate-buffered saline at pH 7.4 may also be used. A thick cell suspension is made and a drop of this suspension placed on each of several slides. The slides are air-dried and immediately fixed in acetone for 10 min at room temperature. Other fixatives which have been tried and found to be unsatisfactory include gluteraldehyde, formalin and methanol–acetic acid.

Cells in suspension, such as those in blood, urine and bile, have been studied by centrifuging the specimen and preparing smears of the cell pellet.

Staining of the slides

A drop of the fluorescent antibody is placed on the slide and the slide incubated in a moist chamber for 45 min at room temperature. The slides are then washed by simple immersion in three separate washes of phosphate-buffered saline, pH 7.4—10 min for the first wash and 5 min each for the two subsequent washes. The slides are then mounted with cover-slips using buffered glycerol.

The slides are examined with a Zeiss microscope under darkfield illumination using the HBO 200W illuminator with either UG I exciter filter and 41 barrier filter or with BG 12 exciter filter and 53 barrier filter. Positive fluorescence consists of apple-green fluorescence which may take any of three forms in the liver preparations studied: (*a*) discrete particles within the nucleus, numbering from 1 to 20 per cell; (*b*) diffuse finely granular fluorescence of the entire nucleus; or (*c*) fluorescence of the nuclear rim. Occasionally diffuse granular fluorescence is found in the cytoplasm. In most of the liver biopsy specimens which give positive fluorescence the majority of the cells on a given slide display fluorescent particles. However, in some cases only 10%–20% of the cells are positively stained.

THE INDIRECT METHOD

Indirect fluorescent staining has been attempted by staining liver biopsy preparations and other cells with unconjugated rabbit antiserum to the Australia antigen for 45 min, washing three times as described, and then staining with fluorescein-conjugated goat *versus* rabbit anti-gamma-globulin for 30 min followed by further washing. This resulted in disruption of the cells and considerable fluorescent debris outside the cells which was difficult to evaluate. By decreasing the staining time to 20 min or 15 min and the time of washing to three 5-min washes, discrete fluorescent granules were detected in some preparations that were also found to be positive by the direct method. It appeared that the particles were " washed " out of the nucleus into the cytoplasm and the direct method of fluorescent staining is therefore preferable, at least for the present time.

X. POSSIBLE SOURCES IN MAN OF ANTIBODY TO THE AUSTRALIA (HEPATITIS-ASSOCIATED) ANTIGEN [1]

The discovery of the relationship between the Australia (hepatitis-associated) antigen and viral hepatitis by Blumberg, Alter & Visnich (1965b) was made using serum from a patient with haemophilia who had received numerous transfusions. The serum contained antibody to this antigen. All epidemiological studies published to date have also employed human antiserum and it seems likely that such serum will remain an important source of antibody for the foreseeable future.

[1] Prepared by Dr Doris Ménaché, Dr R. H. Purcell and Dr F. G. Wewalka.

SOURCES

Antibody to Australia antigen is found with varying frequency in patients who have received multiple transfusions of blood and blood products and rarely found in normal persons, though it appears to be considerably more common in tropical than in non-tropical populations. Antibody, as measured by complement fixation, agar-gel diffusion and immunoelectrophoresis, is most frequently found in the serum of persons who are repeatedly transfused, such as patients with haemophilia, thalassaemia, or aplastic anaemia. Such persons are believed to

undergo repeated exposure to the antigen which may be present in the transfused blood. The development of antibody in repeatedly transfused patients may represent an anamnestic response. Antibody in these patients is composed for the most part of immunoglobulin G.

Others likely to have the antibody are patients who have received massive transfusions, patients with chronic liver disease and persons working in blood banks, haemodialysis units and liver disease units. Other medical staff members and relatives of patients with acute or chronic hepatitis may also develop antibody. In blood transfusion services in which screening for antigen and antibody is carried out a small proportion of healthy blood donors may be found to have antibody.

Antibody detectable by complement fixation, gel diffusion or immunoelectrophoresis is rarely found after primary exposure to the antigen, and when present it may remain detectable for only a few weeks or months.

Persons likely to develop antibody should be tested at weekly intervals and when antibody is detected the patient may be asked to volunteer for plasmapheresis under medical supervision.

Plasma may be used as a source of antibody or it may be converted to serum by the addition of calcium or thrombin or the absorption of fibrinogen. If thrombin is used, it must be human and not animal thrombin, since persons with immunoglobulin A deficiency may have precipitating but not complement-fixing antibodies to bovine and other animal proteins, and such antibodies may lead to false positive results (Alter, personal communication).

The serum or plasma, which must be free of bacterial contamination, is dispensed in small aliquots and stored frozen. It should not be repeatedly frozen and thawed or stored unfrozen for a long time. It may be freeze-dried and stored at 4°C.

CHARACTERIZATION OF THE ANTIBODY

New lots of serum or plasma to be used as a source of antibody should be compared by the immunodiffusion technique with sera known to contain antibody to Australia antigen. In addition the serum or plasma should be further characterized for its ability to react with a panel of sera containing Australia antigen but not with antigen-negative sera.

Human sera containing antibody to Australia antigen differ in their relative usefulness as reagents for detecting this antigen by complement fixation, gel diffusion or immune precipitation electrophoresis. The titre of antibody as measured by gel diffusion seldom exceeds 1 : 8 and is often lower. The complement-fixing titre of an antiserum is usually less than 1 : 64 but titres as high as 1 : 4096 or greater have been recorded. It is recommended that a serum which reacts in complement fixation with the Australia antigen should not be used unless it also forms a line of identity with the antigen in gel diffusion. Weak antisera may be concentrated by vacuum filtration or precipitation of gamma-globulins. The latter method may limit the usefulness of the serum for complement fixation because such gamma-globulin preparations may be anticomplementary. Many antisera which are satisfactory for immunodiffusion may not be satisfactory for use in the complement-fixation test.

Certain other antigen–antibody reactions between human sera have been described. These include reactions of complement-fixing antibodies to blood group substances and precipitating antibodies to lipoproteins. Such reactions can cause confusion. Antibodies to blood group substances can be removed by absorption with appropriate red cells. Precipitating antibodies to lipoproteins and other proteins can be differentiated from antibodies to the antigen by the lack of identity of their precipitin line.

XI. POSSIBLE SOURCES IN ANIMALS OF ANTIBODY TO THE AUSTRALIA (HEPATITIS-ASSOCIATED) ANTIGEN [1]

Early reports of the detection of hepatitis-associated antigen and antibody in haemophiliacs and other persons receiving multiple transfusions led some to believe that there would be an ample supply of these materials for research purposes and, if necessary, for mass screening of blood donors. However, such has not been the case. While human serum containing the hepatitis-associated antigen is not difficult to find, serum containing antibody to this antigen has been extremely scarce.

[1] Prepared by Dr R. H. Purcell.

In efforts to find a convenient source of antibody a number of species of animals have been inoculated with hepatitis-associated antigen. Melartin & Blumberg (1966) prepared hyperimmune serum in rabbits by immunizing the animals with whole human serum containing the antigen and absorbing the resultant antiserum with normal human serum which did not contain it. They obtained an antiserum which gave a single precipitin line with the hepatitis-associated antigen. However, antisera prepared and absorbed in this way cannot be used for complement-fixation tests. Furthermore, they may contain non-precipitating antibodies to normal and abnormal serum components such as transaminase enzymes and other liver cell proteins which would limit their usefulness for certain other techniques.

CHIMPANZEES

Preparation of antibody in chimpanzees

The hepatitis-associated antigen has been found in the sera of chimpanzees (Blumberg, Sutnick & London, 1968; Hirschman et al., 1969). Recently Lichter (1969) prepared hyperimmune serum by immunizing chimpanzees with partially purified hepatitis-associated antigen from the serum of another chimpanzee which was a chronic carrier. Relatively large quantities of antiserum were obtained by repeated plasmapheresis of the chimpanzees.

Purification of immunizing antigen

Chimpanzee serum containing the antigen is passed through a Sephadex G-200 column previously equilibrated with 0.01 M phosphate, 0.15 M NaCl (pH 7.4). The first peak serum fraction of effluent which contains the antigen as well as other serum proteins is used as the immunizing antigen.

Immunization of chimpanzees

Immunizing antigen containing 5 mg of protein is emulsified in a modification of Freund's complete adjuvant (Arlacel 15%, mineral oil 85%, heat-killed *Staphylococcus aureus* 1 mg/ml), and injected intramuscularly into chimpanzees previously shown to be free from the hepatitis-associated antigen. At monthly intervals the chimpanzees are given intramuscular booster injections of the same antigen (5 mg protein) in incomplete Freund's adjuvant. When suitable antibody titres are reached, the animals are repeatedly subjected to plasmapheresis.

Antiserum produced in this way is specific, at least as determined by immunodiffusion in agar-gel. However, chimpanzees are extremely expensive and difficult to obtain and they are susceptible to many human infectious agents, including, perhaps, hepatitis agents not related to the hepatitis-associated antigen, and may therefore, like man, be expected to produce antiserum which is not monospecific.

OTHER PRIMATES

The antigen has been detected in sera from a small number of gibbons, orang-utans (Shulman & Barker, 1969), red spider monkeys, African green monkeys (Blumberg, Sutnick & London, 1968) and marmosets (Blumberg et al., 1970). Such observations suggest that these animals, like chimpanzees, may be susceptible to infection with hepatitis virus B. Orang-utans and gibbons are not practical sources of antibody because of their rarity and cost and spider monkeys and marmosets are also impracticable, partly because of their size. Serial transmission of the hepatitis-associated antigen to African green monkeys has been attempted in several laboratories. London et al. (1970) inoculated the antigen into two infant African green monkeys and passaged it to another. Immunization of African green monkeys with antigen derived from the homologous species may therefore provide a useful and relatively inexpensive reagent.

SMALL NON-PRIMATE ANIMALS

The immunization of guinea-pigs with purified hepatitis-associated antigen yields potent hyperimmune serum which is useful for all currently employed serological tests—immunodiffusion, complement fixation and radioimmunoassay. In contrast, rabbits that have received injections with the same immunizing antigen, according to the same immunization schedule, produced only weak and non-specific antiserum (Purcell et al., 1970). Other laboratories have experienced similar difficulty in immunizing rabbits.

Millman et al. (1970) have recently described the production of antibody in the ascitic fluids of mice. This antiserum has been found to give a reaction of identity with human antiserum by the immunodiffusion test.

Preparation of immunizing antigen

Hepatitis-associated antigen is purified from human serum by two cycles of isopycnic banding

in caesium chloride, followed by rate zonal separation in sucrose (according to the method described by Gerin et al., 1969b). This purification procedure takes advantage of the fact that the antigen has a density of 1.20 g/cm³ in caesium chloride and a sedimentation coefficient of 110 in sucrose, characteristics which serve to differentiate it from all other serum components. It is preferable to use a human serum from a chronic carrier of the antigen with as high a complement-fixing titre as possible (1:1024 or greater).

Isopycnic banding. Five millilitres of human serum containing the antigen are layered on to a 5-ml step-wise caesium chloride density gradient ranging from 1.1 g/cm³ to 1.6 g/cm³ and overlaid with 3 ml of light mineral oil. As many as 6 such tubes may be centrifuged at one time in a Spinco centrifuge by using an SW-40 rotor. The tubes are centrifuged for 8 hours at 152 000 *g*. Three discrete bands are seen; the middle band, which contains the antigen at a density of 1.20 g/cm³, is collected. The 1.20 g/cm³ bands are combined and rebanded in tubes containing 10 ml with 1.1 g/cm³ to 1.4 g/cm³ caesium chloride density gradients. A single sharp band is observed at a density of 1.20 g/cm³ in each tube; these bands are extracted, combined, dispensed in 0.1-ml amounts and stored at 4°C. This preparation, designated hepatitis-associated antigen (Cs-2) is the starting material for the rate separation in sucrose. The antigen (Cs-2) preparation should have a complement-fixing titre approximately 8 times greater than the titre of the starting serum.

Rate separation of the antigen in a sucrose gradient. A 0.1-ml sample of the antigen (Cs-2) preparation is diluted 1/10 with phosphate-buffered saline, pH 7.4, to diminish its density [1] and is centrifuged on a preformed 6-ml 10%–30% linear sucrose gradient at 117 000 *g* for 2.5 hours at 5°C in an SW-40 rotor. The contents of each tube are fractionated and each fraction is tested for the hepatitis-associated antigen by the complement-fixation test. The fractions containing peak complement-fixing activity are pooled, diluted with phosphate-buffered saline, pH 7.4, and stored at −20°C. The complement-fixing titre of the rate-separated antigen is usually much lower than the titre of the Cs-2 preparation. The fall in titre appears to be due to aggregation of the purified antigen which occurs after the rate separation; it does not impair its immunogenicity. If the human serum used for purification contained a high titre of the hepatitis-associated antigen (1 : 1024 or greater by the complement-fixation test) the purified antigen is diluted with phosphate-buffered saline to the volume of the original serum. The diluted purified antigen is stored at −20°C.

[1] Dialysis against phosphate-buffered saline is also a suitable means of diminishing the density of hepatitis-associated antigen (Cs-2).

Immunization of animals (method of Purcell et al., 1970)

After preinoculation samples of serum are obtained, guinea-pigs are inoculated in each hind foot pad with 0.2 ml of a 50% emulsion of Freund's complete adjuvant and purified hepatitis-associated antigen. Six weeks later the animals are inoculated intraperitoneally with 0.2 ml of the purified antigen. One week following the intraperitoneal inoculation and at weekly intervals thereafter, guinea-pigs are bled and their sera tested for antibody. The intraperitoneal inoculation of the purified antigen is repeated every other week until suitable antibody titres to this antigen are obtained, at which time the guinea-pigs are exsanguinated (approximately 2–3 months after starting the immunization schedule).

When tested by agar-gel diffusion and complement fixation, guinea-pig hyperimmune serum to the hepatitis-associated antigen reacts with antigen-containing sera but not with normal sera. Guinea-pig antiserum prepared by the method described is extremely potent (titres as high as 1 : 40 or greater by the immunodiffusion test, 1 : 2048 by the complement-fixation test and 1 : 5000 or more by radioimmunoassay).

LARGE NON-PRIMATE ANIMALS

While antiserum to the hepatitis-associated antigen prepared in guinea-pigs is of excellent quality, the use of such animals is impracticable for large-scale production of reagents, and it would be preferable to use large animals. Attempts to immunize large animals, including goats and donkeys, are in progress. Such animals have proved useful for the preparation of large quantities of reference reagent-quality antisera to a number of viruses. However, these antisera are less suitable for complement-fixation tests than are antisera prepared in guinea-pigs.

XII. APPLICATION OF LABORATORY METHODS [1]

One of the earliest promises held forth by the discovery of the hepatitis-associated antigen was the fulfilment of a long-standing need for a simple and reliable method for screening blood donors in order to eliminate or reduce the risk of post-transfusion hepatitis.

A variety of test methods for the detection in serum or plasma of the hepatitis-associated antigen and antibody to it have been described and several are given in detail in this paper. On the whole there is good evidence that the tests are specific and that false positive results are only rarely encountered. However, it is difficult to make exact quantitative assessments of the relative sensitivity of the tests because of the variations among reagents used by different laboratories or even by the same laboratory at different times. Nevertheless, in certain laboratories comparisons have been carried out and indicate that the complement-fixation test is more sensitive than the immunodiffusion test for the detection of antigen, but not necessarily so for the detection of antibody. Similarly there are claims that various immunoelectrophoretic methods are more sensitive than the immunodiffusion techniques. Preliminary evidence suggests that radioimmunoassay is likely to be the most sensitive method for detection of both antigen and antibody. The complex and specialized nature of the current radioimmunoassay techniques limit their use to a few laboratories. It is not yet possible to establish the order of sensitivity of immune electron microscopy and platelet aggregation tests.

In the screening of blood donors early prospective studies by Okochi & Murakami (1968) and by Gocke, Greenberg & Kavey (1969) using the immunodiffusion techniques indicated that reduction in risk by the elimination of blood containing hepatitis-associated antigen would be less than 25%. A number of studies currently in progress are using more sensitive methods of detection and more critical methods of patient follow-up. It is hoped that these studies will yield reliable data for predicting accurately the preventive efficacy of these screening procedures. At the same time they should provide valuable collections of donor serum and serial serum specimens from recipients for further studies directed at non-antigen-related hepatitis associated with blood transfusions. It is fully realized that variations in methods of testing for the hepatitis-associated antigen and the problems inherent in mass screening procedures necessitate the establishment of appropriate standards before legal requirements for such testing are adopted. Until such time as sufficient quantities of the appropriate reagents become readily available it should be appreciated that a full-scale programme for the screening of donors is not practicable for many blood transfusion services. With this problem in view the production of antisera in animals and the development of reference standards for both antigen and antibody, which would allow valid comparisons to be made, are under way, and initial steps have been taken to set up a collaborative study, involving laboratories from several countries, for the evaluation of a batch of antigen and the corresponding guinea-pig antiserum being prepared under the auspices of the Research Reference Reagents Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, USA.

It is desirable that all countries in which blood transfusion and laboratory services are able to carry out the more practicable tests (immunodiffusion, complement fixation and immunoelectrophoresis) should begin the development of programmes for the screening of blood donors. Application of testing procedures to individual units of plasma used for processing in pools should also contribute to the reduction of the risk of hepatitis associated with plasma fractions. This is of particular importance in the preparation of fibrinogen and other blood derivatives which are already well recognized as carrying the risk of hepatitis.

It must be clearly understood that the techniques for the detection of the hepatitis-associated antigen and antibody, as currently practised, are subject to change. A number of more sensitive and more complex methods have been, or are being, developed and these may be applied where special facilities are already available.

It is stressed, however, that a negative result for the hepatitis-associated antigen, at least as obtained by the currently available tests, does not in any way imply freedom from infection.

It must also be emphasized that all clinical spe-

[1] Prepared by Dr R. W. McCollum and Dr A. J. Zuckerman.

cimens, blood and blood derivatives, materials and reagents used in any of the tests employed for the detection of the antigen or antigens associated with or responsible for hepatitis must be treated with the full precautions usually practised in laboratories undertaking diagnostic microbiology. Some test procedures such as those employing radioactive isotopes have additional safety requirements.

XIII. NEEDS AND TRENDS IN RESEARCH AND DEVELOPMENT [1]

The discovery of a specific serum antigen associated with human viral hepatitis (Blumberg, Alter & Visnich, 1965b; Blumberg et al., 1967; Prince, 1968; Gocke & Kavey, 1969) and the subsequent developments, as detailed in the preceding sections, have given renewed hope that the frustrating puzzle of viral hepatitis may be resolved. Although impressive strides have been made within a relatively brief period, our understanding of the basic virology of hepatitis and of the practical applications to be derived from it is still in its infancy.

It appears that the form of viral hepatitis related to the currently recognized hepatitis-associated antigen represents only type-B (serum) hepatitis. However, it is by no means certain that all infections which might be classified as serum hepatitis on clinical and/or epidemiological grounds are related to the hepatitis-associated antigen. Type-A (infectious) hepatitis is believed to represent a much larger and more broadly based public health problem. The etiology of type-A hepatitis, including the question of single as against multiple agents, is still far from settled although a new lead has been offered in the recently reported finding of a coronavirus both in human case material (Zuckerman, Taylor & Almeida, 1970) and in marmoset-passaged materials derived from previously reported studies (Deinhardt & Holmes, 1968).

The repeatedly confirmed demonstration of the hepatitis-associated antigen in early acute-phase serum specimens obtained from individuals with type-B viral hepatitis and in blood and blood products known to induce the infection, together with its morphological features on electron-microscopic examination, have led many investigators to the tacit assumption that the antigen particles are viral and represent the etiological agent. Others, while acknowledging the intimacy of the relationship, have reservations concerning the specific nature of the particles and their derivation. There is general agreement concerning the diagnostic and epidemiological usefulness of the various hepatitis-associated antigen indicator systems and their potential value in preventing hepatitis associated with the use of human blood and blood products. There is also general agreement that additional knowledge is needed to reach complete fulfilment of the promise generated by Blumberg's discovery.

The needs, as currently viewed, fall into several broad categories:

1. Methodology and materials—e.g., improved methods for detection of the hepatitis-associated antigen and antibodies, including increased sensitivity without loss of specificity, and standardization of reagents, which would allow for more meaningful interpretation and comparison of results from different laboratories.

2. Characterization and etiological significance—e.g., further physical, chemical, biological and immunological characterization of the antigen and its specific relationship to the etiological agent(s) of type-B viral hepatitis.

3. Specific role of the antigen in the infectious process—e.g., the cellular or tissue derivation of this antigen and its role (including antigen–antibody complexes) in the pathogenesis of acute and chronic forms of hepatitis.

4. Diagnostic and epidemiological applications—e.g., further epidemiological studies that would more clearly define the extent of viral hepatitis type-B infection, both apparent and inapparent, in different populations; the relative importance of various potential modes of transmission; the role and significance of the persistent hepatitis-associated antigen carrier state, and the relationship of this antigen to other forms of chronic liver disease.

The physical and chemical characteristics of the hepatitis-associated antigen have been explored rather thoroughly but not exhaustively. For example, the nucleic acid content is unknown and the relationship between the spherical or ovoid particles (approximately 20 nm) and the tubular or filamentous forms remains unsettled. A recent report by Dane, Cameron & Briggs (1970) of another " virus-like "

[1] Prepared by Dr R. W. McCollum.

particle, measuring 42 nm in diameter, provides further problems of characterization and relationships.

Infectivity of the hepatitis-associated antigen *per se* has not yet been demonstrated although its relationship to infectivity appears to be well established. The finding of this antigen in chimpanzees and certain other non-human primates and a recent report of passage in African green monkeys (London et al., 1970) call for more intensive investigation of such animals as model systems which may help to elucidate the role of the antigen in the infectious process and the pathogenesis of hepatic lesions. Additionally, there is urgent need for feasible laboratory methods for propagation of the type-B hepatitis agent(s) and/or the antigen. Relatively standard methods of animal and tissue culture inoculation investigated extensively over the past four decades, with presumably negative results, need to be reconsidered and perhaps re-evaluated in the light of current knowledge.

To date relatively little information is available about the localization of the hepatitis-associated antigen in specific tissues or cells. Immunofluorescence (Millman et al., 1969; Coyne et al., 1970) and electron-microscopic (Nowoslawski et al., 1970) studies of liver tissue obtained from individuals with demonstrable circulating hepatitis-associated antigen have provided presumptive evidence of its presence in hepatocytes. The findings reported so far have been limited to specimens obtained after onset of symptoms, which is relatively late in the course of infection. The sites of early, or primary, viral and/or antigen replication during the incubation period remain a mystery. Similarly the suggested role of antibodies to the antigen and circulating antigen–antibody complexes in determining the clinical course and outcome of infection requires more extensive investigation. On the basis of currently available information it appears that different methods of testing may be measuring different types of antibodies which vary in their temporal relationship to the infectious process.

We also need to know more about the distribution of this antigen and other potentially infectious particles in the various protein fractions derived by currently accepted methods. For example, it would be important to know whether even minute amounts of the antigen may be precipitated with the gamma-globulin fraction and whether the lack of demonstrated infectivity of albumin is related to the absence of the hepatitis-associated antigen or perhaps to heating at 60°C for 10 hours. Although serological reactivity of the antigen is not readily destroyed by heat, little or nothing is known about immunogenicity and/or infectivity following various degrees of heat treatment. Inferences based on prior observations may need to be reconsidered.

Most " epidemiological " studies concerned with the frequency of the hepatitis-associated antigen among large populations are based on single serum sample surveys. The reported results probably reflect in large part the frequency or prevalence of persistent carriers. They offer little in the way of answering more basic epidemiological questions, e.g., the relative roles of parenteral and non-parenteral transmission. Serological methods which would reveal other evidence of prior infection, i.e., immunity, are needed for a more meaningful interpretation of serological surveys and to complete the picture of variations in response to infection in relation to host and environmental factors. A similar need exists in the clinical usefulness of the hepatitis-associated antigen both as a diagnostic and prognostic indicator of type-B hepatitis virus infection. Other areas of clinical and epidemiological need include more extensive studies concerned with both the antigen and antibody in relation to other forms of acute and chronic liver disease, such as chronic active hepatitis, post-necrotic cirrhosis and primary carcinoma of the liver.

Although it is tempting to speculate about the ultimate application of current knowledge of the hepatitis-associated antigen in the prevention and control of type-B viral hepatitis, this knowledge is still too limited and fragmentary for meaningful projection.

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B. S. BLUMBERG, Associate Director for Clinical Research, The Institute for Cancer Research, Philadelphia, Pa., USA

ROBERT J. BYRNE, Chief, Research Reference Reagents Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md., USA

ROBERT M. CHANOCK, Chief, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md., USA

W. CHARLES COCKBURN, Chief Medical Officer, Virus Diseases, World Health Organization, Geneva, Switzerland

Y. KONO, Research Officer, National Institute of Animal Health, Tokyo, Japan
J. KOZA, Head of General Virology Laboratory, Institute of Epidemiology and Microbiology, Prague, Czechoslovakia
ROBERT W. MCCOLLUM, Professor of Epidemiology, Yale University School of Medicine, New Haven, Conn., USA
DORIS MÉNACHÉ, Chef de Laboratoire, Centre national de Transfusion sanguine, Paris, France
K. PENTTINEN, Professor of Virology, University of Helsinki, Finland
ROBERT H. PURCELL, Staff Scientist, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md., USA
PATRICIA E. TAYLOR, Research Associate, London School of Hygiene and Tropical Medicine, London, England
F. G. WEWALKA, Professor, First Medical University Clinic, Vienna, Austria
A. J. ZUCKERMAN, Reader in Virology, London School of Hygiene and Tropical Medicine, London, England

RÉSUMÉ

HÉPATITE VIRALE ET ÉPREUVES POUR LA DÉTECTION DE L'ANTIGÈNE AUSTRALIA (ASSOCIÉ À L'HÉPATITE) ET DES ANTICORPS CORRESPONDANTS

On définit habituellement l'hépatite virale comme une maladie provoquée par l'un ou l'autre de deux agents pathogènes: le virus A, agent étiologique de l'hépatite infectieuse, et le virus B, responsable de l'hépatite sérique.

La découverte de l'antigène « Australia » par Blumberg et ses collaborateurs, et la démonstration indiscutée de son association avec l'hépatite sérique — mais non avec l'hépatite infectieuse —, ont suscité un immense effort de recherche durant les trois dernières années. L'antigène a été rarement décelé dans les populations normales des régions à climat tempéré, mais fréquemment trouvé chez des sujets bien portants vivant sous les tropiques. On le rencontre surtout chez des patients traités par des transfusions répétées de sang ou de fractions du sang ou qui reçoivent des transfusions massives, chez des patients atteints d'une affection hépatique chronique et chez des personnes travaillant dans des banques de sang ou des services d'hémodialyse.

On peut déceler l'antigène Australia et les anticorps correspondants à l'aide de différentes épreuves, les deux méthodes les plus couramment utilisées étant la diffusion en milieu gélifié et la fixation du complément. Le présent mémoire fournit une description détaillée de ces deux tests, ainsi que d'autres techniques plus complexes, de sorte que les hommes de science compétents disposant des réactifs, du matériel et des facilités de travail indispensables puissent les mettre en pratique dans leur laboratoire.

La morphologie de l'antigène, telle qu'elle apparaît au microscope électronique, et ses caractéristiques biochimiques sont décrites. A l'heure actuelle, les données rassemblées ne permettent pas de déterminer avec certitude s'il s'agit d'un virus, d'un virus « lent » ou d'un antigène spécifique produit par le foie en réponse à l'infection. Les tentatives de culture de cet antigène ont jusqu'à présent échoué.

L'importance pratique immédiate de cette découverte est qu'elle permet d'opérer un tri parmi les donneurs de sang et d'exclure ceux qui sont porteurs de l'antigène. Les spécialistes consultés ont émis le vœu de voir les autorités sanitaires des pays qui disposent de services de transfusion et de laboratoires suffisamment développés envisager la mise en place d'un système de sélection des donneurs et d'examen de chaque lot de plasma avant le mélange et le fractionnement. Les recherches prospectives déjà effectuées indiquent que ces mesures préventives ne réduiront le risque d'hépatite sérique que dans une proportion inférieure à 25%; on peut cependant espérer que grâce aux méthodes plus sensibles actuellement disponibles et à une meilleure observation des malades on pourra réviser cette estimation dans un sens favorable.

Pour beaucoup de services de transfusion, un des obstacles à la mise sur pied d'un système efficace de sélection des donneurs est le manque de quantités suffisantes de réactifs standardisés. Au cours de leur travaux, les spécialistes consultés ont pris un certain nombre de mesures visant à l'organisation d'une étude collective, dans les laboratoires de plusieurs pays, d'un lot d'antigène et de l'antisérum correspondant actuellement en cours de préparation aux National Institutes of Health, Etats-Unis d'Amérique.

Le dernier chapitre du mémoire énumère les besoins de la recherche sur de nombreux aspects de la question et contient des suggestions relatives aux moyens à mettre en œuvre pour obtenir les résultats les plus utiles.

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