DIAGNOSTIC ANTIGEN FOR VIRAL HEPATITIS
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9 Claims. (Cl. 167-78)

This is a continuation-in-part of our copending patent applications, Ser. No. 91,322, filed Feb. 24, 1961, and Ser. No. 224,840, filed Sept. 19, 1962, both now abandoned.

This invention relates to a diagnostic antigen for viral hepatitis and to methods for preparing the same.

Viral hepatitis, also known as "acute infectious hepatitis," "dysenterial jaundice," or as "serum hepatitis," is a disease which results from invasion of the liver by the filterable IH and SH viruses. Virus IH (also known as Virus A) causes "epidemic hepatitis." It is usually found in stools and in the blood of infected individuals and is transmitted to others by direct contact as well as by contaminated carriers such as food and water or blood, serum and blood plasma as well as by contaminated medical instruments.

"Serum hepatitis," caused by Virus SH (also known as Virus B) is clinically similar to that caused by Virus IH except that, in the opinion of several authors, it is found principally in circulatory fluids, not in stools. Characteristically, Virus SH evidence may be found in blood, serum, and plasma of diseased individual's mouths, sometimes years, after the infection recedes from its active state.

Virus SH is transmitted from one individual to another during blood or plasma transfusion or serum inoculation. Too, it may be passed during skin puncture or venipuncture through instruments contaminated with carrier blood and since transmission in these cases occurs by means of human sera infections, the disease is frequently referred to as "homologous serum jaundice" or "homologous serum hepatitis."

Several authors are of the opinion that Virus A and Virus B are merely different types of the same virus species or even that they are identical.

The terms "epidemic hepatitis," "infectious hepatitis," and "viral hepatitis" are often used interchangeably to denote viral invasion of the liver resulting in a pathologic state. It is in this context that the terms are employed in defining this invention.

In recent years, viral hepatitis has assumed such enormous world-wide epidemic proportions that the prognosis for curing the disease is not good. Kalk (Helvetia Medicina Acta 1961, 4) notes that the illness has attacked the human population in epidemic waves following certain characteristic laws which depend on the multiplicity and virulence of the causal agent and the degree of prevalence of the disease in a population with consequent changes in their immunity. Europe, for example, was visited by a particularly serious epidemic in the period 1939-1950. (Bundesgesund Heilsblatt, No. 1, Jan. 12, 1962) discloses that in West Germany alone, about 120,000 new cases of viral hepatitis arise annually.

The problem of controlling epidemic hepatitis is becoming more severe not only because of the large number of new cases annually throughout most of the civilized world, but also because about 10% of the cases do not recover completely; about 5% enter a chronic stage; and about 2% become cirrhotic. Thus, the number of liver patients increases from year to year.

It can be readily seen, therefore, that there is an acute need for a simple diagnostic method which facilitates rapid detection of the disease before the onset of clinically observable symptoms, especially prior to the preicteric phase. Rapid diagnosis in cases where spread of hepatitis is suspected throughout a large segment of the population or a large institution such as a hospital is especially desirable.

Further, it is essential to make a quick determination in cases where blood transfusion is contemplated but it is not known with absolute certainty whether the donor is free of the active form of hepatitis or is perhaps a carrier of the latent form.

Presently known methods for the detection of viral hepatitis are cumbersome and time-consuming. One such method, for example, involves the neutralizing effect of immune serum. It is known that blood of individuals who have recovered from viral hepatitis contains antibodies which are specific against hepatitis virus. Therefore, when blood serum of a test individual is added to embryonic cells of a noninfected individual, the tissue culture is inoculated with hepatitis virus, and the inoculated culture is grown for a period of 9 to 14 days at 35° to 40°C, the formed monolayered cells show no cytopathogenic effect. Absence of cytopathogenesis shows that the blood of the test individual contains antibodies. This and other equally well known techniques depend upon human tissue culture or embryonic cells of human origin for cultivation of hepatitis virus.

We have now discovered that human hepatitis virus can be successfully cultivated in tissue cultures of trypsinized animal kidney, or a permanent cell line cultured from animal kidney, and that the lipid component of the virus so propagated yields a highly effective diagnostic antigen for viral hepatitis.

The novel antigen possesses numerous advantages in that it can be employed on a large scale; it is not temperature labile; and is simple to use. (The antigen is an extremely reliable diagnostic tool. If a test individual has active or latent viral hepatitis of either the epidemic or the serum type, a positive reading is readily detectable on the skin of the subject.)

A particularly outstanding advantage of the novel antigen is its function in differential diagnosis of hepatitis.

In cases of suspected liver cell damage in the jaundiced patient where the exact cause of cellular deformation is difficult to establish, liver biopsy is advocated to make a correct diagnosis. This is particularly the case when responsibility for hepato cellular changes due to viral infiltration may be mistakenly assigned to toxic hepatitis.

Until recently, the difference between viral hepatitis, and toxic hepatitis was relatively clear cut, since it could be easily established by means of biopsy that the viral form affects cells throughout the lobule of the liver, whereas liver poisons, such as carbon tetrachloride, affects all metabolically similar cells in a like fashion. Thus, toxic hepatitis caused by true poisons shows zonal injury with fat absent or present. In viral hepatitis, however, fat is absent except during recovery, after steroid therapy, or in children.

Differential diagnosis between viral hepatitis and toxic hepatitis became a serious problem with the advent of widespread use of more complex organic structures, such as phenylidine, pheneme, sulfonamides and others. In such cases of jaundice, findings have been reported as being indistinguishable from viral hepatitis. Toxic hepatitis due to ingestion of certain organic substances produces a clinical picture which completely mimics one typical of viral hepatitis. Moreover, laboratory findings are all too frequently of limited use in establishing differential diagnosis unless combined with needle biopsy and, preferably, the electron microscope.

It will be seen therefore, that the novel viral hepatitis diagnostic antigen can be used to establish absence of toxic hepatitis due to drugs even though the medication
had been administered over a long period of time. This is so because the antigen is protein-specific with respect to the antigen-antibody reaction. (If antibodies are present in the circulating blood, either in the latent or active phase of the disease, administration of the test antigen evokes a positive reaction, and toxic hepatitis is ruled out.) No reaction will occur in the presence of drug hepatotoxicity since administration of the test antigen should not be expected to produce a reaction.

A suitable method for propagating hepatitis virus is as follows: Pigs are slaughtered, the kidneys removed under aseptic conditions, rinsed in phosphate-buffer salt solution, Hank's solution (or a similar medium), cut into pieces of about 2 to 3 mm, edge length, and washed three or four times in one of the above-named solutions. A 0.25% buffered trypsin solution, pH value 7.5, is allowed to react on the tissue pieces at 37°C, while stirring. About 20 to 30 ml trypsin solution are required for approximations of 50 grams of kidney fragments. After about 20 minutes the process is interrupted and the cell-containing liquid removed and discarded. The reaction vessel is charged again with the same volume of trypsin solution. The process is repeated six times every ten minutes, all further cell suspensions obtained being collected and cooled with ice water. The suspension is centrifuged for 10 minutes at about 1000 r.p.m. (180 g x gravity). The supernatant liquid is removed and the sediment is washed with phosphate-buffer salt solution at 37°C and centrifuged three more times. Any erythrocytes present are sucked off as far as possible. An aliquot of the cell suspension is sedimented at between about 100 to 150 times gravity for about ten minutes and the sediment is added to enough prepared nutrient medium to ensure a cell number of about 100,000 to about 250,000 cells/mL. The nutrient medium used may be the "Medium 199" according to Morgan, Morton and Parker (Proc. Soc. Exp. Biol. and Med. 73, 1, 1950) with 10% calf serum and penicillin and streptomycyn in the usual proportions. Bovine amnion liquid with 10% calf serum as well as penicillin and streptomycyn or other cell culture liquids may also be used as a nutrient medium. From the 1 cell suspension cultures are prepared in tubes or flasks by apparition out 2 cc. per tube (1.6 x 16 cm.) or 15 cc. per flask (4.5 x 4.5 x 12 cm.). The culture vessels are incubated at 37°C. The medium is replaced by fresh culture medium every three to four days. After 7 to 9 days of incubation the culture medium is reduced to about 35% and the culture vessels contain a monolayer of epithelial cells and are ready for inoculation with viral material. The inoculation of the tissue culture to be used according to the invention with live hepatitis virus can be effected by using aqueous feces suspensions; blood; blood serum; blood plasma; or suspensions of ground organs from persons infected with virus hepatitis. Culture liquids of tissue cultures of the virus are also suitable. For purpose of inoculation, the nutrient medium is decanted and each tube or flask is inoculated with viral material. After 1 to 4 hours fresh nutrient medium is added, the infectious solution either being left in the culture vessel or removed before the nutrient medium is added. The nutrient medium is changed 2 to 3 times each week. To obtain maximum cell and virus multiplication, the culture medium is kept at a approximately neutral pH. The inoculated tissue cultures are further incubated at temperatures in the range of 34 to 42°C until cytopathogenic effect is observed.

At the end of the incubation period the liquid culture substrates are separated from the tissue culture cells. The cells are separated from the walls of the culture vessels by means of 0.25% trypsin solution in phosphate-buffered salt solution at 37°C with frequent shaking and are exposed to trypsin solution for a further 3 to 20 hours at 37°C or mechanically ground by means of a cell mill. Any virus still cell-bound will be freed by this method.

The cell substrate thus treated is cleansed of the undissolved larger particles by low-speed centrifugation. The virus can be centrifuged out from the excess liquid and the separated nutrient substated by high speed centrifuging for which in general an operation of 60 to 120 minutes at 25,000 to 30,000 times gravity is suitable.

It is to be understood that the processes described above may be advantageously conducted on a variety of animal kidney tissue cultures. Pig kidneys or a permanent cell line prepared from pig kidneys are preferred for this purpose, although, for example, kidney, Guinea pig kidney, etc., may be employed with equally satisfactory results, as exemplified below. In addition, He-La cell tissue cultures, are suitable and advantageous for propagation of hepatitis virus.

It is readily evident, therefore, that tissue cultures prepared either from rabbit or Guinea pig kidney cells as well as from He-La cells represent suitable substrates for propagation of hepatitis virus. Elementary bodies are found on electron microscopic examination of the total experimental material from the same size and type as those which are observable in fecal samples of patients with hepatitis, in inoculated cell cultures and chicken eggs, and in inoculated tissue cultures prepared from pig kidney cells.

As to temperature variation for cultivation of tissue cultures from pig kidneys, incubation at 34°C over a prolonged period of time may be accomplished with some limitations, while 40°C is equivalent for all purposes at 37°C. Tissue cultures from pig kidney are maintained satisfactorily at 34°C for over 32 days.

The cell culture on which the hepatitis virus is grown is preferable one derived from an animal in which human hepatitis is not known to occur. This reference is to preclude the possibility of latent hepatitis virus in the cell tissue which is cultured and to avoid the presence of antibodies which would react with the hepatitis virus and prevent its growth.

The pig kidney is the preferred source of the cells for cultivating. Still further preferred is the use of a permanent cell line prepared from the primary pig kidney cells. The preparation of a permanent cell line from about 30 to 35 passages of subcultures from the primary pig kidney culture provides cells about which there is no need to take into consideration possible latent infections of the pig kidney, and it has been found that the inactivation effect is observed about three days after inoculation with virus material rather than the ten to thirteen days which are required for the destruction of the primary pig kidney cell culture.

Although animal kidney culture is a preferred source of hepatitis virus for propagation of the novel antigens, it has been discovered that the antigen can be prepared with equally satisfactory results from fecal lipid of individuals with clinical signs and symptoms of hepatitis and from choricallantoic membrane of hen's eggs.

**Preparation of fecal suspension of virus**

Stools obtained from patients in the acute stage of infectious hepatitis are used as the source of virus. Control stools are obtained from central personnel and patients without previous history of infectious or serum hepatitis. All stools are stored at refrigerator temperature for no longer than one to four days. Then a 10% suspension of the suspension in tap water is prepared by homogenization in sterile centrifuge tubes. The homogenate is then centrifuged at 5000 r.p.m. for about one hour. The supernate is separated out and treated with penicillin 400 μg/ml and streptomycin v/v/ml. The supernate is then allowed to stand at room temperature for about four hours after which sterile controls are made in Standard-I Bouillon, and incu-
Inoculation of embryonated hen's eggs

The fecal suspension (supra) is transferred to the atlanto-cavity of the developing chick embryo using 0.2 ml. inoculum per egg. The eggs are incubated at 35° C. to 36° C. for five days. Then, candled and the embryos still alive are chilled overnight in the cold room at 4° C. The chorionallantoic membranes are harvested, pooled and emulsified with tap water, 10 ml of water to each chorionallantoic membrane. Control series are initiated either by atlanto-cavity injection of normal fecal suspension or no injection into the chorionallantoic membrane. In all other respects the control eggs are handled like the infectious hepatitis series.

A skin test antigen is prepared in such a way that the virus is inactivated by chemical means, preferably by contact with formalin at a concentration of 1:2,000 to 1:6,000, preferably about 1:4,000. It is desirable to allow the formaldehyde to remain in contact, preferably a slightly elevated temperature, e.g., 34° C. to about 41° C., for about 7 to 15 days. Cell residues are separated by centrifugation under sterile conditions.

Lower molecular weight components and remaining formaldehyde are removed by dialysis against distilled water, ultra-centrifugation, or chromatographic absorption. By working under sterile conditions, an aqueous, sterile solution or suspension (respectively) of the antigen is obtained.

A most important feature of our invention resides in the isolation of the antigenic component from the cultured virus. (We have found that the antigen is obtainable from the lipoid fraction of the live virus.) Accordingly, to accomplish the isolation of the antigen successfully, one may employ an organic solvent which will dissolve the desired component without destroying its antigenic power. We have found that solvents most suitable for this purpose are ethyl ether and chloroform.

The virus particles themselves do not pass into the organic solution layer, together with the infectious nucleo-proteins. The obtained antigen components therefore are not infectious.

It is to be understood, however, that other organic solvents suitable for this purpose may be readily determined by further experimentation. After evaporation of the solvent, the residue is suspended in a physiological solution, such as Ringer's, and isotonic sodium chloride.

If desired, a suitable dispersing agent such as Tween-80 may be added to ensure uniform distribution of the antigen throughout the liquid phase.

Thereupon the suspensions are heated for one hour in a water bath at a temperature between 55° C. and 85° C. preferably at a temperature of 60° C.

Application of the novel antigenic preparation may be cutaneous (topical), on the breast or the upper arm, or intracutaneous (by injection), in the upper arm, the latter in a volume of about 0.1 to about 0.2 cc. About twenty minutes after injection a slight reddening of the skin area is observed which is non-specific and soon subsides. The actual determination of the nature of the response, i.e., negative or positive, is made after twelve to forty-eight hours have elapsed. During that period, at the injection area, there develops a red papula or swelling accompanied by generalized reddening and, occasionally, cockade phenomena. The reaction may last up to about 8 days.

The antigen of this invention is useful as a diagnostic for hepatitis in homologous and heterologous individuals with chemical signs and symptoms of viral hepatitis. The antigen gives a positive reaction when tested against homologous individuals if the virus is present in the starting material. There is a high correlation between skin test results and electron microscopic findings of the corresponding fecal suspension. For example, in all cases where the fecal skin test antigen is negative when tested on homologous individuals, no elementary bodies of viral hepatitis are seen under the electron microscope, for the corresponding fecal suspension.

When the fecal skin test antigens are tested on heterologous individuals, in many cases the antigens elicit positive reactions giving just about the same pattern of reaction whenever tested. There appear to be several strains of hepatitis virus, and it is possible that some individuals harbor more than one strain. For example, it has been observed that two or more heterologous antigens can elicit positive results in one individual, whereas the same antigens are only partially positive in others. This is because each antibody is specific for an individual antigen and is not capable of reacting with antigens which do not contain common reactive chemical groupings.

The following examples are intended to illustrate, but not to limit, the scope of the present invention.

**EXAMPLE I**

**Pig kidney tissue culture**

A kidney is removed from a pig weighing approximately 40 kg. under sterile conditions. The kidney is placed in phosphate-buffered saline solution (PPS) containing 200 I.U. of penicillin and 100 gamma of streptomycin. The solution is kept in a temperature at 37° C. The PPS is made up as follows:

(a):

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8.0 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2 g</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Water</td>
<td>400 ml</td>
</tr>
</tbody>
</table>

(b):

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂HPO₄</td>
<td>1.5 g</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Water</td>
<td>400 ml</td>
</tr>
</tbody>
</table>

(c):

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Water</td>
<td>200 ml</td>
</tr>
</tbody>
</table>

The kidney is cut into pieces about 2 to 3 mm. square. The kidney pieces are washed four times with PPS solution at 37° C. The washed kidney pieces are placed in a 200 ml. Erlenmeyer flask, 25 ml. of 0.25% trypsin solution is added to 50 grams of the kidney pieces in the flask and the flask is placed in a water bath at 37° C. The contents of the flask are stirred for 20 minutes with a magnetic stirrer.

A portion of the trypsin solution is decanted and discarded. Fresh trypsin solution is added to the flask to make the volume back up to 25 ml. Six times, at ten minute intervals the trypsin solution is decanted into a flask and replaced with fresh trypsin solution. The collected fractions are kept in an ice bath.

The trypsin solution is centrifuged for ten minutes at 1000 r.p.m. (180 times gravity) to sediment the cells. The supernatant is decanted and the cells are washed three times with PPS at 37° C. Any erythrocytes present on the sedimented cells are aspirated off.

The cells are added to sufficient tissue culture medium to provide a density 1.25×10⁹ cells/ml. Fifteen million portions of this cell suspension are added to square flasks having the dimensions 4.5 x 4.5 x 12 cm. To suspend the cells a culture medium of the following composition is used:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>800 ml</td>
</tr>
<tr>
<td>Medium 199 (10× concentrate)</td>
<td>100 ml</td>
</tr>
<tr>
<td>2.8% sodium bicarbonate solution</td>
<td>50 ml</td>
</tr>
<tr>
<td>1% streptomycin solution</td>
<td>11.3 ml</td>
</tr>
<tr>
<td>1.33% penicillin solution</td>
<td>11.3 ml</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>11.3 ml</td>
</tr>
</tbody>
</table>

Phenol red is added to the flasks as an indicator. The flasks are incubated at 37° C. for five days. The culture medium is decanted and replaced with fresh culture me-
medium and the flasks are again incubated at 37° C. At the end of a further three day period the culture medium is again decanted and replaced with fresh medium and the flasks are again incubated at 37° C. for three days. The culture medium is again decanted and a mono-layer of epithelial cells is observed in the flasks.

EXAMPLE II

Permanent pig kidney cell line

Flasks with nine day old primary pig kidney cultures obtained by the procedure of Example I are washed with calcium- and magnesium-free Moscona solution at 37° C. The Moscona solution is prepared as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8.00</td>
</tr>
<tr>
<td>KCl</td>
<td>0.20</td>
</tr>
<tr>
<td>NaH₂PO₄.H₂O</td>
<td>0.005</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>1.00</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.00</td>
</tr>
<tr>
<td>Water (deionized)</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

The Moscona solution is sterilized by filtration through a Seitz filter. 10 ml of 0.25% trypsin solution is added to each flask and the flasks are incubated for 20 minutes at 37° C. The trypsinized cell suspension is centrifuged for five minutes at 150 times gravity and the supernatant trypsin solution is discarded. The sedimented cells are washed three times by centrifugation with Moscona solution. The washed cells are added to Difco Medium 199 containing 20% calf serum which has been inactivated for 30 minutes at 56° C. The culture in each starting flask is divided into three flasks for the growth of the subcultures. The culture medium has the following composition:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (deionized)</td>
<td>800 ml</td>
</tr>
<tr>
<td>Medium 199 (10X concentrated)</td>
<td>100 ml</td>
</tr>
<tr>
<td>2.8% sodium bicarbonate solution</td>
<td>50 ml</td>
</tr>
<tr>
<td>1% streptomycin solution</td>
<td>12.8 ml</td>
</tr>
<tr>
<td>1.33% penicillin solution</td>
<td>12.8 ml</td>
</tr>
<tr>
<td>Calf serum</td>
<td>256 ml</td>
</tr>
</tbody>
</table>

15 ml of culture medium is added to each flask and the flasks are incubated in a stationary position at 37° C. Every three days, the culture medium is decanted and fresh medium is added. After nine days a subculture is prepared by transferring a portion of the cells to a fresh flask and 15 ml of fresh culture medium is added. The subculture is grown by the same procedure as described above. A total of thirty-five passages of subcultures is made.

EXAMPLE III

Virus growth on pig kidney cells

A flask of primary pig kidney cells prepared according to Example I is inoculated with 2 ml of inoculum, which is prepared as follows: 3 loops (≈ 3 mm) of faces of a patient suffering from virus hepatitis are stirred in 10 ml of tap water. The suspension is centrifuged for 20 minutes at 2000 times gravity to free it of coarse particles and is filtered through a Schott frint filter No. G5. After being in contact with the cell culture for two hours at 37° C., 13.0 ml of fresh culture medium is added to the flask. The sediment content of the medium is 3%. The flask is incubated at 37° C. and the medium is replaced with fresh medium three times each week. During virus growth the medium is maintained at approximately neutral pH. During the culture process, the cells are observed for cytopathogenic effect. Upon the observation of cytopathogenic effect, the nutrient substrate is separated from the tissue cells and the tissue cells are digested with trypsin solution at 37° C. for 20 hours. The suspension is centrifuged at 2000 times gravity for 20 minutes. The supernate is separated and centrifuged at 27,000 times gravity. The sediment from this centrifugation is suspended in sterile water and is examined for the presence of hepatitis virus by means of an electron microscope.

EXAMPLE IV

Virus growth on pig kidney cells from permanent cell line

A flask of pig kidney cells prepared according to Example II inoculated with 2 ml. of inoculum pooled hepatitis serum prepared from the sera of four patients suffering from virus hepatitis. After being in contact with the cell culture for two hours at 37° C., 13.0 ml. of fresh culture medium containing 3% calf serum is added to the flask. The flask is incubated at 37° C. in a stationary position. Cytopathogenic effect is observed after ten days. The procedure is repeated for fifteen passages, and cytopathogenic effect is finally observed after three days. The nutrient medium and tissue culture cells are worked up as in Example III.

EXAMPLE V

Hela cell tissue culture

Sixteen tissue culture tubes are seeded, each with 2 ml. of a cell suspension harvested from an Earle-T-Flask. The tissue culture tubes are incubated at 37° C. The nutrient medium has the following composition: 65% Hank's solution, 10% medium 199, 10% lactalbumin-yeast solution, 10% calf serum, 5% horse serum, 0.002% phenol red and penicillin and streptomycin as indicated in previous examples. The lactalbumin-yeast solution contains 5 parts by weight lactalbumin hydrolysate and 1 part by weight yeastolate Difco, dissolved in 1,000 parts by volume of a phosphate-buffered saline. Nutrient media are changed every two or three days.

The inoculum is the same as that used in Example III. The tissue cultures are inoculated after 2 days' incubation. The old nutrient media are removed and 8 tubes each with hepatitis pool. The volume of the inoculum is 0.4 cc. each. The inoculated tissue culture tubes are incubated for 2 hours at 37° C. after which time the inoculum is pipetted off and 2 ml. of first nutrient medium are filled into each of the tubes. The tissue cultures inoculated with control pool or with hepatitis pool respectively are processed according to the time scale shown below:

<table>
<thead>
<tr>
<th>Days</th>
<th>Substrate</th>
<th>Electron Microscope Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Pool</td>
<td>Hepatitis Pool</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>--</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>--</td>
</tr>
<tr>
<td>12</td>
<td>M</td>
<td>--</td>
</tr>
<tr>
<td>12</td>
<td>Z</td>
<td>++</td>
</tr>
</tbody>
</table>

M=nutrient media; Z=trypsinized tissue culture.

The number of viruses found in the nutrient medium is much smaller than that of the tissue culture cells. This is demonstrated as follows: Nutrient media of each of 4 tubes are sampled and the tissue culture cells trypsinized. Further treatment of the substrate is accomplished as already described above. Suspensions are prepared from the sedimented material and examined with the aid of the electron microscope.
EXAMPLE VI
Lipid extract from feces

To 15 ml. of a dense suspension of feces (supra) 30 ml. of ethyl ether is added and the material extracted in a separatory funnel. The solvent layer is removed. The procedure is repeated with 30 ml. solvent. The two solvent phases are pooled, and evaporated under reduced pressure. The remaining lipid extract of virus containing material is transferred to a sterile test tube and stored at 4°C. for making the antigen.

EXAMPLE VII
Production of antigen from tissue culture

Tissue culture suspension prepared as described above (Examples III and IV) are inactivated with formaldehyde at a final dilution of 1:4000. The procedure is performed at 37°C. over a period of twelve days. After inactivation the suspension is deformed by dialysis against distilled water at 4°C. for three days, the water being changed once daily. At the end of three days the preparation is tested for bacterial contamination. The deformed preparation is the skin test antigen.

EXAMPLE VIII
Production of antigen from fecal extract

Using sterile test tubes, one loopful of the lipid extract prepared as described in Example VI is dissolved in 1 ml. of ethyl ether. The solvent containing the lipid complex is then evaporated under reduced pressure. The remaining lipid complex is resuspended in 3.5 ml. of physiological saline. The test tube is well shaken to obtain homogeneity, after which inactivation is accomplished by raising and maintaining the temperature to 60°C. for a period of one hour. The resulting antigen preparation is stored in a refrigerator.

EXAMPLE IX
Production of antigen from chloroallantoic

An equal volume of glycerine is added to the emulsified suspension of chloroallantoic membrane (supra), with final addition of 0.25% phenol. The mixture is placed in a water bath and kept at boiling temperature for eight hours. After cooling, 3.5 ml. is transferred to a separatory funnel and 7.0 ml. chloroform is added. The mixture is shaken gently for five minutes and then allowed to stand. The chloroform phase is drawn off and saved. The procedure is repeated, adding 7.0 ml. chloroform to the chloroallantoic glycerine preparation to complete extraction of the lipid complex from the virus containing material. The two chloroform phases are pooled and evaporated under reduced pressure. The remaining lipid complex is resuspended in 3.5 ml. sterile physiological saline and shaken to homogeneity. The suspension is inactivated at 60°C. for about one hour. The resulting antigen preparation is stored at 4°C.

What is claimed is:

1. A preparation useful in the diagnosis of viral hepatitis comprising a physiological suspension of antigenic components of human hepatitis virus, said antigenic components being the residue obtained by extracting human hepatitis virus containing pig kidney cells with an organic solvent selected from the group consisting of ethyl ether and chloroform separating the solvent from the cellular material and subsequently evaporating the organic solvent, and observing the response thereto, a positive response being indicated by swelling and reddening at the site of injection after about 12 to 48 hours.

2. A method of diagnosing viral hepatitis in humans which comprises intracutaneously injecting a composition comprising a physiological suspension of an antigenic component of human hepatitis virus, said antigenic component being the residue obtained by extracting human hepatitis virus containing pig kidney cells with an organic solvent selected from the group consisting of ethyl ether and chloroform separating the solvent from said fecal suspension, evaporating the solvent and suspending the resulting antigenic residue in a physiological fluid.

3. A method of propagating human hepatitis virus which comprises inoculating pig kidney tissue cells with human hepatitis virus, providing the inoculated cells with nutrient fluid capable of supporting growth of the pig kidney tissue cells, and incubating the inoculated culture at a temperature of about 34°-42°C. until cytopathogenic effect is observed.

4. A method of obtaining a diagnostic antigen for viral hepatitis which comprises extracting the antigenic components of human hepatitis virus from an organic solvent selected from the group consisting of ethyl ether and chloroform separating the solvent from the human hepatitis virus, evaporating the solvent and suspending the resulting antigenic residue in a physiological fluid.

5. A method of obtaining a diagnostic antigen for viral hepatitis which comprises extracting the antigenic components of human hepatitis virus with an organic solvent selected from the group consisting of ethyl ether and chloroform separating the solvent from the human hepatitis virus, evaporating the solvent, suspending the resulting antigenic residue in a physiological fluid and heating the suspension for about one hour at a temperature of about 55°-85°C.

6. A method of obtaining a diagnostic antigen for viral hepatitis which comprises inoculating pig kidney cells with human hepatitis virus, providing the inoculated cells with nutrient fluid capable of supporting growth of the pig kidney cells, incubating the inoculated culture at a temperature of about 34° to 42°C. to propagate the virus therein, separating and inactivating the virus without destroying its antigenicity and recovering the inactivated antigenic virus.

7. A method of obtaining a diagnostic antigen for viral hepatitis which comprises inoculating pig kidney cells with nutrient fluid capable of supporting growth of the pig kidney cells, incubating the inoculated culture at a temperature of about 34°-42°C. until cytopathogenic effect is observed, separating and treating the virus by contact with formaldehyde at a concentration of about 1:2000 to 1:6000 at a temperature of about 34°-41°C., for about 7 to 15 days, and de-formalizing and recovering the treated virus.

8. A method of obtaining a diagnostic antigen for viral hepatitis which comprises inoculating pig kidney cells with human hepatitis virus, providing the inoculated cells with nutrient fluid capable of supporting growth of the pig kidney cells, incubating the inoculated culture at a temperature of about 34°-42°C. until cytopathogenic effect is observed, separating and treating the virus by contact with formaldehyde at a concentration of about 1:2000 to 1:6000 at a temperature of about 34°-42°C. for about 7 to 15 days, and separating the treated virus from low molecular weight components and formaldehyde by dialysis against distilled water.

9. A method of obtaining a diagnostic antigen for viral hepatitis which comprises extracting the antigenic components of human hepatitis virus from a human hepatitis virus infected fecal suspension with an organic solvent selected from the group consisting of ethyl ether and chloroform separating the solvent from said fecal suspension, evaporating the solvent and suspending the resulting antigenic residue in a physiological fluid.

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