# Antibody in Hepatitis Patients Against a Newly Isolated Virus. (26891)

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During the past 20 years many investigators have attempted to propagate in the laboratory the etiologic agents of infectious hepatitis and homologous serum hepatitis (1,2).

In the 1940's, several groups of investigators attacked the problems arising from contamination of human plasma with serum hepatitis virus. Their work was concerned in part with studying the effects of heat, irradiation and chemical agents on the virus in contaminated plasma pools. Because of the need for a standard supply of infectious material, the National Institutes of Health (NIH) icterogenic pool was prepared in 1951 from plasma specimens from several patients in the acute phase of clinically typical serum hepatitis(3). Subsequent studies by the group established that parenteral inoculation with this pool material caused hepatitis with jaundice in about 50% of volunteers(3).

This report deals with the preliminary characterization of a cytopathogenic agent recovered on one occasion from primary rabbit kidney cell cultures (RKTC) inoculated with a stored dried aliquot of the 1951 NIH-6* icterogenic plasma pool(3).

*Materials.* The virus isolate (A-1 agent) was passed serially 15 times in RKTC and subsequently 46 times in primary chick embryo cultures (CETC). Most studies described here were conducted with materials from the 25th to 46th CETC passages. Primary cell cultures were prepared by the conventional trypsinization procedure(4). Chick embryo cultures were prepared in this laboratory; all other cell cultures were obtained from the Tissue Culture Section, Division of Biologics Standards (DBS). Tissue cultures were maintained in either medium 199 or Eagle's Basal Medium (BME) with varying amounts of normal calf serum (NCS) added (0 to 10%). All NCS used in these experiments was inactivated at 56°C for 30 minutes. Animals employed were supplied by the Laboratory Aids Branch, NIH. Embryonated eggs from white Leghorn chickens were obtained from a commercial hatchery. The sources of human sera tested for antibody against the A-1 isolate will be dealt with in a subsequent section. The antiviral sera employed in tests to determine the possible relationship of A-1 virus to known viruses were available in this laboratory, obtained from Dr. Wallace P. Rowe or purchased from Microbiological Associates, Inc.

*Methods.* A plaque-reduction neutralization test was utilized for all serologic determinations. CETC monolayers in 2 oz. prescription bottles overlayed with our modified 199 plaque medium (Table I) provided the substrate for plaque formation. Sera to be tested were inactivated at 56°C for 30 minutes. Two-fold serum dilutions were prepared in medium 199 containing 10% NCS. The serum dilutions in 0.2 ml amounts were mixed with equal volumes of an appropriately diluted virus suspension which was to contain about 100 plaque forming units (PFU) of virus. After incubation at 36°C for one hour, 0.2 ml from each mixture was inoculated onto the monolayer contained in a single bottle. Following a one-hour adsorption period at 36°C the cell sheets were washed twice with 1.8 ml of medium 199, after which they were overlaid with the agar-plaque medium mixture and incubated at 37°C. Plaque counts were made on the 8th, 10th and 12th days of incubation. Serum neutralizing antibody titers were expressed as the reciprocal of the highest serum dilution causing a 4-fold or greater reduction in plaques, when the control virus mixture contained 60 to 250 PFU. In tests with smaller amounts of virus, *i.e.*, 30 to 60 PFU, an 8-fold reduction was considered significant; tests with less than 30 PFU were deemed unsatisfactory. Serum

* Aliquots of the NIH infected plasma pool which were stored in the lyophilized state have been coded as NIH-6. The same pool material stored in the frozen state has been referred to in the literature(1) as NIH-8.

TABLE I. Modified 199 Plaque Medium.

| | ml |
|---|---|
| 2× concentrated 199 plaque medium (with 1:30,000 neutral red) | 72 |
| 3% tryptose phosphate broth | 9.0 |
| 1× Eagle's basal medium (without phenol red) | 7.0 |
| 2× concentrated skim milk | 2.0 |
| | 90 |
| 3% Noble agar | 90 |
| | 180 |

Modified 199 plaque medium and Noble agar are mixed immediately prior to use; 7.5 ml of the mixture is poured into each 2 oz prescription bottle.

dilution values represented the final dilution of serum in the appropriate serum-virus mixture.

*Results. Isolation and biologic behavior of agent.* The A-1 agent was originally recovered in the following manner: an aliquot of the NIH icterogenic plasma pool was dialyzed against Earle's balanced salt solution for 72 hours, Seitz filtered, heated for 1 hour at 60°C and then inoculated into RKTC. Four days after inoculation cytopathogenic effect (CPE) was observed in the cell culture; transfer of the fluid from this culture demonstrated that it contained a transmissible agent. The uninoculated RKTC control bottles prepared from the same batch of rabbit cells did not develop CPE on incubation. Moreover, 2 blind passages of the control material in RKTC failed to reveal CPE. The A-1 agent produced CPE (Fig. 1) with regularity 4 to 6 days after inoculation even in early passages. The CPE progressed rapidly to complete degeneration of the cell sheet at which time the virus titers of tissue culture supernates were in the range of $10^6$ to $10^7$ $TCID_{50}/0.2$ ml.

The A-1 virus multiplies in a variety of cell culture hosts. All of the 6 primary and 6 stable cell lines tested to date support its growth (Table II). Titers of $10^6$ are common in each of the cell systems listed in the table. Cytonecrosis is seen in each of the cell cultures listed in Table II except in primary rhesus monkey kidney.

Microscopic examination of Giemsa and hematoxylin-eosin stained collodion prepara-

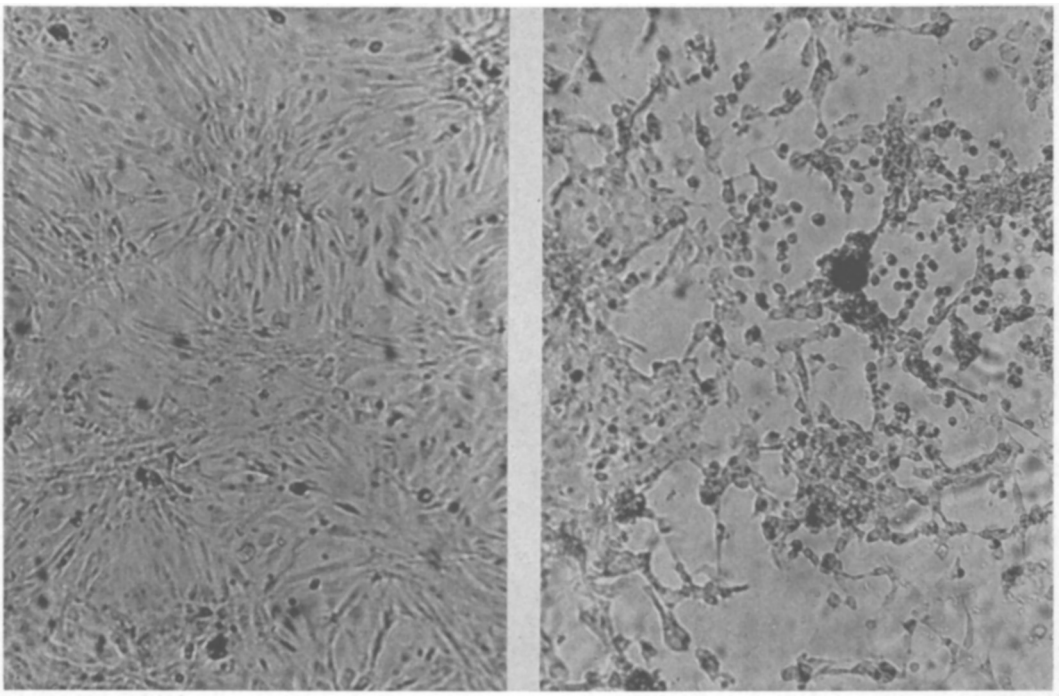


FIG. 1. Primary rabbit kidney—uninoculated control (left); 4 days after inoculation with A-1 agent (right).

TABLE II. Cell Culture Susceptibility to A-1 Agent.

| Host cell | Multiplication | CPE |
|---|---|---|
| Primary | | |
| Monkey kidney | + | 0 |
| Rabbit " | + | + |
| Chick embryo | + | + |
| Human amnion | + | + |
| Rat embryo | + | + |
| Cat kidney | + | + |
| Continuous | | |
| Monkey kidney | + | + |
| KB | + | + |
| HeLa | + | + |
| Human amnion | + | + |
| Hep-2 | + | + |
| Chang liver | + | + |

tions†(5) of infected CETC, RKTC and KB (Eagle) cell cultures revealed no giant cells, inclusions, elementary bodies or other distinctive cytopathological features.

Inoculation of infected cell culture fluids onto blood agar, Sabouraud's agar and in thioglycollate broth gave no evidence of the consistent presence of any bacterial or fungal organisms. Tests for pleuropneumonia-like organisms were negative when a high cell culture passage level of the virus (RK15, CE45) was examined for their presence‡.

Inoculation of the A-1 virus into common laboratory animals and embryonated eggs failed to produce overt disease or gross pathological lesions (Table III). However, blind passage in embryonated eggs established that inapparent infection had occurred. In one instance, after 8 passages in the allantoic sac and in another after 15 in the yolk sac, back titrations of egg materials into CETC revealed amounts of virus comparable to those present in the original tissue culture inoculum ($10^6$ to $10^7$ $TCID_{50}$). Chicks hatched from infected eggs appeared normal. Four serial passages in suckling and weanling mice (IC and IP) showed no evidence of disease and back titrations to cell cultures failed to demonstrate the presence of the virus. Four rhesus monkeys remained healthy during a 6-month observation period following inoculation (Table III). No significant fluctuation was noted in their body temperatures or their white blood cell counts during an 11-week observation period. Tests in these monkeys for viremia and for neutralizing antibody made at weekly intervals for 3 months gave negative results.

Attempts to produce hemagglutination with A-1 virus have been uniformly unsuccessful. Unheated and heat inactivated virus materials obtained from infected RKTC and CETC were mixed with 0.5% suspensions of red blood cells (RBC) from the following animals: man (Groups A, B, and O), monkey (rhesus, cynomologous and vervet), chicken, rabbit, mouse, guinea pig, sheep, hamster, cat and dog. Tubes were observed for 24 hours at 4°, 25° and 36°C. Hemagglutination also failed to occur when RBC from 1-day-old chicks were employed using the method of Havens(7). Infected allantoic and amniotic fluids from the 8th egg passage of the virus likewise produced no hemagglutination.

*Physical properties.* The A-1 agent retains its infectivity for cell cultures after storage in 2% NCS at –20°C for at least 20 months; lyophilized samples in 10% skim milk kept at room temperature are infectious for at least 16 months. Serum free suspensions of the virus lose their infectivity after 14 days at 4°C, 7 days at 25°C and 1 day at 37°C. Addition of NCS or normal human plasma (NHP) to such virus suspensions preserves infectivity over this temperature range for

TABLE III. Absence of Clinical Disease or Gross Lesions in Laboratory Animals Inoculated with $10^4$ to $10^6$ $TCID_{50}$ of A-1 Agent.

| Animal host | Routes |
|---|---|
| Weanling mice | IC–IP |
| Suckling " | " |
| " hamster | " |
| Embryonated chick egg | IC–CAM–YS–All.–Amn. |
| Newly hatched chick | IM |
| Guinea pig | IV–IM–IP–SC |
| Rat | IP–SC |
| Rooster | IV–IM |
| Rabbit | IV–IM–SC–Cornea |
| Monkey | IV–IM–SC–IP–IS–IT |

IC = intracerebral; IP = intraperitoneal; CAM = chorio-allantoic membrane; YS = yolk sac; All. = allantoic sac; Amn. = amniotic sac; IV = intravenous; IM = intramuscular; SC = subcutaneous; IS = intraspinal; IT = intrathalamic.

† Performed by Miss Nancy G. Rogers, DBS.

‡ Performed by Dr. M. F. Barile, DBS, by previously reported technics(6).

FIG. 2. A-1 agent plaque reduction neutralization test photographed on the 10th day following inoculation and agar overlay of primary chick embryo monolayer tissue cultures.

periods of 3 to 8 weeks. The virus is inactivated in less than 30 minutes at 56°C, even in the presence of undiluted NHP. The A-1 virus is ether and desoxycholate(8) sensitive. It passes through millipore filters of an average size of 300 mμ with no appreciable loss in titer, and also passes through filters of a pore size of 100 mμ but with a marked fall in titer (reduction from $10^6$ to $10^1$).

CETC fluids containing about $10^6$ $TCID_{50}$ of the A-1 virus in serum free medium 199 were tested for stability to photodynamic action.§ In the presence of toluidine blue (6 μg/ml), polychromatic light from two 100 volt photoflood lamps 7½ inches apart reduced the virus titer below detectable levels in 60 seconds. In contrast to findings with other viruses(9), the addition of either 5% bovine serum albumin or undiluted human plasma so stabilized the virus that, for practical purposes, the titer was unchanged after 10 minutes of irradiation under identical conditions.

*Serology.* During the course of cell culture passage it was found that the A-1 virus produced discrete plaques in both CETC and RKTC. Since less technical difficulty was encountered with the CETC system, it was chosen for the plaque reduction neutralization test. The results obtained in such a test are illustrated in Fig. 2; the bottle on the left containing the virus control material shows about 60 plaques, that in the middle illustrates a 4-fold reduction of plaques attributable to the neutralizing effect of antiserum, while the bottle on the right illustrates complete neutralization of the challenge dose of virus and no plaques are seen.

The A-1 virus was not neutralized by the various normal and immune sera listed in Table IV.‖ The 400 human sera mentioned in this table were obtained from a group of people accepted as blood donors.

Human and animal sera contain heat labile inhibitors for the agent. These may be detectable in sera diluted as much as 1 to 80, thus it is essential that all sera used either in media or for serologic testing be inactivated for 30 minutes at 56°C.

TABLE IV. Failure of Various Antisera and Normal Sera to Neutralize the A-1 Agent.

| Antiserum | Normal serum | No. tested |
|---|---|---|
| Herpes simplex | Rabbit | 25 |
| Measles | Rooster | 6 |
| Mumps | Rat | 8 |
| Poliomyelitis I–II–III | Guinea pig | 20 |
| Coxsackie A9 | Calf | 25 |
| " B1, 2, 3, 4, 5 | Monkey | 20 |
| ECHO 1–3, 5–19 | Human | 400 |
| Reovirus 1, 2, 3 | Human γ globulin | 20 |
| Influenza A, $A^1$, $A^{11}$ | | |
| " C | | |
| " D | | |
| Parainfluenza 1, 2, 3 | | |
| SV - 5 | | |
| Lymphocytic choriomeningitis | | |

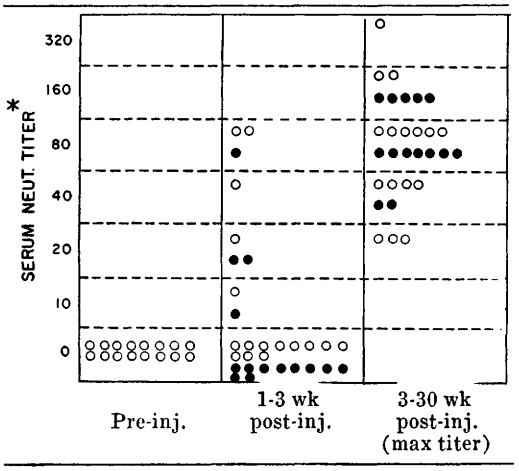
Table V contains data on the development of neutralizing antibody against the A-1 virus in 30 persons with experimentally induced homologous serum hepatitis¶. The volunteers were from 6 different groups. Each group had been inoculated with material from a different serum hepatitis virus pool during the studies undertaken in man some years ago. One of the 6 represented the

§ Courtesy Dr. C. W. Hiatt, DBS, by methods previously described(9).

‖ Great difficulty has been encountered in producing antisera which will neutralize A-1 virus. Hyperimmunization of monkeys, rabbits, guinea pigs, rats and chickens resulted in no detectable neutralizing substances. Low titer sera have been produced in a few guinea pigs inoculated repeatedly with $10^6$ $TCID_{50}$ of infectious virus in incomplete Freund's adjuvant. Each of the antisera listed in Table IV was known to contain homologous antibody in high titer.

¶ Sera kindly supplied by Dr. R. Murray, DBS.

TABLE V. A-1 Agent Antibody in 30 Volunteers Developing Serum Hepatitis.




○ Pre-inj. serum available and tested. ● No pre-inj. serum; first available serum specimen 1 to 3 wk post-inj.

0 neutralization titer = < 10.

* Expressed as reciprocal of serum dilution.

NIH icterogenic plasma pool(3) from which the A-1 agent was subsequently recovered. Four others were icterogenic plasma lots prepared about the same time from individual infected donors(10).** The sixth source of virus was an infected lot of thrombin(11).** Each of these 30 patients developed clinical hepatitis with jaundice. The information in Table V indicates that a 4-fold or greater increase in antibody occurred in all but 2 of these persons. The 2 individuals who failed to show such an increase in antibody did display a 2-fold rise from a titer of 20 to 40 and from 80 to 160, respectively; both were among those from whom the first available serum specimen had been obtained several weeks after inoculation.

Table VI presents serologic results obtained on specimens from 4 different groups of patients whose clinical diagnosis was in each instance infectious hepatitis. These include military personnel who contracted the disease in the United States (IH-I), United Nations personnel who contracted it in Korea (IH-II), a group of American servicemen who became ill after visiting Italy (IH-III) and finally, a group of children who had been inoculated with a strain of infectious hepatitis virus during the course of studies conducted in New York (IH-IV)(12). These groups of sera were graciously supplied by Drs. Ross L. Gauld, J. Anthony Morris, Robert McCollum and Saul Krugman, respectively. The other 17 human sera listed in Table VI were from cases of leptospirosis or leprosy and were included as coded controls. Antibodies against the A-1 virus were found in sera from a small number of individuals but in no instance in which paired sera were tested was a 4-fold or greater rise in titer demonstrated.

*Discussion.* It should be emphasized that the A-1 virus was recovered on only one occasion following inoculation of cell cultures with the NIH icterogenic plasma pool. It has not been recovered in repeated re-isolation attempts from this same pool, nor has it been recovered from other specimens presumed to contain the virus of homologous serum hepatitis.

Five sera from serum hepatitis cases known to neutralize the A-1 virus at high serum dilutions were absorbed with a suspension made from fresh, frozen, normal human liver. The post-absorption neutralizing antibody titers of these samples were identical with those obtained with the untreated sera. Thus, it appears unlikely that antibody against human liver tissue(13) influences the plaque-

TABLE VI. A-1 Virus Neutralizing Antibodies in Sera of Patients with Infectious Hepatitis and Other Conditions.

| Clinical diagnosis | Early sera* No. positive‡ /No. tested | Late sera† No. positive /No. tested | No. with 4-fold rise |
|---|---|---|---|
| IH-I | 1/16 | 2/12 | 0 |
| IH-II | 0/8 | 0/24 | 0 |
| IH-III | 0/4 | 9/128 | 0 |
| IH-IV | 0/3 | 0/3 | 0 |
| Leptospirosis | | 0/5 | |
| Leprosy | | 0/12 | |

* Early sera = Specimen taken before or up to 2 wk after onset of jaundice.

† Late sera = Specimen taken more than 2 wk after onset of jaundice.

‡ Positive = Neutralization at 1:10 or greater serum dilution.

** The 4 individual plasma sources were from donors designated L.H., V.S., H.H. and C.D., respectively, in Table 4 of reference(10). The thrombin referred to was listed as lot 2 in Table 2 in reference (11).

reduction neutralization test employed.

The plasma aliquot from which the A-1 virus was presumably recovered had been heated for 1 hour at 60°C, yet the tissue culture-adapted isolate is inactivated by similar treatment even when suspended in undiluted normal human plasma. The significance of this observation is undetermined.

*Summary.* 1. A virus has been recovered in tissue cultures inoculated with the 1951 NIH icterogenic plasma pool. 2. This agent produces cytopathogenic effects in a wide variety of cell cultures, but induces no overt disease in common laboratory animals. It produces inapparent infection in embryonated eggs. 3. Serologic studies have failed to show any relation between the A-1 virus and a number of recognized viruses. 4. Heat inactivated sera from normal human beings and animals did not neutralize the virus. 5. Volunteers infected with serum hepatitis virus from 6 different sources have, with a high degree of consistency, developed antibodies against the A-1 agent. 6. Sera from patients with infectious hepatitis have not neutralized the virus with comparable regularity.

The technical assistance of Alfred Segee is gratefully acknowledged.

1. Symposium on the laboratory propagation and detection of the agent of hepatitis. Washington, D.C.: Nat. Acad. Sci., Nat. Research Council, 1954, Publication 322, pp. 1-115.

2. Rightsel, W. A., Keltsch, R. A., Tekushan, F. M., McLean, I. W., *Science,* 1956, v124, 226.

3. Murray, R., Oliphant, J. W., Tripp, J. T., Hampil, B., Ratner, F., Diefenbach, W. C. L., Geller, H., *J.A.M.A.,* 1955, v157, 8.

4. Melnick, J. L., Chap. IV in *Diagnostic Procedures for Virus and Rickettsial Diseases,* Am. Public Health Assn., N. Y., 1956, 116.

5. Enders, J. F., Peebles, T. C., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 277.

6. Barile, M. F., Yaguchi, R., Eveland, W. C., *Am. J. Clin. Path.,* 1958, v30, 171.

7. Havens, W. P., Jr., *New England J. Med.,* 1958, v259, 1202.

8. Theiler, M., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 380.

9. Hiatt, C. W., *Trans. N. Y. Acad. Sci.,* 1960, v23, 66.

10. Murray, R., Diefenbach, W. C. L., Ratner, F., Leone, N. C., Oliphant, J. W., *J.A.M.A.,* 1954, v154, 1072.

11. Porter, J. E., Shapiro, M., Maltby, G. L., Drake, M. E., Barondess, J. A., Bashe, W. J., Stokes, J., Jr., Oliphant, J. W., Diefenbach, W. C. L., Murray, R., Leone, N. C., *J.A.M.A.,* 1953, v153, 17.

12. Ward, R., Krugman, S., Giles, J., Jacobs, M. A., *Hepatitis Frontiers,* edited by Hartman, F. W., *et al.,* Little, Brown and Co., Boston, 1957, 227.

13. Hughes, T. P., *J. Immunol.,* 1933, v25, 275.

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## Inactivation of Vacuolating Virus ($SV_{40}$) by Formaldehyde. (26892)

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Sweet and Hilleman(1) recently reported discovery of a new virus of rhesus and cynomolgus monkey origin. Unlike other simian viruses this agent fails to cause detectable cytopathogenic changes in cell cultures of rhesus and cynomolgus monkey kidneys where it multiplies to relatively high titers. In kidney cell cultures derived from the African Green monkey, *Cercopithecus aethiops,* however, the virus causes cytopathic effects characterized by cytoplasmic vacuolations which coalesce with eventual destruction of the cells. For this reason the virus has been named vacuolating virus and has been designated $SV_{40}$ by Hull.

The present report describes studies on formaldehyde inactivation of $SV_{40}$ and its detection in some lots of poliomyelitis and adenovirus vaccines.

*Materials and methods. Tissue cultures.* Kidneys obtained from African Green monkeys were trypsinized and grown at 37°C in