Attempts to Detect Hepatitis B Virus by Negative Hemadsorption Assay (37329)

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The etiologic agent of viral hepatitis type B (long incubation hepatitis, serum hepatitis) has repeatedly eluded detection by conventional virologic means. In 1969 Marcus and Carver (1) described a negative hemadsorption plaque assay for detecting rubella virus grown under noncytopathic conditions. Rubella-infected cells interfered with the replication of Newcastle disease virus (NDV), resulting in the appearance of hemadsorption-negative areas throughout the culture. This phenomenon, termed intrinsic interference, was not mediated by interferon, was brought about by the action of virus-induced proteins and has been observed with other noncytopathic viruses (1). Using this tissue culture technique, Carver and Seto (2) reported that intrinsic interference developed in WI-38 cells, a human diploid cell strain, following inoculation with a human serum (Newhall) containing hepatitis type B antigen (HB Ag). The interference factor could be neutralized by human antiserum to HB Ag and was successfully passed to a second culture at high dilution. It is recognized that there is a close association between HB antigen and type B viral hepatitis.

We cooperatively report here our findings that the apparent interference with hemadsorption production by NDV does not correlate well with the presence of HB Ag in human sera, does not occur uniformly with human or chimpanzee specimens known to contain the virus of hepatitis B, and when seen is poorly reproducible in successive tests of the same specimens.

Materials and Methods. Baylor specimens for inoculation included human sera and plasma contributed by donors or patients from the Ben Taub General Hospital and from regional blood banks. In addition, Newhall serum was kindly provided for testing by Dr. D. H. Carver. HB Ag was initially detected by discontinuous counterimmunoelectrophoresis (DCIE) (3) or complement fixation (4) and confirmed by a sensitive and specific double-antibody radioimmunoassay (RIA-DA) method (5).

Phoenix Laboratories examined seven blood specimens, supplied as follows: Newhall serum (Dr. D. H. Carver, Baltimore); Newell (MS-2) serum (Dr. S. Krugman, Willowbrook State School); NIH-6 human and NIH-197 chimpanzee plasma (Dr. L. F. Barker, Division of Biologics, FDA); two acute illness phase human sera containing HB Ag; and a normal human serum control. The Newell (MS-2) serum and the NIH-6 human plasma pool are known to contain the virus of hepatitis B through the induction of long incubation hepatitis in human volunteers (6, 7). The NIH chimpanzee plasma was derived from animals successfully infected with the original NIH human plasma pool and has been proven infective through further successful chimpanzee subpassages (8). HB Ag was initially detected in these specimens by complement fixation. Specimens in both laboratories were stored at 4° or —20° until used.

WI-38 cells utilized at Phoenix were obtained from Flow Laboratories at passage level 16. These cells were repassaged in screw-capped tubes and utilized for testing at no higher than the 20th passage level. At Bay-

lor, WI-38 cells grown in screw-capped tubes were obtained from the same source at passage levels between 20 and 22. In addition, human embryonic lung fibroblasts (HEL—Baylor) were prepared and utilized at passage levels 12 through 15 in Linbro multidish trays (FB-16-24-TC, Linbro Chemical Co., New Haven). Cells at Baylor were maintained in Eagle's minimal essential medium (MEM) containing 3% fetal bovine serum or calf serum, penicillin and streptomycin (100 units and 100 μg/ml, respectively), and sodium bicarbonate (2.25 g/liter). Cells were grown at 37° and maintained at 35° in a humidified atmosphere of 5% CO_2–95% air. Cells in Phoenix were maintained in a similar fashion with the exception that Hepes buffer[1] (*N*-2-hydroxyethylpiperazine-N^1-2-ethane sulfonic acid) was used without CO_2 incubation. The California strain of NDV used by both groups was prepared from the allantoic fluid of infected 9–10-day-old chick embryos.

Inoculation of specimens and performance of the assay were similar to methodology described by Carver and Seto (2). Initially, 0.2 ml of serum diluted 1:10 or greater was added to cells pretreated with 25 μg/ml DEAE-dextran and incubated at 37° on a roller apparatus. Later, DEAE-dextran and serum were mixed and applied to the cells simultaneously (Carver, personal communication). After adsorption for 1 hr, the cells were fed with 1.5 ml of Eagle's MEM and further incubated at 35°. The medium was changed at 3-day intervals.

Representative inoculated and control cultures were challenged at 1-day intervals with NDV from Days 6 through 14 at Baylor and from Days 6 through 15 at Phoenix. The medium was removed and 0.2 ml of NDV containing 25 μg/ml of DEAE-dextran was added at a concentration 2- to 4-fold greater than that required to give confluent hemadsorption on WI-38 monolayers (multiplicity of infection = 10–40 plaque-forming units/cell). Cultures were placed on a roller apparatus for 1 hr at 37°. The monolayers were then washed twice with phosphate buffered saline (PBS), pH 7.2. Residual NDV was neutralized with 0.2 ml of rabbit-anti-NDV at a dilution sufficient to neutralize ≧98% of the input virus. Finally, the cells were washed three times with PBS, refed with medium and incubated for 15–18 hr at 37°. The medium was removed and 1.5 ml of washed guinea pig red blood cells (1%, v/v) were added to each tube. Following a period of red blood cell adsorption for 30 min at 4°, cells were washed twice with cold PBS and examined under low power magnification. About 100 tube cultures were used to test a single serum to allow for proper sampling and controls.

Results and Discussion. At Baylor, 23 individual sera containing HB Ag were evaluated. Seventeen specimens revealed some areas of nonhemadsorbing cells at some stage during the incubation period in HEL or WI-38. Of the six HB Ag containing specimens evaluated at Phoenix, two showed patterns of nonhemadsorption in WI-38. Basically three types of negative hemadsorption were seen by both laboratories as demonstrated in Fig. 1. Most frequently observed were small areas of 20–30 cells having discrete margins surrounded by apparently healthy cells (Fig. 1B). These plaques were scattered randomly throughout the monolayer, ranged in number from 0 to 5 per tube, and were observed in cultures inoculated with HB Ag containing serum and in those inoculated with diluent alone. The second pattern was somewhat larger, up to 1 mm^2, with irregular margins and appeared to follow the growth pattern of the cells in the monolayer (Fig. 1C). The morphological appearance of the cells was usually normal, although occasionally they became granular and contracted, leaving holes in the cell sheet. This pattern of clearing was seen most often in cultures inoculated with HB Ag positive serum, but was sometimes observed in the HB Ag negative serum and diluent controls. The third type of negative hemadsorption demonstrable in the monolayers was a diffuse swirling or streaking pattern which closely paralleled the growth formation of the cells (Fig. 1D). This configuration may have resulted from a metabolic abnormality or a nutritional deficiency of the

[1] Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or the U.S. Department of Health, Education, and Welfare.

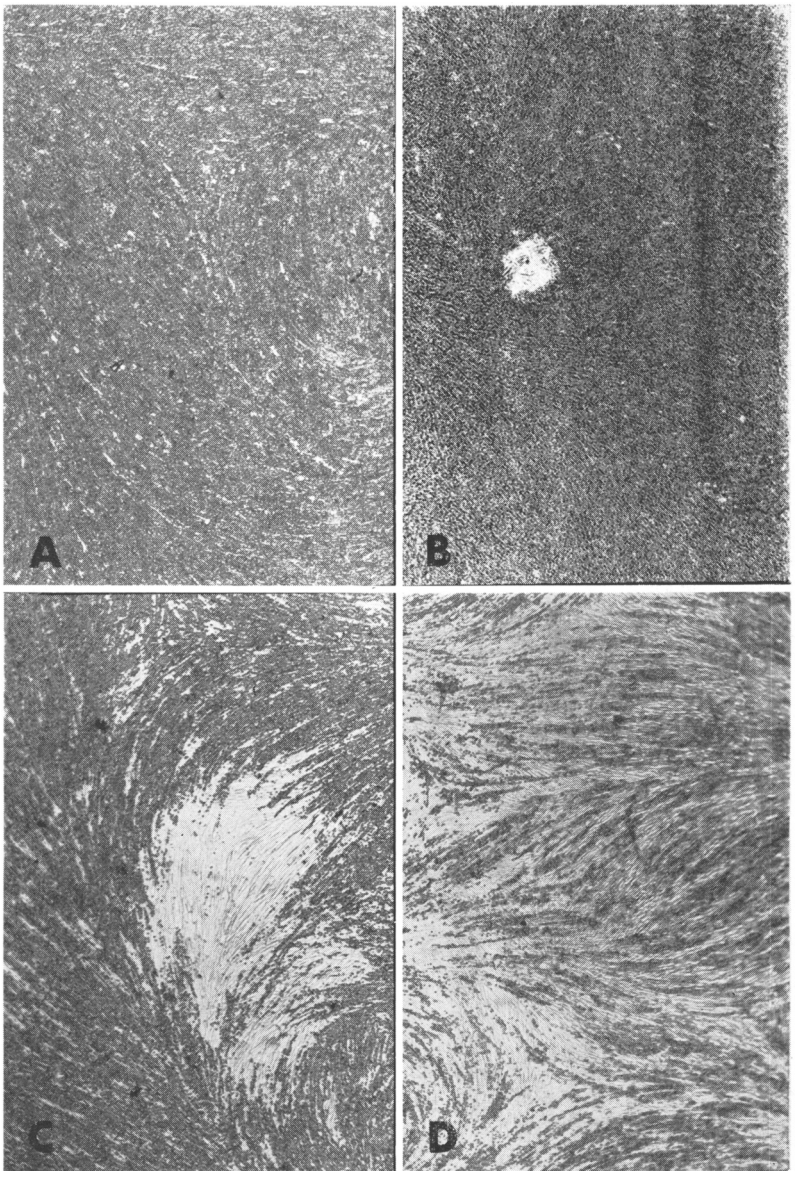

FIG. 1. Hemadsorption interference in WI-38 cells. (A) Diluent control—100% hemadsorption; (B) small "plaque" with discrete margin; (C) large area with irregular margins; (D) diffuse streaky interference.

cells, since it was invariably seen in older cultures or in cultures fed at intervals exceeding 3–4 days.

Disconcerting and undesirable features of this assay system included nonreproducible results which occurred when retesting the same sample, difficulties in determining when a specimen was positive, the large number of operations to contend with in the assay itself and the inexplicable "positive" results observed in presumably normal human sera as well as in cell control tubes inoculated with diluent only. Sera were regarded as positive if any dilution showed areas of negative hemadsorption distinctly differing from that observed in the HB Ag negative serum or diluent controls on one or more days. Of the 17 HB Ag positive specimens tested at Baylor, 9

were cultured once, 4 were cultured twice, and 4 were tested three times. Reproducible results were observed in only three of the eight specimens retested one or more times. Frequently, nonhemadsorbing areas would first appear in the higher dilution tubes, then at the lower dilutions, and might or might not occur in the intermediate dilutions. In addition, sera which were repeatedly tested often differed significantly in the time that interference appeared following initial infection and in the dilution at which it was seen. Thus, a specimen which was positive at a 10^{-1} dilution on Days 7, 8, and 9 in one test might show interference at dilutions 10^{-1}, 10^{-2} and/or 10^{-3} on Day 7 only upon retesting. Furthermore, the hemadsorption patterns were frequently dissimilar between duplicate tubes. At Phoenix, the initially positive results with Newhall could not be reproduced upon retesting.

We have included as controls in these experiments four human sera which were negative for HB Ag and HB Ab by a sensitive RIA-DA (5). In three out of four of these sera, areas of hemadsorption interference were seen at least once during multiple tests. In addition, the same inexplicable dilutional and temporal discrepancies noted previously with HB Ag positive sera occurred.

At Baylor, five specimens were tested simultaneously under code to eliminate observer bias. The inocula included the Newhall serum, an acute phase serum from a patient with HB Ag positive hepatitis, an acute phase serum from a volunteer who received known infectious hepatitis type A (MS-1) material, and two control sera negative for HB Ag by RIA. The results of this test failed to distinguish between specimens containing HB Ag and negative controls. While the Newhall serum was found to be positive on several consecutive days, one of the control sera was also found to be positive. The other specimens gave equivocal results. Both laboratories participated in another experiment utilizing WI-38 cells, NDV, and Newhall serum, all kindly supplied by Dr. Carver. Although some hemadsorption interference was again observed with the Newhall serum, the results failed to correspond in time or by dilution with those obtained in Dr. Carver's laboratory using the same serum and lot of WI-38 cells, or with results obtained from previous tests with this specimen.

Our inability to consistently distinguish between normal human serum and infectious HB Ag positive serum could indicate that unknown toxic substances or extraneous viral agent(s), completely unrelated to viral hepatitis type B, may be present in human serum. This is supported by the negative or inconsistent results found when testing specimens known or suspected to contain the hepatitis B virus. Conversely, the results obtained could be due to intrinsic factors such as the physiological state of the cells. In this regard we have observed that WI-38 cells remain viable and readily transferable for up to 7 days in the medium employed; however, the maintenance of a 100% hemadsorption baseline requires that the cells be fed at least every 3 days. Thus, the apparently delicate nature of maximum hemadsorption production may give false positive results similar to the pattern seen in Fig. 1D. In fact, we found that some lots of WI-38 and HEL cells were incapable of maintaining a 100% hemadsorption baseline, regardless of how often they were fed. Small areas of nonhemadsorption (Fig. 1B) were found randomly scattered throughout the monolayers and closely resembled clones of NDV-resistant cells. Although we were aware of this particular plaque type, it was not always possible to distinguish it from other patterns described.

Summary. Our results indicate that random areas of hemadsorption interference can be demonstrated in cultures inoculated with normal human serum and diluent alone, similar to that observed with HB Ag positive sera. We have concluded that the negative hemadsorption test is not specific for the agent of type B viral hepatitis.

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1. Marcus, P. I., and Carver, D. H., *in* "Fundamental Techniques in Virology" (K. Habel, and N. P. Salzman, eds.), p. 161. Academic Press, New York (1969).

2. Carver, D. H., and Seto, D. S. Y., Science **172**, 1265 (1971).

3. Wallis, C., and Melnick, J. L., Appl. Microbiol.

21, 867 (1971).

4. Melnick, J. L., *in* "Analytical Serology of Microorganisms" (J. B. G. Kwapinski, ed.), p. 411. Wiley, New York (1969).

5. Hollinger, F. B., Vorndam, V., and Dreesman, G. R., J. Immunol. **107**, 1099 (1971).

6. Krugman, S., Giles, J. P., and Hammond, J., J. Amer. Med. Ass. **200**, 365 (1967).

7. Murray, R., Bull. N. Y. Acad. Med. **31**, 341 (1955).

8. Maynard, J. E., Berquist, K. R., and Krushak, D. H., *in* "Hepatitis and Blood Transfusion" (G. N. Vyas, H. A. Perkins, and R. S. Schmid, eds.), p. 393. Grune and Stratton, New York (1972).

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