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8. Supported by grants from the National Institutes of Health (NS 8839 and RR 5632), the California Department of Mental Hygiene (RP 14-54), and the National Association for Retarded Children. We thank Drs. Aubrey Milunsky and Hugo W. Moser for supplying MLD cultures, and Mr. Joe Trammell and Miss Marilyn Scott for their technical assistance. The opinions or conclusions stated in this report are those of the authors and are not to be construed as official or as necessarily reflecting the policy of the California Department of Mental Hygiene.

11 January 1971

Production of Hemadsorption-Negative Areas by Serums Containing Australia Antigen

Abstract. *Exposure of human Wi-38 cells to human serums containing Australia antigen, and presumably serum hepatitis virus, renders the cells refractory to infection by Newcastle disease virus as detected by the hemadsorption-negative plaque test for intrinsic interference. Induction of the Newcastle disease virus refractory state could be passed in cell culture with up to a 1 : 100,000 dilution of material obtained from cells "infected" with serums containing Australia antigen after filtration (0.45-μm pores) and heating to 60°C for 1 hour. Human antiserums to the Australia antigen prevented induction of the Newcastle disease virus refractory state.*

Australia antigen (Au) has been found specifically in the serums of patients with serum hepatitis (*1*), and serums containing this antigen have been found to transmit serum hepatitis (*2*). It appears that Au [and the SH antigen which is probably identical with it (*3*)] is either a component of the serum hepatitis virus or intimately associated with this virus. Serums containing this antigen were used in our attempt to develop a tissue culture assay system for serum hepatitis virus. Since cytopathic changes caused by the virus have not been described, a noncytopathic assay system was sought and is described herein.

Inhibition of the replication of Newcastle disease virus (NDV) has been described as an assay system for rubella virus growing under noncytopathic conditions (*4*). Bovine erythrocytes are absorbed by tissue culture cells in which NDV replicates, whereas the rubella-infected cells are refractory to NDV superinfection and therefore do not absorb the erythrocytes. This noninterferon-mediated interference against superinfection with NDV has been called intrinsic interference (*5*). Subsequently, Sindbis and West Nile viruses, poliovirus (*5*), lymphocytic choriomeningitis virus (*6*), infectious bronchitis virus (*7*), cytomegalovirus (*8*), and reovirus (*9*) have also been shown to induce intrinsic interference when growing under noncytopathic conditions. We have sought interference with the development of NDV hemadsorption as an assay system for serum hepatitis virus.

When Au was found in the plasma contributed by a 42-year-old blood donor to the Children's Hospital Medical Center in Boston, Dr. Sherwin Kevy arranged for the donor to be bled again and we were sent 200 ml of Au-positive serum. This donor developed a severe case of hepatitis 4 weeks after the collection of blood. His serum (Newhall specimen) was used as a virus source while optimal conditions for the assay system were developed.

At present, the test is done as a modification of that previously described (*10*). Wi-38 cells in screw-top tubes are treated with phosphate-buffered saline (PBS) containing 25 μg of diethylaminoethyl (DEAE) dextran per milliliter. The tubes are kept at 37°C for 1 hour while on a roller wheel. After aspiration of the dextran, 0.3 ml of specimen fluid (containing Au and presumably containing serum hepatitis virus) is placed in each test tube and the tubes are kept at 37°C on a roller wheel for another hour. Each tube then receives 1.5 ml of minimal essential medium (MEM) with 3 percent calf serum and the tubes are incubated at the optimal temperature of 35°C. All tubes are aspirated after 24 hours and receive fresh medium. The cells remain at 35°C with medium changed every 4 days until the time of challenge with NDV. Medium is removed from the cells and NDV (California) is added at a multiplicity of 10 plaque-forming units (assayed on chick embryo fibroblasts) per cell in 0.3 ml of medium containing 25 μg of DEAE dextran per milliliter. The tubes are placed at 37°C for 1 hour on a roller wheel, and the unattached NDV is removed by aspiration. The monolayers are rinsed once with 3 ml of PBS. To preclude spurious binding of red cells due to the presence of residual input NDV, the rinsed monolayer is exposed to 0.3 ml of NDV antiserum for 30 minutes at 37°C. Antiserum is aspirated and the monolayers are washed twice with 3 ml of PBS, flooded with 1.5 ml of MEM + 3 percent calf serum, and incubated at 37°C for 15 hours. The medium is removed and replaced with 3 ml of a suspension of washed bovine erythrocytes in cold PBS at a concentration of 6×10^7 cells per milliliter. Red cell adsorption is carried out for 30 minutes at 4°C. The tubes are then gently rinsed with cold PBS to remove unattached erythrocytes and 1.5 ml of cold PBS is added to each tube after the last rinsing. The monolayers are then examined under the microscope.

Serial tenfold dilutions of the specimen were each inoculated into replicate tubes for each experiment. One tube for each dilution was then challenged with NDV each day. Experiment with the Newhall specimen revealed that areas of nonhemadsorbing cells first appeared on the 8th to 12th days after infection. These areas were surrounded by hemadsorption positive cells. The hemadsorption negative areas were first seen in tubes inoculated with higher dilutions of the specimen (at a 100,000-fold dilution) and 2 to 3 days later were seen in tubes inoculated with more concentrated specimens. The effect was produced with serum diluted as great as 10,000,000-fold. The hemadsorption negative cells were normal in appearance in two-thirds of the experiments and appeared nonviable in one-third of the experiments. In all cases control cells which had not been inoculated with hepatitis specimens but were otherwise treated in the same manner as the infected cells showed complete hemadsorption of red blood cells following challenge with NDV. Monolayers infected with a given dilution of specimen have hemadsorption negative areas after three to five consecutive daily challenges with NDV, and then subsequent challenges of further monolayers in-

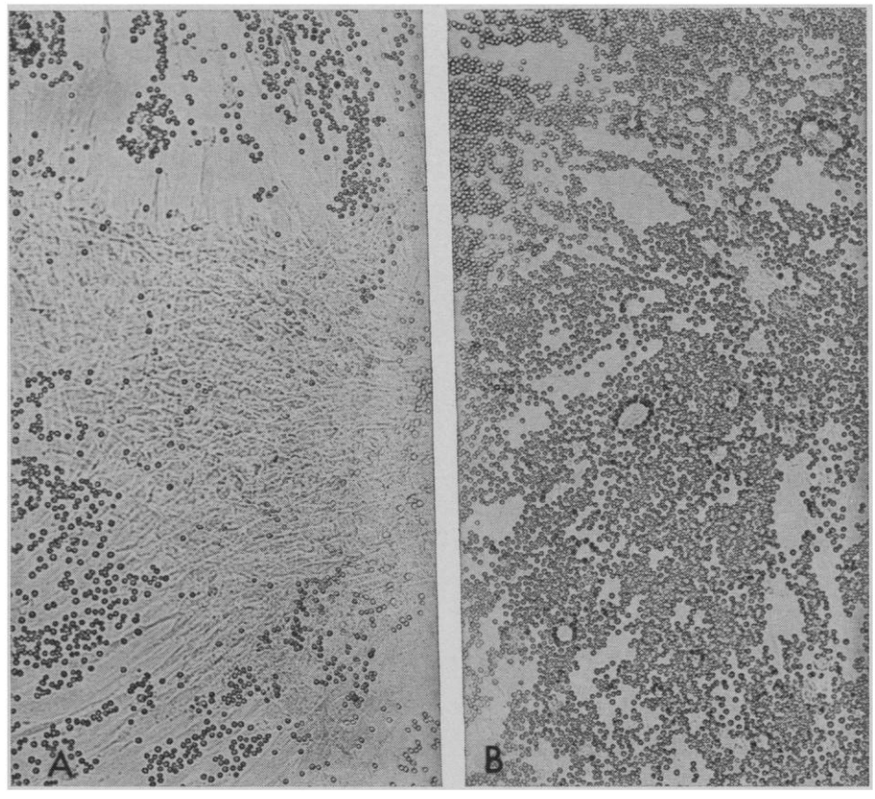

Fig. 1. Hemadsorption following NDV challenge of human placental fibroblasts. (A) Previously infected with a 10,000-fold dilution of prototype Newhall serum containing Australia antigen. (B) Cell control (× 140).

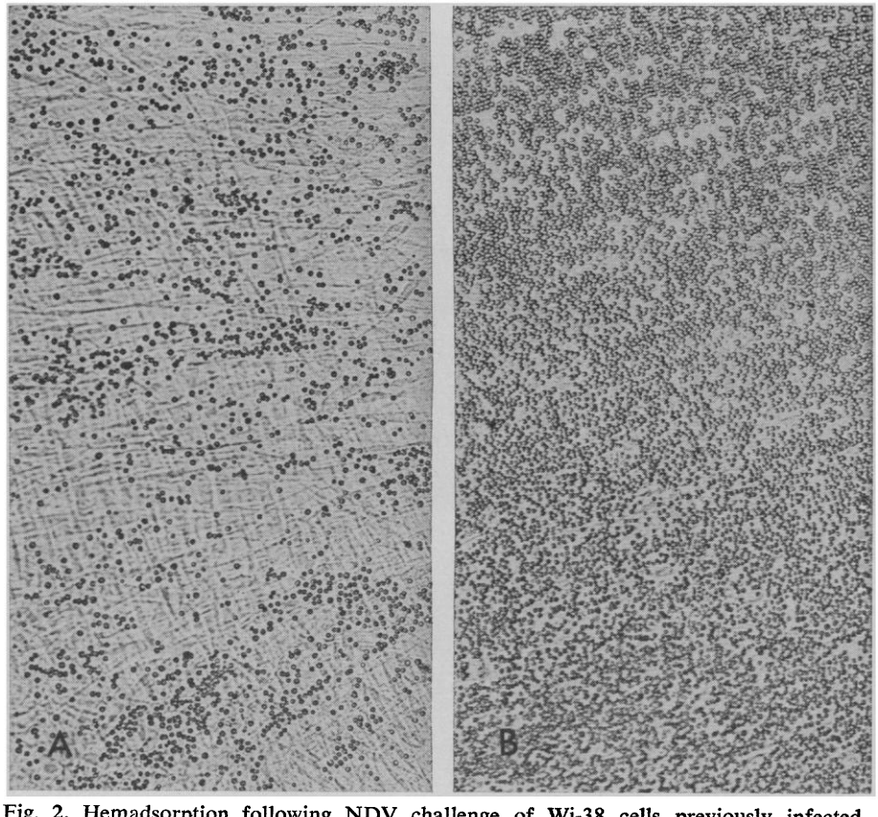

Fig. 2. Hemadsorption following NDV challenge of Wi-38 cells previously infected with (A) passage material diluted 10,000-fold and (B) passage material diluted 10,000-fold treated with serum containing antibody to Australia antigen (× 140).

fected with the same dilution reveal only hemadsorption positive cells.

The Newhall specimen produced hemadsorption negative cells in ten different experiments with Wi-38 cells. In each experiment multiple dilutions produced the effect repeatedly for at least three consecutive days. Heating of the Newhall serum at 60°C for 1 hour did not reduce the titer of specimen which produced the hemadsorption negative cells. The Newhall specimen also produced the effect in four different experiments utilizing a line of human placental fibroblast cells (Fig. 1) obtained from Dr. John Enders (*11*). A second specimen of Au positive blood obtained from Dr. Alfred Prince of the New York Blood Center produced the effect on Wi-38 cells in seven separate experiments.

Attempts were made to pass this effect from Wi-38 cells infected with the prototype serum to other Wi-38 cells. At the time that similarly inoculated tubes exhibited hemadsorption negative areas a tube of cells inoculated with a 10,000-fold dilution of the initial serum was frozen and thawed three times with the medium retained in the tube. The medium was passed through a Millipore filter with a pore size of 0.45 μm and heated at 60°C for 1 hour. A tissue culture control tube was similarly treated. Serial tenfold dilutions of each fluid were inoculated onto new Wi-38 cells in the manner described above. By the 5th day after initial infection extensive hemadsorption negative areas were seen in tubes inoculated with dilutions of the test material as great as 100,000-fold and not with greater dilutions. The effect was most marked with 10,000-fold and 100,000-fold dilutions. This effect persisted with the NDV challenges that occurred on the 6th, 7th, and 8th days after infection. The effect did not occur in tubes inoculated with the passage tissue culture control material.

The passage material from the tube of cells infected with the prototype serum described above was prepared in 10,000- and 100,000-fold dilutions. These dilutions were mixed in equal amounts with 1 : 4 and 1 : 8 dilutions of each of two human serums used by Dr. Sherwin Kevy as standard antiserums to the Au antigen in the agar diffusion technique. The mixtures were heated at 37°C for 1 hour with agitation and then inoculated onto Wi-38 cells. Virus controls consisted of the same dilutions of passage material

which were mixed with equal parts of medium and kept at 37°C for 1 hour with agitation. Hemadsorption negative areas were seen on the tubes inoculated with virus control material after challenge on the 5th through the 8th days after infection. They were not seen in tubes inoculated with material treated with either 1 : 4 or 1 : 8 dilutions of one of the serums or in those inoculated with material treated with a 1 : 4 dilution of the second serum after any of the four NDV challenges (Fig. 2). Tubes inoculated with material treated with a 1 : 8 dilution of the second serum had hemadsorption negative areas which were definite but smaller than those in the virus control tubes following all four challenges.

Serum specimens from 17 blood donors whose serums were negative for Au and whose blood did not produce icteric hepatitis in recipients were obtained from Dr. Sherwin Kevy of the Children's Hospital Medical Center in Boston. These serums were diluted 10,000-fold and used to infect Wi-38 cells. In the same experiments 10,000-fold dilutions of the prototype Newhall serum and two other Au positive serums (Perkins and Matheson) obtained from Dr. Kevy were also used to infect Wi-38 cells. The Perkins serum was obtained from a donor whose blood was taken at another institution where donor blood was not tested for Au. The recipient of this blood developed hepatitis. This donor's serum glutamic oxalacetic transaminase level was over 300 at the time of the blood donation. The Matheson serum was contributed by a technician who developed serum hepatitis after processing Au positive serum. All tubes were challenged daily with NDV starting with the 10th day after infection.

In the first experiment tubes infected with Matheson serum exhibited extensive hemadsorption negative areas after NDV challenge on three successive days. Following the NDV challenges an equivocal amount of hemadsorption negative areas occurred on a single day in tubes infected with five other specimens (including the Perkins serum). Cells infected with the other serums in the experiment demonstrated the same extent of hemadsorption as the control tubes.

In the second experiment cells infected with the prototype Newhall serum had extensive hemadsorption negative areas after two challenges, while cells infected with the Matheson serum had equivocal hemadsorption negative areas on two occasions and cells infected with Perkins serum had equivocal hemadsorption areas on one occasion. None of the cells infected with control serums developed hemadsorption negative areas after any of the NDV challenges in this experiment.

In the third experiment cells infected with Matheson serum had extensive hemadsorption negative areas (involving over 50 percent of the cell sheet) after three challenges with NDV, and cells infected with the Perkins serum had hemadsorption negative areas after two challenges (involving 30 percent of the cell sheet). On one occasion cells infected with the prototype Newhall serum had many plaques of hemadsorption negative cells which appeared nonviable. After one challenge cells infected with serums from one of the "normal" controls had hemadsorption negative areas in about 30 percent of the sheet. Five other control serums had produced equivocal changes following one of the challenges.

Cells treated with Au positive serums repeatedly exhibited hemadsorption negative areas after daily challenges with NDV and thus contrasted with both cell controls and cells treated with control serums. The equivocal findings on single occasions for the control serums might be due either to artifact or possibly the presence of other viral agents. The findings that dilute specimens produced the effect more rapidly than concentrated specimens may be due to the presence in the initial specimen of either a specific inhibitor (such as antibody) or a nonspecific inhibitor.

Although the NDV challenge system was selected as a means of demonstrating intrinsic interference in cells treated with Au-containing serums, another mechanism may account for the cells being refractory to superinfection with NDV. The nonviable hemadsorption negative cells seen on the occasions noted above may be due to a different interaction between the serums hepatitis virus and NDV.

Hemadsorption negative areas were seen after NDV challenges for three to five successive days. Further NDV challenges of cells treated with test serums resulted in normal hemagglutinin formation. At present we cannot account for this. Our report indicating that human cytomegalovirus produces intrinsic interference in Wi-38 cells noted that this interference was lost at the time that cytopathic changes occurred (*8*). In the current system cytopathic changes are not seen at the time hemadsorption negative areas are no longer noted nor at any other time.

Recent publications described two different Au-containing particles (*12*). A large spherical particle (about 43 nm) and smaller (about 20 nm) rod- and sphere-shaped particles have been seen. The large particles which have a core may be the actual virus while the smaller particle may be excess capsid. Further work testing the different particles in the tissue culture system described is needed.

The findings indicate that treatment of cells either with serums containing the Australia antigen or passage fluid heated at 60°C for 1 hour interferes with subsequent hemadsorption by cells challenged with NDV and that this effect is prevented by serum containing antibody to the Au antigen. Nevertheless, it must be emphasized that additional experiments are needed before a specific role is delineated for serum hepatitis virus in this phenomenon.

Additional experiments are also needed to extend our preliminary work indicating that infectious hepatitis virus probably interferes with hemadsorption by NDV-challenged cells.

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13. Supported by PHS research grant CC 00499 from the Center for Disease Control, Atlanta, Georgia, and also grants AI 08225, FR 5378, and HD 00091 from NIH. We thank Jean Simkins for excellent technical assistance. D.H.C. is a Kennedy scholar.

12 February 1971