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Separation of Hemagglutinating and Non-Hemagglutinating Variants of Coxsackie A-21 Virus. (27607)

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A previous communication(1) describes certain properties of a hemagglutinin found in strains of Coxsackie A-21 virus. Many Coxsackie A-21 virus strains recovered in North Carolina in 1960, as well as several isolated in England in 1959, were capable of agglutinating human erythrocytes at 4°C. In contrast the Coe strain originally described by Lennette *et al.*(2), failed to exhibit hemagglutination under these conditions. It was noted that all hemagglutinating (HA+) strains had been tested after not more than 3 tissue culture passages, whereas the non-hemagglutinating (HA–) strain had been passed 10 times in continuous type malignant cells of human origin prior to study. It seemed possible that passage of a virus strain in malignant cells had led to loss of the hemagglutinating capacity. This report provides evidence in support of this hypothesis.

Materials and methods. Tissue culture. Four types of cell cultures were employed. Primary human embryonic kidney (HEK), human epidermal carcinoma of the larynx (HEp-2), and human carcinoma of the nasopharynx (KB) were grown as previously described(3). These tissues were obtained from Microbiological Associates. Semi-continuous diploid cells derived from human embryonic lung (WI-26) were obtained from Dr. L. Hayflick, and were grown in culture according to the method of Hayflick and Moorhead(4). All cultures were maintained in Eagle's Basal Medium to which was added 0.3 mg glutamine, 100 μg streptomycin, and 100 units of penicillin per ml. In certain in-

stances either 2% or 5% heat inactivated (56°C for 30 minutes) chicken serum was included in the medium.

Virus. The virus used was a representative throat swab isolate (#48560-Bouma) obtained from a Marine recruit with symptoms of an acute upper respiratory infection. This strain was found to be serologically indistinguishable from the Coe strain. Tissue culture passages were made in 2 or 4 tube cultures. When cytopathic effects were complete, cultures were frozen and thawed, pooled and stored at −20°C until assayed. Virus was reidentified by neutralization or hemagglutination-inhibition tests after completion of each series of experiments. Antiserum used was prepared in guinea pigs using the Coe virus strain. Hemagglutination tests were performed with washed human type O red cells by the method previously described by Rosen (5). Hemagglutinin titers of tissue culture harvests comprising a comparative series were determined in a single test. For studies on the effect of pH on hemagglutination, 0.01 M 5-5 diethylbarbituric acid in 0.14 M NaCl was used as diluent for both erythrocytes and virus. The pH of this buffer was preadjusted at 0.5 pH unit intervals between pH 5.5 and 8.5 with IN KOH. Infectivity titrations were done in primary human embryonic kidney cultures employing serial 10-fold dilutions. Two or 4 tubes at each dilution were inoculated with 0.2 ml of material, and presence or absence of cytopathic effects was noted over a 7-day period. End points were calculated according to the method of Reed and Muench(6).

Results. Loss of hemagglutinin after serial passage in malignant cell culture. Coxsackie A-21 virus was isolated from a throat specimen (#48560) in HEK, HEp-2, and KB cultures. Each of these isolates was passed 8 times without dilution in the homologous type of tissue culture. Human embryonic tissue cultures were maintained without serum, whereas the malignant cultures contained 5% chicken serum. It was noted (Fig. 1) that hemagglutination titers at each HEK passage level remained essentially unchanged. Virus grown in both HEp-2 and KB tissues showed a definite and progressive loss of hemagglutinating activity. The KB_8 virus (now HA–) was passed 6 times without dilution in HEK cultures and retested for hemagglutinin at each passage. The virus remained HA– at all passage levels. Hemagglutinin-positive and hemagglutinin-negative tissue culture harvests were assayed for infectivity. In all cases at least $10^{6.5}$ $TCID_{50}$/ml of virus was present. The possible effect of serum on he-

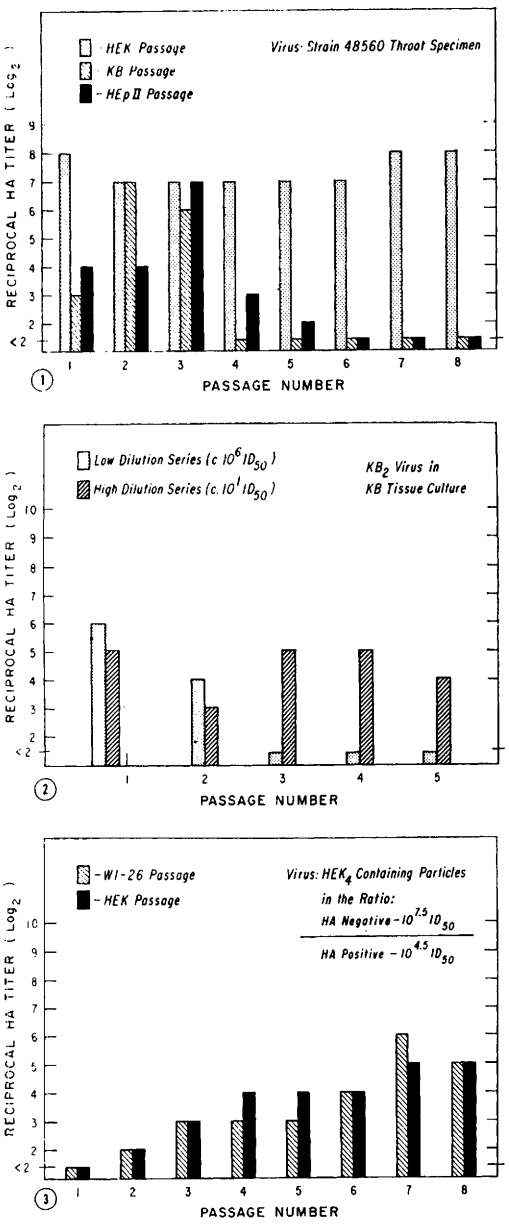

FIG. 1-3

magglutination was evaluated by maintaining KB cultures without added serum and HEK cells in 5% chicken serum. The initial experiment was then repeated. Patterns of hemagglutination were not different from those obtained originally, indicating that serum was not responsible for the loss of hemagglutinin. Since it was possible to maintain cultures for longer periods using serum, subsequent work was carried out with Eagle's medium supplemented with chicken serum.

Demonstration of distinct HA+ and HA– virus particles. To determine whether virus grown in continuous cell lines was being altered in a non-specific fashion or whether an HA– mutant was being selected, passages were made in KB cultures at low and high dilution. Second KB passage virus (HA+) was serially passed at concentrations of approximately 10^6 and 10^1 $TCID_{50}$ respectively. As noted previously, passage of large quantities of virus resulted in rapid loss of the hemagglutinin. By passage of very dilute virus, however, the hemagglutinating property was preserved (Fig. 2). These results suggested that 2 types of particles were present in the KB_2 pool; an HA+ particle and an HA– particle. Malignant cell cultures apparently favored the multiplication of the latter particle.

An attempt was made to separate HA+ and HA– particles by adsorption onto erythrocytes. It was assumed that if separate particles existed, the HA+ virus would adhere to the red cells while the HA– virus would be recovered in the supernatant fluid. One ml of washed, packed, human type O red cells was added to 0.6 ml of undiluted HEK_2 virus. This mixture was incubated for 30 minutes at 4°C and centrifuged at 2000 rpm for 10 minutes at 4°C. The supernatant fluid was removed and the erythrocytes were washed 3 times with cold saline. Titration of the red cell fraction in HEK revealed 10^7 $TCID_{50}$/ml whereas 10^4 $TCID_{50}$/ml were present in the supernate. Fluids from cultures of the highest positive dilution in each case were assayed for hemagglutinin and infectivity; virus obtained from the erythrocytes was HA+ and that derived from the supernatant fluid was HA–. Both harvests contained at least 10^6 $TCID_{50}$/ml.

Attempts to separate both types of virus particle from Coxsackie A-21 positive throat specimens were unsuccessful. In 2 cases 10^2-10^3 $TCID_{50}$ of HA+ virus were obtained from erythrocyte fractions and $10^{0.5}$-10^1 $TCID_{50}$, also HA+, were found in the supernatant fluids.

The effect of pH on hemagglutination by both HA+ and HA– virus was studied. The HA– virus failed to agglutinate human erythrocytes when tested at 0.5 pH unit intervals from pH 5.5-8.5. Tests were run at both 4°C and 33°C. The HA+ virus which titered 1:64 when tested in 0.15 M NaCl at 4°C, also titered 1:64 between pH 5.5 and 7.5. At pH 8.0 complete agglutination occurred at 1:8 and partial agglutination was observed at 1:16. At pH 8.5 no definite hemagglutination could be ascertained at a dilution of 1:4. No agglutination occurred when tests were run at 33°C.

Selection of HA+ particles in non-malignant tissue cultures. Following separation of the 2 types of virus particles, a pool of HA– (HEK_4) was prepared. Adsorption of this pool with human erythrocytes revealed that it contained about 3×10^7 $TCID_{50}$ HA–, and 3×10^4 $TCID_{50}$/ml HA+. Passage of this material without dilution in both HEK and WI-26 cultures resulted in the appearance of detectable hemagglutinin (Fig. 3). Titers achieved, however, were not as high as those seen after passage of the original throat specimen. It may be that the growth advantage of HA+ over HA– particles in non-malignant cells was not as great as that of the HA– over HA+ particles in malignant cells.

Conclusions. The work described confirms the initial hypothesis that passage of Coxsackie A-21 virus in malignant human tissue results in progressive loss of the hemagglutinin. Similar observations have recently been reported for ECHO virus types 3, 11 and 19(7), suggesting the possibility that this may be a not uncommon phenomenon among human enteroviruses. Since hemagglutination provides a useful epidemiologic tool for study of members of this large virus family,

these results suggest the importance of avoiding the use of malignant human tissues in cultivating enterovirus isolates when a hemagglutinin is being sought.

The loss of hemagglutinin appears to depend upon the presence of 2 types of infectious virus particle, with selection of the HA– variant by cells of malignant origin. Human cells of primary origin favored multiplication of the HA+ particle. It was of interest that the semi-continuous human embryonic fibroblastic cells also selected HA+ particles. These cell strains have been shown to have many properties in common with primary cultures(4). Differential multiplication of Coxsackie A-21 variants, therefore, may provide another marker relating the diploid cell strains to "normal" rather than malignant human cells.

Although they described rapid loss of the hemagglutinin of ECHO 19 virus following passage in HeLa cells, Moscovici and La Placa(8) were unable to demonstrate 2 types of particles in either the initial virus pool (HA+), or the HeLa passaged material (HA–). These authors, however, did not attempt to separate variants from the early HeLa passages which showed definite but reduced HA titers as compared to the original pool. In cases where parent and derived strains appear to be "pure", such material might offer the best opportunity for initial recognition of distinct infectious particles.

The actual mechanism whereby malignant cells select HA-particles is unknown. More precise genetic studies, as well as investigation of various stages of virus replication in relation to possible differences in metabolism of primary and malignant cells, would be facilitated by the development of more sensitive methods for detecting progeny of individual HA+ and HA– particles. Attempts to develop a plaque assay suitable for study of both mutants are in progress.

With respect to the hemagglutination marker, the present findings are similar to those of Burnet and Bull(9) who described O-D variation by strains of influenza A virus. These authors found that virus obtained after very few pasages in embryonated eggs agglutinated human or guinea pig erythrocytes to higher titer than fowl cells (O phase). After several further passages, however, all 3 species of cells were generally agglutinated to about the same titer (D phase). They found that this shift was due to selection of particles having greater capacity to react with fowl erythrocytes. They were able to maintain their strains in O phase by passage of virus at high dilutions.

The predominant Coxsackie A-21 particle in nature appears to be HA+, but there is ample evidence for the existence of the HA– variant as well. Eleven of a total of 238 virus strains recovered from young adults during a 1960 outbreak were HA– after isolation in HEK(10). Differences in virulence of the 2 types of virus for humans could not be detected in that study. The separation of pure pools of HA+ and HA– virus, however, will permit comparison of pathogenic properties of these 2 variants in both suckling mice and human volunteers.

Summary. Coxsackie A-21 virus (strain 48560) lost the capacity to agglutinate human erythrocytes after passage in KB or HeLa cell cultures. Hemagglutinin could be retained by passage at high dilution in such cells. By adsorption onto erythrocytes it was possible to separate distinct hemagglutinating (HA+) and non-hemagglutinating (HA–) particles. It was found that malignant cells favored multiplication of the HA– particles, whereas primary human embryonic epithelial cells and semi-continuous diploid human embryonic fibroblastic cells favored growth of the HA+ particles.

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Demonstration of Two Kinds of Fat Particles in Alimentary Lipemia with Polyvinylpyrrolidone Gradient Columns.* (27608)

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It has long been known that the fat particles of blood plasma are not all alike. Gage and Fish(1) distinguished large and small particles with the dark-field microscope. More recently, Albrink(2) classified the particles as "light chylomicrons" and "heavy chylomicrons". The "light chylomicrons" floated from plasma into isotonic saline after centrifugation at 20,000 G $\times$ 1 hr (1.2×10^6 G min), while the "heavy chylomicrons" floated up only after additional centrifugation at 105,000 G $\times$ 22 hrs (1.32×10^8 G min). Kuo *et al.*(3) centrifuged the fat particles of alimentary lipemic plasma into saline at 58,000 G $\times$ 30 min (1.7×10^6 G min). Some of the particles were packed into a thick "cream" layer at the top of the centrifuge tube, and others floated as a less turbid "saline" layer just below the "cream" layer. The lipid in these 2 layers differed in fatty acid composition. Centrifugation, however, did not sharply separate one group of particles from another. This paper describes how plasma fat particles may be separated into 2 distinct groups by differential flocculation in a polyvinylpyrrolidone gradient column.

Fat particles must be aggregated in order to float spontaneously in plasma. Burstein and co-workers(4,5) showed that 5% polyvinylpyrrolidone (PVP) in turbid plasma flocculates the fat particles, but not the soluble lipoproteins. Eight per cent PVP flocculates the low-density lipoproteins also, but even at this PVP concentration, flocculation is limited to the fat particles if the plasma contains in addition 100 mg NaCl/ml.

Materials. Stock solutions, stable at room temperature for several weeks, should be sparkling clear. A. 10% NaCl containing 0.01% sodium ethylene diamine tetraacetate (EDTA). The solution should be filtered to remove undissolved solute. B. 5% polyvinylpyrrolidone (average molecular weight 40,000) in solution A. (Polyvinylpyrrolidone powder obtained from General Aniline and Film Co., New York City, N. Y.) C. 25% polyvinylpyrrolidone in solution A. Cellulose nitrate centrifuge tubes (20 ml). These are transparent and can be sliced with a tube slicer.

Procedure. A PVP density gradient is made by displacing fluid of continually increasing PVP concentration from a mixing bottle(6) into the bottom of a test tube. Fifteen ml of solution A is placed in a mixing bottle. From a syringe containing 20 ml of solution B, a plastic tube leads through the mixing bottle stopper to a point above the fluid in the bottle. The tip of a second plastic tube is immersed in this fluid and leads through a second perforation in the stopper to the bottom of a cellulose nitrate tube. The contents of the syringe are infused slowly into the mixing bottle while the fluid in the bottle is stirred. Six gradients can be made at once if a large bottle replaces the syringe, and volumes of the solutions are increased proportionately: 90 ml of solution A is put in the mixing bottle, and the outflow is delivered simultaneously into 6 tubes.

Tubes containing the gradient mixture are warmed in an incubator at 37°C before use.

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