Examination by Chromatography and Immunodiffusion of an Adenovirus 3 Isolated from Humans with Infectious Hepatitis

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Received for publication 7 October 1968

Prototype adenovirus 3 and adenovirus SC8, which was found in feces from a patient with infectious hepatitis and which was classified as adenovirus 3 by standard procedures, were compared by chromatography and immunodiffusion techniques. When the radioactive adenovirus moiety in SC8 had been separated from other radioactive components of tissue culture by gel filtration, a smaller infectious agent was detected, whereas with prototype adenovirus 3 one infectious agent was found. The large agent from SC8 was classified as adenovirus type 3 by serum neutralization tests, but results from homologous and heterologous immunodiffusion tests and heat sensitivity tests indicated that this agent was different from the classical prototype adenovirus 3. Similar precipitin patterns obtained in homologous and heterologous reactions by immunodiffusion suggested a similarity between the smaller particle and an unidentified agent isolated without adenoviruses from blood clots from overt cases of hepatitis. With the present evidence, it was not possible to relate the smaller agent to adeno-associated viruses; however, its similarity to an agent isolated from blood of overt cases implies a possible relationship with hepatitis. The continued recovery of the variant strain of adenovirus type 3 from patients with hepatitis, although at relatively low rates of isolation, suggested a possible undetermined relation to the disease.

Davis (5) isolated several adenoviruses from fecal specimens from cases of infectious hepatitis which occurred in 1959 on the San Carlos Apache Indian Reservation in Arizona. Eight of the agents were identified as adenovirus type 3 (8). All eight agents showed the same differences from the prototype in their hemagglutinating properties and in their homologous and heterologous neutralization titers. These differences suggested that the eight probably represented a variant or strain.

Biological patterns, similar to those caused by infectious hepatitis in humans, were reported by Douglas and Berge (6) in chimpanzees inoculated with one of the eight isolates designated as SC8. Although this positive evidence was not supported by the low rate with which adenoviruses were isolated subsequently from humans with the disease; it stimulated additional studies in our laboratory; in these studies radioactive viruses were used to determine the initial site of infection in chimpanzees and the materials from which virus might be isolated most readily. The initial steps in these studies were the preparation and purification of 14C-labeled SC8 grown in cell cultures with media fortified with thymidine-2-14C. Study of the material obtained by fractionation of the radioactive SC8 preparations provided additional information on differences between this viral isolate and the prototype adenovirus 3. These data are reported here.

Materials and Methods

Virus preparations. Viruses were isolated from fecal specimens obtained from patients with infectious hepatitis or were obtained from commercial sources as prototype agents. Viruses were propagated in Blake bottles or stationary tubes of human embryonic lung tissue culture (HEL; E. V. Davis and V. S. Bolin, Federation Proc., p. 386, 1960) or chimpanzee liver tissue culture (CHL; 7). Medium 199 with 15% agamma calf serum or basal medium Eagle's (BME) with 10% agamma calf serum was used as a growth medium, and the same medium with 2% calf serum was used for maintenance. Procedures for plaque
formation were those described by Hsiung (9), with bottle cultures of HEL and CHL.

**Tagging procedures.** Radioactive preparations of virus were obtained by adding 0.25 µC of carbon as thymidine-2-14C for each millilitre of maintenance medium. The radioactive medium was placed on the cells at the time the virus was inoculated.

**Chromatographic fractionation.** Cells grown in Blake bottles were prepared for chromatography by rapid freezing and thawing three times, moderate centrifugation to remove cellular debris, and then ultrafiltration through cellophane membranes at 4 C. Chromatographic fractionations of 1- to 2-ml quantities of crude viral preparations, concentrated 50 to 100 times were made at 4 C on columns packed with 5% Noble agar beads prepared according to the method of Bengtsson and Philipson (3). The void volume of columns measured by displacement of Blue Dextran (Pharmacia Fine Chemicals Inc., Piscataway, N.J.) was 16 ml. The elution rate with phosphate saline solutions buffered at pH 7.4 was 1 ml per min, and serial samples containing 1.5 to 2.0 ml were collected for subsequent analyses. The quantities of radioactive carbon in alternate samples were determined by liquid scintillation counting; total protein in each sample was measured by the method of Daughaday (4). Preparations of uninfected tissue culture maintained in radioactive medium were also fractionated and analyzed, as were nonlabelled preparations of adenovirus type 3.

**Immune sera.** Immune sera were prepared in rabbits by inoculating five 1-ml doses of concentrated tissue culture material, each containing $9 \times 10^6$ TCID$_{50}$ of virus, into the marginal ear vein; the first four doses were administered on alternate days and the fifth after an interval of 3 weeks. Immune serum was obtained by cardiac puncture 1 week after the fifth injection and was stored at -20 C until used.

**Serum titration.** Antisera used for serum neutralization titrations were absorbed by incubating portions of each antisera with homologous and heterologous antigens. Antigens used for absorption were grown and concentrated as described for chromatographic fractionation. Each preparation was incubated with excess antigen for 1 hr at 37 C and 1 hr at room temperature, centrifuged at 10,000 x g for 10 min to remove suspended solids, and filtered through 100- and 50-nm filters. After inactivation by heating at 56 C for 30 min, sera were sterilized by filtration through sterile 220-nm filters. Neutralization titers of absorbed and unabsorbed antisera were measured in HEL with serial twofold dilutions with 200 TCID$_{50}$ of homologous virus. The neutralization titer of each serum was the reciprocal of the highest dilution of sera which neutralized 200 TCID$_{50}$ of virus.

**Immunodiffusion studies.** Immune sera used in the immunodiffusion studies were absorbed with concentrates of homologous normal tissue culture to remove antibodies induced by calf serum and cell components. Viral antigens were prepared from material used for antibody production by further concentration as described for the chromatographic fractionation. Comparison of precipitin patterns formed by homologous and heterologous antigen-antiserum reactions was made in accordance with the technique described by Auernheimer and Atchley (2).

**Hemagglutination studies.** Hemagglutination titers were measured with African green monkey erythrocytes employing twofold dilutions.

**RESULTS**

Neutralization titers of antisera against adenovirus types 3, 7, and 16 were compared with that of SC8 since protection against SC8 has been reported for these sera. Similarities between adenovirus type 3 and SC8 were clearly indicated by the amounts of neutralizing antibodies measured by tissue culture neutralization tests in antisera absorbed with homologous and heterologous antigens (Table 1). Absorption of antisera to adenovirus 3, 7, 16, or SC8 with its homologous antigen caused a complete loss of neutralizing antibody. Similar results were observed in heterologous absorptions only when antisera to adenovirus 3 were absorbed with SC8 antigen or when antisera to SC8 were absorbed with adenovirus 3 antigen. Antisera to adenovirus 16 were partially neutralized by absorption with SC8 antigen, but absorption of these antisera with adenovirus 3 or 7 antigen caused no change. No changes in the neutralization titer of antisera to adenovirus 7 were caused by similar absorption with antigens from adenovirus 3, 16, or SC8.

Antigenic differences between the SC8 virus and the prototype adenovirus 3 were demonstrated in sketches of the immunodiffusion tests (Fig. 1A and B). Common precipitins were indicated by continuous lines and differences by the number of lines which formed with the homologous and heterologous systems. Precipitin lines between the wells of antisera in Fig. 1A and B were caused by the excess heterologous antigen used in absorbing the sera in the number 2 wells. Differences in precipitin patterns similar to those observed with adenovirus 3 and SC8 were ob-

**Table 1. Neutralization titers of antisera measured in homologous systems after absorption with homologous and heterologous antigens**

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<thead>
<tr>
<th>Antisera*</th>
<th>Absorbing antigen</th>
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<tr>
<td></td>
<td>Adeno-virus 3</td>
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<tr>
<td>Adenovirus 3</td>
<td>&lt;20</td>
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<tr>
<td>Adenovirus 7</td>
<td>320</td>
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<tr>
<td>Adenovirus 16</td>
<td>320</td>
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<tr>
<td>SC8</td>
<td>&lt;20</td>
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* Before absorption, neutralization titers were: adenovirus 3, 160; adenovirus 7, 320; adenovirus 16, 320; and SC8, 320.
Fig. 1. Precipitin lines formed by homologous and heterologous reactions between antisera to adenovirus 3, SC8, and agent Y and antigens from SC8, agents X and Y, and adenoviruses 3, 7, and 16. (A) 1. Tissue control antigen. 2. SC8 antisera absorbed with excess adenovirus type 3. 3. SC8 antiserum absorbed with tissue control antigens. 4. Adenovirus type 3 antigen. 4. Adenovirus type 3 antigen. 5. SC8 antigen. 6. Adenovirus type 16 antigen. (B) 1. Tissue control antigen. 2. Adenovirus type 3 antiserum absorbed with excess SC8 antigen. 3. Adenovirus type 3 antiserum absorbed with tissue antigen. 4. SC8 antigen. 5. Adenovirus type 3 antigen. 6. SC8 antigen. (C) 1. Tissue control antigen. 2. Adenovirus type 16 antigen. 3. SC8 antigen. 4. Adenovirus type 7 antigen. 5. SC8 antigen. 6. Adenovirus type 16 antigen. 7. SC8 antiserum absorbed with tissue control antigen. (D) 1. Mixture of SC8 and adenovirus 3 antigens. 2. Agent Y antiserum absorbed with tissue control antigen. 3. Agent X antigen. 4. Agent Y antigen.

Precipitin patterns formed in homologous and heterologous reactions by immunodiffusion with adenovirus type 3 from different sources were compared with those formed with SC8. This group included five of the eight from the San Carlos group (8), six which were isolated from sporadic cases of hepatitis or their contacts between 1962 and 1966 in San Carlos and Phoenix, Ariz., three recovered from children in acute respiratory disease studies by T. D. Y. Chin, U.S. Public Health Service, Kansas City, Kan., and three prototype cultures of adenovirus 3 from commercial sources. The prototype viruses and the agents furnished by T. D. Y. Chin formed similar precipitin patterns. Differences between these agents and those from the San Carlos group and subsequent cases of hepatitis were similar to differences observed with SC8 and adenovirus type 3.

Attempts to purify SC8 by plaque formation were unsuccessful. Plaques did not develop in bottle cultures of ChL or HEL which had been inoculated with the agent.

Specific antigens associated with individual precipitin lines could not be separated chromatographically from preparations of SC8 or adenovirus 3 on the agar columns used. Each major fraction, discussed below, that contained one of the antigenic components contained all of them.

Measurements of total radioactivity in consecutive chromatographic fractions showed little difference between the prototype adenovirus 3, SC8, or uninfected tissue culture material used for the control (Fig. 2). Hemagglutinating activity in the fractionated SC8 preparation was concentrated primarily in fractions obtained prior to those containing the protein. Most of the radioactivity was found in the same fractions; however, considerable quantities of radioactivity persisted in a few fractions after hemagglutinating activity was greatly diminished, and a second minor peak of radioactivity was found in fractions obtained after most of the protein had been eluted (Fig. 2). On the basis of these observations, consecutive fractions were combined arbitrarily into major fractions A, B, C, and D, so that A contained all of the eluate prior to the appearance of radioactivity, protein, or hemagglutination activity. Fraction B contained those portions with the highest hemagglutination titers and easily detectable amounts of radioactivity and protein. C contained the major portion of the protein, less radioactivity than B, and a little hemagglutination activity. D contained the remaining radioactivity and protein, but no hemagglutination activity.

For purposes of comparison, similar serial chromatographic fractions of the prototype virus and the uninfected tissue culture material were combined quantitatively into major fractions on the basis of eluted volumes. The marked differences observed in radioactive content of comparable serial samples from SC8 and uninfected radioactive tissue culture were also found in the major fractions. Fraction B of SC8 contained about five times as much radioactivity as the control, and fractions C and D each contained two times as much (Fig. 2).

Hemagglutination studies indicated that the radioactivity was incorporated into the SC8 virus. When African green monkey erythrocytes were
reacted with samples of fraction B from SC8, then lysed and the cell stroma removed by centrifugation, this debris contained easily detectable quantities of radioactivity. However, when the sample was first neutralized with antiserum against SC8, no radioactivity was found on the cell membranes. Similar tests with uninfected tissue culture material were also negative.

Differences were observed in the infectivity titers of fractions of SC8 and the prototype adenovirus 3. The infectivity titers of the prototype virus in HEL decreased progressively from $10^{-3.5}$ for fraction B to $10^{-1}$ for fraction D. The infectivity titers of the SC8 preparation were $10^{-3}$ in fraction B, $10^{-1}$ in fraction C, and $10^{-4}$ in fraction D. Tubes inoculated with fractions C or D of SC8 developed, on initial passage, cytopathic effects (CPE) unlike those characteristic of adenoviruses, but frequently on subculture characteristic adenovirus CPE were produced. CPE produced in the early passages were difficult to read but occurred with sufficient consistency and persistency to suggest the presence of a second infectious agent. The presence of a second agent could
also account for the differences in distribution of hemagglutination activity and radioactivity found in the fractionated SC8 preparation and for the smaller second peak of radioactivity shown in Fig. 2.

Evidence that the SC8 isolate might be a mixture of a variant or strain of adenovirus 3 and a second agent, agent X, was indicated by the persistence of the atypical appearance in tissue cultures inoculated with preparations of SC8 which had been neutralized either by antiserum to fraction B from the SC8 virus or by antiserum to the prototype adenovirus 3. Further evidence of a second infectious particle was indicated by an immunodiffusion study which showed that agent X, when reacted with antiserum to an unidentified agent, Y, isolated without adenoviruses from blood of other hepatitis patients, formed precipitin patterns identical to those obtained with the homologous reaction (Fig. 1D). Neither agent X nor agent Y formed precipitin lines common to SC8 or other adenoviruses. When the SC8 virus, agent X, and agent Y were cultured in media containing radioactive thymidine and then fractionated by chromatography, the SC8 preparation again had most of the radioactivity in fractions B and C, whereas both agent X and agent Y contained most of the radioactivity in fraction D. Each of these differed from preparations made from an uninfected tissue culture control.

**DISCUSSION**

An adenovirus 3 isolated from patients with hepatitis was a mixture of a variant strain of adenovirus and a smaller infectious agent. Highly purified, viable preparations of radioactive adenovirus were obtained by chromatography on agar beads, and evidence associating radioactivity with the adenovirus was obtained from hemagglutination studies with fraction B. The presence of the smaller particle was recognized when it had been separated from the adenovirus moiety of the mixture.

Results of neutralization tests with type-specific antisera indicated that the SC8 agent contained an adenovirus type 3, but precipitin patterns formed by immunodiffusion tests provided visual evidence of differences in the antigenic composition of the adenovirus component of SC8 and adenovirus type 3. Four lines were formed in each homologous reaction and two in each heterologous reaction between SC8 and adenovirus type 3 (Fig. 1A and B). These precipitins probably represented the complement-fixing antigen common to mammalian adenoviruses, the type-specific antigen, and two subgroup antigens. Since precipitins did not form in reactions between SC8 and agent Y antisera or between SC8 antisera and agent X, the precipitins formed in heterologous reaction between SC8 and adenovirus 3 must be associated with antigens from the adenovirus moiety of these agents. After homologous and heterologous absorptions with the respective adenovirus antigens, antisera against SC8 or adenovirus type 3 did not protect tissue culture cells from infection in serum neutralization tests (Table 1). These observations suggested that the two precipitins formed by heterologous reactions represented the complement-fixing antigen and the type-specific antigen of adenovirus type 3. Two precipitins were also observed in heterologous reactions between SC8 antiserum and adenovirus types 7 and 16 which were used for comparisons (Fig. 1C). One of these lines represented the complement-fixing antigen. However, the other, the relationship of which was not understood from these studies, did not represent the respective type-specific antigens, since appreciable changes did not occur in neutralizing capabilities of antisera to adenovirus types 7 and 16 after heterologous absorption with other adenovirus antigens.

Physical differences between SC8 and adenovirus 3 were also suggested by thermal inactivation studies (R. G. Bird et al., in press). The prototype virus was inactivated after heating at 50°C for 15 min, but the SC8 resisted this treatment for 40 min. The differences in thermal lability and the precipitin patterns of SC8 and prototype adenovirus 3 supported the suggestion of Hatch and Siem (8) that the adenovirus moiety of SC8 may be a variant or hybrid strain of adenovirus 3.

Other evidence supporting the proposal that SC8 is a variant strain of adenovirus 3 was indicated when adenovirus 3 from different sources was examined by immunodiffusion. Agents studied included other members of the San Carlos group studied by Hatch and Siem, several isolates classified as adenovirus 3 (obtained between 1962 and 1966 from patients with infectious hepatitis in Arizona), adenovirus 3 from another geographical area, and prototype viruses from three commercial sources. Identical precipitin patterns characteristic of one group were formed in homologous and heterologous reactions with the San Carlos strains of adenovirus 3 and with those strains isolated from cases of infectious hepatitis between 1962 and 1966. A second set of similar precipitin patterns was associated by similar reactions with the three prototype viruses and the agents from Kansas City. The consistent difference in precipitin patterns formed by all of the San Carlos adenovirus type 3 strains and the prototype virus supports the proposal of Hatch and Siem. The continued recovery of the San Carlos adenovirus 3 from patients with hepatitis...
suggested that this agent may be indigenous to this area or that it may be associated with the disease.

The amounts of tissue culture infectivity in similar chromatographic fractions indicated additional differences in the antigenic constituents of the SC8 preparation and prototype adenovirus 3. Comparison of the titers suggested that fraction D of SC8 contained about 10^4 times the infectivity of fraction D of the prototype preparations. Since the agar columns used for the chromatographic fractionation function as molecular sieves from which larger molecules are eluted first, particles in fraction D must be smaller than particles in fraction B.

The greater infectivity in fraction D of the SC8 preparation seemed to be due to a smaller infective particle not present in the prototype preparation. Visual support for this premise was furnished (R. G. Bird et al., in press) when a particle measuring 40 nm was found in electron micrographs to be associated with an adenovirus type 3 from the San Carlos group. Data obtained from the chromatographic fractionations of the radioactive preparations and from the immunodiffusion studies also supported this suggestion. The CPE produced in tissue culture by agent Y were similar to those observed in the first passage material from chromatographic fractions C and D of SC8. Since typical adenovirus CPE reappeared after a number of subpassages, the original inoculum obviously did contain some adenoviruses, but in insufficient quantity to mask the contaminating smaller agent on the initial passage.

Results of immunodiffusion and chromatographic studies demonstrated that neither agent X, obtained from SC8 material, nor the unidentified agent isolated from blood clots, agent Y, was adenovirus. No precipitin lines common to adenovirus were formed when these antigens were reacted with antisera to the original SC8 or with antisera to other adenoviruses. However, both X and Y formed precipitin lines with antisera to agent Y. When radioactive preparations of agent Y were separated by chromatography, the distribution of radioactive material in the different fractions was similar to that observed with agent X.

Other investigators studying the adenoviruses (1) have found small agents associated with adenovirus infections in tissue culture and have designated them as adeno-associated viruses (AAV). Present evidence does not relate this small particle to the AAV. In most instances, the AAV were considered to be completely dependent upon the adenovirus and unable to replicate without them. Our studies suggested that agent X, isolated with adenovirus from cases of hepatitis, can multiply without the adenovirus and, in fact, is similar, if not identical, to agent Y, which was isolated without adenoviruses from other cases of hepatitis.

LITERATURE CITED