

Description of a Viral Agent Found in Blood Obtained from Patients with Infectious Hepatitis

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A small, double-stranded deoxyribonucleic acid viral agent (agent Y) has been found in blood from patients with infectious hepatitis. The agent was propagated in chimpanzee liver tissue culture which had been grown and maintained on unheated agamma calf serum. A causal relationship between this agent and the occurrence of human disease cannot be determined from the data presented.

Cytopathic effects (CPE) suggesting the presence of an infectious agent (agent Y) were observed in tissue culture inoculated with blood clots obtained from patients and their contacts during an outbreak of infectious hepatitis which occurred on the Sacaton Indian Reservation in Central Arizona in 1966. Similar cytopathology was observed with fractions prepared by gel filtration (4) from an adenovirus type 3 which had been isolated from stool specimens from Apache Indians with infectious hepatitis in 1959 (2). Evidence suggesting the identity of these two agents was obtained by immunodiffusion tests when identical precipitin patterns were formed in homologous and heterologous systems. This report describes procedures used and results obtained from the subsequent propagation and identification of this agent.

MATERIALS AND METHODS

Virus preparations. Agent Y was detected by inoculating blood clots into stationary tubes of human embryonic lung (HEL; E. V. Davis and V. S. Bolin, *Fed. Proc.*, 1960, p. 386) or chimpanzee liver (ChL) (3) by procedures previously described (5). Medium 199 with 15% unheated agamma calf serum was used for growth of HEL, and Basal Medium Eagle with 10% unheated agamma calf serum was used for growth of ChL. The same medium with 2% calf serum was used for maintenance. Unheated calf serum was checked for toxicity and viral contamination by subculturing 0.2 ml of each 500-ml unit through a minimum of five serial passages in ChL. Subcultures of the serum were continued as control specimens for comparison with infected tissue. Plaque formation in bottle cultures of HEL and ChL was carried out by the procedures described by Hsiung (6), by using media fortified with 0.3 mg of arginine per ml as suggested by Wallis and Melnick (10).

Prototype adenovirus 3 and poliovirus 3 were obtained from commercial sources for comparison and for system control.

Immune sera. Immune sera to agent Y, prototype adenovirus type 3, and poliovirus type 3 were prepared in rabbits by inoculating five doses of concentrated tissue culture material into the marginal ear vein. The first four doses were administered on alternate days and the fifth was administered after an interval of 3 weeks. Immune serum was obtained by cardiac puncture 1 week after the last injection and stored at -20°C until used. Antigens were prepared in ChL or HEL cells grown in Blake bottles. When cell sheets inoculated with the viral agents had disintegrated, tissue culture was frozen at -50°C and thawed rapidly three times. The supernatant fluid was decanted from the debris after centrifugation at $10^4 \times g$ for 10 min and was concentrated $100 \times$ by filtration through cellophane tubing at 4°C . Antigen used for the first inoculation was equivalent to one Blake bottle of tissue culture fluid (4.5×10^6 TCID₅₀); subsequent inoculations contained one-half that amount. Rabbit antisera against other prototype agents were prepared by a commercial supplier.

Immunodiffusion studies. Precipitin patterns formed by homologous and heterologous antigen-antisera reactions were compared in accordance with the technique described by Auernheimer and Atchley (1). Antisera were absorbed with concentrates of uninfected tissue culture to remove antibodies induced by cell components and calf serum. Viral antigens were prepared from material used for antibody production by further concentration as described for immune sera.

Hemagglutination studies. Hemagglutination tests were performed with sheep, green monkey, rat, guinea pig, and type O human erythrocytes, employing serial twofold dilutions of antigen.

Nucleic acid determination. Nucleic acid type was determined by comparing results from chemical inhibition and enzyme digestion procedures with agent Y, adenovirus type 3, and attenuated poliovirus type 3. In chemical inhibition tests, 5-fluorodeoxyuridine (FUDR) or 5-bromodeoxyuridine (BUDR) was

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incorporated into maintenance media at a final concentration of 10^{-4} M. Enzymatic digestions were conducted by the procedures described by Lin and Maes (8) by using commercially prepared ribonucleases and deoxyribonucleases. After each treatment, duplicate tubes or plaque bottles of ChL were inoculated with serial 10-fold dilutions of infected tissue culture fluid.

Physical characteristics. Procedures proposed by Hsiung (6) for characterizing animal viruses were used. Size and sensitivity to heat, pH, and chloroform of agent Y were determined by comparing results from these tests with those obtained from adenovirus 3 and attenuated poliovirus type 3. Size was estimated from the change of infectivity titers of filtrates which passed through 100-, 50-, and 10-nm membrane filters. Sensitivity to heat was determined by measuring infectivity of preparations after heating for 1 hr at 56, 60, and 65 C. In pH sensitivity tests, fluid from tissue culture was adjusted to pH 3 with HCl and incubated for 1 hr at 37 C before inoculation with tissue culture. Chloroform sensitivity tests were performed by standard procedures. After each treatment, all preparations were filtered through sterile 220-nm filters.

Mouse inoculation. Litters containing 6- to 8-day-old Wistar white mice were inoculated intracranially with 0.03 ml of a 10^{-1} dilution of infectious tissue culture fluid which had been clarified by centrifugation at $10^4 \times g$ for 10 min. Inoculated animals were sacrificed after 21 days, and the brains were removed aseptically. The brains from each litter were combined, frozen, and homogenized in saline at 4 C. Aqueous extracts were clarified by centrifugation at

$10^4 \times g$ for 10 min, and 0.03 ml was inoculated into each of a litter of 1-day-old mice.

RESULTS

Table 1 presents a description of the source of the viral agents used in this study; their infectivity titers in ChL; their size as estimated from infectivity titers of filtrates which passed through 100-, 50-, and 10-nm membrane filters; their sensitivity to pH, heat, and chloroform; and their nucleic acid type. Similar CPE were caused in ChL and HEL by all isolates designated as agent Y (H127, H131-7, H159, H174, or 3312). The infection was difficult to recognize but usually appeared 3 days after inoculation as separate areas or clumps of rounded cells. Typical CPE in ChL 4 days after infection with agent Y (H174) are seen in Fig. 1A. By the 5th day, infected and uninfected tissue cultures were easily differentiated since foci of rounded cells had enlarged and had separated progressively from the glass. The virus killed the cells without further disintegration and disengaged clumps appeared crenated. At the 5th day, uninfected monolayers were still intact. The CPE were reproduced in ChL by serial transfer of infected tissue culture fluid diluted to 10^{-5} . Similar effects were not observed in HEL with dilutions greater than 10^{-3} . Further visual evidence of cytopathology caused by this agent was obtained in plaquing trials. Plaques, 0.3 to 1.0 mm in di-

TABLE 1. Source of viral agents used, infectivity titers, size, sensitivity to pH, heat, and chloroform, and nucleic acid type

Agent ^a	Source	Titer ^b	Size (nm)	Sensitivity ^c			Nucleic acid type
				pH 3	65 C	Chloroform	
H127	7-year-old male	10^{-5}	<50 >10	—	—	—	DNA
H131-7	7-year-old female	10^{-5}	<50 >10	—	—	—	DNA
H159	22-year-old male	10^{-5}	<50 >10	—	—	—	DNA
H174	8-year-old male	10^{-5}	<50 >10	—	—	—	DNA
3312	11-year-old female	10^{-5}	<50 >10	—	—	—	DNA
Adenovirus 3		10^{-4}	<100 >50	—	+	—	DNA
Poliovirus 3		10^{-7}	<50 >10	—	+	—	RNA ^d

^a H127, H131-7, H159, H174, and 3312 were isolated from blood clots taken from patients during the acute phase of infectious hepatitis. Adenovirus 3 was the prototype and poliovirus 3 was the Sabin oral vaccine type.

^b Greatest dilution which caused 2 + CPE in ChL.

^c Symbols: +, sensitive; —, not sensitive.

^d Ribonucleic acid.

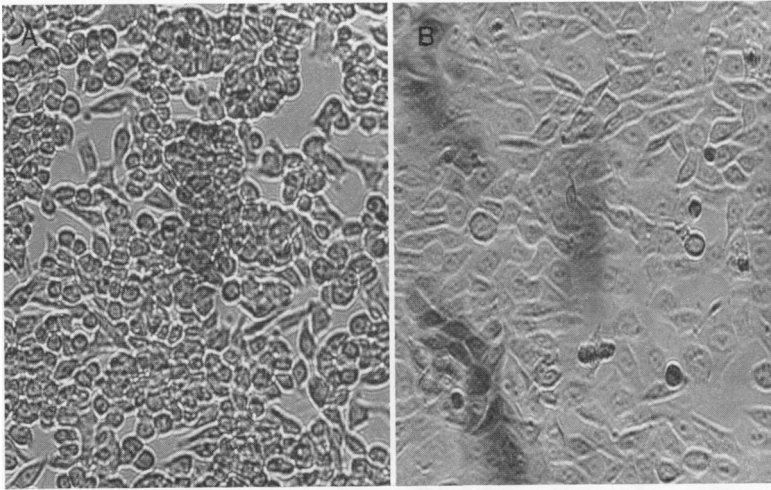


FIG. 1. Chimpanzee liver tissue culture 4 days after inoculation. (A) Infected with agent Y. (B) Uninfected control. $\times 260$.

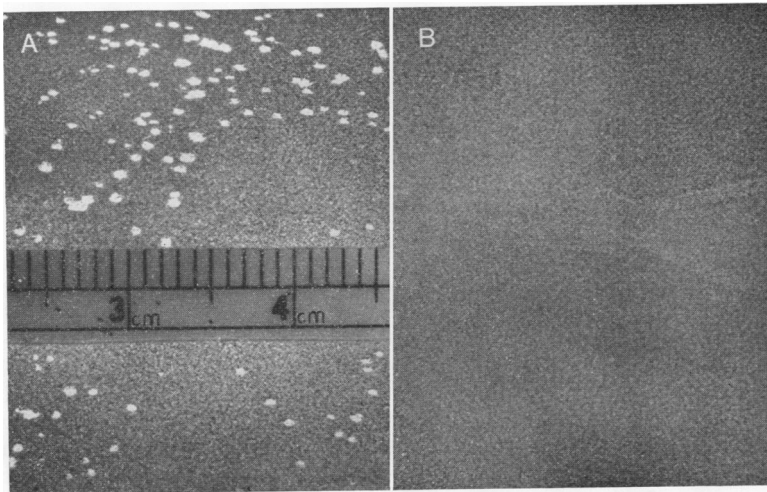


FIG. 2. Monolayers of chimpanzee liver cells 5 days after inoculation for plaque formation. (A) Infected with agent Y. (B) Uninfected control.

ameter (Fig. 2), were observed 5 days after inoculation in ChL grown and maintained on unheated calf serum. The number of plaques decreased progressively with each serial 10-fold dilution so that one to five plaques were apparent in bottles inoculated with 0.5 ml of 10^{-5} dilutions of infected material. The plaquing phenomenon could be reproduced serially.

Serum neutralization tests were performed with each agent Y isolate and its type-specific antiserum. Antisera diluted 1:80 neutralized 200 TCID₅₀ of each agent in homologous systems. Similar results were obtained in heterologous

systems. Greater than 80% reduction in plaque-forming units was observed with agent Y after neutralization with homologous sera or with heterologous sera against other members of the agent Y group. Agent Y was not neutralized in tube titrations or plaque-forming procedures by pools of antisera to echovirus types 1 through 32, which include reovirus type 1, formerly echovirus type 10, and rhinovirus type 1A, formerly echovirus type 28, antisera to human adenovirus types 1 through 18, antisera to poliovirus types 1, 2, and 3, and antisera to coxsackieviruses A1, A2, and A9 or to any of the six coxsackie B viruses.

No evidence of infection was observed in 1-day-old mice inoculated intracranially with 0.03-ml of preparations of agent Y. Extracts made of brains of these animals 21 days after initial inoculation did not produce visible effects when inoculated into 1-day-old mice.

Filtration studies and sensitivities to heating, pH, and chloroform were conducted with adenovirus 3, poliovirus 3, and agent Y. Before filtration, the greatest dilutions causing recognizable CPE were 10^{-4} for adenovirus 3, 10^{-5} for agent Y, and 10^{-7} for poliovirus 3. No changes in infectivity were noted in filtrates of agent Y, adenovirus type 3, and poliovirus 3 which passed through 100-nm filters. In filtrates which passed through 50-nm filters, dilutions of adenovirus 3 greater than 10^{-1} did not cause infection, and no change was noted in infectious properties of agent Y or poliovirus 3. Adenovirus 3 did not pass through 10-nm filters. Dilutions of filtrates from 10-nm filters which caused infections were 10^{-1} for poliovirus and 10^0 for agent Y. These three agents were not inactivated at pH 3 or by treatment with chloroform. However, only agent Y resisted heating at 60 C for 1 hr.

Infectivities of agent Y, adenovirus 3, and poliovirus 3 were measured after treatment with FUDR, BU DR, ribonucleases, and deoxyribonucleases. FUDR or BU DR at a concentration of 10^{-4} M in maintenance medium had no apparent effect on poliovirus 3. After similar treatment, some decrease in infectivity was noted in tube titration tests with agent Y and adenovirus 3, but no plaque reduction for agent Y was observed. Hydrolysis with 10 Kuntz units of ribonuclease per ml at 37 C for 30 min reduced maximum dilutions of poliovirus 3 which caused recognizable CPE from 10^{-7} to 10^{-2} . This treatment did not alter the infectious properties of adenovirus 3 or agent Y. No decrease in infectivity of tissue culture fluids containing poliovirus 3, adenovirus 3, or agent Y was noted after treatment with deoxyribonuclease I. However, treatment with deoxyribonuclease II destroyed the infective properties of agent Y and adenovirus 3, as measured by tube titrations and plaque reduction, with no apparent effect on poliovirus 3.

Agent Y did not induce hemagglutination in sheep, green monkey, rat, guinea pig, or type O human erythrocytes.

Tracings of characteristic precipitin lines formed by immunodiffusion between agent Y antigens and type-specific antisera are presented in Fig. 3. Identical patterns suggesting similarity of the agents were formed in homologous and heterologous reactions between type-specific

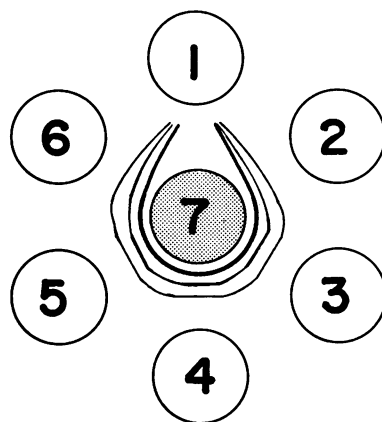


FIG. 3. Precipitin patterns formed in homologous and heterologous reactions with agent Y from different sources. (1) Tissue culture control antigen; (2) H127 antigen; (3) H131-7 antigen; (4) H159 antigen; (5) H174 antigen; (6) 3312 antigen; (7) H174 antisera absorbed with excess tissue culture control antigen.

antisera and each antigen. In each case, precipitins could be inhibited by absorbing the antigen with heterologous antisera against other agent Y isolates before homologous reactions. Characteristic precipitin patterns were not formed in heterologous reactions between agent Y antigen and antisera against adenovirus types 1 through 18 or poliovirus types 1, 2, or 3.

Electron photomicrographs of a portion of a cell from ChL, 4 days after infection with H174, are presented in Fig. 4. The nuclei of infected cells were enlarged and contained clusters of material not found in normal cells (Fig. 4A). These clusters contained small bodies with dark-staining centers, believed to be agent Y, which were 14 to 18 nm in diameter. Similar structures were found in one fraction, at a buoyant density of 1.38 to 1.42, which was obtained when crude preparations of infected tissue culture were separated on cesium chloride density gradients. Portions of this fraction did not induce CPE in ChL on first passage; however, precipitin patterns similar to those previously described were formed in immunodiffusion trials with this fraction.

DISCUSSION

A small deoxyribonucleic acid (DNA) virus has been detected in blood obtained from patients with infectious hepatitis. Infections from agent Y were first recognized in tissue cultures grown and maintained in media which contained calf serum that had been inactivated by heating. However, this agent could not be subcultured under these conditions, and classical tests for its

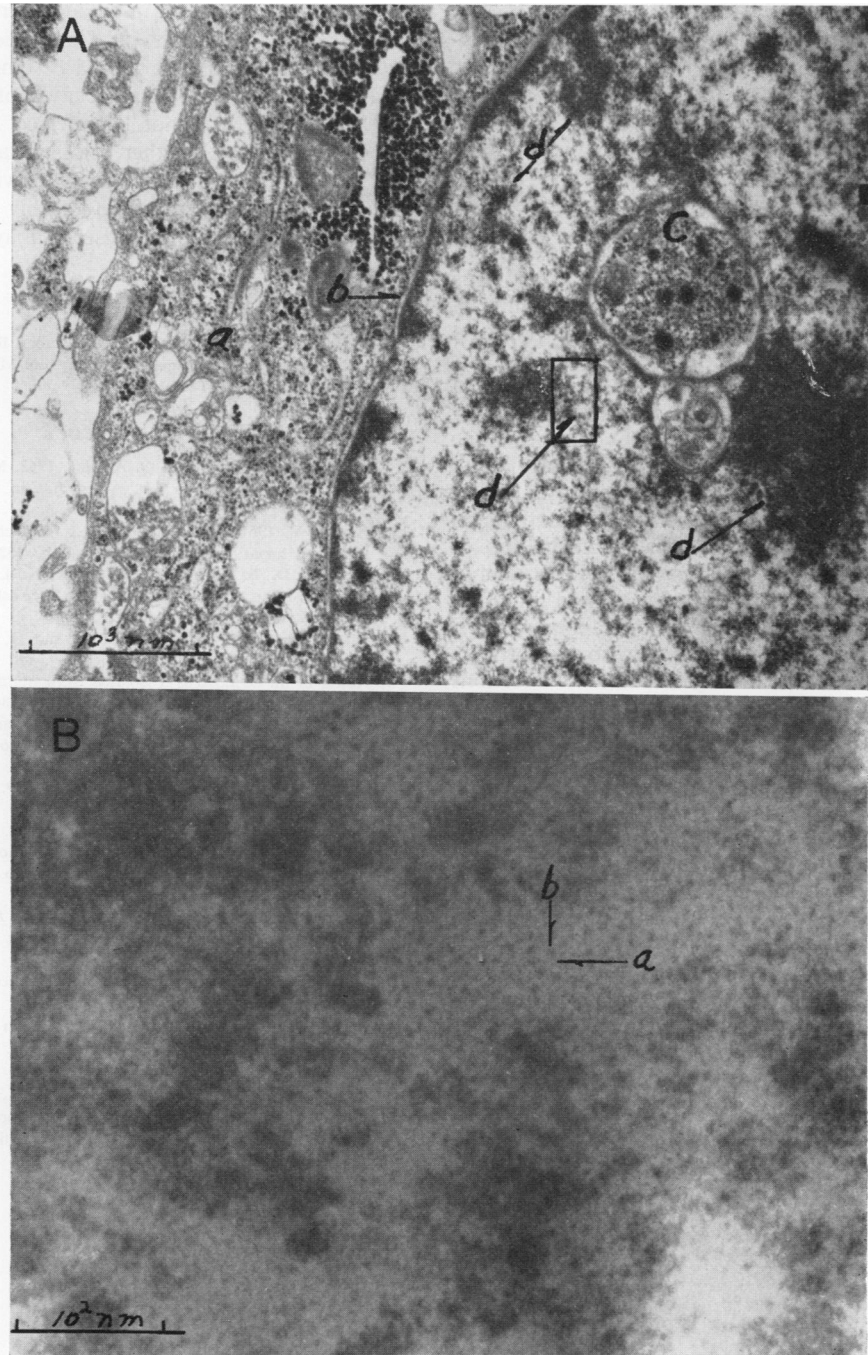


FIG. 4. Electron photomicrograph of chimpanzee liver cells 4 days after infection with H174. Sections were doubly stained with uranyl acetate and lead citrate. A: (a) cytoplasm; (b) nuclear membrane; (c) nucleus; (d) clumps of particles. B: enlargement of inset in Fig. 4A. (a) Particle; (b) dark-stained center.

identification could not be performed. Reproducible results were obtained when subculture was attempted in cell cultures which were grown and maintained with unheated agamma calf serum.

The possibility that agent Y may have been introduced into tissue culture as an exogenous agent such as a pleuropneumonia-type organism (PPLO) or as a latent virus in unheated calf serum must be considered. However, attempts to relate this agent to PPLO or to a latent virus were unsuccessful. Incorporation of kanamycin, streptomycin, or tylosin into maintenance media did not prevent plaque formation. The plaques formed by agent Y lacked the morphological characteristics recently described for PPLO (7, 11), and known cultures of PPLO did not plaque in the systems described above. Adsorption of antisera against agent Y with concentrated preparations of PPLO isolated from contaminated tissue culture did not inhibit the formation of characteristic precipitin patterns in immunodiffusion tests. In addition, serum used for tissue culture was subcultured without apparent CPE through five serial passages. Also, to provide additional protection against latent infections interfering with the recognition of CPE caused by agent Y, subculturing of the serum was continued in parallel with subsequent tests.

Results of tests performed to characterize agent Y suggest that this agent is not related to any of the known DNA viruses. Poxviruses, herpesviruses, and adenoviruses are larger than 50 nm in diameter. Conditions for the culture and for the recognition of papovaviruses (9) differ from those for agent Y. Antisera against agent Y which protected ChL from 200 TCID₅₀ at dilutions of 1:80 afforded no protection against the H viruses, the RV viruses (Helene W. Toolan, *private communication*), or the associated adenovirus particles (R. V. Atcheson, *private communication*).

Agent Y has been found as a single agent in blood from humans with infectious hepatitis

and as a contaminant (4) in an adenovirus type 3 isolated earlier by Davis (2) from feces of patients with the disease. It should be emphasized, however, that an etiologic association of agent Y with the disease may not be inferred from the data presented here. Further field and laboratory work, which is already in progress, will be required before the relationship of this agent to the occurrence of human disease may be determined.

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