HEPATITIS A: IN VITRO ISOLATION OF AN AGENT WITH UNUSUAL GROWTH REQUIREMENTS FROM A CLINICAL CASE OCCURRING IN COSTA RICA

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An enterovirus-like agent with atypical properties has been isolated in vitro from a hepatitis case in Costa Rica. Many of its properties are consistent with hepatitis agents isolated in marmosets and observed in human feces.

Introduction

This communication describes a viral agent isolated in cell cultures that had been maintained under special conditions following inoculation with a fecal specimen from a case of clinical hepatitis in Costa Rica. Investigations have indicated that this agent possesses several characteristics which distinguish it from known enterovirus types.

Picornaviruses are small (15-30 nm) ribonucleic acid-containing viruses which are further subdivided into rhinoviruses and enteroviruses (1). The former are differentiated from the latter on the basis of significant reductions in infectious virus titer following exposure to pH 3.0 for 1-3 hours (2). Despite one unconfirmed report of rhinovirus isolation from feces, attempts at isolation from fecal samples collected from infected patients and volunteers have not been successful (3). In addition, all rhinovirus types tested have been found to be acid-labile (2).

To date, despite persistent efforts, all attempts at in vitro isolation of the HeA virus or viruses responsible for hepatitis A have been unsuccessful. However, an agent having picornavirus-like characteristics has been transmitted to marmosets from HeA cases (4, 5). Also, a spherical virus-like antigen (diameter 27 nm) was reported recently in the feces of a human volunteer with HeA (6).

Current enterovirus isolation methods (7) have not succeeded in detecting the HeA agent. Since these employ culture media containing 0.11 to 0.22 per cent NaHCO₃, the use of a more stable acid environment for HeA virus isolation was explored. The rest of this article is devoted to reporting the course and outcome of that work.

Methods and Results

Fecal Samples Tested

Fecal samples were collected for a double-blind study by the staff of the Louisiana State University International Center for Medical Research and Training (LSU-ICMRT) located in San José, Costa Rica. Specimens were obtained from 35 hepatitis A cases occurring in a geographic area found to be endemic for the disease (8, 9). Fifty-five control specimens were also collected from individuals living outside the endemic area. All specimens were prepared as 10 per cent extracts.
**Virus Isolation Studies**

All fecal samples were inoculated into primary cultures of Rhesus monkey renal cells and into cultures of HEp-2 cells for enterovirus isolation (7). Because of our interest in viruses that could not be isolated in this way, no further attention was given to specimens from which virus isolations were obtained. The remaining specimens were tested in cultures of human embryonic diploid lung cell WI-38.

**Medium I.** Confluent cultures of cells were maintained in Eagle’s basal medium (EBM), which was prepared in Earle’s balanced salt solution (Earle’s BSS) with 10 per cent fetal calf serum (FCS) and 0.11 per cent NaHCO₃ (final concentrations). The cultures were then inoculated, incubated at 36°C, and observed for cytopathic effects (CPE). The pH of the medium ranged from 7.4 at the time of inoculation to 6.6 following three days of incubation.

**Medium II.** In a parallel study, cell cultures were maintained in a serum-free EBM, which was prepared in a glucose-free Hanks’ BSS, gassed with CO₂ and inoculated. Following incubation at 36°C, the acidity of this medium stabilized at approximately pH 6.8 and remained unchanged during the period of incubation. Fecal extracts frequently were toxic to cells maintained in this serum-free acidic environment, necessitating the passage of fluids from degenerated cultures into fresh cell cultures being maintained under similar conditions.

**Findings.** Most of the fecal extracts were found to produce CPE only in the conventional medium (I), or in both I and II. In one instance, however, CPE were observed only among cultures maintained in the stable glucose-free acid environment of Medium II. This result was obtained in cultures inoculated with a fecal extract designated CR69(076) (10). It was later determined that the specimen in question had been collected from a seven-year-old male, whose case had been clinically diagnosed as hepatitis A. The agent responsible for the observed CPE in Medium II was successfully reisolated from this same fecal specimen, utilizing the procedures described below.

**Replication of CR69(076) Virus**

Difficulties in propagating the CR69(076) agent in medium I or II caused us to adopt the following methodology, which permitted rapid and consistent replication: Confluent monolayer cultures of WI-38 cells were maintained in an EBM prepared from 100X concentrates of vitamin and amino acid mixtures and from a 200 millimolar solution of glutamine in modified Hanks’ BSS containing an equivalent quantity of galactose in lieu of glucose (11, 12). This medium (Medium III), which was gassed with CO₂, also included 0.035 per cent NaHCO₃, 0.6 per cent FCS, sodium pyruvate (55 mg/liter) (11), alanine (45 mg/liter) (11), and 5-bromodeoxy-uridine (BrDUR) (20 μg/ml). Two ml of the medium were added to each culture, and the cultures were allowed to incubate at 36°C for 24 hours prior to inoculation. Following gassing, the pH of the medium was 6.0. Following incubation, the medium reached an equilibrium at pH 6.7-6.8.

With this method, infectious virus titers of CR69(076) were found to range from 10^6.9 to 10^7.4 tissue culture infectious doses (TCID₅₀) per ml. Maximum titers were usually reached by the fourth or fifth day of incubation following inoculation.

In brief, few or no CPE were observed in media containing the usual proportions of NaHCO₃ (0.11 or 0.22 per cent) or in media which had become alkaline owing to improperly sealed culture tubes. It was also observed that virus proliferation would occur, but at a lesser rate, if a medium containing a high concentration of NaHCO₃ was first saturated with CO₂ and then added to cell cultures and inoculated.

These observations indicated that an acid environment, as provided by Medium III, was advantageous to the replication of CR69(076). Yet, as mentioned earlier, the use of Medium I resulted in poor replication of this agent—even though the hydrogen ion concentrations of
inoculated cultures ranged from pH 7.4 at the time of inoculation to pH 6.6 following incubation. To clarify this apparent inconsistency, parallel infectious virus titrations were conducted with cell cultures maintained in Medium I and with others maintained in Medium III, as described above. Because of the excessive acid pH that developed in the course of incubation, Medium I was changed on the third and sixth days of incubation.

The results of these titrations are presented in Table 1. It may be noted that the maximum infectious virus titer was almost attained after the third day of incubation in Medium III, while the amount of virus detected in the Medium I cultures was substantially less. After this latter medium was changed, the virus titer determined on the sixth day indicated that the amount of infectious virus had doubled; but only a slight increase was noted in the cultures containing Medium III during the same period. A second change of Medium I on the sixth day resulted in additional increase of the infectious virus titer, though to a lesser degree, by day nine. It should be mentioned here that if medium changes were not made, the CPE would regress; infected cells would become detached from the glass and enter the medium, leaving behind a cell sheet of normal appearance.

**TABLE 1- Influence of different maintenance media upon the infectious virus titer of CR69(076).**

<table>
<thead>
<tr>
<th>Days of incubation</th>
<th>Medium I</th>
<th>Medium III</th>
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<tbody>
<tr>
<td>3</td>
<td>10^2.5^c</td>
<td>10^6.27</td>
</tr>
<tr>
<td>6</td>
<td>10^4.95</td>
<td>10^6.7</td>
</tr>
<tr>
<td>9</td>
<td>10^5.35</td>
<td>10^6.87</td>
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<tr>
<th>Medium changed in inoculated cultures on days 3 and 6 of incubation.</th>
</tr>
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<tbody>
<tr>
<td>^aEBM in Earle's BSS, 10% FCS, 0.11% NaHCO₃. Medium changed in inoculated cultures on days 3 and 6 of incubation.</td>
</tr>
<tr>
<td>^bEBM and Hank's BSS (galactose), 0.6% FCS, 0.035% NaHCO₃, BrDUR (20 µg/ml), Na pyruvate (55 mg/l), alanine (45 mg/l), and gassed with CO₂. Medium not changed during incubation.</td>
</tr>
<tr>
<td>^cTo determine more precisely the 50% tissue culture dose endpoint, each virus dilution was inoculated into 10 WI-38 tube cultures (0.2ml/tube), and the TCID₅₀ endpoint calculated by the method of Reed and Muench.</td>
</tr>
</tbody>
</table>

These findings suggest that the CR69(076) agent not only requires an acid environment for its replication but replicates best inside a narrow acid pH range. In Medium I, cellular metabolism results in a rapid shift of the hydrogen ion concentration from the alkaline side of neutrality to the relatively acid side, quickly passing through that range optimal for viral replication and permitting only limited infection by CR69(076) to occur.

The replication of rhinovirus types requires an acid environment; but it also proceeds better at an incubation temperature of 33°C than at one of 36°C. Therefore, studies were conducted to determine whether a similar situation existed with CR69(076). Simultaneous duplicate titrations were carried out with CR69(076) virus stock. One titration was incubated at 33°C, the other at 36°C. Similar titrations were also carried out with ECHO 25 and these were incubated in the same manner. In both instances, higher infectious virus titers and more rapid virus proliferation were observed at 36°C than at 33°C.

Efforts to detect viral hemagglutinins with human “O” erythrocytes at various temperatures (4°C, 20°C, and 37°C) utilizing normal saline and phosphate buffers (pH 5.7, 6.1, 6.5, 7.1, 7.5, and 8.0) as diluents were unsuccessful.

**Cytopathic Effects of CR69(076)**

The CPE produced by CR69(076) in cultures of WI-38 cells were similar to those produced by enteroviruses. Infected cells stained with Giemsa, and with hematoxylin and eosin—often showed swollen nuclei which later became pyknotic. Although neither cytoplasmic nor nuclear inclusions were apparent, the cytoplasm of some cells contained eosinophilic masses like those seen in enterovirus-infected cells.

Limited investigation of the spectrum of suitable host cells indicated that CR69(076) was unlike the majority of the enteroviruses. Moreover, there was no evidence of CPE in primary cultures of Rhesus monkey renal cells, either under culture conditions normally employed for enterovirus isolation or under those
found to be optimal for CR69(076). Similar negative results were obtained with cultures of VERO, HEp-2, HeLa, and Chang liver cells. However, CPE were noted in cultures of a diploid human foreskin fibroblast cell line (HuFs-6)6, utilizing the methodology described for the replication of CR69(076).

**Physical and Chemical Characteristics of CR69(076)**

Unlike the enterovirus types, samples of CR69(076) showed a significant loss of infectious virus titer following storage at -70°C.

Infected culture fluid, clarified by centrifugation, was distributed in screw-capped vials in 1 ml aliquots and stored in a mechanical deep freezer at -70°C. The vials were then removed periodically and their contents was titrated. As shown in Table 2, a substantial loss of infectious virus titer was observed following storage for three months. Subsequent lowering of the storage temperature to -90°C reduced the rate of loss.

The stability of infectious CR69(076) virus following exposure to 60°C for one hour was also investigated. Dilutions of CR69(076) and ECHO 25 were made with sterile deionized water. These were then incubated in a water bath at 60°C for one hour, placed in an ice bath, and inoculated into cultures of WI-38 cells, utilizing the medium most suitable for each virus type. No evidence of CPE was observed in cultures inoculated with dilutions of ECHO 25 exposed to 60°C, though CPE were observed with similar dilutions maintained at 20°C. In contrast, CPE were observed (and confirmed by passage) in one of four cultures of WI-38 cells inoculated with a 10-2 dilution of CR69(076), following its exposure to 60°C for 1 hour. CPE were not observed with other dilutions. A similar lack of complete inactivation following exposure to 60°C for 1 hour has been reported for the marmoset HeA Agent (5).

CR69(076) samples showed no loss of infectivity titer in comparison to untreated controls following overnight exposure to 20 per cent diethyl ether at 4°C or following three-hour exposure to pH 3.0 at 20°C. Proliferation in cells previously exposed to BrDUR (20 μg/ml) confirmed the ribonucleic acid composition of this agent.

Electron micrographs of concentrated cell culture fluids infected with CR69(076) revealed icosahedral particles having diameters of approximately 29 nm (see Plates 1 and 2). This is within the picornavirus size range and is consistent with the description of the virus-like antigen previously reported (6).

**Relationship of CR69(076) to Known Enteroviruses**

When tested against type-specific enterovirus antisera by neutralization, CR69(076) produced no inhibition with antisera for ECHO virus types 1-24, 26, 27, 29-32; Coxsackie group A, types 1-3, 9; Coxsackie group B, types 1-6; and poliovirus, types 1-3. However, rabbit antiserum for ECHO virus type 25 was found to neutralize 100 TCID₅₀ of CR69(076) at a dilution of 1:400. By comparison, it was determined that this same antiserum would neutralize 1,000 TCID₅₀ of the homologous ECHO virus (type 25, strain JV-4) at a 1:10,000 dilution. The significance of this observation is uncertain, since it has been reported that ECHO virus type 25 antiserum inhibits ECHO virus type 12 at a dilution of 1:100 (13). Also, it should be mentioned that cross-relationships have been reported between Coxsackie group A types 3 and 8, 11 and 15, 13 and 18; ECHO virus types 1 and 8, 12 and 29, 6 and 30; and,

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6 A product of North American Biologicals, Inc.
PLATES 1 and 2—Representative samples of virus particles obtained from frozen/thawed cell cultures centrifuged to settle cellular debris. The supernatant was then centrifuged at 150,000 revolutions per hour for three hours, and the resulting pellet was suspended in 10 per cent formalin and negatively stained with 2 per cent phosphotungstic acid. The bar in Plate 1 represents 100 nm and that in Plate 2 represents 60 nm.

to a minor degree, poliovirus types 1 and 2 (7). Cross-neutralization studies of CR69(076) are in progress.

Relationship of CR69(076) to HeA

Paired sera collected from disease cases that occurred during epidemics of hepatitis A (1967) and hepatitis B (1964) in Costa Rica (8, 9) were examined by neutralization and complement-fixation procedures using CR69(076) as the antigen (see Table 3). Of 20 paired sera collected during the 1964 (hepatitis B) epidemic, only one pair showed a significant rise in the antibody level of the convalescent serum. In contrast, when paired sera from the 1967 (hepatitis A) epidemic were investigated, eight out of 24 serum pairs (33 per cent) showed significant increases in the antibody levels of the convalescent sera. It is of interest to note

<table>
<thead>
<tr>
<th>Year</th>
<th>Hepatitis type of each epidemic</th>
<th>No. of paired sera tested</th>
<th>No. of paired sera with significant antibody responses$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1964</td>
<td>B</td>
<td>20</td>
<td>1 (5%)</td>
</tr>
<tr>
<td>1967</td>
<td>A</td>
<td>24</td>
<td>8 (33%)</td>
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</tbody>
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$^a$An antibody level in the convalescent serum representing at least a fourfold rise.

that subsequent investigations of sera for Australia antigen revealed the nature of the epidemics (9).

Discussion

The enterovirus-like nature of the HeA agent isolated in marmosets (5), the demonstration
by electron microscopy of an enterovirus-like antigen in feces of a human volunteer with hepatitis A (6), and the absence of any in vitro isolation of the etiologic agent, indicate that current enterovirus isolation procedures have limitations despite the success achieved in isolating many enterovirus types. The need for new approaches to virus isolation has also been brought out by electron microscopic demonstration of orbivirus-like particles in fecal samples from infants with diarrhea and the failure to isolate such agents from positive-appearing samples by conventional techniques (14, 15).

This report presents data regarding an agent whose presence would have remained undetected if only conventional isolation methods were employed. Regardless of the role of CR69(076) in disease, these observations are felt to be significant and should be given further consideration in those instances where efforts to isolate a causative disease agent have been unsuccessful.

The CR69(076) agent's need of a limited hydrogen ion concentration in order to achieve optimum replication is a unique characteristic, unlike any of the features attributed to known enterovirus types. Conceivably, it may apply in principle to many of the Coxsackie A virus types which infect man, but which to date have only been isolated in newborn mice, as well as to newly found virus-like agents such as the orbiviruses.

From our investigations, it appears that extreme acidity does not affect adsorption of the virus on the cell surface, interfering instead with virus penetration. Although the way this penetration is accomplished remains uncertain, enzymatic activity has been considered a possible mechanism. This activity would be influenced by the existing hydrogen ion concentration. Once penetration is accomplished, virus replication does not appear to be affected by the existing extracellular environment. It is presumed that a similar situation exists inside the cells when the extracellular environment is within the alkaline range of neutrality as when it is in the acid range.

Our investigations indicate that CR69(076) is an enteric virus. This conclusion is based on its size, particle morphology, nucleic acid composition, ether stability, optimal replication at 36°C, resistance to acid pH, and CPE produced. However, CR69(076) also possesses characteristics unlike those of known enteric virus types. This has been shown by its limited spectrum of host cell types, its limited replication in a narrow pH range on the acid side of neutrality, its failure to replicate under alkaline or extremely acid conditions, and its relative instability at -70°C under the storage conditions described. On the basis of these observations, it is felt that CR69(076) may be the prototype strain of a new group.

CR69(076) is markedly similar in its physical-chemical properties to those described for the HeA virus isolated in marmosets (5). Filtration studies of the latter show it to have a particle size less than 50 nm but greater than 25 nm. Further support for the enterovirus-like nature of the HeA virus was obtained by immune electron microscopy studies of fecal samples from a volunteer with HeA (6). These detected a virus-like particle having an icosahedral morphology and an average size of 27 nm. Electron micrographs of CR69(076) (Plates 1 and 2) reveal a particle with an icosahedral morphology and an average diameter of 29 nm, two features showing similarity to other enteroviruses and indistinguishable from those of the reported virus-like antigen (5).

Consideration of these data, together with the source of CR69(076) and this virus-like antigen, indicate that further study of the relationship between these agents is needed even without prior demonstration of antigenic similarity.

It is not possible to claim an etiologic relationship between CR69(076) and HeA on the basis of a single virus isolation from a case of HeA and nine immunologic responses from cases of the disease. There is thus an obvious need for additional investigation along the lines indicated here.

Among other things consideration should be given to the possible existence of multiple HeA virus types in evaluating relationships between
the various reported HeA isolates. One need only recall the search for the virus of the common cold, its initial isolation by the senior author and colleagues (16), and the existence today of at least 100 distinct serologic types. Conceivably, CR69(076) may not have been the prevailing virus type involved in the 1967 epidemic; and the serologic responses obtained in 33 per cent of the paired sera from cases of clinical hepatitis may represent infections caused by a second virus type occurring at a low level within a geographic area known to be endemic for the disease (9).

Consideration should also be given to the disease itself, for the very nature of hepatitis may make serologic diagnosis difficult, particularly if the infected individual experienced a prolonged acute or preicteric phase of the syndrome. Under such conditions, it is reasonable to assume that the immune response already has been elicited by the time jaundice becomes apparent, medical attention is sought, and appropriate serum samples are collected for study. Under such conditions it is possible that maximum levels of antibody may already have been attained; or, if antibody increases were detected, these may not be within a range that should be considered significant. It is obvious, though perhaps not possible in practice, that initial or acute serum samples should be collected early in the preicteric stages of the disease in order to permit a true serologic evaluation.

The desirability of isolating additional strains of the CR69(076) virus should also be considered. It is possible that a loss of infectious virus titer, as the result of prolonged storage of specimen material, may have occurred during our investigations. Such a development could have a most critical effect, particularly if the virus were excreted at a low level at the time the specimen was obtained and if the specimen were collected in the later stages of the disease. It is therefore felt that investigation of fresh fecal samples, collected as close to the onset of jaundice as possible, would be more productive.

Volunteer studies have indicated that virus excretion ceases about one week after the onset of jaundice (17). It has been our experience that patients often have jaundice for several days before seeking medical attention. Because of its clinical course, the collection of a proper specimen appears to be an even more critical problem in etiologic investigations of HeA than in the investigation of other enterovirus-associated illness. Obviously further investigations in this area are urgently required.

**SUMMARY**

An enterovirus-like agent, designated CR69(076) and possessing properties unlike the known enteroviruses as well as an unusual growth requirement, was isolated from a fecal sample collected from a seven-year-old male Costa Rican experiencing clinical hepatitis. The agent, isolated in cultures of WI-38 cells, replicates best within a stable and relatively narrow pH range provided by the maintenance medium. Little or no replication has been observed when the medium has a pH above 7.0 or an excessively acid pH.

The agent is not a rhinovirus, being acid-stable and replicating best at a temperature of about 36°C. Moreover, it failed to infect a spectrum of cells usually susceptible to infection by enterovirus types, and proved relatively unstable when stored at a temperature of -70°C under conditions described in the text.

CR69(076) has a particle diameter of approximately 29 nm and an icosahedral morphology. It is ether-resistant and possesses a ribonucleic acid core. Although some antigenic crossing was noted when it was tested with antiserum to ECHO virus type 25, no inhibition was noted when it was tested with antisera to other enteroviruses.

Besides being isolated from a case of hepati-
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tis A, the agent prompted an increased antibody response in paired sera from nine cases of hepatitis A in Costa Rica. Furthermore, many of the properties of CR69(076) were found to be similar to those described for the hepatitis A agent isolated in marmosets and previously observed in the feces of a volunteer with hepatitis A by investigators at the United States National Institutes of Health.

ADDENDUM

In an effort to obtain additional virus isolates, 160 specimens of feces, urine, and liver biopsies were collected from cases of clinical hepatitis occurring in Costa Rica and the United States from 1963 to the present. These were inoculated into WI-38 cell cultures, utilizing the methodologies previously described. Although studies are still in progress, virus isolations have already been obtained from one liver biopsy specimen, one urine specimen, and two fecal specimens. Past efforts to isolate agents from these same specimens were unsuccessful when they were inoculated into cultures of WI-38 and Rhesus monkey cells maintained under the usual culture conditions employed for enteric virus isolation. The relationship of these recent isolates to CR69(076) has not yet been determined.

REFERENCES

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**HEPATITIS A OUTBREAK IN MINNEAPOLIS, U.S.A.**

Twelve cases of hepatitis A were reported to the Minneapolis Health Department in the week ending 22 April 1974. Investigation revealed that seven of the individuals were employees of a large retail store. Since the outbreak appeared to have a common source centered in the store, practicing physicians, hospital admission directors, medical records librarians, and the general public in the Minneapolis-St. Paul area were informed of the outbreak and asked to report all cases of hepatitis A. Between 22 April and 11 May an additional 136 cases were reported, bringing the total to 148. Twenty-eight of the 148 patients were employees of the department store and 77 were other persons who had eaten in the store’s restaurants.

Results of food history questionnaires from 66 store-associated cases and 482 controls implicated lunch served in the store’s basement restaurant as the vehicle of infection; cold sandwiches with lettuce and tossed salads carried the greatest risk. Of 12 patients who ate in the restaurant only once, 10 ate there on 15, 16, or 18 March. One of the employees who regularly prepared sandwiches and placed salad mix in bowls by hand became ill on 18 March.

All persons who had eaten at the store since 15 February were advised to get injections of immune serum globulin; the Minneapolis Health Department gave almost 10,000 injections and private physicians gave many more. Health department officials discussed their findings with store managers and offered a series of recommendations which stressed more frequent hand washing for food handlers and less handling of food and ingredients where possible. (U.S. Center for Disease Control, *Morbidity and Mortality, Weekly Report*. Volume 23, No. 19, 1974.)