Human volunteer and tissue culture studies of viral hepatitis

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One of the problems that investigators regularly have encountered in studies of viral hepatitis is the lack of sufficient numbers of controlled and documented specimens obtained from human beings known with certainty to be in the pre-exposure phase, the incubation period, or the early acute phase of the illness. In an effort to meet this need, studies were undertaken with the collaboration of the Walter Reed Army Institute of Research (Drs. Marcel E. Conrad, Bertram F. Felsher, and Allen Ginsberg, Division of Medicine). In adult male volunteers the infectiousness was studied of a serum pool (the MS-1 pool) which previously had been proved[1] to produce in children mild infectious hepatitis, negative for Australia/SH antigen.[2]

Studies in volunteers

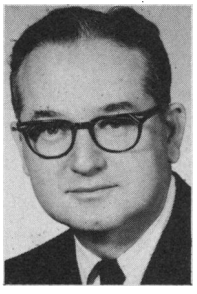
In the first of these studies, MS-1 serum was given to volunteers in a prison population.[3] The dosage was 0.05 ml. administered orally. The volunteers were fully informed concerning the purposes and procedures of the study. They were screened by extensive physical and biochemical testing before admission to the study group, and further pretesting was performed after the men were admitted to the isolation unit and prior to introduction of the test material.

The MS-1 serum and the control, uninfectious material (bovine plasma) were administered under code so that neither the investigators nor the volunteers knew which material each individual received; liver biopsy specimens also were examined under code. Five volunteers received control material and none developed evidence of hepatitis (Fig.1). Three of the 10 men who received MS-1 serum (subjects C, F, and K) developed clinical, biochemical and liver biopsy evidence of hepatitis. A typical course of the illness is shown in Fig.2 which depicts the responses of subject K.

In the second study, plasma from one of the men who developed hepatitis (F, day 29) was tested. It did not produce hepatitis when given orally in a 0.1 ml. dose to 10 additional volunteers (Table I); this specimen had been taken during prodromic symptoms, but eight days before the period of rising transaminase levels. In the third study, plasma obtained at the time of maximum transaminase levels from another of the men who became infected in the first study (K, day 30) produced hepatitis in five of 10 volunteers (dosage 0.1 ml. administered orally).

Further studies with these volunteers have been conducted.[4] Three of the men who had developed hepatitis in the first and third studies after receiving MS-1 or K-30 materials orally (K, B, Mc) showed immunity to the disease when challenged one-and-a-half to three years later by subcutaneous inoculation with serial 0.1-ml. doses of K-30 plasma (study IV in Fig.3). Unexpectedly it was found that resistance sufficient to protect against development of hepatitis after oral administration of the virus could be overwhelmed by subcutaneous inoculation of the agent. Among four men parenterally challenged who previously had developed no illness after oral administration of MS-1 or K-30 infectious material, two (RW and PMc) developed clinical hepatitis following the parenteral inoculation of K-30 plasma. Two others, PM and RMa, continued to show resistance to infection even by the more sensitive parenteral route. It thus appears that immunity can be developed, but that it is not solid, all-or-none, and is dependent upon the dose or route by which exposure occurs.

Support for this concept was gained from our experience in studying the large epidemic of hepatitis in



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Delhi, India, in December 1955.[5] During the epidemic period, one of us (JLM) was fortunate to have been in India, working as a staff member of the Rockefeller Foundation at the Virus Research Centre in Poona, and participated in studying the epidemic. During this outbreak, about 35,000 cases occurred, a case rate of 2000 per 100,000 in the single month of the epidemic. The epidemic was found to have been caused by heavy contamination of the city's water supply by sewage during a single week in mid-November, 1955. The peak of the epidemic occurred 40 days later; thus the incubation period was somewhat longer than that which has been noted for infectious hepatitis. A possible explanation is that the disease was produced in partially immunized persons. At the time, it was stated:[5]

"That the population of Delhi was partially immune is known from the occurrence of cases in the community

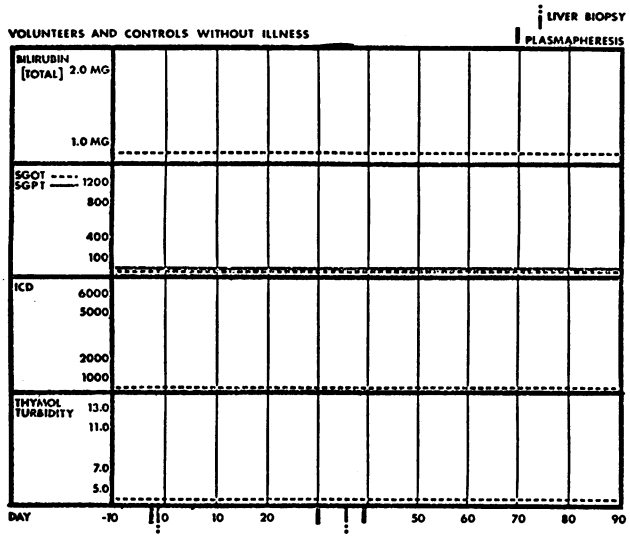

FIG.1—Laboratory test record typical of the observations with volunteers who received control materials by the oral route. Serum fractional bilirubin, isocitric dehydrogenase (ICD), serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), and thymol turbidity remained at normal levels from 10 days before introduction of the test material to 90 days post-feeding. Days of plasmapheresis and liver biopsies are indicated at the top and bottom of the figure.[3]

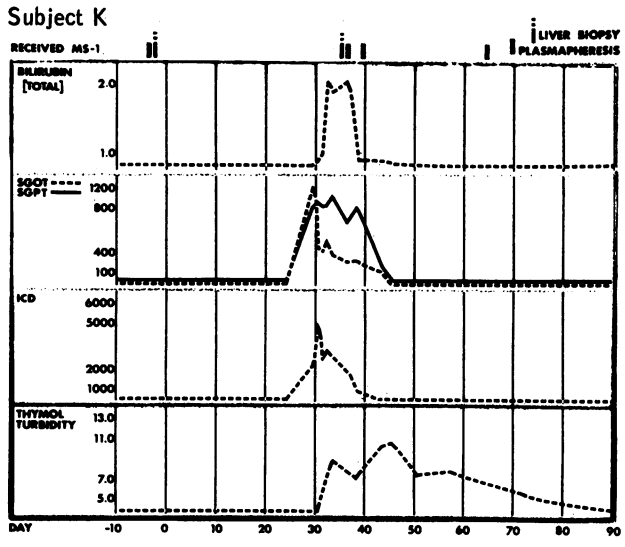

FIG.2—Record of laboratory results with volunteer K who developed hepatitis after oral administration of MS-1 serum, and whose 30-day plasma was infectious in subsequent tests. This infectious plasma was obtained at the time of maximum transaminase elevation. A liver biopsy taken on day 36 was interpreted as acute hepatitis, mainly portal.[3]

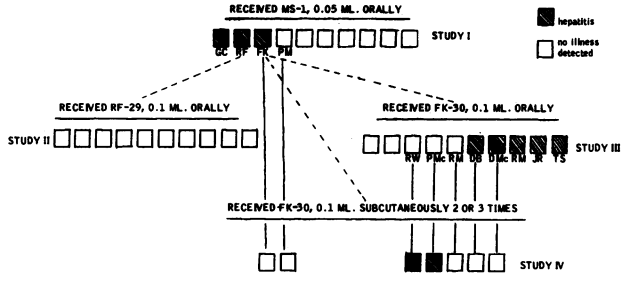

FIG.3—Diagram of hepatitis transmission studies I through IV, in adult male volunteers. In studies I, II, and III, MS-1 or K-30 material was administered orally, while in study IV, K-30 plasma was administered subcutaneously.

Table I.
Transmission to volunteers

	Results after oral administration			Results after subcutaneous administration	
				Phase 4 0.1 ml. K-30	
Exposed and control groups studied	Phase 1 0.05 ml. MS-1 serum	Phase 2 0.1 ml. F-29 plasma	Phase 3 0.1 ml. K-30 plasma	Men infected after oral administration of MS-1 or K-30	Men resisting oral exposure to MS-1 or K-30
Infected exposed	3/10	0/10	5/10	0/3	2/4
Infected controls*	0/5	0/5	0/5		

*Received bovine plasma or pre-exposure volunteer serum.

before the epidemic. Immunity is probably relative to the challenge dose, and may be overridden by exposure to a massive dose of virus. However, in such individuals, the onset of disease may be delayed and the disease may be mild."

A large majority of cases occurred in the age group between 20 and 40 years. The explanations offered included the fact that severe hepatitis with jaundice is not often seen in the young. Secondary cases would not be expected because few persons would have remained susceptible after the widespread and massive exposure. However, there were no observed secondary cases, even in areas which were not on the contaminated water lines. Moreover, jaundice epidemics have been recorded which included a substantial number of cases in the ages between eight and 15 years, a group that largely escaped in the Delhi 1955-56 epidemic.

A more attractive hypothesis is that the epidemic was imposed on a partially immunized population — one sufficiently immunized by the endemic prevalence of the disease to prevent major outbreaks from infection that occurs in response to normal exposure. Thus hepatitis in this epidemic would have occurred only in those in whom the massive exposure was such that it overrode the immunity present in the individual. Widespread immunity in the population would have prevented secondary cases from occurring at a rate above the normal one at which cases occur in Delhi. This explanation fits the observations in an area such as South Delhi, which, like the other areas of the city, experienced no secondary cases. The people in South Delhi were not generally exposed to the water of the city, and cases occurred only among those daily migratory workers who worked in parts of the city where they drank the Delhi water, and then returned at night to their homes. No secondary cases occurred in South Delhi, indicating that its inhabitants had sufficient immunity to protect them against normal exposure to infected persons.

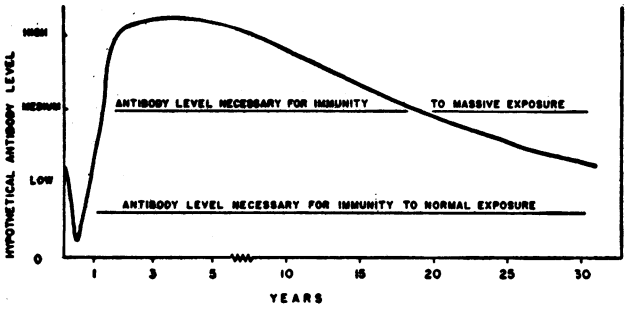

FIG.4—Theoretical explanation of age distribution of cases in the Delhi hepatitis epidemic. Early acquisition of antibody and its fall over a period of years is shown. A low level of antibodies suffices for protection against the usual dose of virus to which a person is exposed, but a high level of antibody is needed for protection against high doses of virus, such as occurred in Delhi in November, 1955. High antibody levels would be expected in those most recently infected, namely the young, and therefore cases might be expected to occur with much higher frequency in those over the age of 15 to 20 years.[5]

We have drawn a hypothetical curve (Fig.4) for antibody levels to an enteric virus in an area like Delhi where hepatitis is endemic. One might expect that under conditions of life in Delhi young children would develop antibodies to high titres and that these would be maintained for a period of some years, perhaps by multiple exposures and infections; then, because solid immunity would be present to protect against further exposure to usual doses, antibodies would fall steadily with the course of time. A low level of antibody seems sufficient for immunity to normal exposures. Immune phenomena not being all-or-none, we would expect that higher antibody levels would be necessary for immunity to massive exposures of the type which took place in Delhi, and the cut-off point of antibody titre for protection would be correspondingly raised. If this concept is true, then we would expect to find cases occurring chiefly after the age of 15 or 20 years, which agrees with the observations in the Delhi epidemic.

Studies in tissue culture

Many attempts have been made over the years to isolate hepatitis viruses, but with no recognized success.[6]

Table II.

Results of D6 testing of coded plasma from patients who were infected orally with K-30 day plasma

Patient and day		Code	Primary passage	Second passage	Third passage	Fourth passage	Fifth passage	Sixth passage
R	0	7	NSD*	±	±	—	—	—
R	27	8	—	±	+	+	+	+
Mc	0	5	—	±	±	±	±	—
Mc	32	6	±	±	+	+	+	+
Mi	0	4	NSD	—	—	—	±	—
Mi	36	3	NSD	—	—	±	—	—
B	0	2	—	—	—	±	—	—
B	40	1	NSD	+	+	+	+	+
S	0	9	NSD	—	—	—	—	—
S	49	10	NSD	—	+	+	+	+
Passage control			—	—	—	—	—	—

*NSD = Nonspecific degeneration

Among the most determined efforts were those made by McLean and his associates using Detroit 6 (D6) cells; the results were irregular, however, and the attempts were abandoned.[7,8]

With the availability of plasma proven to be infectious upon human passage, it was decided to attempt to isolate and propagate, in tissue culture, agents from the serum and plasma of the volunteer participants. A cloned line of D6 cells obtained originally from McLean[9] was used. When acute-phase plasmas of two of the volunteers (K and C) were inoculated and passed in the cloned D6 cells, cytopathic effects occurred. No cytopathogenic agents were present in the plasmas of the same volunteers before inoculation, or in the cell controls carried in parallel with each culture passage of the material from the patients.[3]

Since an agent cytopathogenic for D6 cells was repeatedly isolated in both the Houston and the Chicago laboratories from specimens of subject K, studies have been continued particularly with the specimens from this subject and from the further sets of volunteers who received his plasma.[10] A cytopathogenic agent was isolated from the K-30 specimen after two passages in D6 cells. Additional passages in these cells were required to reveal virus in the specimens taken later in the course of disease; with the day 37 and day 39 samples, virus could be detected only after the fourth and fifth culture passages. This suggests that the concentration of virus in the plasma decreased in the course of the illness. The K virus was carried for more than 20 passages in tissue culture, and yielded titres of about 10^4 TCD_{50} per ml.

Samples taken from the volunteers who developed hepatitis after oral administration of the K-30 plasma were coded and studied in D6 cells. The samples included specimens taken before exposure and during each patient's period of elevated transaminase. As shown in Table II, four of the hepatitis patients (R, Mc, B and S) yielded a cytopathogenic agent in their acute phase specimens, whereas all five pre-infection samples were negative.

The cytopathic effect of the virus on the cloned D6 cell line was similar to that described by McLean.[7] Cells in the infected cultures became small, round, and sharply outlined, and the confluency of the monolayer was lost, sometimes in two to three days (Fig.5). Specific degeneration could be read only through day 6 or 7, since control cultures also begin to undergo nonspecific changes on day 4 or 5.

Table III.

Assay for echovirus 11 neutralizing antibodies and for interfering factor in plasma of patient K

Sample	Echovirus neutralization CPE*	Echovirus interference CPE*
Pre-serum	4	4
30-day plasma	3	0
37-day plasma	3	0
100-day plasma	3	4
Echovirus control	4	4

*Numbers represent degree (on a 0 to 4 scale) of echovirus CPE at 36 hours after challenge.

Differential filtration of the infectious agent through gradacol membranes indicated its size to be approximately 18 to 20 nm. Virus particles of similar diameter were also observed by electron microscopy of cell lysates from the infected cultures (Fig.6). A number of empty particles were observed in many of the preparations studied (Fig.6c). Empty particles which are devoid of nucleic acid are well known to occur when viruses replicate, and they are usually an indication that the preparation is low in infectivity.

Like other small viruses, the K virus was found to resist overnight treatment with ether at 4°C[10] and in subsequent studies[11] it has also been shown to be heat-stable (56°C for 30 minutes) and resistant to acid pH. It contains DNA, as shown by results in our laboratory and independently by Overby *et al.*[12]

During safety testing of the plasmas from the first set of volunteers, it was observed that acute phase plasmas from one of these subjects (K) interfered with the replication of echovirus 11.[3] No interference was produced by the corresponding specimens from patients C and F. Since measurement of interference with a superinfecting virus is a useful method for assay of viruses of limited cytopathogenicity, we attempted to utilize this property for further studies of the specimens.[10] To determine whether the interference was specific for the acute phase sample, cell cultures were inoculated with pre-infection

Table IV.

Interference after passage of K virus in D6 cells

Plasma day	Number of passages in D6 cells	Echovirus CPE* 24 hours	36 hours	48 hours
30	11	0	0	1
30	15	0	0	0
30	11 + 1 in GMK	2	4	4
30	11 + 1 in HEK	2	4	4
D6 cell control	Passage 11	3	4	4
D6 cell control	Passage 15	3	4	4
Echovirus		3	4	4

*Same legend as in Table III.

Table V.

Interference and virus particles in D6 isolates from volunteers infected with K-30 plasma

Source of isolate (Patient and day)		CPE in D6 cells	Echovirus interference by D6 isolates	20 nm. particles by electron microscopy
R	0	neg.	neg.	neg.
	23	+	neg.	
	25	+	+	
	27	+	+	+
Mc	0	neg.	neg.	
	27	+	neg.	
	30	+	+	
B	0	neg.		neg.
	40	+		+

Note. Blanks indicate a test was not run.

serum of subject K, and with plasmas from post-infection days 30, 37 and 100. Ten days later the cultures were challenged with an echovirus. Results are shown in Table III. No interfering effect was found in the pre-exposure serum nor in the plasma taken 100 days after oral administration of the MS-1 serum pool. However, both the day 30 and the day 37 plasmas interfered with the development of echovirus CPE.

Experiments were conducted to determine if the agent isolated in D6 cells from K's plasma also interfered with the echovirus. Human embryonic kidney (HEK) or green monkey kidney (GMK) cells were inoculated with various passage levels of K virus or with harvests of uninoculated D6 passage control cells. From the results shown in Table IV it can be seen that the K virus grown in D6 cells interfered with echovirus but that after one passage in primary human or monkey cell cultures the ability to interfere was lost. Control material from uninoculated D6 cells had no interfering effect. The titre of the interfering agent was 10^4 interfering doses per ml. — about the same as the TCD_{50} infectivity of these passages when tested by CPE in D6 cells.

As described earlier, several acute phase plasma samples from volunteers who developed hepatitis after oral administration of the K-30 day plasma yielded a cytopathic agent in D6 cells. As in the original K-30 material, an interfering factor was detected. It could be demonstrated in isolates from the acute phase plasmas of three of the passage patients, but not in their pre-infection plasmas (Table V). The tissue culture fluids from the third or fourth passage in D6 cells were tested for interference. Isolates from day 23, 25, and 27 plasma samples from R were cytopathic, but only the day 25 and 27 isolates were found to interfere with echovirus replication. Mc-27 and 30-day plasma isolates were both cytopathic, but only the day 30 isolate produced echovirus interference. Table V also indicates that 20-nm. particles similar to those detected in K-30 day isolate were found by electron microscopy in the R-27 and B-40 day isolates.

In further tissue culture studies[11,12] the acute phase plasma of K has been found to contain a virus which shares some properties with the H-3 member of the parvovirus (picornavirus) group. The K agent is small, 18 to 20 nm. in diameter, contains DNA, is resistant to heating at 56°C. for 30 min., to acid pH and to ether. As found with some other members of the parvovirus group, K virus agglutinates red blood cells of various species. In this study it was found that K virus agglutinated human O and guinea pig cells but not rhesus monkey cells. H-3 virus did not agglutinate human O or rhesus cells but did agglutinate guinea pig cells. Thus on the basis of the hemagglutination reaction K seemed to differ from H-3.

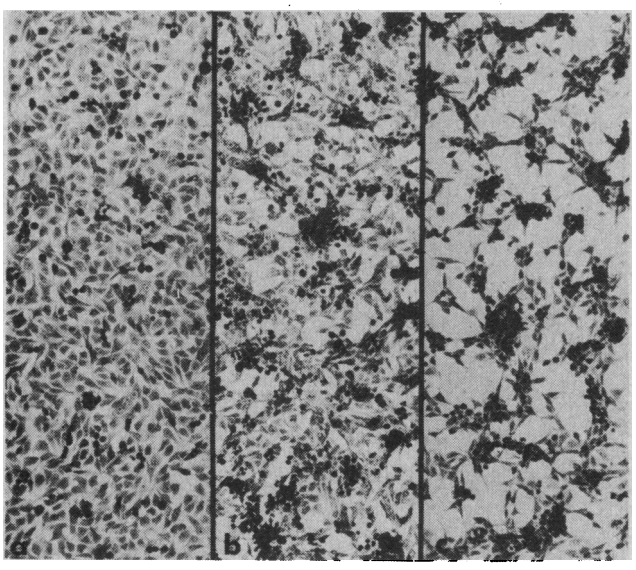

FIG.5—D6 cells infected with K-30 day isolate. (a) normal cells; (b) cells showing partial CPE; (c) cells with 4+CPE

Additional evidence which suggests that K virus is a unique parvovirus is seen in the differences in susceptibility of the different cell lines tested. Toolan *et al.*[13] have shown that H-1 and H-3 replicate in permanent human cell lines but not in primary cells. H-1 can replicate in primary human lung cells when cells are co-infected with a helper adenovirus. We have found that K does not replicate in primary human kidney cells when inoculated alone or with adeno 7 as helper. Since Toolan *et al.*[13] used adeno 12, the failure of adeno 7 to enhance K in HEK cells may be a property of the adenovirus used as helper, since differences in enhancement of the adenosatellite viruses have been noted with different adenovirus helpers.[14] It is noteworthy that adeno 7 does enhance the multiplication of K virus in D6 cells a hundred-fold. The hemagglutination-inhibition and neutralization data indicate K virus is antigenically related to H-3 but not to H-1. Early antiserum prepared in rabbits against K virus reacted only with K virus, and not with the parvoviruses H-3, RV, or X-14. Antiserum prepared by inoculating young hamsters with K-30 virus reacted with all the viruses tested (RV, X-14, H-3), with highest titres against K itself. Titres were two-fold lower against H-3 and five-fold lower against RV and X-14. In the neutralization tests, K was not neutralized by immune sera prepared against H-1 and HB antigens, but was neutralized by antiserum against H-3. These findings suggest a strong relationship between H-3 and K but not an identity. In some respects the relationship is like that observed within some of the enterovirus serotypes.[15]

Summary

The MS-1 infectious hepatitis serum pool, previously shown to produce hepatitis in children, produced hepatitis in three of 10 adult male volunteers when 0.05 ml. of the serum was administered orally. Plasma obtained from one of the infected volunteers, K, during his period of maximum serum transaminase levels (day 30) produced hepatitis in five of 10 additional volunteers after oral administration. Three of the men who had developed hepatitis in these initial studies, and four who had resisted the oral exposure, were challenged one-and-a-half to three years later by subcutaneous inoculation of the K-30 plasma. The three who had developed hepatitis after the oral challenge were now immune, whereas two of the four men previously resistant now developed hepatitis.

A virus was isolated from K-30 plasma. The isolate produced a cytopathic effect (CPE) in a cloned line of Detroit 6 (D6) cells. Differential filtration of the infectious agent through gradacol membranes indicate its size to be approximately 18 to 20 nm. Spherical virus particles of similar diameter were observed by electron microscopy of cell lysates from infected cultures. In addition to yielding the virus in D6 cells, the K-30 plasma, but not the pre-exposure or convalescent phase plasma of this subject, produced interference against

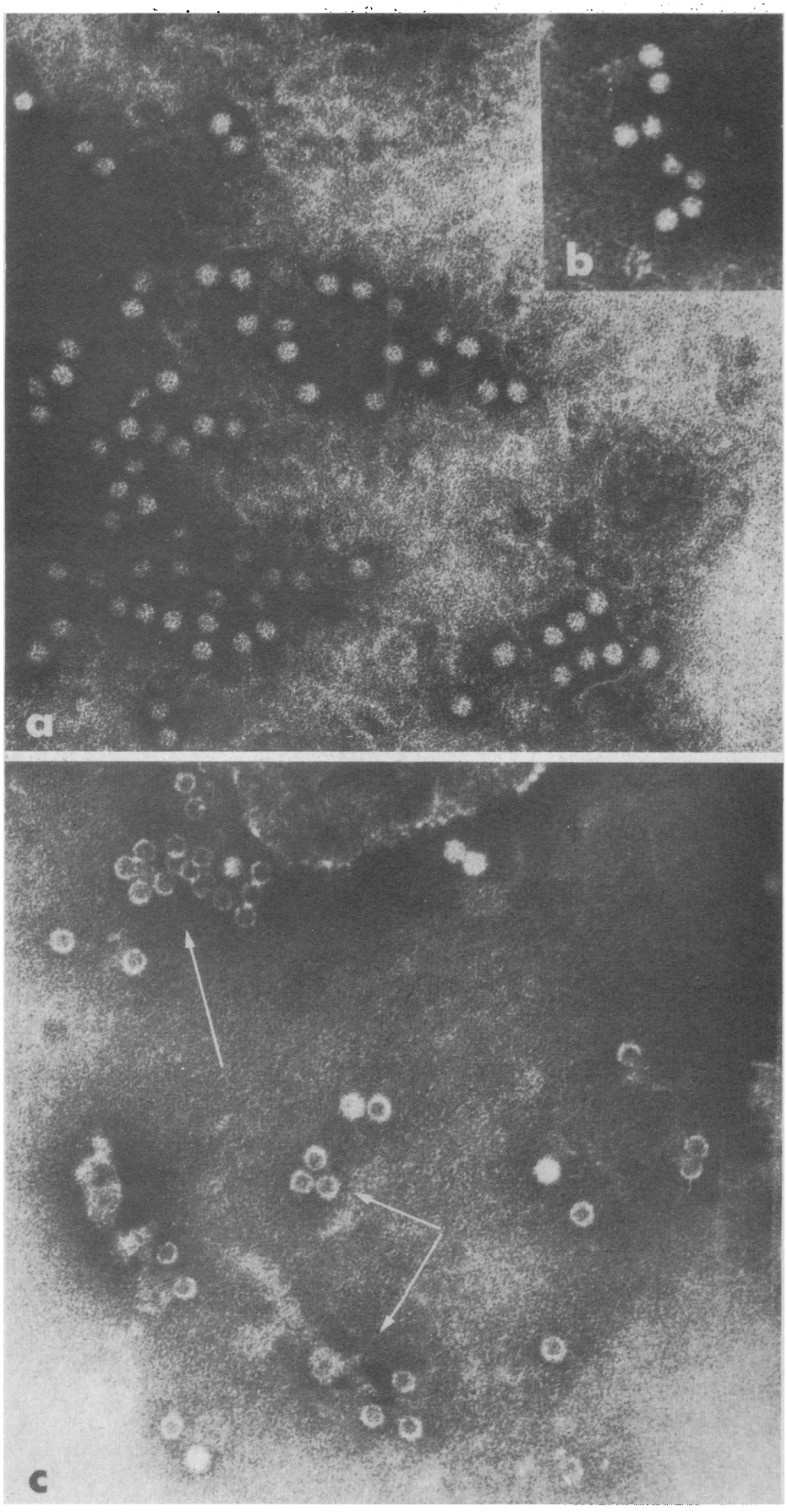

FIG.6—Electron micrographs of (a) virus isolated in D6 cells from K-30 day plasma; (b) virus isolated from R-37 day plasma; (c) numerous empty particles (arrows) seen in some K-30 preparations (X 145,000)

replication of echovirus 11 in primary cultures of human or monkey kidney cells.

Similar virus isolates were obtained from acute phase plasmas of four of the men who developed hepatitis after oral administration of the K-30 plasma; an interfering factor was found in acute phase plasmas of three of these men.

Further tissue culture studies have shown K virus to contain DNA, and to be heat-stable, ether-resistant and stable to acid pH. It shares properties with the parvoviruses (picornaviruses), and is antigenically related to certain members of the group.

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Discussion

DR. KRUGMAN: I would like to comment about the interpretation of Dr. Melnick's study in which four men were given MS-1 by mouth; subsequently, two developed hepatitis and two did not. Later, when the four men were challenged by a parenteral inoculation of MS-1 serum, only the two who had developed hepatitis previously were protected. Our experience has indicated that the amount of MS-1 serum used for this study represents a 50% infective dose. Therefore, I believe that two men were not infected by mouth, and they were still susceptible when they received the larger dose of MS-1 serum parenterally. We have not observed the "partial-type" of resistance which you suggest as characteristic of the Delhi epidemic of hepatitis. One attack of viral hepatitis, type A, MS-1 strain has been followed by solid immunity. I don't know how to explain the phenomenon which was observed in Delhi, but I don't believe that you demonstrated evidence of partial immunity in the two men who were not resistant to challenge with MS-1 serum given parenterally.

DR. MELNICK: The Delhi outbreak can be explained on the basis of a large dose of virus overwhelming the decreased immunity expected in adults. The volunteers in the current studies were prisoners. Coming from the lower socio-economic group, it is highly improbable that they had not been exposed to the common viruses for which antibodies are found in normal human gamma globulin. Therefore, the new data on the volunteers suggest that oral resistance to hepatitis virus A may be overcome by administering the virus parenterally.

DR. MAYNARD: A question has arisen as to whether Kirk agent is a tissue culture contaminant. Many of you remember the experience some years ago in which a number of adenoviruses, particularly adenovirus type 3, cross type 16, was isolated from a number of cases of hepatitis in two Indian reservation epidemics in Arizona. We have subsequently recovered a small agent very similar to Kirk agent from human embryonic lung tissue culture passage material. This material was derived from cells originally inoculated with a stool sample from a hepatitis patient and from which an adenovirus type 3 was isolated. The small agent emerged after the adenovirus was destroyed by heating. This agent grows quite well in D-6 cells. From another patient, whose acute phase serum was inoculated into our continuous line of chimpanzee liver cells, an adenovirus type 3 was again isolated. After several passages in ChL the adenovirus was destroyed by heating, and another small particle, which we called "agent Y", emerged. We have made antibody to Kirk agent as well as to these other small agents in guinea pigs, and by hemagglutination inhibition, neutralization, and gel diffusion procedures, all three agents seem to be very similar, if not identical. Since the adenovirus helper effect has been demonstrated for these agents, their emergence from cultures after primary isolation of adenoviruses becomes reasonable.

DR. CONRAD: We brought a large number of acute hepatitis specimens back from American soldiers in Korea in 1962 and 1964, and cultivated adeno 11 or adeno 16 double prime out of the majority of those specimens. There were two specimens out of which Burkey cultivated no adenovirus, but a small virus came out of the culture. When he suppressed the adenovirus in others he found again this small virus. The two patients who did not have the adenovirus in their serum were the two mildest patients we had ever seen.