# Recovery of Hepatitis Agents in the Marmoset from Human Cases Occurring in Costa Rica (37005)

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Deinhardt *et al.* (1, 4) and Holmes *et al.* (2, 3) have presented convincing evidence that marmosets of the species *Saguinus nigricollis* (white-lipped marmoset) and *S. oedipus* (cottontop marmoset) develop signs of hepatitis when inoculated with serum or plasma from patients with acute infectious hepatitis A but not with serum hepatitis B. Hillis (5) and Lorenz *et al.* (6) reported similar findings in tests in marmosets of the species *S. mystax* (white mustached marmoset). Parks *et al.* (7, 8) carried out tests in *S. oedipus* and suggested that the phenomenon represented activation of latent "marmoset hepatitis" rather than the transmission of human disease based on the development of hepatitis findings (elevated transaminase, histologic hepatitis) in control as well as test animals and on a reported immunologic relationship between the "marmoset hepatitis virus" and Deinhardt's GB strain of hepatitis virus propagated in marmosets.

The present study records the induction of hepatitis in mystax species marmosets given bloods from cases of hepatitis A among patients in Costa Rica. Such hepatitis was not induced in marmosets given a variety of normal control materials or on serial passage in marmosets of normal marmoset serum. The findings are in full accord with those of Deinhardt, Hillis, and Lorenz and their associates (1–6); they are in disagreement with those of Parks *et al.* (7, 8).

*Materials and Methods. Marmosets.* Marmosets of the *S. mystax* species were used mainly although a few animals of the *S. nigricollis* species were employed. The animals were wild caught and transported and held under conditions designed to minimize contact with man and other animal species. The marmosets were allowed to "condition" for several weeks after receipt in open cages that housed one animal each. Thereafter, they were transferred to Horsfall-type isolator units that housed two animals each in separate cages and that were equipped with controlled filtered air flow at negative pressure. Blood samples were drawn and injections of materials were made via the femoral artery or vein. Bleedings were usually 2 ml in volume and the dose of inoculum was 0.2–1.0 ml. Bleedings of injected animals were usually made weekly. Bleedings and percutaneous liver biopsies were made under phencyclidine·HCl anesthesia, the latter using a Menghini-type pediatric liver biopsy set. Necropsies were performed on all animals that died or were sacrificed and appropriate specimens were taken for histopathologic examination. The animals were commonly found to be infected with filaria and other parasites.

*Handling and testing of marmoset specimens.* Liver tissue specimens taken at autopsy or biopsy were fixed with formalin, stained with hematoxylin and eosin, and examined blind by qualified pathologists. The sera collected from blood samples were stored frozen at −20° if not tested immediately. Tests for serum isocitric dehydrogenase (SICD) and for serum glutamic oxaloacetic transaminase (SGOT) were performed by modifications of standard procedures (9, 10). Values in excess of 2000 and 200, respectively, based on criteria established by Deinhardt *et al.* (1–4) and confirmed here, were considered to be

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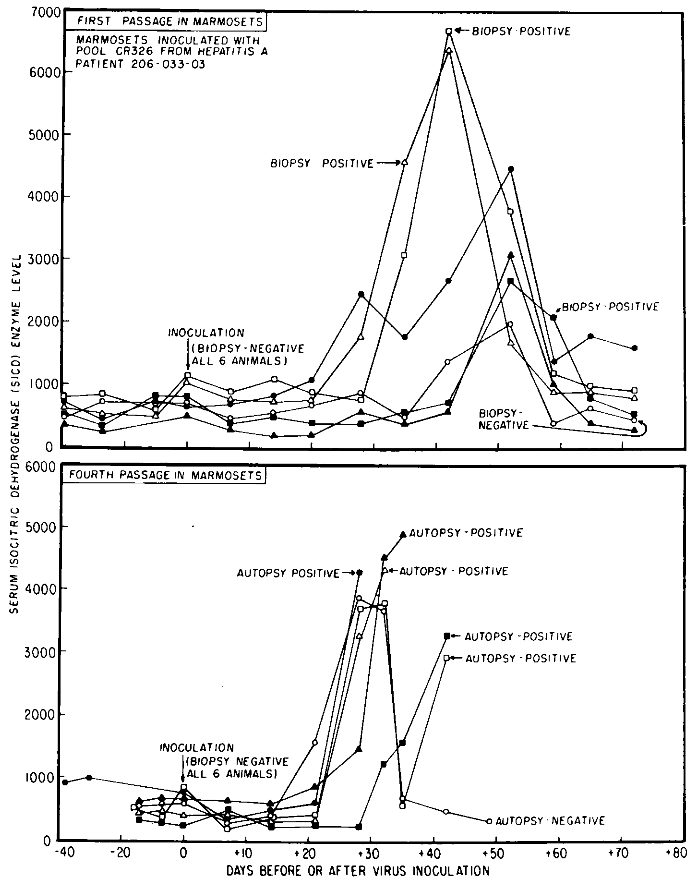


FIG. 1. Enzyme determinations and biopsy–autopsy histopathologic findings in first and fourth passages of hepatitis strain CR 326 in marmosets.

abnormally elevated. Tests for hepatitis-associated antigen (HAA), *i.e.,* Australia antigen or hepatitis B antigen, were carried out by standard agar gel diffusion (11), complement-fixation (CF) (12, 13), and counter-immunoelectrophoresis (14) techniques. Tests for antibody were done by CF and indirect hemagglutination (15).

*Human hepatitis specimens.* The laboratory studies were ancillary to a large-scale epidemiologic study of hepatitis carried out by two of us (V.V. and J.A.) at the International Center for Medical Research and Training, Louisiana State University, School of Medicine, San Jose, Costa Rica (16). The study was conducted in semiurban and rural areas of the counties of San Ramon and Palmares in the province of Alajuela. Hepatitis was followed in the families. According to the study plan, all members of each family were bled immediately upon occurrence of a case of viral hepatitis in the family and at weekly intervals for 2 mo thereafter or for a longer period if secondary cases of hepatitis occurred. The sera were separated from the clot and standard clinical laboratory tests for SGOT, serum glutamic pyruvic transaminase (SGPT), bilirubin, thymol turbidity and cephalin cholesterol flocculation were performed immediately. The remaining serum and the corresponding blood clots were frozen and stored at —20° until used. Other kinds of clinical specimens, not pertinent to the present report, were also taken. The hepatitis patients were studied in the hospital and all the family members were visited and observed weekly by qualified nurses. Complete medical records were kept for all persons who

TABLE I. Attempts to Recover Human Hepatitis A Virus in Marmosets from Bloods of Patients in Costa Rica.

| Hepatitis patients | | | | Marmosets | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Passage 1 | | | Passage 2 | | | Passage 3 | | | |
| | | | | | Enzyme elev. | | Histol. | Enzyme elev. | | Histol. | Enzyme elev. | | Histol. | |
| Case no. | Age (yr) | Clinical findings[a] | Laboratory findings[b] | Species and no. | No. pos./total | Day began | No. pos./total | No. pos./total | Day began | No. pos./total | No. pos./total | Day began | No. pos./total | |
| Hepatitis A cases | | | | | | | | | | | | | | |
| 206-033-03 | 9 | f,h | c,d,e | 6m[c] | 6/6 | 28–50 | 3/3 | 6/6 | 24–28 | 5/5 | 3/3 | 28–30 | 3/3 | |
| 206-033-07 | 2 | a,b,h,i | a–f | 4m | 4/4 | 48–61 | 4/4 | ND[d] | ND | ND | ND | ND | ND | |
| 206-033-02 | 11 | b,c,e,f,h,i | c–f | 6m | 2/6 | 42–64 | 2/2 | ND | ND | ND | ND | ND | ND | |
| 068-330-08 | 7 | a,b,e,f,h | a–f | 6m | 6/6 | 55–102 | 5/5 | 4/4 | 25–39 | 4/4 | 6/6 | 25–38 | 6/6 | |
| 203-035-09 | 8 | a–d,f,h | a–f | 4m, 1n | 1/5 | 77 | 0/5 | ND | ND | ND | ND | ND | ND | |
| 036-153-05 | 5 | a–f,h | c–f | 2n | 0/2 | — | 0/2 | ND | ND | ND | ND | ND | ND | |
| 211-156-04 | 6 | a,b,e,f,h | a–f | 1m, 3n | 0/4 | — | 0/3 | ND | ND | ND | ND | ND | ND | |
| Control: hepatitis B case | | | | | | | | | | | | | | |
| 202-039-05 | 20 | a–h | a–f | 6m | 0/6 | — | 0/6 | ND | ND | ND | ND | ND | ND | |

[a] a = asthenia; b = anorexia; c = nausea; d = vomiting; e = jaundice; f = choluria; g = acholia; h = hepatomegaly; i = fever.

[b] Positive laboratory test results for: a = direct bilirubin; b = total bilirubin; c = SGOT; d = SGPT; e = cephalin cholesterol flocculation; f = thymol turbidity.

[c] m = mystax; n = nigricollis.

[d] ND = not done.

TABLE II. Specificity of Marmoset Hepatitis Test.

| | Marmoset test results | | |
|---|---|---|---|
| | | No. pos./total no. | |
| Inoculum | No. animals | Enzyme elev. | Pos. histopath. |
| Hepatitis A blood samples | | | |
| Preacute illness (8 patients) | 70 | 0/70 | 2/70 |
| Acute illness (7[a] patients) | 33 | 19/33 | 14/24 |
| Convalescent (9[a] patients) | 86 | 5/86 | 6/86 |
| Hepatitis B blood samples: | | | |
| Preacute illness (2 patients) | 22 | 0/22 | 1/22 |
| Acute illness (1 patient) | 6 | 0/6 | 0/6 |
| Convalescent (2 patients) | 20 | 0/20 | 1/20 |
| Miscellaneous: | | | |
| Phosphate buffered saline | 33 | 1/33 | 1/33 |
| Goat serum | 32 | 0/32 | 0/32 |
| Human immune globulin | 11 | 0/11 | 0/11 |

[a] Five of the 7 acute illness and 3 of the 9 convalescent patients' specimens gave positive tests. The rest were negative.

were entered into the study.

*Results. Induction of hepatitis in marmosets.* There was a critical shortage of marmosets and experiments were designed with this handicap. Samples of serum and/or extracts of clotted blood from 7 cases of hepatitis A (hepatitis-associated antigen negative) and 1 case of hepatitis B (hepatitis-associated antigen positive) among children in Costa Rica were inoculated into groups of 2 to 6 marmosets as shown in Table I. The diagnosis of hepatitis A was based on proper clinical and laboratory criteria for hepatitis plus the failure to find either HAA antigen or antibody in the specimens. The samples, for each patient, represented pools of material collected from 42 days prior to onset of clinical disease and to 11 days after such onset. The dose was 0.25 to 0.5 ml of material diluted as much as 1:5. It is seen that hepatitis occurred in marmosets given materials from 5 of the 7 hepatitis A patients. This was confirmed by histopathologic observation in all but 1 case (203-035-09) using the general criteria defined by Deinhardt *et al.* (1). Serial marmoset passage was carried out for 2 of the patients (206-033-03 and 068-330-08) with positive results in all animals in all passages. Specimens from the hepatitis B case, confirmed by demonstration of HAA antigen in the serum, did not induce hepatitis in marmosets.

Special reference is made to hepatitis case 206-033-03. The pool of human serum used to initiate passage in marmosets was designated CR326. This number has since been used to designate the strain of virus which was recovered from the sample and which is being studied extensively in these laboratories. Figure 1 records the SICD enzyme levels and the liver biopsy or autopsy findings in the mystax marmosets on first and fourth passages of serum from hepatitis case 206-033-03. It is seen that all animals in first passage were normal by enzyme and biopsy determination prior to inoculation with pool CR326 and all showed significant enzyme elevations within Days 25 to 55 thereafter with peak levels within days 40 to 55. Tests for SGOT performed simultaneously corresponded entirely with the SICD test results. Supporting evidence for hepatitis was given in the biopsy histopathologic findings in all 3 animals tested in the acute phase of the disease. Similar results were obtained in the second and third passage in marmosets and in the fourth passage also as shown in Fig. 1. The only significant change on passage was a shift in time of occurrence of peak enzyme levels to as early as Day 28. The presence of hepa-

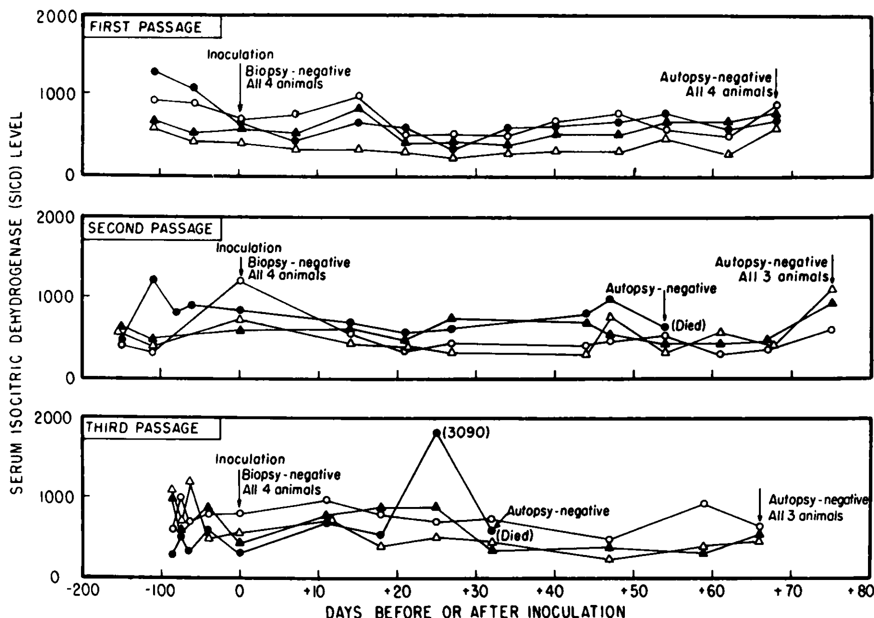


FIG. 2. Enzyme determinations and biopsy–autopsy histopathologic findings on serial passage in control marmosets of marmoset serum taken just prior to inoculation of specimen from hepatitis patient 206-033-03.

titis was histologically confirmed in 5 of the 5 animals that were examined at the appropriate time. Infectivity titrations of the CR326 agent carried out in serial 10-fold steps in 2 to 4 animals/group gave 50% infectivity dose values of $10^{2.5}$ to $10^{4.5}$/ml in pools of serum from marmoset passage levels 3 and 4.

The histomorphologic changes in the marmosets were of variable severity particularly when needle biopsy specimens were examined. A composite picture, however, was drawn for the CR326 agent. The hepatic cells showed moderate degeneration with variation in size, glycogen depletion, diffuse cytoplasmic vacuolation, and occasional necrosis predominantly in the region of the central vein. There was distention of the sinusoids with prominence of the sinusoidal cells (Kupffer cells). The infiltrate was primarily mononuclear, but occasional granulocytes were seen. Megakaryocytes and large blast cells were found occasionally suggesting hematopoiesis. Inflammatory foci were frequently seen adjacent to the central vein and predominantly mononuclear infiltration was observed in the periportal area. Microfilaria were commonly observed without associated inflammatory reaction. Nonspecific inflammatory changes, occasionally seen in the livers of both control and test marmosets, could nearly always be differentiated from those attributable to viral hepatitis.

*Validity of the marmoset test for human hepatitis A.* During the course of 5 yr, 274 marmosets were administered human bloods from cases of hepatitis A or hepatitis B, goat serum, human immune globulin or saline solution for control purposes (data to be published). A composite result of experience with these animals, as well as those used in virus isolation attempts (Table I), is given in Table II. The indispensable element for induction of serum enzyme elevations in the marmosets, *i.e.*, hepatitis, was the introduction of blood from patients with hepatitis A. None of the 92 animals that received hepatitis B specimens or goat serum or human immune globulin showed enzyme elevation. One of 33 recipients of saline solution did show such change and this persisted for 7 days with a maximum SICD level of 2218.

Blood specimens taken from hepatitis A patients 22 to 29 days before onset of illness gave negative findings in 70 marmosets. Pools of blood containing specimens from acute illness (as in Table I) or during convalescence (29 to 113 days after onset) gave positive serum enzyme findings in 24 of 119 animals. Histopathologic and serum enzyme findings were not always in total agreement as evidenced by the appearance of lesions in 4 of 112 animals which showed no significant enzyme change.

For special control purpose, serial passage was carried out in marmosets of a pool of sera taken, prior to inoculation, from the marmosets in which the CR326 hepatitis agent (patient 206-033-03) was initiated. Inocula for passages 2 and 3 were pools of sera from bloods taken at appropriate times from passages 1 and 2, respectively. The findings are shown in Fig. 2. All marmosets in all passages retained SICD levels that were within normal range except for one animal in passage 3 (3090 SICD units). This elevation was likely nonspecific since the animal died shortly thereafter and the histopathology findings were negative for hepatitis. The histopathologic examinations of the livers from the animals in the various passages taken at autopsy were negative for viral hepatitis.

*Discussion.* The findings in the present studies of propagation in marmosets of hepatitis A from human cases in Costa Rica are in close agreement with those obtained in similar studies of cases of hepatitis A in the United States as reported by Deinhardt *et al.* (1–4) and by Lorenz *et al.* (6). Thus, blood specimens from a majority of hepatitis A cases studied (5/7) caused hepatitis in marmosets. The negative findings in 2 cases of hepatitis might have been the result of improper collection time or improper handling of specimens, relative insensitivity of the marmoset test, or absence of virus. The hepatitis B case gave expected negative results. The tests for SGOT gave essentially identical results as those for SICD and, therefore, were not recorded in the report. The histopathologic findings generally confirmed the tests for enzyme elevation though the former kind of assay was less reliable because of inability, in every instance, to differentiate between viral and nonviral lesions. This was due in part to the generally mild hepatitis that occurs in the marmosets and to the random nature of biopsy sampling that may or may not include affected areas of the liver.

The pathologic changes seen in the liver of marmosets infected with the CR326 virus, and the other positive human specimens as well, were similar to those described by Deinhardt *et al.* (1) and by Lorenz *et al.* (6). Characteristically, the principal lesion was focal or diffuse degeneration of hepatocytes that progressed to necrosis in some of the cells. There was distention of the sinusoids with prominence of the sinusoidal cells. Infiltration, when present, was mononuclear and was seen predominantly in the region of the central vein and the periportal area. Occasional granulocytes were seen, especially in instances of severe liver damage. The extent of damage was generally small, compared with hepatitis in man. The nonspecific inflammatory changes seen occasionally in the control and test marmosets could usually be differentiated with certainty from viral hepatitis but this was not true in a small proportion of animals.

The marmoset appeared to be highly reliable for detecting and propagating human hepatitis A virus and for use as a model in studies of this disease. Though similar hepatitis-inducing viruses might be indigenous to the marmoset, they are of no practical importance when due care is exercised in field and laboratory to minimize human contact and the chance of cross-contamination. Marmoset hepatitis agents such as described by Parks and Melnick (7) were not encountered in the present studies.

*Summary.* Marmosets inoculated with blood specimens from human cases of hepatitis A but not from cases of hepatitis B developed hepatitis with elevation in serum enzymes and characteristic histopathologic changes. The marmoset was highly reliable for detecting, propagating and studying human hepatitis A with minimal spurious results.

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