Hepatitis B Antigens in Serum and Liver of Chimpanzees Acutely Infected with Hepatitis B Virus

K. R. BERQUIST,* J. M. PETERSON, B. L. MURPHY, J. W. EBERT, J. E. MAYNARD, AND
R. H. PURCELL

Center for Disease Control, Phoenix, Arizona 85014,* and National Institute of Allergy and Infectious Diseases,
Bethesda, Maryland 20014

We report the temporal patterns of various immunohistological and serological parameters of acute self-limited hepatitis B virus infection of two chimpanzees, and we provide evidence that the synthesis of hepatitis B core antigen precedes that of hepatitis B surface antigen. Our data suggest that existence of a biphasic hepatitis B virus infection involving a hematogenous reinfection of the liver and indicate that recruitment of liver cells to produce hepatitis B virus may occur in a pattern consistent with a replicative cycle of about 8 days.

The discovery of hepatitis B surface antigen (HB$_s$ Ag) (2, 6, 7) and hepatitis B core antigen (HB$_c$ Ag) (3) and the subsequent development of animal models for hepatitis B virus (HBV) infection (4, 16) have facilitated the study of this important disease of humans. Our current knowledge owes much to the development of sensitive radioimmunoassays for the measurement of these antigens and their corresponding antibodies in serum and to the application of immunofluorescence techniques for their detection in tissues obtained during the course of infection. Nevertheless, the details of HBV production and pathogenesis remain to be elucidated.

Several investigators have applied immunofluorescence to the detection of HB$_s$ Ag and/or HB$_c$ Ag in liver. Edgington and Ritt (10) clearly demonstrated the presence of (what is now known as) HB$_s$ Ag in the cytoplasm of liver cells in a number of human patients. Others found hepatitis B-associated antigens in both the cytoplasm and the nuclei of hepatocytes (17, 18). Almeida and co-workers (3) resolved the confusion by identifying and distinguishing the two antigens. Subsequently, Barker and co-workers (4) demonstrated the exclusively nuclear localization of HB$_c$ Ag and the exclusively cytoplasmic localization of HB$_s$ Ag by immunofluorescence.

In none of these studies, however, were all of these serological and histological parameters available for intercomparison at each point throughout the time course of infection. This paper presents such a comparison.

MATERIALS AND METHODS

Two 10-year-old male chimpanzees, chimps 86 and 87, were inoculated intravenously with plasma from chimpanzees infected with the MS-2 strain of HBV. The infective titers of these inocula are not known; they represented a second passage in chimpanzees of the original MS-2 serum of Krugman et al. (13). Blood samples obtained three times a week were tested by Abbott-Ausria-125 for HB$_s$ Ag and by Abbott AUSAB and passive hemagglutination (Electro-Nucleonics) for anti-HB$_s$. Liver biopsies were obtained by Meneghini needle weekly during the incubation period and every 2 weeks after the first detection of HB$_s$ Ag in the tissue. One section of the biopsy (approximately 2 cm) was washed once in 5 to 10 ml of tissue culture medium and then minced in 0.5 ml of the medium. The fluid portion was tested for HB$_s$ Ag by Ausria (this technique proved as sensitive as immunofluorescence for detection of HB$_s$ Ag in the liver). A second fragment (approximately 4 mm) was immediately mounted in Tissue-Tac (Dade) on a cork disk, frozen with liquid nitrogen, and then stored at −70 C. Sections of 4-μm thickness were cut in a cryostat for testing by immunofluorescence. A third fragment was processed for staining with hematoxylin and eosin.

The immunoglobulin G fraction of guinea pig anti-HB$_s$ and human anti-HB$_c$ was purified on diethylaminoethylcellulose columns, labeled with fluorescein isothiocyanate according to the method of Clark and Shepard (9), and used for direct immunofluorescent staining of liver sections. After removal from the freezer, slides holding cryostat sections warmed for 5 min at 37 C and fixed in acetone at room temperature (30 s for HB$_s$ Ag, 3 min for HB$_c$ Ag). Each section was flooded with 20 μl of an appropriate dilution of the antibody preparation and incubated for 40 min at room temperature in a humid box. A brief dip wash and three 5-min washes in phosphate-buffered saline removed unreacted antibody. Cover slips were applied over tris(hydroxymethyl)aminomethane-buffered 90% glycerol, pH 9.5. All specimens were read under code by using a Zeiss Universal microscope equipped with primary filters BG12 or KP500, secondary filter 50, and an HBO 200 W/4 mercury light source. Positive and negative controls were included in each test. Specificity of the

staining with anti-HB$_s$ was established for each positive specimen by blocking with purified HB$_s$ Ag. A quantity of HB$_c$ Ag (kindly furnished by J. Hoofnagle) permitted specificity testing of the fluorescein-labeled anti-HB$_c$, but not of the reactions of individual specimens.

RESULTS

The courses of HBV infections in the two chimpanzees were basically similar. The salient features are displayed in Fig. 1.

We first detected HB$_s$ antigenemia approximately 7 weeks after inoculation in both animals. From this point on, the parameters we followed became positive sooner in chimp 86 and reverted to normal sooner in chimp 87. Detection in the liver of HB$_c$ Ag at 7 and 10 weeks and of HB$_s$ Ag at 12 and 14 weeks preceded detection of histological abnormalities on sections stained with hematoxylin and eosin. This last parameter approximately coincided with the rise in serum glutamic oxalacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) and reverted to normal only after they had dropped. In reverse sequence, hepatic HB$_c$ Ag disappeared before or simultaneously with hepatic HB$_s$ Ag, biochemical tests became normal with the loss of circulating HB$_s$ Ag, and finally anti-HB$_s$ was detected at 28 weeks in chimp 86 and 33 weeks in chimp 87.

The level of serum HB$_s$ Ag fluctuated markedly early in the course of infection (Fig. 2). This was reproducible when all specimens were retested simultaneously with the same Ausria kit. Both animals were plasmapheresed (approximately 200 ml in each case) on day 52, but in only one case was this followed by loss of antigenemia. These fluctuations occurred in cycles of about 8 days, and in the case of chimp 87 there was also a hiatus of 18 days without detectable antigenemia.

In another study, one of five chimpanzees successfully infected with HBV clearly showed an early isolated antigenemia. Serum was positive by radioimmunoassay on days 63 and 70, negative on days 77 and 84, and then positive from days 91 through 196. Elevated SGPT was first detected on day 119 (Purcell, unpublished data).

At the beginning and end of the period during which HB$_c$ Ag and HB$_s$ Ag were found in the liver, gradations of postivity were seen. The number of positive nuclei increased from "rare" to "many" and later decreased to "few" before HB$_c$ Ag was lost entirely. The most intensely positive specimen (in terms of number and brightness of positive nuclei) for HB$_s$ Ag was from chimp 86 on day 112, at the approximate midpoint of the infection. Positive nuclei had a lacy or granular pattern of fluorescence. Positivity for HB$_s$ Ag started with rare single cells showing completely fluorescent cytoplasm in the case of both chimpanzees. It progressed to a pattern of staining of the periphery of nearly all cells and of the entire cytoplasm of a few cells, and then in the case of chimp 87 it returned to the pattern of isolated positive cells.

In the case of chimp 86, there was an abrupt transition from the fully positive pattern to negativity, but an intermediate stage may not
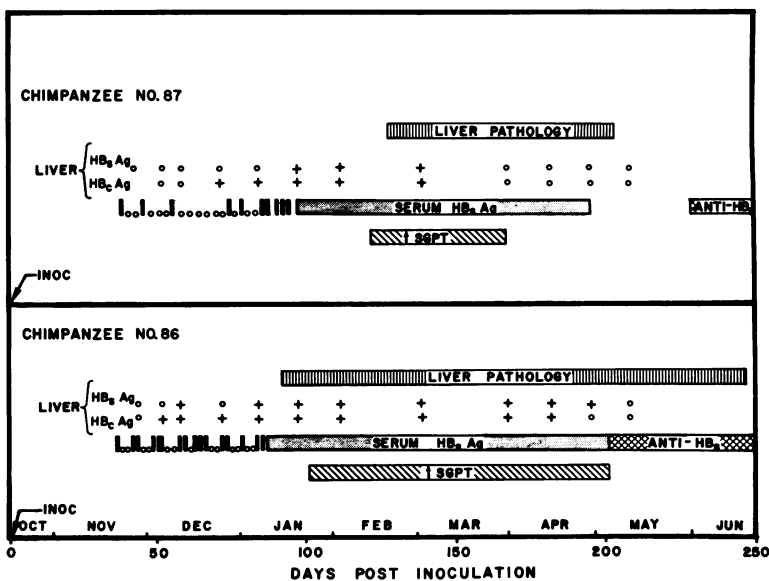

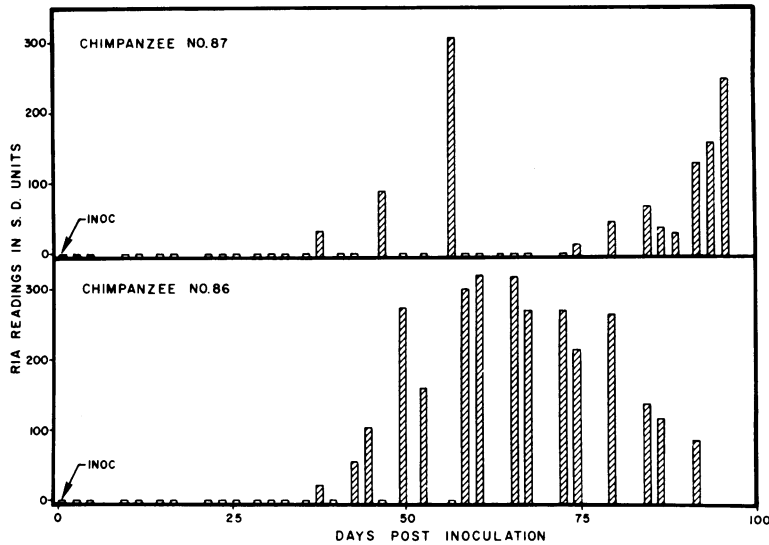FIG. 1. *Time course of parameters of hepatitis B virus infection in chimpanzees.*

FIG. 2. *Variation of serum HB$_s$ Ag in infected chimpanzees as detected by radioimmunoassay early in the course of disease.*

have been seen because of the 2-week interval between biopsies. The nucleus was always spared by staining for HB$_s$ Ag.

DISCUSSION

One of the most striking observations was that detection of antigenemia preceded detection of HBV antigens in the liver. We regard this as probably due to sampling error, because the amount of liver tissue examined by biopsy was a small fraction (approximately 10^{-7}) of the whole liver (even when an average of one cell per unit tissue examined is positive, the Poisson distribution gives the probability of finding no positive cells as 37%). The pattern at this stage is probably one of rare isolated infected cells, since this is the pattern seen when HB$_s$ Ag-positive cells are first detected. It is also unlikely that any extrahepatic synthetic site exists to explain these results, since Murphy et al. (Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, S 104, p. 231) have found HB$_s$ Ag in various tissues but HB$_c$ Ag in the liver only.

Other patterns have been found in human disease. Akeyama et al. (1) identified solitary, focal, disseminated, and lobular distributions of HB$_s$ Ag-containing cells and made a rough correlation with the degree and type of liver disease. They found only the first three types in acute hepatitis, but did not correlate these data with the stage of disease at time of biopsy. Our own findings are of solitary and disseminated patterns, if one ignores the perimembranous staining which affects nearly all the cells. If one considers this staining, then the pattern is not

merely lobular, but total. These patterns may correlate with stages of infection or with size of inoculum. The finding of foci or lobules of contiguous HB$_s$ Ag-containing cells may represent cell-to-cell spread of virus, whereas a disseminated noncontiguous distribution may represent a large inoculum or a secondary, hematogenous spread of the virus.

Support for the existence of such a biphasic disease is found in the data of Bradley et al. (8), who observed two peaks of serum polymerase activity, one preceding or paralleling the development of antigenemia, the other following it. Our own data on chimp 87 (Fig. 2) support this: after three definitely positive serum specimens there was a period of 18 days during which six separate specimens were negative for HB$_s$ Ag. If one postulates nonspecific clearing mechanisms to account for loss of serum HB$_s$ Ag after its first appearance, then its second appearance can be seen as the expression of a secondary infection. The start of the second phase of antigenemia is 35 days after the start of the first phase, and this is close to the incubation period of 39 days for initial antigenemia. Focusing even more on the details of antigenemia shown in Fig. 2, we note that the fluctuations occur with a periodicity of about 8 days. This could represent the time required for successive infections, each involving more cells and producing a larger total burst of virus particles. A replicative cycle of this length is certainly more commonly seen than is one of several weeks to several months.

The detection of HB$_c$ Ag before the detection of HB$_s$ Ag in the liver is also consistent with the

findings of others (8, 14) that deoxyribonucleic acid polymerase appears in the serum during or before early antigenemia. Unlike tests of serum, however, our examination of the liver by immunofluorescence reflects the number of cells containing a given antigen more than it does the amount of antigen produced. Moreover, since it involves tests of roughly equal sensitivity applied to the same tissue, it more probably gives a valid indication of the sequence in which the two antigens are synthesized. This sequence, as well as the persistence of cellular HB_s Ag until or after the disappearance of cellular HB_c Ag, is consistent with the postulated central role of the Dane particle in the synthesis of HB_s Ag coat material.

We have found, as others have (5), that histological and chemical evidence of liver damage is not detectable until long after the detection of HBV antigens in liver and the peak of HB_s Ag in serum. The pathogenesis of hepatitis, therefore, may not be a function of virus production, although it probably depends on the presence of virus antigens within or on the cell. In particular, we see that the SGPT becomes normal only after HBV antigens are no longer detected in the liver. Rises in SGOT and SGPT bear various relationships to the appearance of HB_s Ag and occur before, with, or after the detection of antibody (4, 11, 15, 19). In general, however, the biochemical abnormalities occur at a time when anticomplementary activity in the sera is to be expected, and, in cases of hepatitis B, hepatitis A, and non-A-non-B hepatitis, anticomplementary activity has indeed been found to coincide with enzyme elevations (19). All of this implicates humoral immune mechanisms in the pathogensis. There is also evidence that cellular immune responses play a role in hepatitis B. Irwin and co-workers (12) found that lymphocytes of patients with acute hepatitis B produce macrophage migration inhibitory factor when stimulated with purified HB_s Ag. Moreover, in one case, followed through the course of illness, the production of migration inhibitory factor peaked as the SGOT began to rise and returned toward normal as the patient recovered (12).

LITERATURE CITED

1. Akeyama, T., T. Kamada, M. Koyama, and H. Abe. 1974. Distribution patterns of hepatitis B antigen in the liver. Arch. Pathol. 98:252-256.
2. Allison, A. C., and B. S. Blumberg. 1961. An isoprecipitation reaction distinguishing human serum-protein types. Lancet 1:634-637.
3. Almeida, J. D., M. Rubenstein, and E. J. Stott. 1971. New antigen-antibody system in Australia-antigen-positive hepatitis. Lancet 2:1225-1227.
4. Barker, L. F., F. V. Chisari, P. P. McGrath, D. W. Dalgard, R. L. Kirschstein, J. D. Almeida, T. S. Edgington, D. G. Sharp, and M. R. Peterson. 1973. Transmission of type B viral hepatitis to chimpanzees. J. Infect. Dis. 127:648-662.
5. Barker, L. F., M. R. Peterson, M. R. Shulman, and R. Murray. 1973. Antibody responses in viral hepatitis, type B. J. Am. Med. Assoc. 220:1079-1082.
6. Blumberg, B. S., B. J. S. Gerstley, D. A. Hungerford, W. T. London, and A. I. Sutnik. 1967. A serum antigen (Australia antigen) in Down's syndrome, leukemia and hepatitis. Ann. Intern. Med. 66:924-930.
7. Blumberg, B. S., A. I. Sutnick, and W. T. London. 1968. Hepatitis and leukemia: their relation to Australia antigen. Bull. N.Y. Acad. Med. 44:1566-1586.
8. Bradley, D. W., J. E. Maynard, K. R. Berquist, and D. H. Krushak. 1974. Hepatitis B and serum DNA polymerase activities in chimpanzees. Nature (London) 251:356-357.
9. Clark, H. F., and C. C. Shepard. 1963. A dialysis technique for preparing fluorescent antibody. Virology 20:642-644.
10. Edgington, T. S., and D. J. Ritt. 1971. Intrahepatic expression of serum hepatitis virus-associated antigens. J. Exp. Med. 134:871-885.
11. Hoofnagle, J. H., R. J. Gerety, and L. F. Barker. 1975. Antibody to hepatitis B core antigen. Am. J. Med. Sci. In press.
12. Irwin, G. R., Jr., W. J. Kierholzer, Jr., R. Cimis, and R. W. McCollum. 1972. Delayed hypersensitivity in hepatitis B: clinical correlates of in-vitro production of migration inhibiton factor. J. Infect. Dis. 130:580-587.
13. Krugman, S., J. P. Giles, and J. Hammond. 1967. Infectious hepatitis: evidence for two distinctive clinical, epidemiological and immunological types of infection. J. Am. Med. Assoc. 200:365-373.
14. Krugman, S., J. H. Hoofnagle, R. J. Gerety, P. M. Kaplan, and J. L. Gerin. 1974. Viral hepatitis, type B DNA polymerase activity and antibody to hepatitis B core antigen. N. Engl. J. Med. 290:1331-1340.
15. Lander, J. J., P. V. Holland, H. J. Alter, R. M. Chanock, and R. H. Purcell. 1972. Antibody to hepatitis-associated antigen: frequency and pattern of response as detected by radioimmunoprecipitation. J. Am. Med. Assoc. 220:1079-1082.
16. Maynard, J. E., K. R. Berquist, D. H. Krushak, and R. H. Purcell. 1972. Experimental infection of chimpanzees with the virus of hepatitis B. Nature (London) 237:514-515.
17. Millman, I., V. Zavatone, B. J. S. Gerstley, and B. S. Blumberg. 1969. Australia antigen detected in the nuclei of liver cells of patients with viral hepatitis by the fluorescent antibody technique. Nature (London) 222:181-184.
18. Nowoslawski, A., W. J. Brzosko, K. Madalinski, and K. Krawczynski. 1970. Cellular localization of Australia antigen in the liver of patients with lymphoproliferative disorders. Lancet 1:494-498.
19. Purcell, R. H., J. H. Walsh, P. V. Holland, A. G. Morrow, S. Wood, and R. M. Chanock. 1971. Seroepidemiological studies of transfusion-associated hepatitis. J. Infect. Dis. 123:406-413.