

Detection of Hepatitis A Antigen by Immunofluorescence

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Received for publication 16 May 1977

Hepatitis A antigen (HA Ag) was demonstrated by immunofluorescence (IF) in liver biopsies from chimpanzees with experimental hepatitis A virus infection. Blocking experiments with paired sera from patients with hepatitis types A, B, or non-A, non-B, as well as with purified HA Ag, showed that the fluorescence was specific for HA Ag. HA Ag could be demonstrated only in biopsies from chimpanzees inoculated with hepatitis A virus. In two of four chimpanzees biopsied weekly, HA Ag could be detected by IF before stool shedding of HA Ag, elevation in serum alanine aminotransferase (SGPT), or histopathological evidence of liver disease was seen. The HA Ag was detected for 4 to 5 weeks; the last IF-positive biopsy was obtained after SGPT activity had returned to normal. In the two other chimpanzees, HA Ag could be detected only in the biopsy taken at the time of SGPT elevation. In the early IF-positive biopsies, HA Ag was diffusely distributed in the cytoplasm of many cells, but it later accumulated in a focal distribution in the cytoplasm of a few of the hepatocytes and Kupffer cells. This cytoplasmic distribution agrees with previous electron microscopic data.

The first successful transmission of well-characterized hepatitis A virus to animals (marmosets) was convincingly demonstrated in 1969 by Holmes et al. (13). In 1973, the technique of immune electron microscopy (IEM) was used by Feinstone et al. to identify a 27-nm virus-like particle in the stools of patients with acute type A hepatitis and to demonstrate specific antibody to the particle (anti-HA) in convalescent sera (9). In 1975, Dienstag et al. (5) and Maynard et al. (14) reported that chimpanzees without preexisting anti-HA were susceptible to hepatitis A virus infection and had an illness that was biochemically, histologically, and serologically similar to that of humans.

More recently, Schulman et al. were able to demonstrate hepatitis A antigen (HA Ag) by IEM in chimpanzee liver, bile, and stool taken during acute infection with hepatitis A virus (24). They also demonstrated 27-nm particles in the cytoplasm of chimpanzee hepatocytes during acute illness by thin-section electron microscopy. The particles resembled the HA Ag particles found by IEM, but antigenic specificity was not determined. HA Ag had previously been demonstrated by IEM in homogenized marmoset liver taken during acute infection with type A hepatitis, and 27-nm particles were seen in the cytoplasm of hepatocytes from one of these

marmosets by thin-section electron microscopy (20).

Since 1973, there has been rapid progress in the development of tests for detection of HA Ag and anti-HA, in part as a result of applying techniques previously developed for the detection of hepatitis B antigens and antibodies (12, 15, 19, 21, 22), but attempts to detect HA Ag by immunofluorescence (IF) have not been successful. In type B hepatitis infection, it is possible to demonstrate intrahepatic hepatitis B surface antigen (HB_s Ag) in the cytoplasm and hepatitis B core antigen (HB_c Ag) in the nucleus of hepatocytes by IF (2, 8). The localization of these antigens has led to better understanding of the nature and course of the disease (1, 11, 17, 18). Described herein is the demonstration and cellular localization of HA Ag by IF.

MATERIALS AND METHODS

Chimpanzees. Experimental type A hepatitis in the chimpanzees used in this study was described previously (5, 24).

Biopsies. Open biopsies of liver and small bowel were performed on chimps 786 and 883 on the 2nd and 3rd day, respectively, after the first detected elevation in serum alanine aminotransferase (SGPT). Weekly percutaneous liver biopsies were performed on chimpanzees 714, 753, 755, and 756 starting before inoculation and continuing during the convalescent period. Needle biopsies from chimpanzees with acute or chronic hepatitis B virus infection served as addi-

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tional negative controls.

Fluorescein-conjugated reagents. Immuno-globulin G (IgG) purified by ammonium sulfate precipitation and diethylaminoethyl-cellulose column chromatography was conjugated with fluorescein isothiocyanate (FITC) by the method of Clark and Shepard (4). Three sera were labeled: a convalescent serum from a chimpanzee with naturally occurring type A hepatitis (7), a convalescent serum from a human volunteer who was experimentally infected with the MS-1 strain of hepatitis A virus (3), and a hyperimmune serum from a chimpanzee (753) that had been experimentally infected with hepatitis A virus and reinoculated with partially purified HA Ag to produce an anamnestic antibody response (5, 6). These sera had anti-HA titers of 1:10,000, 1:320,000, and 1:128,000 respectively, as measured by immune adherence hemagglutination (IAHA) (16), and the molar FITC to protein (F/P) ratios of the conjugates were 3.2, 6.8, and 6.0. For detection of HB_s Ag and HB_e Ag, FITC-labeled anti-HB_s and anti-HB_e were used as previously described (17).

Purified HA Ag. HA Ag for use in blocking experiments was partially purified by the method of Moritsugu et al. (16) from the stool of a patient with naturally acquired type A hepatitis (7).

Immunofluorescence. Snap-frozen biopsy tissues were cut on a cryostat in 4- μ m-thick sections and placed on glass slides. The slides were stored at -70°C until used, when they were air dried for 5 min at 37°C. Slides were stained unfixed by layering on the tissue 20 μ l of the FITC-conjugated antibody appropriately diluted in phosphate-buffered saline (pH 7.4) containing 5% human serum albumin. The slides were incubated at room temperature for 30 min in a humidified chamber. They were then rinsed in phosphate-buffered saline for 15 s, followed by three washings of 5 min each. After washing, excess saline was drained off, and a cover slip was applied over a mounting medium consisting of 90% glycerin in tris-(hydroxymethyl)aminomethane buffer (pH 9.5).

The slides were examined immediately with a Zeiss Universal fluorescence microscope equipped with an HBO 200 W/2 mercury light source and vertical illuminator with 500 dichroic mirror reflector for fluorescence microscopy and with a 12-V 60-W bulb and dark-field condenser for transmitted-light microscopy for orientation. The exciter filter was a KP 490 interference filter; to diminish the autofluorescence and increase the contrast, an additional 2-mm LP 455 glass filter was used. A 2-mm LP 530 or LP 510 glass filter was used as the barrier filter. Most observations were made with a X40 Neofluor or a X40 oil Apoplan objective and X10 oculars. The microscope was equipped with a Zeiss 35-mm camera. Photographs were made on Kodak Ektachrome EPD daylight ASA 200 film with up to 3 min of exposure time. The film was processed by the Kodak ESP-1 method to increase its effective ASA rating to 800. All experiments included appropriate controls, and the slides were read under code.

Blocking tests. Blocking experiments to confirm the specificity of the IF reactions were performed, using paired sera from humans experimentally or naturally infected with type A hepatitis (3, 7) and chimpanzees experimentally infected with the virus (5). In

addition, specificity of the reaction was confirmed with serum pairs from patients with type B or non-A, non-B hepatitis (10). Blocking experiments were performed by mixing 25 μ l of a 1:5 dilution of the serum to be tested with 25 μ l of a 1:800 dilution of the FITC-labeled hyperimmune chimpanzee anti-HA (F-anti-HA). Final concentration of the conjugate was 1:1,600, its end point dilution. The slides were then stained with 20 μ l of the mixture as described above. The slides were coded before reading.

An antigen-blocking experiment was also performed. Twenty-five μ l of a 1:800 dilution of the F-anti-HA was mixed with 25 μ l of a preparation of HA Ag partially purified from a human stool. The mixture was incubated for 1 h at room temperature and then overnight at 4°C. The HA Ag and anti-HA/HA Ag complexes were pelleted by centrifugation in an SW56 rotor, using (0.38 by 1.66 in [0.95 by 3.74 cm]) tubes with Delrin adaptors in a Beckman L5-50 ultracentrifuge at 23,000 rpm for 90 min.

Serological testing. Anti-HA was measured by IEM (9) and IAHA (16). HA Ag was detected by IEM (9) and solid-phase radioimmunoassay (22). Antibody to hepatitis B surface antigen (anti-HB_s) was measured by passive hemagglutination test or radioimmunoprecipitation test (10).

RESULTS

In initial experiments with F-anti-HA, cytoplasmic fluorescence was observed in acute-phase liver biopsies from chimpanzees 786 and 883. This fluorescence was consistently observed in all sections studied from three different tissue blocks from each animal. Figure 1 is an example of the typical fluorescence pattern. The appearance was granular and concentrated in the cytoplasm of hepatocytes scattered throughout the tissue. Nuclear staining was never observed. Smaller cells with the appearance of Kupffer cells occasionally contained fluorescence. Positively stained cells were frequently observed in small clusters in a focal distribution. The number of fluorescing cells seldom exceeded 5 to 10% of the total and was often considerably less. Superior positive staining was consistently observed in sections of one block from chimpanzee 786. This block was used as a positive control in all subsequent experiments and as the substrate for all blocking experiments.

The F-anti-HA had a titer of 1:1,600 when tested against the most positive tissue, but in dilutions higher than 1:400 the fluorescence was not consistently positive when tested against tissue with fewer positive staining cells. A 1:200 dilution of this reagent was therefore used in all studies except for the blocking experiments.

To test the specificity of the IF reaction, blocking experiments were performed, using, as potential blocking antibodies, sera in which the presence or absence of anti-HA had been determined by IEM, IAHA, or both.

Only sera containing anti-HA blocked the IF

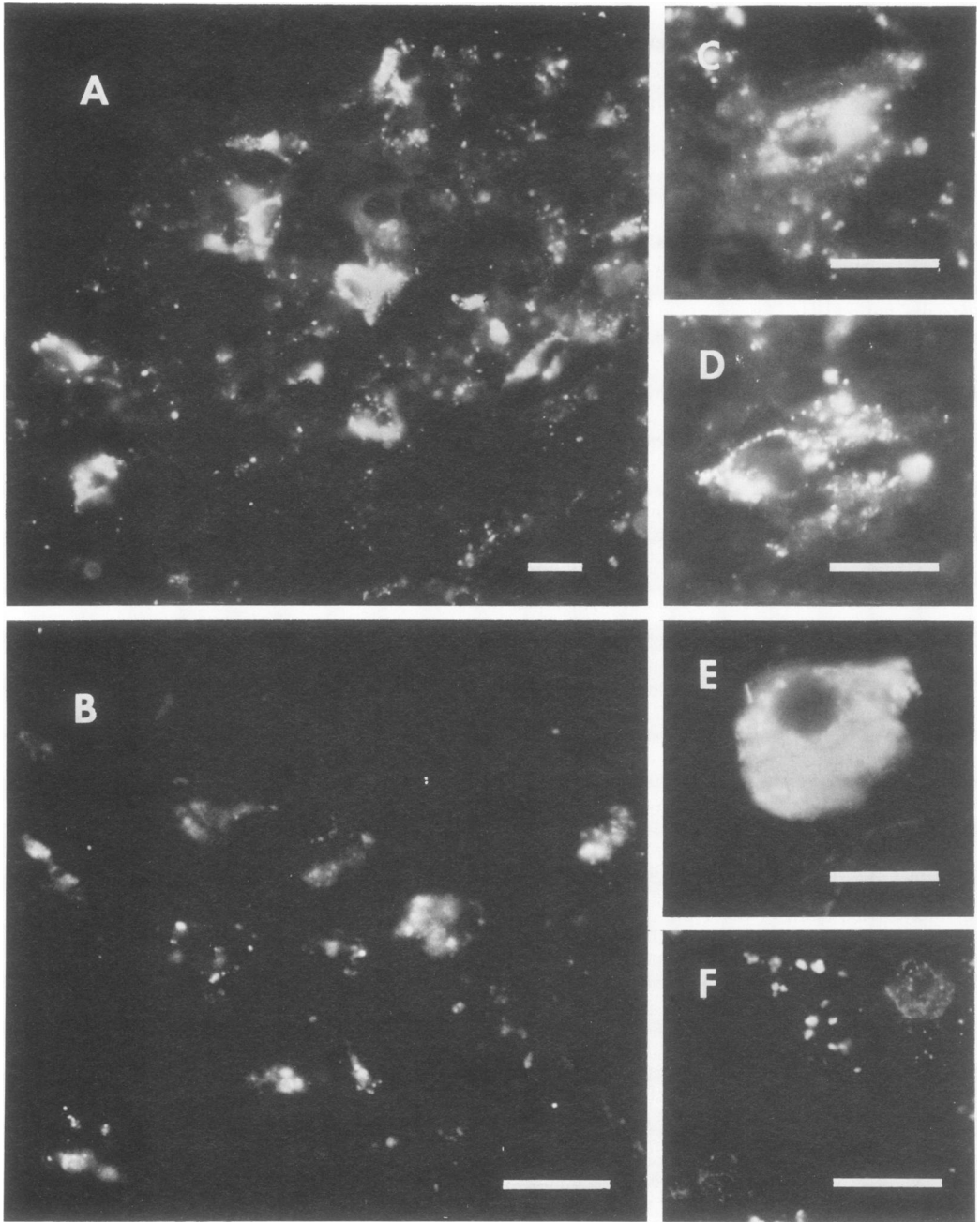


FIG. 1. A-D, Hepatitis A antigen in chimpanzee liver detected by IF with FITC-labeled anti-HA (A, C, and D, chimpanzee 786; B, chimpanzee 756). E and F, HB_sAg and HB_cAg in chimpanzee liver (chronic hepatitis B) by IF with FITC-labeled anti-HB_sAg and anti-HB_cAg. Bar = 20 μ m.

reaction (Table 1). Acute-phase sera which had no detectable antibody by IAHA, but did have antibody when tested by IEM, also neutralized the fluorescence. This indicated that the fluorescence-blocking test could detect early anti-

body as does the IEM test (but not IAHA) (16, 23).

Neither the preexposure nor the convalescent sera from the two non-type A hepatitis cases contained blocking activity. The sera from the

TABLE 1. Specificity of immunofluorescence as measured by inhibition by sera from hepatitis cases

Sera from given cases	Fluorescence detected ^a	Anti-HA status		Anti-HB _s by PHA ^b
		By IAHA ^c	By IEM ^d	
Type A hepatitis				
B, day 0	+	<1:10	0	<1:4
B, day 40	+/-	<1:40 ^f	2+	<1:4
B, day 105	0	1:16,000	NT	<1:4
K, day 0	+	<1:10	0	<1:4
K, day 37	+/-	<1:10	1+	<1:4
K, day 100	0	1:5,000	3+	<1:4
We, acute	0	<1:10	1+	<1:4
We, 3 mo	0	1:16,000	3-4+	<1:4
Chimp 753 day 4	+	<1:10	0	<1:4
Day 33	0	<1:10	2-3+	<1:4
Day 153	0	1:3,200	3-4+	<1:4
Chimp 714 day 5	+	<1:2	NT	<1:4
Day 153	0	1:3,200	NT	<1:4
Type B hepatitis				
W, preexposure	+	<1:10	0	<1:4 ^g
W, convalescent (7 mo)	+	<1:10	0	1:32 ^g
Type non-A, non-B hepatitis				
S, acute serum	+	<1:40 ^f	NT	<1:4
S, convalescent serum (2 mo)	+	<1:100 ^f	NT	<1:4

^a Fluorescein-labeled anti-HA was mixed with the indicated serum and applied to a section of HA Ag-containing liver. Incubation and washing were as described in the text. +, Fluorescence not diminished, no blocking; +/-, diminished fluorescence, partial blocking; 0, no fluorescence, complete blocking.

^b Tested by passive hemagglutination (PHA).

^c Titer.

^d Antibody rating on a scale of 0 to 4+. 0, No antibody, and 4+, heavily coated with antibody. NT, Not tested.

^e B, K, We, W, and S refer to patients.

^f Nonspecific reaction at this dilution.

^g Tested by radioimmunoprecipitation.

type B hepatitis patient had no detectable anti-HA by IAHA or IEM, whereas the non-A, non-B hepatitis case had a nonspecific reaction by IAHA at 1:40 in the acute serum and at 1:100 in the convalescent serum; therefore, low levels of anti-HA could not be excluded.

The specificity of the IF was further confirmed by a blocking experiment with purified HA Ag, as described above. The F-anti-HA mixed with purified HA Ag completely lost its staining ability when tested on a HA Ag-positive liver biopsy. A control preparation of F-anti-HA, treated in the same manner with buffer instead of HA Ag, gave clearly positive fluorescence. The pellet from the HA Ag blocking test was examined in the electron microscope after negative staining with 2% phosphotungstic acid. Typical 27-nm HA Ag particles complexed by antibody were seen.

Weekly liver biopsies were available on chimpanzees 714, 753, 755, and 756 from before inoculation through convalescence. Figure 2 summarizes the laboratory and clinical events which occurred in chimpanzee 753. The liver first became positive for HA Ag by IF at about the same time that fecal shedding of HA Ag was

detected by IEM and solid-phase radioimmunoassay. However, the HA Ag was detectable in the liver 1 week prior to both SGPT elevation and earliest histopathological changes in the liver. Antigen was detected in the liver for 5 weeks, beginning the 2nd week after inoculation and extending about 3 weeks beyond the end of detectable HA Ag shedding into the stool.

The pattern of fluorescent staining changed during the course of the disease. In the earliest positive biopsies, very fine granular fluorescence was seen in the cytoplasm of the hepatocytes with only a few granules in each cell, but usually with many cells involved. In the succeeding biopsies, the fluorescence became brighter but accumulated in the cytoplasm of a few scattered hepatocytes and Kupffer cells or small clusters of hepatocytes. Nuclear staining was never observed. In the final positive biopsy of the series, the intensity of the staining appeared markedly decreased.

Table 2 summarizes the results for all four chimpanzees studied. In chimpanzee 756 the HA Ag could be detected in the liver by immunofluorescence for 4 weeks; its temporal relationship to other indicators of infection, as well as the

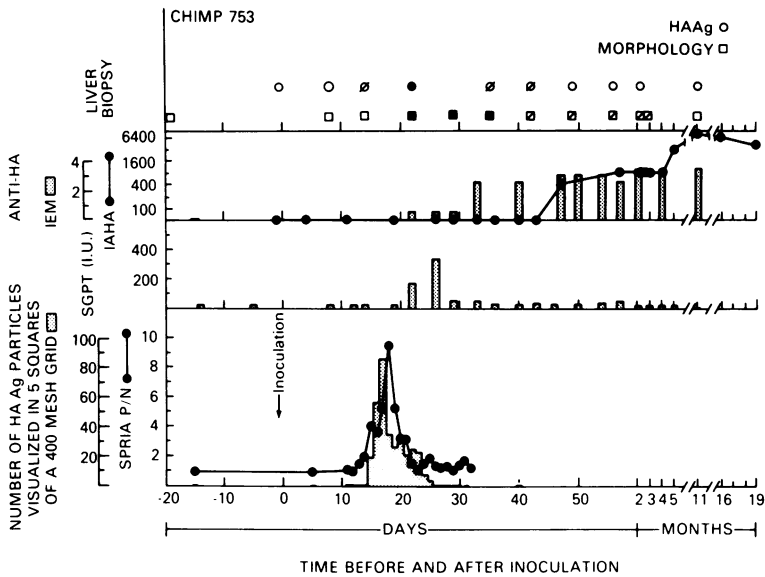


FIG. 2. Temporal relationship of HA Ag as measured by IF to liver histopathology, anti-HA, changes in SGPT, and shedding of HA Ag in feces of chimpanzee 753. Biopsy data: (○), no fluorescence; (◊), weak fluorescence; (●), bright fluorescence; (□), normal liver; (■), acute hepatitis, and (◑), resolving hepatitis. Anti-HA rating on a scale 0 to 4 as determined by IEM: 0, no antibody; 1, particles with distinct surfaces, lightly coated with antibody; 2, particles with moderately blurred surfaces, moderately coated with antibody; 3, particles with indistinct surfaces, heavily coated with antibody; 4, particles with surfaces obscured by a very heavy antibody coating. Anti-HA (inverse titer) as determined by IAHA. Solid-phase radioimmunoassay (●). Data were converted to ratio of counts per minute bound in the presence of HA Ag to counts per minute bound in negative control test (positive/negative, P/N). P/N \geq 2.1 is considered positive.

TABLE 2. Type A hepatitis in chimpanzees: temporal relationships^a

Chimpanzee no.	Inoculum (route)	Source of infection ^b	Fecal HA Ag shedding ^c	SGPT elevation ^c	First detection of anti-HA ^c	Acute histopathology	Liver HA Ag by IF	Incubation period ^d
753	Acute-phase stool (i.v.)	Primary inoculation	15-25 (17)	22-33 (26)	22	21-35	14-42	22/22
714	Acute-phase stool (oral)	Primary inoculation	27-28 (27)	29-36 (36)	26	28-42	35 ^e	29/29
756	Preinoculation stool (i.v.)	Secondary exposure	49-51 (51)	54-57 (57)	47	49-63	42-63	54/26
755	Preinoculation stool (oral)	Secondary exposure	ND	50-57 (54)	54	49-63	49 ^e	50/22

^a HA Ag, hepatitis A antigen; SGPT, serum alanine aminotransferase; anti-HA, serum antibody to hepatitis A antigen by IEM; IF, immunofluorescence; i.v., intravenous.

^b Primary inoculation: day 0 of the study. Secondary exposure: chimpanzees 755 and 756 were exposed to chimpanzees 714 and 753, respectively, on day 28 of the study.

^c Number of days (peak) after initiation of the study. ND, Not detected.

^d Observed incubation period (numerator) was the number of days after initiation of the study that SGPT was first elevated; the calculated incubation period (denominator) was the number of days between primary inoculation or secondary exposure and the first elevation of SGPT.

^e Biopsy obtained 7 days earlier not available.

fluorescence pattern, was similar to that of chimpanzee 753. In chimpanzees 714 and 755, HA Ag could be detected only in one biopsy, but in both cases the biopsy obtained 1 week previously was exhausted and could not be evaluated.

Small-bowel biopsies taken during acute hepatitis A virus infection of chimpanzees 786 and 883 were negative for HA Ag when tested with F-anti-HA.

Fluorescein-conjugated IgG from two convalescent type A hepatitis patients with high titers of anti-HA produced results similar to that seen with the chimpanzee F-anti-HA when tested against the HA Ag-positive chimpanzee biopsies, but no fluorescence was seen when FITC-labeled anti-HB_s and anti-HB_e were used.

No fluorescence could be detected in liver biopsies from chimpanzees with acute or chronic

type B hepatitis when tested with F-anti-HA. In contrast, specific fluorescence was present in the cytoplasm and nucleus of hepatocytes of these biopsies when tested with fluorescein-conjugated anti-HB_s and anti-HB_c, respectively (Fig. 1).

DISCUSSION

The marmoset was the first nonhuman animal species shown to be susceptible to hepatitis A virus infection (13). Subsequently, Dienstag et al. (5) and Maynard et al. (14) showed that chimpanzees without preexisting anti-HA developed acute hepatitis after inoculation with stools taken from humans during acute infection with hepatitis A virus. The chimpanzees excreted HA Ag in the stools, had liver biochemical and histological abnormalities typical of type A hepatitis, and developed anti-HA.

Although HA Ag has been detected in homogenates of chimpanzee liver taken during acute hepatitis A virus infection, and HA Ag-like particles were identified in liver cell cytoplasm by thin-section electron microscopy, previous attempts in our laboratory to demonstrate HA Ag by IF had failed, although HB_s Ag and HB_c Ag could be readily demonstrated in appropriate liver tissue by IF (17).

By modifying the illumination and optical filtration systems of the microscope, we were able to demonstrate cytoplasmic fluorescence in liver biopsies taken from chimpanzees during the period of acute type A hepatitis. FITC-conjugated hyperimmune chimpanzee IgG and human convalescent IgG with high anti-HA titers both produced this fluorescence. Blocking experiments with paired sera from types A, B, and non-A, non-B hepatitis cases, as well as blocking experiments with purified HA Ag, showed that the fluorescence was specific for HA Ag. Furthermore, the appearance of this fluorescence was temporally related to hepatitis A virus infection and disappeared during the convalescent phase. Liver biopsies containing HB_s Ag and HB_c Ag by IF from chimpanzees with acute or chronic hepatitis B virus infection contained no fluorescing material when tested with anti-HA fluorescein-conjugated reagents.

The fluorescence test appeared to be quite sensitive for detecting HA Ag in liver. Intrahepatic HA Ag could be detected prior to SGPT elevations or fecal shedding of HA Ag in at least two of four chimpanzees from whom serial biopsies were available. Since it is known that HA Ag is excreted into the bile (24), the detection of HA Ag in the liver prior to its detection in feces suggests that the primary site of viral replication is the liver and not the bowel. When we tested small-bowel biopsies by IF for HA

Ag, the results were negative. Although this suggests that there is no intestinal phase of replication, it does not completely rule it out, since serial intestinal biopsies from different levels of the gut were not obtained. Furthermore, HA Ag existed in the liver in large enough quantities to be detected by IF a week before liver pathology and SGPT elevations were first detected (Fig. 2). The latter coincides with the first appearance of antibody as detected by IEM and suggests the possibility that an immunological mechanism may play a role in hepatic injury. In this respect, type A hepatitis resembles type B hepatitis, in which antigen is found both in liver and in serum before evidence of liver damage appears.

The pattern of the fluorescence in hepatitis A virus infection is one of HA Ag diffusely distributed in most areas of the liver biopsies early in infection and later focally accumulating in a few of the hepatocytes and Kupffer cells; antigen was detected only in the cytoplasm during both phases. This pattern was observed with all three FITC-conjugated anti-HA sera used and in biopsies from all four chimps and agrees with the previous demonstration by electron microscopy of 27-nm virus-like particles in the cytoplasm of hepatocytes taken from chimpanzees and marmosets during acute hepatitis A virus infection. The finding of HA Ag only in the cytoplasm supports other findings (20) that suggest a ribonucleic-acid genome in hepatitis A virus. However, a nuclear phase of replication cannot be ruled out; early descriptions of the localization of HB_s Ag in the cytoplasm of liver cells (8) suggested that the hepatitis B virus contained ribonucleic acid, but the subsequent detection of HB_c Ag in hepatocyte nuclei (2) confirmed an intranuclear phase of replication for this virus consistent with its present classification as a deoxyribonucleic-acid virus.

Although it is probable that HA Ag can be detected by IF in human liver during acute hepatitis A virus infection, liver biopsy during acute illness is rarely indicated. Therefore, the problem of making an early diagnosis of type A hepatitis remains. The use of IEM for detecting HA Ag in stools is extremely cumbersome, and very early specimens are usually required. The IAHA test for anti-HA is very sensitive for detecting seroconversions, but it appears to measure only late-developing antibody. It has been suggested (23) that if a serum has antibody detectable by IEM or solid-phase radioimmunoassay (which can detect early antibody) but not by IAHA, this is good evidence for acute hepatitis A infection. Although this might be true generally, results obtained by this type of combined test must be interpreted with caution

when applied to an individual case. Although an IF blocking test can detect early antibody, is quite sensitive, and requires only a fluorescence microscope and fluorescein-labeled antibody, the limiting factor is the availability of liver tissue containing HA Ag. At present, the IF test is primarily useful as a research tool.

ACKNOWLEDGMENTS

We thank Virginia Frye and Carol Armiger for clerical assistance.

L. R. M. was supported by grants from Michaelsen Foundation, Copenhagen, and the Danish State Medical Research Council.

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