Natural and Experimental Infection of Woodchucks with Woodchuck Hepatitis Virus, as Measured by New, Specific Assays for Woodchuck Surface Antigen and Antibody

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Solid-phase radioimmunoassays for woodchuck hepatitis virus (WHV) surface antigen (WHsAg) and antibody to it (anti-WHs) were developed. The test for WHsAg could detect as little as 10 ng/ml. In both tests it was necessary to employ radiolabeled WHsAg instead of anti-WHs as the probe because the latter appeared to be labile to the conditions of labeling. The tests were used to characterize naturally acquired and experimental WHV infections of woodchucks. Forty-three of 72 wild-caught woodchucks had serological evidence of WHV infections; 16 of these resulted in chronic infection, and the remainder were self-limiting. All chronically infected animals were positive for WHsAg and DNA polymerase activity. During 3 years of observation, 11 of the 16 WHsAg-positive animals and 3 of the 27 anti-WHs-positive animals, but none of the 21 uninfected animals, developed hepatocellular carcinoma. Seroconversion, possibly resulting from infection with WHV, was documented in a chimpanzee inoculated with WHV. An immune adherence hemagglutination test for WHsAg was also developed by using anti-WHs of chimpanzee origin as a reagent, but the test was not useful for detecting anti-WHs of woodchuck origin because of the lability of the latter.

The value of the woodchuck as a model for the study of hepatitis and its course of infection has been well established (9, 10). The natural occurrence of hepatitis in the animals with the subsequent development of chronicity and hepatocellular carcinoma may closely resemble what happens in humans who are infected with hepatitis B virus (HBV) and subsequently develop liver cancer.

Previous studies have shown evidence of shared characteristics between the human hepatitis B virus and the woodchuck hepatitis virus (WHV). Both are DNA viruses, have similar morphologies, and possess analogous antigen-antibody systems which cross-react serologically (1, 9, 10, 11). This close antigenic relationship between WHV and HBV has made it possible to detect markers of WHV infection in woodchucks by using established tests for HBV infection. However, such tests can detect only cross-reactive WHV antigens and antibodies. We report our attempts to develop specific tests for WHV surface antigen (WHsAg) and antibody to it (anti-WHs) by adaptation of woodchuck reagents to the well-defined laboratory techniques of solid-phase radioimmunoassay (SPRIA) and immune adherence hemagglutination (IAHA).

MATERIALS AND METHODS

Woodchuck samples. Woodchuck sera and plasma were obtained from a colony of wild-caught woodchucks (Marmota monax) maintained for a period of up to 3 years (7).

Woodchucks were trapped in the wild, and therefore exact ages could not be determined. Upon receipt the animals ranged in weight from 6,700 to 850 g. Those heavier than 3,500 g were considered "adults," and those weighing less than 3,500 g were considered "yearlings." As could be determined from available records, this colony consisted of approximately 30% yearlings and 70% adult animals. Males and females were equally distributed in the two groups. These animals were bled monthly and monitored for markers of hepatitis infection on a routine basis. The monitoring included tests for serum glutamic oxalacetic transaminase and serum glutamic pyruvic transaminase levels and the presence of DNA polymerase (an indirect indicator of WHV virions) and a commercial test (AusRIA; Abbott Laboratories, Chicago, Ill.) for human HBV surface antigen (HBsAg). Selected sera were tested for antibody to HBsAg (anti-HBs) by a commercial SPRIA (AusAb; Abbott Laboratories). All

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sera and plasma samples were stored at −70°C. In addition, sera from 82 woodchucks trapped in New York for the purpose of establishing a breeding colony were kindly supplied by B. C. Tappel.

**Purification and radiolodination of WHSAg.** Serum from a chronic carrier woodchuck with WHV was partially purified by isopycnic banding in CsCl followed by flotation in CsCl as previously described for HBsAg (2).

The total protein and the proportion of protein attributable to WHSAg was determined by Lowry assay of the partially purified material and the particulate fraction of a sample that was subjected to rate zonal centrifugation in sucrose. The purity and identity of the particulate fraction were, in turn, determined by labeling it with 125I (see below) and demonstrating only two polypeptides, analogous to the P1 and P2 polypeptides of HBsAg, by polyacrylamide gel electrophoresis. Identical results were obtained when radiolabeled WHSAg was precipitated with woodchuck convalescent anti-WHS and subjected to analysis by polyacrylamide gel electrophoresis. The concentration of total protein in the partially purified preparation was 240 μg/ml; the concentration of WHSAg was 84 μg/ml. WHSAg was labeled with 125I by the chloramine T method of Hunter and Greenwood (3). Normal woodchuck serum and normal chimpanzee serum (final concentrations of 1%) were added to the [125I]WHSAg to prevent nonspecific reactions.

**Radioimmunoassays (RIAs).** SPRIAs for antigen and antibody were performed by the methods of Peterson et al. (6) and Purcell et al. (8) for markers of HBV infection.

(i) RIA for antibody. Wells of polyvinyl microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) were precoated with 75 μl of a 1:12,800 dilution of WHSAg. Both partially purified antigen and a high-titered WHSAg-positive serum from a naturally infected chronic carrier animal proved satisfactory and comparable as the "capture" substrate. Plates were covered and incubated in a humidified box at 4°C for a minimum of 4 h. Precoat reagent was recovered for reuse, and wells were washed two times with normal saline containing 0.1% sodium azide. The wells were postcoated with 250 μl of saline containing 1.0% bovine serum albumin and incubated overnight at 4°C. Plates were again washed two times, and 25-μl test samples, diluted 1:10, were inoculated into duplicate wells and allowed to incubate for 18 to 42 h at 4°C. After washing five times, the wells were inoculated with 50 μl of [125I]WHSAg and incubated for 4 h at 37°C. Plates were washed five times, and the individual wells were cut apart, transferred to Kimble poly styrene gamma counting tubes, and counted in a gamma counter. Results of duplicate wells were averaged and divided by the mean counts per minute obtained with a normal woodchuck serum; the ratios were expressed as sample/negative (S/N) counts. Values of 2.1 or greater were considered positive.

(ii) Blocking RIA for antigen. Microtiter wells were precoated with the reagent antigen and postcoated as above. To this substrate was then bound a quantity of standard reagent woodchuck antibody (25 μl per well of a pool diluted 1:50) sufficient to give an S/N value of 10. The next day, the plates were washed five times, and the test samples (diluted 1:2) were inoculated in duplicate into the wells and incubated overnight at 4°C. Wells were washed five times, and labeled WHSAg was added (50 μl per well). After 4 h of incubation at 37°C, the wells were washed five times, cut apart, and counted. If WHSAg was present in the sample, it competed for binding to the immobilized antibody and blocked the attachment to the labeled antigen. The results were expressed as a ratio of negative control (unblocked) counts per minute to sample counts per minute (N/S).

**IAHA.** A test for WHSAg by IAHA was also developed, by a modification of the method of Mayumi et al. for HBsAg (4) and of Moritsugu et al. for hepatitis A antigen (5). In 96-well microvolume polyvinyl microtiter plates (Dynatech Laboratories; 1-220-70, bottom) 10 μl of 10-fold dilutions (1:10, 1:100, 1:1,000) of samples to be tested for WHSAg were mixed with an equal volume of anti-WHS prepared in a chimpanzee (see below) and diluted to contain 4 to 8 μg of antibody. Duplicate sets of dilutions were incubated with buffer only. Plates were covered and incubated overnight in a humidified box at 4°C. Ten microliters of freshly diluted (1:65) guinea pig complement (NIH guinea pigs were bled, and sera were pooled and stored at −70°C in small volumes) was added to all wells, and the plates were shaken and then incubated at 37°C for 40 min. Ten microliters of diethiothreitol (3 mg/ml) was added, immediately followed by 10 μl of a 2% human O erythrocyte suspension, added to the plate while on the shaker. The test was incubated at room temperature for approximately 60 to 80 min until hemagglutination patterns could be read. On a scale of 0 to 4, +, 3+ and 4+ were considered positive when read against a buffer control.

**Test of effect of reducing agents on woodchuck antibody.** Four samples of a standard woodchuck antibody pool (diluted 1:5) were incubated with equal volumes of one of the following: 0.1 M 2-mercaptoethanol in phosphate-buffered saline, pH 7.4, for 60 min at 37°C; phosphate-buffered saline, pH 7.4, for 60 min at 37°C; 1.5 mg of diethiothreitol per ml in phosphate-buffered saline, pH 7.4, for 60 min at room temperature; or phosphate-buffered saline, pH 7.4, for 60 min at room temperature. These mixtures were then diluted in a 10-fold series (to 10⁻⁴) and tested for anti-WHS endpoint titrations by SPRIA.

**RESULTS AND DISCUSSION**

The RIA test for WHSAg on an early and late serum from each of the animals in the colony revealed two distinct populations of results. One group of N/S values was 1.0 or less, and the other group of N/S values was 1.8 or greater (Fig. 1). The one value which fell between these two groups (1.5) was an early serum from a woodchuck that subsequently became antigen positive and DNA polymerase positive, developed a chronic WHV infection followed by hepatocellular carcinoma, and eventually died. This animal is believed to have been developing antigen at the time of the early bleeding.

When these same woodchuck serum serum pairs were tested for anti-WHS by direct SPRIA, the antibody-containing sera (depicted in Fig. 1 as shaded blocks) were seen to lie only within the
antigen-negative population and had an average N/S value of 0.6. The additional antibody in the serum test samples had an additive effect with the reagent antibody already in the wells, thus producing S values higher than normal. Sera negative for both antigen and antibody had a mean N/S value of 0.9.

Figure 2 shows the results of titration by RIA of the partially purified WHsAg. This test detected antigen at a concentration as low as 10 ng/ml. On the basis of the data in Fig. 1 and this titration we chose an N/S value of 1.5 (164 ng/ml) as the cutoff point for positivity (33% suppression). Generally, this is a somewhat lower suppression level than is required in most blocking tests which utilize labeled antibody and is dictated by the less complete blocking of antigen when compared to antibody in such tests.

In attempts to set up a direct SPRIA by radiolabeling the woodchuck antibody and to perform the IAHA test to look for antibody in woodchuck sera, we encountered difficulties in both instances, which suggested to us that the anti-WHs was labile to a common or similar reagent employed in both tests. Since a reducing agent is used in both procedures, we constructed the experiment to test the effect of 2-mercaptoethanol and dithiothreitol on anti-WHs. Our data show that when antibody was exposed to either one of these reducing agents, there was a 10 to 30-fold fall in titer (Fig. 3). This sensitivity to reducing agents strongly suggests that the
anti-WHs may be of the immunoglobulin M class, but no further attempts were made to classify this antibody. In a subsequent experiment (data not shown), it was determined that the WHsAg precoat was not affected by similar treatment with these reducing agents, thus confirming that it was the antibody and not the antigen that was labile to the reducing agents.

The 72 feral woodchucks from the mid-Atlantic region (Maryland, Delaware, and Pennsylvania) comprising the colony were studied prospectively by SPRIA for markers of WHV infection for up to 3 years. Sixteen of these animals (22.2%) developed chronic WHV infection with detectable WHsAg and DNA polymerase activity in their serum. Twenty-seven of the 72 animals (37.5%) had evidence of transient naturally acquired infection with subsequent development of anti-WHs. The remaining 29 animals remained free of WHV infection; some of these were used for experimental transmissions of the virus. Eleven of the 16 WHsAg carriers and three of the 27 anti-WHs-positive animals, but none of the 21 seronegative animals, developed hepatocellular carcinoma during the course of the study. The incidence of naturally acquired WHV infection and the incidence of hepatocellular carcinoma in the feral woodchucks described herein are similar to those reported previously for Pennsylvania woodchucks (9, 10).

In a preliminary study of the 82 woodchucks trapped in New York, none was positive for WHsAg, and only two had detectable anti-WHs by the SPRIA tests. Thus, there appeared to be regional differences in the prevalence of WHV infection and subsequent hepatocellular carcinoma, observations similar to those of HBV infection and associated hepatocellular carcinoma in humans (10).

Utilizing the sensitive and specific RIA tests described above for evaluation of animal transmission studies, we determined that we were able to transmit the viral infection from woodchuck to woodchuck. Two susceptible animals were experimentally infected in this preliminary study. Woodchuck no. 12 was inoculated intravenously with 1 ml of a $10^{-3}$ dilution of a serum from a woodchuck chronically infected with WHV and responded with detectable antigenemia after 4 weeks, peaking at 6 to 7 weeks, with subsequent seroconversion to anti-WHs at 10 weeks (Fig. 4). Woodchuck no. 13, which received the same inoculum, seroconverted after 12 weeks, but WHsAg could not be detected during the course of the study. The same sera from woodchuck no. 12 that were positive for WHsAg by SPRIA were also weakly positive by AusRIA. All of the sera from both woodchucks were negative for anti-WHs when tested by AusAb. Serum glutamic oxalacetic transaminase and serum glutamic pyruvic transaminase values did not become elevated during WHV infection, and this and other studies (7) have led us to the conclusion that such tests, as currently performed, are not useful for detecting hepatitis in woodchucks.

Since WHV and HBV are serologically related, we thought it would be of interest to see whether primates were susceptible to WHV infection. In January 1979, chimpanzee no. 51 was inoculated intravenously with a $10^{-1}$ dilution of serum from the same antigen-positive woodchuck that was the source for the woodchuck transmission experiment. A specific anti-
WHs response was demonstrated after 8 weeks in this chimpanzee (Fig. 5). Although the S/N ratios were not high, there was, by contrast, no anti-HBs antibody detected by the Abbott AusAb test during this period. When inoculated with a partially purified preparation of WHsAg, this animal responded with a typical anamnestic elevation of antibody (Fig. 6). Whereas the serological response of this chimp was consistent with experimental infection with WHV, we were unable to detect any WHsAg and could not exclude a late immune response to the inoculum. A repeat of this experiment in another chimpanzee did not result in a similar seroconversion.

We have not yet developed a sensitive anti-core assay with which to distinguish between immunization and infection with WHV.

Relative sensitivity of the SPRIA and AusRIA for detecting WHsAg was difficult to assess because of frequent false-positivity with the latter test. In general, however, SPRIA and AusRIA detected the same positive sera and were equally sensitive for detecting dilutions of the partially purified WHsAg preparation. The SPRIA did not detect HBsAg in human plasma that were strongly positive by AusRIA (136 and 33 µg of subtypes adw and ayw HBsAg per ml, respectively). Many false-positive results with
woodchuck sera were also obtained with AusAb; this test virtually never detected anti-WHs. Thus, the SPRIAs appeared to detect an antigen-antibody system unique to WHV. In contrast, AusRIA detected an antigen common to both viruses.

We were able to use serum from the peak of chimp anti-WHs in an IAHA test for WHsAg in woodchuck sera. This was dictated by the fact that woodchuck antibody could not be used because of its lability to the reducing agent dithiothreitol as reported above. The limitation of the IAHA test, therefore, is that it can be used for the detection of WHsAg only. The test facilitates the quantitation of antigen, however.

Data on the comparative sensitivity of the RIA and IAHA test for WHsAg are, at this time, only preliminary. Of 137 sera tested, 28 were positive by both tests, 104 were negative by both tests, and 5 were negative by RIA but appeared to be positive by IAHA. These five sera gave partial hemagglutination patterns, as did five sera in the antigen-positive group of 28. We do not yet know why there was an incomplete hemagglutination of the erythrocytes. Since the hyperimmune chimp was immunized with only partially purified antigen, a degree of non-specificity may be due to an immune response to woodchuck proteins.

In summary, we have developed a sensitive specific RIA to detect WHsAg and anti-WHs as markers of WHV infection and an IAHA test which simplifies the quantitation of the antigen. With these tests we have been able to evaluate studies of naturally and experimentally infected animals. The development of similar assays for WHV core antigen and antibody to it will facilitate the use of the woodchuck animal model for furthering our understanding of the relationship of this group of viruses to the development of hepatocellular carcinoma in their respective hosts.

FIG. 6. Anamnestic response in the chimpanzee described in the legend to Fig. 5. The chimpanzee was boosted with partially purified WHsAg.
LITERATURE CITED


