JOURNAL OF VIROLOGY, Dec. 1986, p. 895–901
0022-538X/86/120895-07$02.00/0

Vol. 60, No. 3

Protection of Chimpanzees from Type B Hepatitis by Immunization with Woodchuck Hepatitis Virus Surface Antigen

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Received 6 February 1986/Accepted 27 August 1986

Two chimpanzees immunized with woodchuck hepatitis virus (WHV) surface antigen (WHsAg) developed antibodies cross-reactive with hepatitis B virus (HBV) surface antigen (HBsAg). After challenge with HBV, one animal was completely protected and the other experienced a subclinical infection, without evidence of liver disease. Three woodchucks immunized with HBsAg developed antibodies to HBsAg which did not cross-react with WHsAg. After challenge with WHV, all three woodchucks developed typical acute infections with associated hepatic lesions. Serological studies with the cross-reactive antibodies raised in chimpanzees suggested that the protective epitopes of WHsAg were related to the group *a* specificity of HBsAg. These studies indicated that cross-protective epitopes are shared by HBV and WHV; however, the humoral response to these epitopes can vary among species.

Hepatitis B virus (HBV) and the woodchuck hepatitis virus (WHV) are related hepadnaviruses (31, 35) and can establish persistent infections leading to a variety of disease sequelae in their natural hosts (e.g., chronic hepatitis and hepatocellular carcinoma) (36, 37). Although the chimpanzee has long served as a surrogate host for humans in modeling HBV infection, the chronic disease spectrum in this model is less severe and hepatocellular carcinoma has not been observed (41). In contrast, there is a high incidence of active hepatitis and hepatocellular carcinoma in woodchucks chronically infected with WHV (27). Therefore, the woodchuck and WHV can provide a useful model of virus-induced liver disease similar to that observed in humans (9).

Both the chimpanzee and woodchuck models are potentially useful for investigating cross-species infection and immunity to hepadnaviruses. For example, reciprocal transmission and immunization studies involving both models can further define the extent of host-virus specificity and disease susceptibility and also improve the possibilities for developing and testing alternative vaccine sources. In the present study, we conducted a cross-vaccination experiment with these two models to determine whether shared epitopes of HBV and WHV elicit protective immunity.

MATERIALS AND METHODS

Preparation of vaccines. The 20-nm forms of HBV surface antigen (HBsAg) and WHV surface antigen (WHsAg) were purified from serum specimens by ultracentrifugation (11). An HBV vaccine was prepared from HBsAg by Formalin treatment and adsorption to alum, as described previously (19). The immunogenicity and efficacy of the HBV vaccine used in these studies were demonstrated previously (28, 29, 45, 46). A WHV vaccine was prepared from WHsAg by using the same procedure and has been shown to protect adult and newborn woodchucks from experimental WHV challenge (J. L. Gerin, B. C. Tennant, R. H. Purcell, H. Popper, and F. J. Tyeryar, manuscript in preparation).

Cross-vaccination protocol. Two adult HBV-susceptible chimpanzees (A176 and A167) were immunized with the WHV vaccine (DMVI/WC59; 20 μg/0.5 ml intramuscularly at weeks 0, 5, and 25). The chimpanzees were subsequently challenged intravenously at week 30 with $10^{3.5}$ chimpanzee infectious doses of HBV (National Institute of Allergy and Infectious Diseases [NIAID] lot MS-2; HBV/*ayw* subtype); this dose of virus routinely produces acute HBV infections characterized by HBs antigenemia and liver disease (1, 21, 40; Table 1). Four adult WHV-susceptible woodchucks (137, 138, 141, and 143) were immunized with the HBV vaccine (NIAID lot A9A; *adw* subtype; 20 μg/0.5 ml intramuscularly at weeks 0, 5, 25, 29, and 33). Three of the woodchucks were then challenged intravenously at week 34 with $10^{3.5}$ woodchuck infectious doses of WHV (DMVI/WHV8); at this dose of virus, adult woodchucks develop acute WHV infections with associated hepatitis (26, 44; Gerin et al., in preparation).

Serological assays. Serum samples were obtained from all animals at weekly intervals during the pre- and postchallenge periods. Antibodies to HBsAg (anti-HBs), antibodies to HBV core antigen (anti-HBc), and HBsAg were assayed, respectively, by the Ausab, Corab, and Ausria II radioimmunoassays (RIAs) (Abbott Laboratories, North Chicago, Ill.). WHsAg and antibodies to WHV core antigen (anti-WHc) were assayed by RIA as described previously (4, 25). Antibodies to WHsAg (anti-WHs) were assayed by double-antibody radioimmune precipitation (RIP). Briefly, 5 μl of a 1:100 dilution of test serum and 5 μl of ^{125}I-labeled WHsAg (5,000 cpm) were incubated at room temperature in microtiter wells for 3 h; 100 μl of a 1:20 dilution of rabbit anti-woodchuck immunoglobulin serum or goat anti-human immunoglobulin G was then added, and the samples were incubated overnight at 4°C. The samples were then centrifuged at 1,300 × *g* for 25 min, and the amounts of ^{125}I-labeled WHsAg in the supernatants and precipitates were determined. The percentage of ^{125}I-labeled WHsAg in the precip-

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itate was calculated, and net precipitation was determined by subtracting the percentage precipitated by normal woodchuck or chimpanzee serum (8 to 12%); samples with net precipitation values greater than 20% were considered positive for anti-WHs.

Liver pathology. Clinical hepatitis was assessed in chimpanzees by serological assay of serum alanine aminotransferase (ALT) activity, as described previously (1, 40; Table 1). For woodchucks, liver biopsies were obtained at 9 to 12 weeks postchallenge and were examined histologically for hepatic lesions by one of the investigators (H.P.), as described previously (27).

Competitive inhibition RIA. The serological specificity of antibodies elicited in one of the vaccinated chimpanzees was studied by a modification of the commercial Ausab assay for anti-HBs. Briefly, 75 μl of the test serum was preincubated for 8 h with an equal volume of either 1% bovine serum albumin (BSA), normal human or chimpanzee serum (negative controls), or one of several preparations containing HBsAg or WHsAg (competitive inhibitors; see below). The mixtures were then assayed for free anti-HBs activity by incubation with HBsAg-coated beads (for 18 h) and detection of bound antibody with ^{125}I-labeled HBsAg. The percent inhibition of Ausab reactivity was used as a measure of the presence of competing epitopes in the antigen preparations (see below). Percent inhibition was derived by dividing the bound counts in the presence of inhibitor by the bound counts obtained in the presence of BSA. Antigen preparations producing greater than 50% inhibition relative to BSA were considered positive for competing epitopes.

Competing antigen preparations. All inhibitor preparations containing alum were adjusted to 1% BSA 24 h before use to block nonspecific binding sites. Reagents used as competitive inhibitors were as follows: (i) alum alone (NIAID lot P-3; placebo) (ii) the NIAID lot A9A HBV vaccine (HBsAg/*adw*; 40 μg/ml; alum adsorbed), (iii) NIAID lot A8 HBV vaccine (an aqueous form of the A9A vaccine which is not alum adsorbed; HBsAg/*adw*; 40 μg/ml), (iv) serum antigens of the MS-2 strain of HBV (HBsAg/*ayw*; HBs antigenemic serum from an MS-2-infected chimpanzee), (v) serum antigens from a patient infected with the *adr* subtype of HBV (11), (vi) purified 20-nm WHsAg (50 μg/ml), and (vii) Heptavax-B vaccine (20 μg/ml; alum adsorbed; Merck Sharp & Dohme, West Point, Pa.) (38, 39). This vaccine differs from the NIAID vaccines in that the 20-nm HBsAg is pretreated with pepsin; pepsin treatment has been shown to eliminate both the polyalbumin-binding activity and the antigenic reactivity associated with pre-S2 epitopes in the subviral particles (22, 23).

RESULTS

The serological profile of chimpanzee A176 after vaccination with WHsAg and HBV challenge is shown in Fig. 1A. The animal developed anti-WHs antibodies by 3 weeks and sustained the response for most of the prechallenge period. Cross-reactive anti-HBs antibodies were detected transiently after the priming immunization and first boost (sample-to-negative [S/N] ratios of 2.1 to 10.4). After the final boost at week 25, the animal developed a stronger and sustained anti-HBs response. After challenge with HBV, the chimpanzee did not develop HBs antigenemia, anti-HBc, or elevations in serum ALT, indicating complete protection from HBV infection.

The serological profile of another chimpanzee vaccinated with WHsAg and challenged with HBV is shown in Fig. 1B. The animal (A167) developed anti-WHs antibodies after two doses of WHsAg and remained seropositive for most of the prechallenge period. Cross-reactive anti-HBs antibodies were transiently detected at week 4 (S/N = 2.6) and then between weeks 6 and 10. After the last boost with WHsAg at week 25, chimpanzee A167 developed a weak cross-reactive anti-HBs response (S/N = 2.1 to 8.2) that was sustained until the time of challenge. Five weeks after challenge, there was an increase in anti-HBs followed by a transient and low-level anti-HBc response; HBs antigenemia and elevations in serum ALT were not detected in this animal. These serological events indicated a modified and limited course of infection, with absense of clinical hepatitis, when compared with the patterns obtained with the historical challenge controls (Table 1).

All four woodchucks receiving the HBV vaccine developed anti-HBs antibodies, but no cross-reactive anti-WHs antibodies were demonstrated throughout the prechallenge period (Table 2). The RIP with ^{125}I-labeled WHsAg was unable to detect cross-reactive anti-WHs antibodies in woodchuck serum specimens even at lower dilutions (e.g., 1:25). In contrast, anti-HBs antibodies detected in chimpanzees in the Ausab RIA were readily detected by an analogous RIP which used ^{125}I-labeled HBsAg (data not shown).

Three of the four vaccinated woodchucks were challenged with WHV at week 34, even though repeated immunization with HBsAg had failed to elicit cross-reacting anti-WHs antibodies. All three woodchucks developed WHV infections characterized by seroconversion to anti-WHc and histologic evidence of hepatitis (Table 2). Two of the animals developed WHs antigenemia and severe hepatitis despite high levels of circulating anti-HBs; both woodchucks eventually recovered and seroconverted to anti-WHs (Table 2 and Fig. 1C). In the third animal, WHs antigenemia was not detected, but the animal developed anti-WHc antibodies and mild hepatitis (Table 2). Seroconversion to anti-WHs was not demonstrated in this animal; however, a liver biopsy obtained at 10 months postchallenge was negative for WHcAg, indicating that recovery from WHV infection had occurred (26). We concluded that vaccination with HBsAg did not protect these animals from WHV infection or from WHV-induced hepatitis.

Antigens in the MS-2 HBV inoculum and the present HBsAg vaccine were analyzed further with antibodies in the prechallenge serum from chimpanzee A176 (Table 3). Both the alum-adsorbed and aqueous forms of the NIAID HBV vaccine inhibited the cross-reactive anti-HBs activity in the chimpanzee serum; the commercially available Heptavax-B vaccine was also an effective inhibitor of the anti-HBs activity. Both vaccines contain HBsAg of the *adw* subtype. The cross-reactive antibodies were also inhibited by two different HBsAg-positive serum specimens, one from a chimpanzee that was previously infected experimentally with the MS-2 HBV (*ayw* subtype) and another from a patient who was infected with the *adr* subtype of HBV. Purified 20-nm WHsAg was also able to inhibit the cross-reactive anti-HBs antibodies in the serum from the WHsAg-vaccinated chimpanzee. In another experiment (data not shown), cross-reacting antibodies from chimpanzee A176 were reacted with HBsAg-coated beads and were detected equally well with either ^{125}I-labeled HBsAg or ^{125}I-labeled WHsAg. This further indicated that in chimpanzees, the WHsAg vaccine elicited functionally cross-reactive antibodies that recognized both HBsAg and WHsAg. The data in Table 3 indicate that the corresponding epitopes were present in the HBV given to the chimpanzees and in the HBsAg

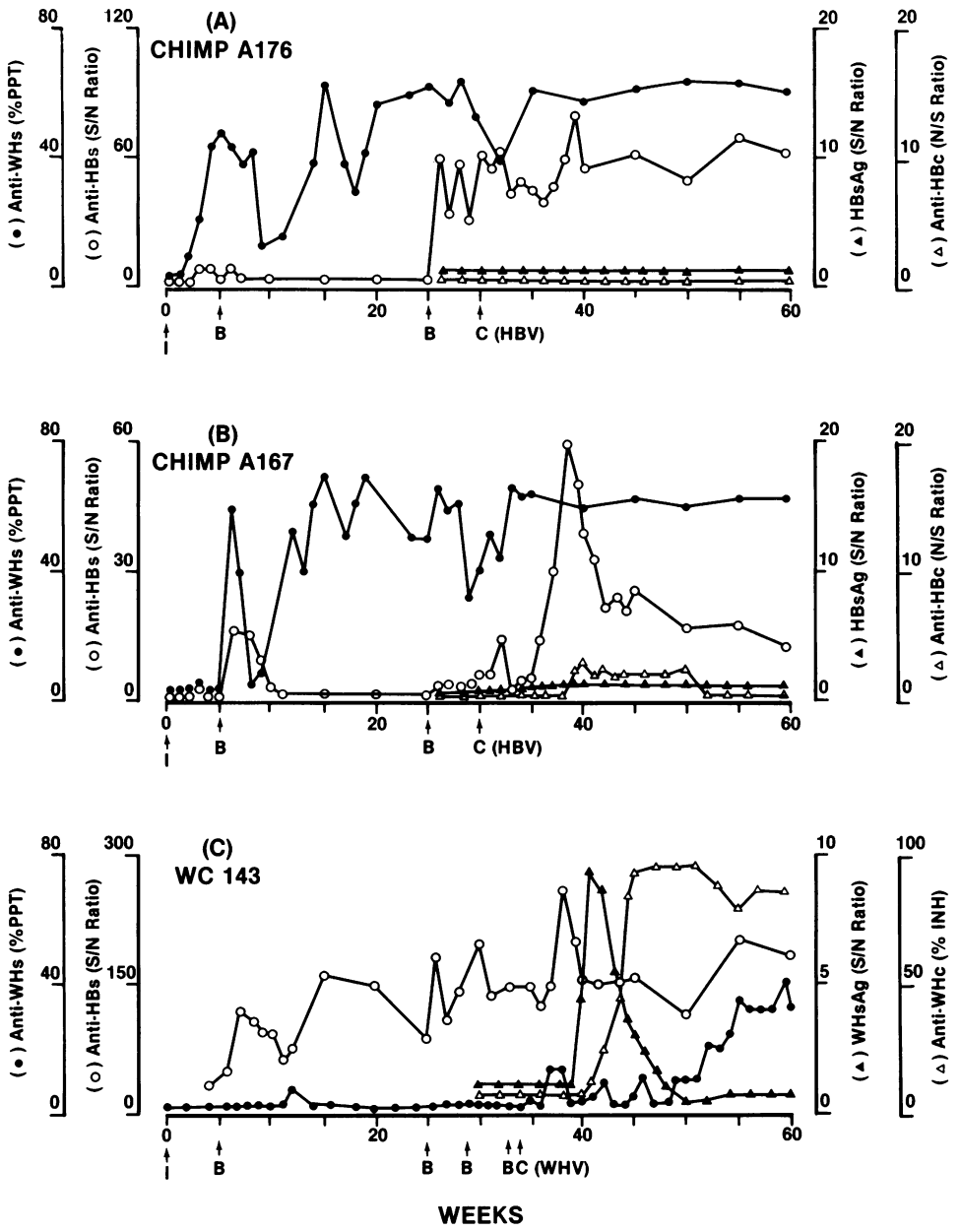

FIG. 1. Serological profiles for chimpanzees A176 (A) and A167 (B) after vaccination with WHsAg and challenge with HBV and for woodchuck (WC) 143 after vaccination with HBsAg and challenge with WHV (C). I, Initial immunization; B, booster immunization; C, challenge with infectious virus; PPT, precipitated; INH, inhibition. To facilitate graphical presentation, the serological data were plotted at 1-, 2-, or 5-week intervals to demonstrate the virological events after vaccination and challenge. Serum ALT levels did not rise above normal in either chimpanzee and were therefore omitted from the plot.

vaccine used to immunize the woodchucks. The analysis further suggested that the cross-reactive epitopes are related to the classically defined group *a* determinants of HBV.

DISCUSSION

Vaccine studies in humans and in the chimpanzee model have shown that protection from HBV infection correlates with the development of an anti-HBs response (19, 28, 29, 38, 39, 45, 46). In the chimpanzee model of experimental HBV infection, suboptimal anti-HBs responses can also afford some degree of protection, as indicated by the absence of or significant decrease in HBs antigenemia, anti-HBc response, and liver disease (12, 13, 21, 28). The results of the present study show that chimpanzees developed anti-HBs antibodies in response to vaccination with WHsAg and were protected from type B hepatitis. Conclusions based on small numbers of animals in this study and others (12, 16, 17, 21) appear to be valid in view of the uniform patterns of chimpanzee response to experimental infection with standardized inocula (Table 1).

The degree of protection afforded by cross-vaccination appeared to be related to serological levels of cross-reactive antibodies present at the time of HBV challenge. Chimpan-

TABLE 1. Challenge of chimpanzees with HBV (strain MS-2)[a]

Chimp no.	Year	HBsAg			ALT		
		First appearance (wk)	Maximum S/N ratio	Duration (wk)	First appearance (wk)	Maximum S/N ratio	Duration (wk)
9	1974	7	92	25	16	640	9
777	1974	9	61	35	20	208	23
778	1974	7	60	40	15	752	32
755	1976	6	36	8	11	2,178	6
42	1981	7	188	8	14	1,470	7
60	1981	7	139	20	14	1,494	15
61	1981	8	131	19	14	963	16
963	1981	8	131	13	11	1,740	10
1098	1983	6	155	15	10	1,251	11
1072	1985	6	165	30	11	690	10

[a] HBV-susceptible chimpanzees were experimentally challenged with the MS-2 strain of HBV/*ayw* during the time period 1974 to 1985. Each animal was given an intravenous inoculation of $10^{3.5}$ chimpanzee infectious doses of HBV (40) and was monitored weekly for serological and biochemical evidence of HBV infection. All animals developed acute type B hepatitis with subsequent seroconversion to anti-HBc and anti-HBs. The incubation periods to the first appearance of HBsAg (S/N ratio >2.1) and an abnormal ALT value (international units/liter; normal values, <60), the maximum values of HBsAg and ALT, and the durations for their detection are shown for each of 10 experimental chimpanzees. Serum HBsAg and ALT values were determined by commercial assays as described in Materials and Methods. Typical patterns of individual chimpanzee response to experimental HBV (strain MS-2) infection were previously reported by this laboratory (21) and others (16, 28).

zee A176 sustained a relatively high level of cross-reacting anti-HBs and was completely protected from HBV infection, whereas chimpanzee A167 had lower anti-HBs levels and developed a modified course of infection without disease (Fig. 1A and B and Table 2). Further studies are required to determine whether there are differences in the fine specificities of the cross-reactive antibodies detected in the two chimpanzees.

The lack of hepatitis in chimpanzee A167 was probably related to the rapid rise in anti-HBs shortly after challenge. This anamnestic response was most likely stimulated by early replicative HBV infection, which was associated with seroconversion to anti-HBc (Fig. 1B). The above observations suggest that in this animal, the WHsAg vaccine induced a suboptimal antibody response to cross-reactive epitopes of HBV, resulting in partial protection upon experimental challenge.

In contrast, woodchucks vaccinated with HBsAg developed only HBsAg-specific antibodies and were not protected from WHV infection. The humoral anti-HBs response did not alter the course of WHV infection and disease, which was consistent with the lack of a cross-reacting antibody response. Thus, cross-vaccination had no apparent priming effect against WHV in these woodchucks.

Hepadnavirus surface antigen particles have a complex quaternary structure and polypeptide composition. The polypeptide composition of 20-nm HBsAg could vary depending on the isolate (33) or type of preparation (e.g., RIA antigens versus vaccine antigens). The cross-reactive antibodies from one protected chimpanzee (A176) were not detected when assayed in the presence of the HBsAg vaccine given to woodchucks (Table 3); therefore, the HBsAg vaccine given to woodchucks contained the appropriate cross-reactive epitopes. The data suggest that the lack of

TABLE 2. Cross-vaccination and virus challenge of chimpanzees and woodchucks

Animal	Vaccine/ challenge virus	Prechallenge		Postchallenge			
		Anti-WHs[a]	Anti-HBs[b]	HBsAg or WHsAg[c]	Anti-HBc or Anti-WHc[d]	Hepatitis[e]	Outcome[a,f]
Chimpanzee							
A176	WHsAg/HBV	+ + +	+ +	−	−	−	CP
A167	WHsAg/HBV	+ + +	+	−	+	−	PP
Woodchuck							
143	HBsAg/WHV	−	+ + +	+	+ + +	+	Anti-WHs (+ +)
137	HBsAg/WHV	−	+ + +	+	+ + +	+	Anti-WHs (+ +)
141	HBsAg/WHV	−	+ + +	−	+ + +	+/−	Anti-WHs (−)
138[g]	HBsAg	−	+ + +				

[a] Anti-WHs was determined by RIP. Net values >20% precipitation were considered positive; the maximum precipitation attained for the prechallenge period in chimpanzees and postchallenge outcome in woodchucks were 70 to 80% (+ + +) and 30 to 40% (+ +), respectively.

[b] Anti-HBs was determined by the Ausab RIA. Maximal S/N ratios are designated as follows: +, 2.1 to 50; + +, 51 to 100; + + +, >100.

[c] HBsAg was determined by the Ausria II RIA, and WHsAg was determined by site-specific monoclonal RIAs (4); S/N values greater than 2.1 and 3.1, respectively, were considered positive.

[d] Anti-HBc was determined by the Corab RIA at 1:2 serum dilutions; anti-WHc was determined at 1:10 serum dilutions by specific competition inhibition RIA (25). S/N values greater than 2.1 and inhibition greater than 50% were considered positive in the respective tests. +, N/S ratio between 2.1 and 4, corresponding to 50 to 80% inhibition for anti-HBc; + + +, >90% inhibition for anti-WHc.

[e] Hepatitis was assessed in chimpanzees by elevations in serum ALT (Table 1) and in woodchucks by histological examination of liver biopsies taken at 9 to 12 weeks postinfection. +/−, Mild; +, severe.

[f] The outcome of experimental challenge of chimpanzees is designated as follows: CP, complete protection; PP, partial protection. The outcome of challenge for woodchucks was infection and recovery and is designated by seroconversion to anti-WHsAg (see footnote *a*); follow-up liver biopsy from woodchuck 141 at 10 months postchallenge was negative for WHcAg, indicating recovery from infection (26).

[g] Woodchuck 138 died before challenge.

TABLE 3. Competitive inhibition of cross-reacting anti-HBs activity by HBsAg and WHsAg

Final serum dilution[a]	Bound ^{125}I-labeled HBsAg (cpm) with BSA[b]	% Inhibition of binding with competitive inhibitor (HBsAg subtype)[c]:						
		Alum	HBV vaccine A9A (*adw*)	HBV vaccine A8 (*adw*)	MSD vaccine (*adw*)	MS-2 (*ayw*)	Patient serum (*adr*)	WHsAg (20 nm)
1:2	3,715	0	86	60	72	98	77	98
1:10	2,460	0	87	92	93	ND	ND	ND

[a] Prechallenge serum samples were obtained from chimpanzee A176 at weeks 26 through 30 after vaccination with WHsAg. The antiserum contained both WHsAg-specific antibodies and cross-reactive anti-HBs antibodies (Fig. 1A). The serum samples were pooled, adjusted to the appropriate dilution, mixed with equal volumes of competitor (75 μl of sample plus 75 μl of competitor), and preincubated for 8 h at room temperature. The sample was then assayed by the Ausab procedure.

[b] The counts per minute of bound ^{125}I-labeled HBsAg reflected the free anti-HBs activity in the serum.

[c] The percent inhibition of binding relative to that obtained for BSA. Normal human or chimpanzee sera did not competitively inhibit the reaction (data not shown). The competing preparations were adjusted to 1% BSA 1 day before use. The preparations shown are alum, the A9A HBV vaccine, the A8 HBV vaccine (an aqueous form of the A9A vaccine), the Merck Sharp & Dohme (MSD) Heptavax-B vaccine, the standard NIAID MS-2 HBV inoculum, undiluted HBs antigenemic serum from a patient infected with the *adr* subtype of HBV (11), and purified 20-nm WHsAg. See Materials and Methods for further description. ND, Not done.

cross-reacting anti-WHs in the woodchucks was due to the lack of a humoral response to the cross-reacting determinants of HBsAg. Therefore, the surface antigens of HBV and WHV share antigenic domains that potentially elicit antibodies associated with protection, although the immune response to these epitopes can vary among species.

Several investigators have shown cross-reactions between HBsAg and anti-WHs sera from convalescent woodchucks (32, 43). These studies indicate that the woodchuck immune system can ultimately respond to cross-reactive surface determinants (at least in the context of WHV). The occurrence of cross-reactive antibodies is most likely dependent on intrinsic (antigen) and extrinsic (host) factors (2) that together influence the relative immunogenicity of hepadnavirus group epitopes. This may limit efforts to study these determinants by lowering the probability of obtaining suitable antibody reagents. For example, anti-WHs sera from convalescent woodchucks (20, 44) and antisera from BALB/c mice hyperimmunized with WHsAg (5) were not generally cross-reactive with HBsAg. In addition, although we have been able to produce a subclinical WHV infection in one chimpanzee (26, 44), the convalescent anti-WHs did not cross-react with HBsAg. In the present study, repeated immunization with WHsAg protected chimpanzees against hepatitis B and is therefore one potential approach to study protective functions of shared hepadnavirus epitopes.

The cross-reactive anti-HBs elicited by WHsAg in chimpanzee A176 recognized antigens of three major HBV subtypes (the *ayw* challenge virus antigen, the *adw* vaccine antigen, and an *adr* serum antigen; Table 3); this indicated that the antibodies react with group *a* determinants of HBV, rather than with subtype *d/y* or *w/r* determinants. The group *a* specificity of HBV is known to elicit antibodies associated with protective immunity, in that vaccination with *ad* vaccines protects against HBV of the *ay* subtype (29, 38). Recent studies with monoclonal antibodies (24, 42) and antibodies to synthetic peptides (3, 10, 15, 22) suggest that the *a* determinant is actually a set of several nonoverlapping epitopes shared by different strains of HBV. Additional evidence obtained with monoclonal antibodies and polyclonal antisera indicates that at least one of the HBV *a* epitopes is shared by WHV (and also by the ground squirrel hepatitis virus) (5–7, 43). Our present results extend these types of observations and indicate that hepadnavirus group determinants can play a role in host immunity. Although some of the observations suggest that shared epitopes are minor determinants on the whole, their role in immunity further indicates that they should be included in the construction of chemical and recombinant vaccines (18, 30).

The cross-reactive antibodies from chimpanzee A176 recognized the commercial HBV vaccine (Heptavax-B; Table 3). The Heptavax HBsAg is pretreated with pepsin and, although it retains group and subtype antigenicity (38, 39), it does not react with antibodies to synthetic peptides representing the HBV pre-S2 region (14, 22, 23, 34). The pre-S2 amino acid sequences, although conserved among HBV strains (22), share little homology with the corresponding sequences of WHsAg (8). Together, these observations suggested that cross-reactive epitopes recognized by chimpanzee A176 are not pepsin-sensitive pre-S2 determinants. There is a greater degree of predicted amino acid sequence homology in the S region of HBV and WHV (8), and therefore the shared epitopes of HBsAg and WHsAg may well be specified within the S region of the envelope genes. More definitive immunochemical studies are in progress to determine the polypeptide location and conformational stability of the cross-protective epitopes identified in this study.

In this study, we used a cross-vaccination protocol to identify at least one shared epitope that stimulates antibodies in chimpanzees that are associated with protection from type B hepatitis. The question remains therefore whether woodchucks can be protected from WHV-induced hepatitis by developing cross-reactive antibodies to the conserved epitopes. The lack of protection observed in the woodchucks in this study could be explained if antibodies were produced mainly against pre-S2 determinants in the NIAID HBV vaccine (23, 45). For example, if pre-S2 epitopes were more immunodominant than others (22), then a virus-specific response would be expected due to the greater differences in pre-S2 amino acid sequences between HBV and WHV (8). The WHV-woodchuck model might therefore be useful in evaluating new synthetic or recombinant HBV vaccines consisting only of those determinants that are conserved in WHV.

ACKNOWLEDGMENTS

This research was supported by contract NO1-AI-22665 between NIAID and Georgetown University and contract NO1-AI-02640 between NIAID and SEMA, Inc.

We thank C. A. Langer for her assistance in conducting serological assays, Cynthia French for her secretarial assistance in the preparation of the manuscript, and D. W. Taylor and B. H. Hoyer for their editorial comments.

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