JOURNAL OF VIROLOGY, July 1983, p. 15–23
0022-538X/83/070015-09$02.00/0

Vol. 47, No. 1

Nonoverlapping Antigenic Sites of Woodchuck Hepatitis Virus Surface Antigen and Their Cross-Reactivity with Ground Squirrel Hepatitis Virus and Hepatitis B Virus Surface Antigens

PAUL J. COTE, JR.,†* AND JOHN L. GERIN

Division of Molecular Virology and Immunology, Georgetown University Medical Center, Rockville, Maryland 20852

Received 22 December 1982/Accepted 28 March 1983

Five nonoverlapping antigenic sites (sites I through V) of woodchuck hepatitis virus surface antigen were identified with competitive binding assays involving monoclonal antibodies. Site I contributed to cross-reactions among surface antigens of hepatitis B-like viruses infecting woodchucks, ground squirrels, and humans. At least three distinct sites (sites I, II, and III) are responsible for cross-reactions between woodchuck and ground squirrel hepatitis virus surface antigens. Sites IV and V of woodchuck hepatitis virus surface antigen are not major cross-reactive sites, suggesting that these elicit virus-specific antibodies. There were no cross-reactions with duck hepatitis B virus surface antigen.

Woodchucks (*Marmota monax*) are naturally infected by an hepatotropic DNA virus, or hepadnavirus (23), that is morphologically and structurally similar to hepatitis B virus of humans (27, 28). Infection of marmots by woodchuck hepatitis virus (WHV) has been viewed as a naturally occurring animal model for virus-induced liver disease and its long-term sequelae, which include chronic hepatitis and hepatocellular carcinoma (2, 21). The surface antigen of these and other hepatitis B-like viruses is found mainly in the form of noninfectious subviral particles that circulate in the blood of infected hosts in vast excess of the virion (26). The particles share antigenic determinants with viral coat proteins (12, 24) and are therefore useful in the preparation of subunit vaccines (9, 10, 19, 22, 29). In the hepatitis B virus system, genetic variation in the hepatitis B surface antigen (HBsAg) can be detected with serum antibodies recognizing group (*a*) and subtype (*d/y*, *w/r*) antigenic determinants (1, 9, 15, 25). In contrast, serological specificities corresponding to different surface antigenic determinants of WHV have not yet been identified. In the present study, we used monoclonal antibodies to identify five nonoverlapping antigenic domains of woodchuck hepatitis virus surface antigen (WHsAg). The five sites were delineated in competitive binding assays with monoclonal antibodies and were further characterized for their cross-reactivity with HBsAg and with surface antigens of the ground squirrel hepatitis virus (GSHsAg) (17) and duck hepatitis B virus (18).

† Address reprint requests to: Paul J. Cote, Jr., Division of Molecular Virology and Immunology, Georgetown University Medical Center, 5640 Fishers Lane, Rockville, MD 20852.

MATERIALS AND METHODS

MCABs. Monoclonal antibodies (MCABs) predefined by immunization of BALB/c mice with WHsAg have been described previously (4). One antibody reacts with WHsAg, GSHsAg, and HBsAg (MCAB no. 101-2), several react with WHsAg and GSHsAg, and others react only with WHsAg. MCABs are herein identified by their primary clonal identification numbers and their antigenic site specificity (e.g., 101/I, 3/II, etc.; see below). Antibodies were partially purified from ascites fluids by ammonium sulfate salt precipitation and were radioiodinated by a solid-phase lactoperoxidase method (14).

Antigens. The 22-nm form of WHsAg was isolated from plasma of WHV chronic carrier woodchucks by established ultracentrifugation procedures (9, 10). Solid-phase antigen adsorbents and radioiodinated antigen (3, 4) were prepared with purified WHsAg obtained from two different woodchucks (no. 7 and 8, respectively; National Institute of Allergy and Infectious Diseases Animal Colony, Meloy Laboratories, Inc., Rockville, Md.). The standard WHsAg-positive serum was that obtained from woodchuck no. 8. A serum sample positive for GSHsAg was a gift of P. L. Marion and W. S. Robinson (5, 11, 17). A serum sample positive for duck hepatitis B virus surface antigen was a gift of J. Summers (18). Sera positive for various HBsAg subtypes were obtained from the Research Resources Branch of the National Institute of Allergy and Infectious Diseases (Bethesda, Md.). Standard control serum was obtained from a colony woodchuck that was negative for all markers of WHV infection. Normal human serum was obtained from the

TABLE 1. Nonoverlapping antigenic sites of 22-nm WHsAg defined by CAB assays[a]

Site	Clone	% Inhibition										
		I	II		III			IV		V		
		101	3	14	114	19	172	71	181	41	31	151
I	101	82	28	25	0	27	19	36	40	26	18	11
II	3	8	91	92	8	27	14	14	15	32	1	23
II	14	17	83	89	13	33	19	6	18	24	1	1
III	114	37	16	16	80	52	46	32	31	31	8	22
III	19	36	21	35	44	90	85	18	23	17	17	13
III	172	31	27	33	61	91	95	29	32	33	19	9
IV	71	14	25	27	14	29	3	76	87	32	11	12
IV	181	18	24	20	22	37	11	74	93	27	10	4
V	41	32	30	37	10	24	8	15	17	80	54	76
V	31	13	33	41	11	33	13	18	18	84	94	77
V	151	10	15	14	23	35	27	20	32	91	87	89

[a] MCABs are listed by site (i.e., I through V) and clone identification number (e.g., 101, 3, etc. [4]). The matrix shows the competitive binding between antibody in ascites fluids and radioiodinated antibody probes for antigenic sites of WHsAg. Values are the percent inhibition of labeled antibody binding by the corresponding ascites fluid sample. The data represent the averages for two or three separate experiments. Strong inhibition was 74 to 95%); moderate inhibition was 44 to 61%; values less than or equal to 35% inhibition were considered negligible. If the average of reciprocal tests was less than 35% inhibition, the antibodies were considered noncompetitive. See the text for details.

commercial Ausab kit (Abbott Laboratories, Inc., Chicago, Ill.).

CAB assay. Purified WHsAg was diluted to 5 μg/ml in phosphate-buffered saline (0.85% NaCl, 0.01 M phosphate buffer, pH 7.3) and was absorbed to wells of polyvinylchloride plates (Dynatech, Inc., Alexandria, Virginia) (60 μl/well, 18 h, 4°C). Wells were then washed and coated with 1% bovine serum albumin in phosphate-buffered saline (albumin-buffer) (18 h, 4°C). For competitive antibody-binding (CAB) assays, ascites fluids containing MCABs were diluted 1:5 in albumin-buffer, and 50-μl samples were incubated in antigen-coated wells (30 min, 25°C). This was followed by the addition to the fluid phase of an appropriate radioiodinated MCAB and further incubation (2×10^5 cpm/5 μl, 2 h, 37°C). Wells were then washed, and bound counts were determined. CAB experiments between different MCABs were performed two to three times each and in both directions (i.e., *X* ascites versus ^{125}I-*Y* and *Y* ascites versus ^{125}I-*X*). Control samples consisted of the probe plus albumin-buffer containing 2% normal mouse serum (blank). The percent inhibition or the degree of competition between labeled and unlabeled antibodies was calculated as 100% × 1.00 − (counts bound in the presence of test antibody/counts bound for the blank).

Site-specific RIAs. Solid-phase sandwich radioimmunoassays (RIAs) for antigen detection were developed based on antigenic site specificities of the MCABs (Cote et al., submitted for publication). In one RIA system, WHsAg- or GSHsAg-positive serum was incubated with a cross-reactive antibody immunoadsorbent, and bound antigen was detected by site-specific radioiodinated MCAB probes. In another RIA system, the group-specific 101 antibody was used to probe for hepadnavirus surface antigens reacted with site-specific immunoadsorbents. Respective incubation conditions for sera and probes were 18 h at 25°C and 3 h at 37°C; sera were diluted to 1:25 or greater for the RIA, and all probes were used at 2×10^5 cpm/50 μl. The results are expressed as the positive or test sample counts per minute (*P* value) divided by counts for a negative control (*N* value). Normal woodchuck and human sera served as negative controls. The avidity of the 101 antibody is lower for HBsAg than for WHsAg and GSHsAg (4); therefore, immunoadsorbents able to bind HBsAg were prepared from a 1:50 dilution of ascites fluids rather than the 1:500 dilutions used for binding WHsAg and GSHsAg.

Immunocomplex mapping of viral antigens by RIA. Five microliters of ascites fluid was incubated with 50 μl of antigen-positive serum diluted 1:125 in albumin-buffer (30 min, 25°C). The mixture of immunocomplexes was then transferred to an assay well coated with MCAB to an overlapping or nonoverlapping site relative to the blocking antibody (18 h, 25°C). After washing, the presence or absence of bound immune complexes was detected with the 101 probe. For controls, the 1:125 dilutions of antigen-positive sera were assayed similarly in the absence of blocking antibody. Normal woodchuck serum was used as the negative control.

Terminology. For the interpretation of present results, we assume that MCABs define individual epitopes of antigens, also referred to as single antigenic determinants. Competitive inhibition between two MCABs indicates that they are directed against the same antigenic site, or domain, which may or may not correspond to a single antigenic determinant. The serologically defined (13) group and subtype determinants of HBsAg have not yet been characterized in relation to the above definitions. Application in the HBsAg system of terms such as group or subtype determinant is by convention (1, 15) and need not imply that either specificity is restricted to a single epitope.

RESULTS

Antigenic domains of WHsAg. Analysis of WHsAg in CAB assays revealed at least five nonoverlapping antigenic sites (or domains)

(sites I through V; Table 1). To construct the matrix, antibodies were ordered so that strong reciprocal inhibition reactions fell on either side of the central diagonal. Three levels of competition inhibition reactions were discerned by histographic analysis (data not shown); these were qualitatively designed as strong, moderate, and low level (i.e., negligible). The mean percent inhibition (± standard error) for strong competition reactions was 86 ± 1% (*n*, 22; range, 74 to 95% inhibition). The mean for moderate competitions was 51 ± 3% inhibition (*n*, 5; range 44 to 61%); that for negligible competition was 20 ± 1% inhibition (*n*, 88; range, 0 to 35%). A few of the assays resulted in borderline competition between antibodies when tested in one direction (*n*, 6; range, 36 to 41%); however, the reciprocal test demonstrated little or no competition (e.g., 31/V ascites versus ^{125}I-14/II and 14/II ascites versus ^{125}I-31/V). In such cases, the average of reciprocal tests was always less than 35% inhibition, and thus the antibodies were considered mutually noncompetitive. One-way observations of this type could be explained if an antibody specific for one epitope was able at higher concentrations to interact weakly with a heterologous epitope or to induce partial allosteric changes elsewhere in the antigen. Weak reciprocal competition between antibody groups mapping to distinct sites could suggest either partial recognition phenomena or immediate proximity relationships between two sites.

Strong reciprocal inhibition reactions in CAB assays suggested that domains mapped by two or more MCABs are defined by steric inhibition between antibodies directed against the same epitope (or determinant) (sites II through V, Table 1). On the other hand, this level of inhibition need not imply that a given antigenic domain corresponds to a single determinant; that is, competition between two antibodies against two distinct epitopes may be strong if the determinants are spatially close together within a reactive domain. We noted that the reciprocal inhibition reactions between antibody 114 and antibodies 19 and 172 were consistently within the moderate range (site III, Table 1). This could suggest that the 114 determinant is distinct from the 19 and 172 epitope(s) although they are all spatially close enough to be resolved as a single domain by antibody competition. Other factors could also influence the relative strengths of reciprocal inhibition reactions, including the balance and affinities of labeled and unlabeled antibody species in the reaction mixture. Molecular size differences, as in immunoglobulin M (IgM) versus IgG competition, were not a complicating factor because the competing species were all of the same isotype (IgG1, κ). Enhancement of antibody binding at distinct sites was not observed for any of the antibody combinations tested (16). At present, allosteric inhibition of antibody binding to topographically distinct sites of WHsAg cannot be differentiated from steric inhibition. More detailed knowledge of the structure and location of determinants in the 22-nm WHsAg particle is required to resolve this issue.

Antigenic analysis with site-specific RIAs. Site-specific solid-phase RIAs were developed for the detection and analysis of WHsAg and related antigens (Cote et al., submitted for publication); two types of assay systems were most useful in this regard, and they are detailed below. RIA positive/negative ratios greater than 3.1 were obtained consistently for known positive samples having WHsAg concentrations between 16 and 80 ng/ml (i.e., the level of assay sensitivity); thus, the 3.1 mark was used as the positive-negative cutoff point. WHsAg and GSHsAg bound to a cross-reactive antibody immunoadsorbent could be detected with different radioiodinated MCABs. WHsAg was detected by all antibody probes directed against each of its five antigenic domains; however, GSHsAg was only detected by antibody probes corresponding to sites I, II, and III of WHsAg (Table 2). Thus, sites I, II, and III of WHsAg cross-react with GSHsAg, whereas sites IV and V are not major antigenic sites of GSHsAg (the latter being most likely WHsAg-specific sites). The same general pattern of detection was observed when these two antigens were assayed on site-specific immunoadsorbents followed by a probe with the 101 antibody; in this system some low-level reactivity of GSHsAg with the site IV and V immunoadsorbents was detected by the 101 probe (Table 3). Such reactivity was only associated with assay of GSHsAg-postive serum at lower dilutions (Fig. 1). Overall, the data clearly show site-specific antigenic differences between WHsAg and GSHsAg. HBsAg was detected with the site I immunoadsorbent-probe combination, but not with any of the others (Table 3). These observations were further confirmed in double-antibody RIP assays against highly purified, ^{125}I-HBsAg/ *ad* and */ay* ligands; of the present antibodies, only the 101 antibody reacts with HBsAg in RIP (data not shown). In addition, HBsAg bound to HBsAg-specific monoclonal immunoadsorbents can be readily detected with the 101 probe at positive/negative ratios comparable to or greater than that given in Table 3 (Cote et al., submitted for publication). Duck hepatitis B virus surface antigen was not detected in any of the present assay systems (data not shown).

Immunocomplex mapping of WHsAg and GSHsAg domains by RIA. Although sites I, II, and III of WHsAg are shared by GSHsAg, this

TABLE 2. RIA of WHsAg and GSHsAg with site-specific ^{125}I-labeled antibody probes[a]

Probe	*P/N* ratio	
	WHsAg	GSHsAg
101/I	24.4	23.4
3/II	16.2	7.8
14/II	21.4	6.9
114/III	17.6	27.3
19/III	20.2	7.4
172/III	57.9	24.3
71/IV	12.3	0.8
181/IV	22.8	1.1
41/V	18.3	0.2
31/V	47.8	0.7
151/V	22.8	0.9

[a] Serum positive for either WHsAg or GSHsAg was diluted 1:25 and incubated on an immunoadsorbent composed of MCAB 3 (a site II antibody that reacts with both antigens). Bound antigen was detected with radiolabeled antibody probes as shown. Values are positive/negative (*P/N*) ratios (*N*, 30 to 90 cpm), and those greater than or equal to 3.1 are considered positive. See the text for details.

need not imply that the cross-reactive antibodies will define analogous nonoverlapping domains of GSHsAg. The following indirect analysis of WHsAg and GSHsAg sites by an immunocomplex mapping technique obviates the need for purified ligand and antigen immunoadsorbents. Blocking of antigen with excess 101/I antibody would theoretically interfere with antigen binding to a 101/I immunoadsorbent and also with binding of the 101/I probe. Blocking of immunocomplex detection by excess 101/I antibody would occur at the level of the homologous probe in other instances where 101-antigen complexes are bound to immunoadsorbents of nonoverlapping specificity. These conditions can be demonstrated for the present immunocomplex assay of either WHsAg or GSHsAg (Table 4). Except for such cases, specific blocking would occur at the level of the immunoadsorbent (see below). Whenever either antigen was prereacted with MCABs other than 101/I, the immune complexes were bound to a 101/I immunoadsorbent, and the antigens were detected at or near control levels with the 101/I probe (Table 4). Thus, antibodies to sites II through V of WHsAg did not map to site I of WHsAg which is defined by the 101/I antibody (see also CAB assay in Table 1); the comparative immunocomplex mapping data in Table 4 indicate that domain I is distinct from other sites on GSHsAg also.

In keeping with this approach, we found that site II antibodies could produce an inhibition of immunocomplex binding to a site II solid phase; antibodies to other sites (with the exception of the 101 antibody) did not block antigen detection by the 101 probe. Similarly, site III antibodies could block immunocomplex binding to a site III solid phase, whereas others did not (except for the 101 antibody; see above). The immunocomplex mapping of sites II and III on WHsAg and GSHsAg is given in Table 4. GSHsAg in positive serum at 1:125 dilutions was not consistently detected at significant levels when site IV and V immunoadsorbents were used to bind the antigen; however, WHsAg was readily detected in those assay systems (Table 4, Table 3, Fig. 1F, G, and H). Therefore, only WHsAg was detected in the immunocomplex mapping of sites IV and V (Table 4). In general, results for WHsAg domains obtained with immunocomplex and competitive antibody mapping were in agreement (Tables 1 and 4). Thus it can also be concluded that antibodies to sites I, II, and III of WHsAg map to and define analogous antigenic domains of GSHsAg. The site IV and V antibodies do not block GSHsAg detection in the cross-reactive RIA systems because they do not react strongly with the antigen (Tables 2 and 3). Second, any weak interactions would probably be directed against sites that do not overlap with site I, II, or III. Note that GSHsAg prereacted with site II or III antibodies is not detected with site IV and V immunoadsorbents, nor are any of the immunocomplexes bound by substrates consisting of bovine serum albumin alone (Table 4; data for bovine serum albumin substrate are not shown). Thus the interference by blocking antibody at the level of the immunoadsorbent is

TABLE 3. RIA of WHsAg, GSHsAg, and HBsAg with site-specific antibody immunoadsorbents[a]

Substrate	*P/N* ratio		
	WHsAg	GSHsAg	HBsAg
101/I	8.7	16.8	(4.5)
3/II	16.3	20.5	
14/II	9.7	8.2	
114/III	18.1	25.2	
19/III	17.4	27.2	
172/III	19.5	21.0	
71/IV	15.1	3.1	
181/IV	18.6	3.1	
41/V	8.7	2.1	
31/V	25.8	4.1	
151/V	24.2	3.5	

[a] Serum positive for either WHsAg, GSHsAg, or HBsAg was diluted 1:25 and incubated on immunoadsorbent substrates shown; bound antigen was detected by the 101/I antibody probe. Values are positive/negative (*P/N*) ratios (*N*, 70 to 90 cpm; except for site I system where *N* was 145 cpm); those greater than or equal to 3.1 may be considered positive values. The value in parentheses for HBsAg was determined by using a slight modification of conditions for the preparation of the 101/I immunoadsorbent. See the text for details.

TABLE 4. Immunocomplex mapping of WHsAg and GSHsAg by solid-phase RIA[a]

Immunoadsorbent	Antigen	*P/N* ratio with indicated blocking antibody											
		None	I	II		III			IV		V		
			101	3	14	114	19	172	71	181	41	31	151
101/I	W	4.6	–	4.1	5.3	6.2	4.7	4.7	4.1	5.6	4.4	4.8	3.6
	G	10.9	–	7.9	8.0	7.2	9.7	4.9	5.9	8.0	6.8	7.9	7.6
3/II	W	13.2	–	–	–	9.2	6.5	11.5	8.3	8.5	9.0	10.6	10.7
	G	12.3	–	–	–	10.6	8.7	7.2	10.1	10.4	9.3	10.4	10.6
114/III	W	8.7	–	6.7	7.8	–	–	–	7.3	6.4	7.7	6.6	6.7
	G	15.2	–	16.6	13.6	–	–	–	13.5	12.7	12.8	13.5	11.9
181/IV	W	9.4	–	10.3	7.8	6.6	5.7	5.7	–	–	8.1	5.8	9.2
	G	–	–	–	–	–	–	–	–	–	–	–	–
31/V	W	10.8	–	7.5	7.9	9.4	5.9	8.0	7.9	5.7	–	–	–
	G	–	–	–	–	–	–	–	–	–	–	–	–

[a] Excess MCAB was prereacted with either WHsAg (W) or GSHsAg (G) to block specific determinants, and the mixtures were then assayed to detect immune complexes. The solid-phase RIA system involved site-specific immunoadsorbents and the 101/I antibody probe. Values in the table are positive/negative (*P/N*) ratios (*N*, 95 to 175 cpm); for comparison, the P/N ratios obtained in the absence of blocking antibody are also given (none). A dash (–) indicates no significant antigen or immunocomplex detection ($P/N < 3.1$). Note that GSHsAg was not detected in the site IV and V RIA systems. Also, blocking of antigens by excess 101/I antibody interferes with binding by the labeled 101/I probe regardless of which immunoadsorbent is used; otherwise, the data reflect interference (or lack of interference) with immunocomplex binding at the level of the immunoadsorbent antibody. See the text for details.

specific. The data suggests that up to three noncompeting antibodies can bind simultaneously to their respective antigenic sites on either antigen.

Enumeration and cross-reactivity of WHsAg domains. The five nonoverlapping antigenic domains of WHsAg are shown in Fig. 2A (adapted from Table 1). The clones producing the MCABs that define these sites were isolated independently from three different hybridization experiments (i.e., from three different mice immunized similarly [4]). Different hybridomas defining up to four sites could be derived from the same mouse (Fig. 2B). More importantly, hybridomas defining the same WHsAg site could be derived independently from different mice (Fig. 2B). The probability of obtaining such antibody sets out of 11 antibodies would be low if WHsAg possessed a large number of nonoverlapping domains. This, coupled with the observation that five distinct sites could be defined by a total of only 11 antibodies, suggests that 22-nm WHsAg is characterized by repeat antigenic structure involving only a limited set of nonoverlapping antigenic specificities.

In a previous study we demonstrated at least three different determinants of WHsAg based on antigenic competition assays involving WHsAg, GSHsAg, HBsAg, and the present MCABs (4). Those results were adapted for comparison with RIA results obtained in the present study, and all are presented in Fig. 2B. The present analysis involves three distinct but related antigens. We cannot yet identify nonoverlapping epitopes within antigenic domains of WHsAg by using homologous variants (7, 31, 32), because such variants have not yet been identified. However, the data do show that the five nonoverlapping antigenic domains of WHsAg identified in competitive antibody mapping exhibit characteristic specificity and cross-reactivity patterns in comparative analyses with related antigens (Fig. 2A and B). The 101/I antibody defines a surface antigenic site that contributes to cross-reactions among the mammalian hepadnaviruses. It does not react with duck hepatitis B virus surface antigen, which is known to be antigenically distinct (18). Regarding woodchuck and ground squirrel viruses, the results indicate that at least three analogous nonoverlapping antigenic sites contribute to moderate and strong cross-reactions between WHsAg and GSHsAg (sites I, II, and III; Fig. 2A and B; Table 4). Antibodies reacting poorly with GSHsAg mapped to sites IV and V of WHsAg, suggesting that at least two nonoverlapping antigenic domains of WHsAg are involved in eliciting virus-specific antibodies. The present results do not rule out the possibility of eventually isolating strongly cross-reactive antibodies that map competitively to sites IV and V of WHsAg, nor do they rule against the mapping of WHsAg-specific antibodies to sites characterized by cross-reactive antibodies (such as sites I, II, and III). These types

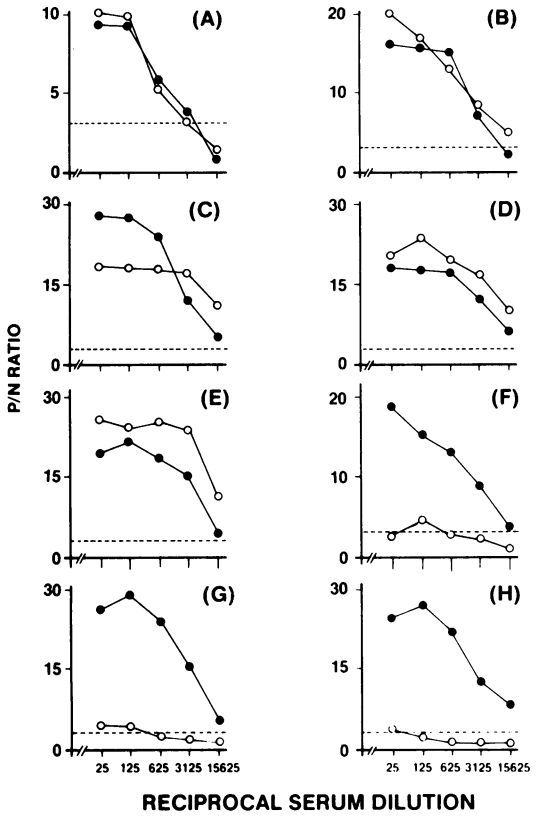

FIG. 1. Titration of sera positive for WHsAg (●) and GSHsAg (○) in immunoadsorbent-based, site-specific RIAs. The probe was ^{125}I-101/I antibody; substrate antibodies and their site specificities were (A) 101/I, (B) 3/II, (C) 19/III, (D) 172/III, (E) 114/III, (F) 181/IV, (G) 31/V, and (H) 151/V. *P/N* is the ratio of counts per minute for the antigen-positive sample to that for an antigen-negative woodchuck serum assayed at a 1:25 dilution; *N* ranged from 70 to 90 cpm for (B) through (H) and was 145 cpm for (A).

of observations would argue in favor of nonoverlapping epitopes within antigenic domains defined by competing antibodies.

DISCUSSION

The molecular relationship between MCAB cross-reactivity and shared antigenic determinants among mammalian hepadnaviruses is not yet fully understood. For example, differences in MCAB avidity for related surface antigens may result from partial antibody recognition of a similar epitope on heterologous antigens, or from differences in the copy number and distribution of an epitope on related subviral particles (or both). During our studies with WHsAg, we isolated the 101/I murine hybridoma, whose antibody clearly reacts with WHsAg and GSHsAg and also reacts significantly, but to a lesser extent, with HBsAg (4) (present results). Antibodies other than 101/I do not react to any extent with HBsAg. On the other hand, antibodies specific for WHsAg could react to a slight extent with GSHsAg, depending on the analytical assay used. For example, site IV and V antibodies react poorly with GSHsAg and exhibit much greater specificity for WHsAg than for GSHsAg. This is demonstrated in the liquid-phase RIP, where antigenic competition is measured against a single antibody species (Fig. 2B). Low-level reactivity of these antibodies with GSHsAg is also apparent in site-specific immunoadsorbent systems (Fig. 2B). In contrast, absolute specificity of these antibodies for WHsAg is suggested by RIA with site-specific probes (Fig. 2B). Of the antibodies demonstrating significant cross-reactivity with WHsAg and GSHsAg, monoclonal antibodies 3/II and 14/II reacted preferentially with WHsAg in competition RIP, and both mapped to site II. In comparison, WHsAg-defined antibodies 19/III and 172/III preferred GSHsAg to WHsAg, and both mapped to site III (Fig. 2A and B). Therefore, competitive antibody mapping confirmed the presence of at least two cross-reactive domains between WHsAg and GSHsAg that were suggested initially by heteroplicity in antigen competition assays (4). The reason why a subset of site III antibodies preferentially reacts with a heterologous antigen in the liquid-phase system is not clear at present.

Antigenic relatedness among hepadnavirus surface antigens requires at least some degree of homology at the level of DNA and amino acid sequences. WHsAg and HBsAg share up to 78% gene sequence homology and nearly 60% homology in their predicted amino acid sequences (6); however, there is somewhat less overall homology suggested by shared tryptic peptides (5, 11). This is because the latter technique gives a minimum estimate of structural relatedness when compared to sequencing data. The antigenic relationships between hepadnavirus surface antigens may correlate closely with the minimal estimates of structural homology derived from peptide maps. For example, WHsAg can be detected in commercial assays designed for HBsAg detection; however, the reactivity in the system is at considerably lower signal/noise ratios when compared with HBsAg (8, 20). This indicates that cross-reactivity between the two surface antigen-antibody systems is not as extensive as the DNA and amino acid sequence homologies might predict. In fact, Werner et al. (30) found only 3 to 5% homology between hepatitis B virus and WHV DNA by using hybridization techniques and subsequently demonstrated very little in the way of cross-reactiv-

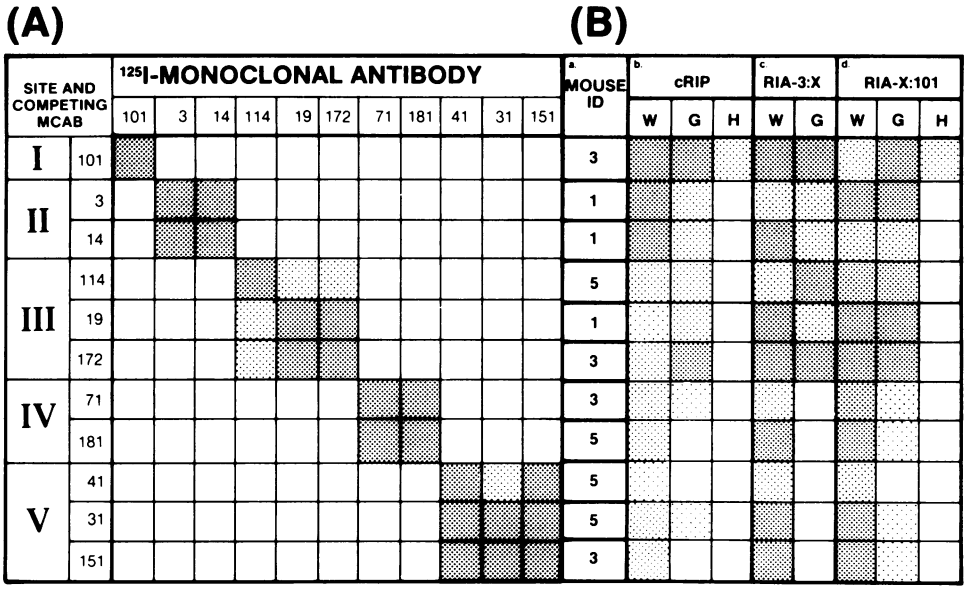

FIG. 2. (A) Nonoverlapping antigenic sites of WHsAg deduced from CAB assays (adapted from Table 1). The data are further supported by immunocomplex mapping of WHsAg by RIA (Table 4). (B) Cross-reactivity of WHsAg (W) antigenic sites with GSHsAg (G) and HBsAg (H) by different immunoassay systems. MCABs corresponding to column headings in B (a, b, c, and d) are those listed by identification number and site at the far left of the figure. RIA 3:X and X:101 refer to immunoadsorbent-probe combinations where "X" represents the MCAB listed at the far left of the figure (see also below). The shading of squares refers qualitatively to strong, moderate, or slight reactivity with the antigen; unshaded squares indicate negligible reactivity for the test. (a) Hybridomas were derived from three independent fusions (i.e., three different mice [no. 1, 3, and 5] that were immunized in the same manner); hybridomas to the same site from the same mouse were derived from different culture wells. (b) Results of double antibody competition radioimmune precipitation assays (adapted from reference 4); reactivities are the relative inhibition of MCAB-mediated precipitation of ^{125}I-WHsAg in the presence of excess WHsAg, GSHsAg, and HBsAg. (c) Site-specific cross-reactivity of WHsAg and GSHsAg by RIA; RIA-3:X refers to the immunoadsorbent-probe combination as adapted from Table 2. (d) Site-specific cross-reactivity of the mammalian hepadnavirus surface antigens by RIA; RIA-X:101 refers to the immunoadsorbent-probe combination as adapted from Table 3.

ity between the respective antigen-antibody systems. Relative to our results, Feitelson et al. (5) have shown that WHsAg, GSHsAg, and HBsAg together exhibit only 25% homology in their tryptic peptide maps, and this correlates with present observations suggesting that one of five (20%) of WHsAg domains may be shared by all three viruses. In the comparison by Feitelson et al. of GSHsAg and WHsAg (5), the major polypeptides of the two antigens shared 55% homology in their tryptic peptides, and our data suggest that three of five (60%) of WHsAg domains are shared with GSHsAg. Although these observations correlate structural and cross-reactive antigenic features among related surface antigens, the present cross-reactivity profile based on murine antibodies may only reflect the extent to which mice respond to shared determinants and not the actual presence or absence of the shared determinants (4). It would appear that antigenic regions of the animal and human viral proteins exhibit more structural variability than conserved "framework" regions.

The tryptic peptide maps derived from different WHsAg isolates indicate that there is at least 75% homology (similar observations were evident for different GSHsAg samples) (5). On these grounds, one might expect to find WHsAg with only four of the five antigenic domains described in the present study; the potential fifth site would be unique to WHsAg and could thus correspond to an antigenic subtype. One would not expect potential subtypic sites to involve the more conserved cross-reactive domains. In our antigenic analysis of over a dozen chronic carrier woodchucks from the National Institute of Allergy and Infectious Diseases colony, we did not detect WHsAg variants lacking any of the five domains presently identified (Cote et al., submitted for publication). The 25% nonhomology among different WHsAg noted by others (5) may not be evident in WHsAg from our colony

woodchucks, or it may reflect structural microheterogeneity unrelated to the presence or absence of specific antigenic determinants. Reciprocal antigen-antiserum absorption studies with WHsAg and GSHsAg and with nonhomologous WHsAg are required to further clarify these points. In any event, the present RIA systems will be useful in screening for WHsAg variants in other areas where WHV infection is endemic. The identification and isolation of potential variants lacking specific epitopes within sites defined by competitive antibody mapping will enable the definition of nonoverlapping epitopes within such sites.

The information concerning antigenic domains of WHsAg will be of value in further developing the WHV system as a model for hepatitis B virus-induced liver disease in humans. The results of the present study show the antigenic similarity of WHV used as a standard infectious pool by our laboratory (woodchuck no. 8) and the WHsAg used to prepare our standard vaccine (woodchuck no. 7) (i.e., MCABs were predefined and selected with WHsAg from woodchuck no. 8 and were used to map antigenic domains of WHsAg from woodchuck no. 7). The antibodies can be used to ascertain the presence and distribution of defined sites on the actual virion. This is important because only those determinants of the 22-nm particle that are accessible on the virion surface are necessary to stimulate protective antibodies. Passive immunization studies in woodchucks can determine which of these antibodies may be immunoprotective, thus implicating the corresponding surface antigenic domain. Of futher interest is the idea that we may be able to produce antibodies against synthetic WHsAg peptides and then map these specificities to antigenic domains associated with protection. This would provide a method to screen for synthetic peptides with vaccine potential. The present immunocomplex RIA can be adapted for the analysis of woodchuck antibody responses to vaccination. In this regard, antiidiotypic antibodies prepared against affinity-purified, site-specific MCABs should also prove useful. The MCABs can be tested for their ability to modify the natural course of persistent infections so as to halt progressive changes favoring the development of hepatocellular carcinoma. The use of these antibodies as therapeutic agents or carriers in the treatment of woodchuck hepatoma can likewise be investigated. The present antibodies may also prove useful in combined passive and active immunotherapy, especially in neonatal woodchucks born to mothers with chronic viral hepatitis. In comparison to the human system, the ability to interdict the process of maternoneonatal virus transmission by such methods may in turn prevent the development of chronic viral hepatitis in young animals and thus eliminate the risk of hepatocellular carcinoma in later life (2, 19).

ACKNOWLEDGMENTS

This work was supported by Public Health Service contract N01-AI-82572 between Georgetown University and the National Institute of Allergy and Infectious Diseases.

We thank Sherrie Baxter for her secretarial assistance in the preparation of this manuscript.

LITERATURE CITED

1. **Bancroft, W. H., F. K. Mundon, and P. K. Russell.** 1972. Detection of additional antigenic determinants of hepatitis B antigen by immunoprecipitation. J. Immunol. **109**:842–848.
2. **Beasley, R. P.** 1982. Hepatitis B virus as the etiologic agent in hepatocellular carcinoma: epidemologic considerations. Hepatology **2**(Suppl.):21–26.
3. **Chisari, F. V., J. L. Gerin, and T. S. Edgington.** 1974. Immunochemistry of the hepatitis B virus: ^{125}I-HBsAg ligand. J. Immunol. **113**:543–553.
4. **Cote, P. J., Jr., G. M. Dapolito, J. W.-K. Shih, and J. L. Gerin.** 1982. Surface antigenic determinants of mammalian "hepadnaviruses" defined by group- and class-specific monoclonal antibodies. J. Virol. **42**:135–142.
5. **Feitelson, M. A., P. L. Marion, and W. S. Robinson.** 1981. Antigenic and structural relationships of the surface antigens of hepatitis B virus, ground squirrel hepatitis virus, and woodchuck hepatitis virus. J. Virol. **39**:447–454.
6. **Galibert, F., T. N. Chen, and E. Mandart.** 1981. Localization and nucleotide sequence of the gene coding for the woodchuck hepatitis virus surface antigen: comparison with the gene coding for the human hepatitis B virus antigen. Proc. Natl. Acad. Sci. U.S.A. **78**:5315–5319.
7. **Gerhard, W., J. Yewdell, M. E. Frankel, and R. Weber.** 1981. Antigenic structure of influenza virus hemagglutinin defined by hybridoma antibodies. Nature (London) **290**: 713–718.
8. **Gerin, J. L.** 1979. The eastern woodchuck (Marmota monax): a potential animal model for virus-induced disease in man, p. 171–175. *In* E. Bartoni, L. Chandusi, and S. Sherlock (ed.), Systemic effects of HBsAg immune complexes. Piccin Medical Books, Padova, Italy.
9. **Gerin, J. L., R. M. Faust, and P. V. Holland.** 1975. Biophysical characterization of the *adr* subtype of hepatitis B antigen and preparation of anti-*r* sera in rabbits. J. Immunol. **115**:100–105.
10. **Gerin, J. L., P. V. Holland, and R. H. Purcell.** 1971. Australia antigen: large-scale purification from human serum and biochemical studies of its proteins. J. Virol. **7**:569–576.
11. **Gerlich, W. H., M. A. Feitelson, P. L. Marion, and W. S. Robinson.** 1980. Structural relationships between the surface antigens of ground squirrel hepatitis virus and human hepatitis B virus. J. Virol. **36**:787–795.
12. **Hess, G., J. W.-K. Shih, P. M. Kaplan, and J. L. Gerin.** 1977. The demonstration of subtype (*d* or *y*)-specific determinants on the surface of the presumed hepatitis B virus. J. Immunol. **119**:1542–1544.
13. **Hoofnagle, J. H., R. J. Gerety, L. A. Smallwood, and L. F. Barker.** 1977. Subtyping of hepatitis B surface antigen and antibody by radioimmunoassay. Gastroenterology **72**: 290–296.
14. **Kennel, S. J., B. C. Del Villano, R. L. Levy, and R. A. Lerner.** 1973. Properties of an oncornavirus glycoprotein: evidence for its presence on the surface of the virions and infected cells. Virology **55**:464–475.
15. **LeBouvier, G. L.** 1971. The heterogeniety of Australia antigen. J. Infect. Dis. **123**:671–675.

16. **Lubeck, M., and W. Gerhard.** 1982. Conformational changes at topologically distinct antigenic sites on the influenza A/PR/8/34 virus HA molecules are induced by the binding of monoclonal antibodies. Virology **118**:1–7.
17. **Marion, P. L., L. S. Oshiro, D. C. Regnery, G. H. Scullar, and W. S. Robinson.** 1980. A virus of Beechy ground squirrels that is related to hepatitis B virus of humans. Proc. Natl. Acad. Sci. U.S.A. **77**:2941–2945.
18. **Mason, W. S., G. Seal, and J. Summers.** 1980. Virus of Pekin ducks with structural and biological relatedness to human hepatitis B virus. J. Virol. **36**:829–836.
19. **Maupas, P. H., J. P. Chiron, F. Barin, P. Coursaget, A. Goudeau, J. Perrin, F. Denis, and I. Diop Mar.** 1981. Efficacy of hepatitis B vaccine in prevention of early HBsAg carrier state: controlled trial in an endemic area (Senegal). Lancet **i**:289–292.
20. **Millman, I., T. Halbherr, and H. Simons.** 1982. Immunological cross-reactivities of woodchuck and hepatitis B viral antigens. Infect. Immun. **35**:752–757.
21. **Popper, H., J. W.-K. Shih, J. L. Gerin, D. C. Wong, B. H. Hoyer, W. T. London, D. L. Sly, and R. H. Purcell.** 1981. Woodchuck hepatitis and hepatocellular carcinoma: correlation of histologic with virologic observations. Hepatology **1**:91–98.
22. **Purcell, R. H., and J. L. Gerin.** 1978. Hepatitis B vaccines: on the threshold. Am. J. Clin. Pathol. **70**:159–169.
23. **Robinson, W. S.** 1980. Genetic variation among hepatitis B and related viruses. Ann. N. Y. Acad. Sci. **354**:371–378.
24. **Shih, J. W.-K., G. Hess, P. M. Kaplan, and J. L. Gerin.** 1980. Characterization of the hepatitis B virus (Dane particles). J. Virol. Methods **1**:47–59.
25. **Shih, J. W.-K., P. L. Tan, and J. L. Gerin.** 1978. Antigenicity of the major polypeptides of hepatitis B surface antigen. J. Immunol. **120**:520–525.
26. **Summers, J.** 1981. Three recently described animal virus models for human hepatitis B virus. Hepatology **1**:179–183.
27. **Summers, J., J. M. Smolec, and R. L. Snyder.** 1978. A virus similar to human hepatitis B virus associated with hepatitis and hepatoma in woodchucks. Proc. Natl. Acad. Sci. U.S.A. **75**:4533–4537.
28. **Summers, J., J. M. Smolec, B. G. Werner, T. J. Kelly, Jr., G. V. Tyler, and R. L. Snyder.** 1980. Hepatitis B virus and woodchuck hepatitis virus are members of a novel class of DNA viruses, p. 459–470. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Szmuness, W., C. E. Stevens, E. J. Harley, E. A. Zang, W. R. Oleszka, D. C. William, R. Sadovsky, J. M. Morrison, and A. Kellner.** 1980. Hepatitis B vaccine: demonstration of efficacy in a controlled clinical trial in a high-risk population in the United States. New Engl. J. Med. **303**:833–841.
30. **Werner, B. G., J. M. Smolec, R. L. Snyder, and J. Summers.** 1979. Serological relationship of woodchuck hepatitis virus to human hepatitis B virus. J. Virol. **32**:314–322.
31. **Wiktor, T. J., and H. Koprowski.** 1980. Antigenic variants of rabies virus. J. Exp. Med. **152**:99–105.
32. **Yewdell, J., and W. Gerhard.** 1982. Delineation of four antigenic sites on a paramyxovirus glycoprotein via which monoclonal antibodies mediate distinct antiviral activities. J. Immunol. **128**:2670–2675.