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Glycoproteins of Natural Origin with an Affinity for Hepatitis B Surface Antigen

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Sera from certain animal species contain a substance(s) which binds hepatitis B surface antigen. The hepatitis B binding substance found in animals is not antibody, but appears to be a glycoprotein which reacted with antigen-coated beads and produced a "false positive" test for antibody. This glycoprotein could be selectively and quantitatively removed by reaction with purified hepatitis B surface antigen and centrifugation. Pili fractions isolated from *Neisseria gonorrhoeae* and *Escherichia coli* bound to hepatitis B surface antigen and produced false positive anti-hepatitis B surface antigen reactions. Mouse anti-bovine hepatitis B binding substance and rabbit anti-*E. coli* pili were capable of neutralizing bovine hepatitis B binding substance.

Recently we observed that sera from certain animal species contain a substance(s) which binds to hepatitis B surface antigen (HBsAg) and can be detected by radioimmunoassay (RIA) for antibody to HBsAg, anti-HBs (Abbott Ausab). In this assay purified HBsAg, absorbed to the surface of a polystyrene bead, is ordinarily used to detect anti-HBs. The substance in the animal sera is not antibody but a binding substance, "hepatitis B binding substance" (HBBS), which reacts with the coated antigen and produces a false positive test. HBBS can be selectively and quantitatively removed by reaction with purified HBsAg and centrifugation. It does not produce a precipitin reaction by immunodiffusion when reacted with HBsAg.

We first became aware of this HBsAg binding substance as a result of a search for possible insect vectors of the hepatitis B virus. We reasoned that animals with anti-HBs might have become sensitized from insect bites, the insects having fed on human hepatitis B virus carriers. All of the animal sera tested appeared to contain anti-HBs when measured by the RIA method. This report gives preliminary evidence that HBBS is a glycoprotein and may be related to some bacterial lectins.

MATERIALS AND METHODS

Animal sera. Animal sera were collected from various sources, as indicated in Table 1, and stored frozen in screw-capped vials. The majority of sera were from the Division of Clinical Research serum collection and had been stored at −20°C for as long as 15 years. Several bovine sera were collected and donated by J. F. Ferrer of the University of Pennsylvania School of Veterinary Medicine, New Bolton Center, Pa. Bulk lots of bovine, dog, horse, swine, and sheep sera were purchased from Microbiological Associates (Bethesda, Md.) and from Pel-Freez Biologicals, Inc. (Rogers, Ark.). Mouse sera (from a DBA/2 inbred strain) were collected on the day of testing by heart puncture. Woodchuck sera were kindly supplied by Robert L. Snyder, Penrose Research Laboratory, Philadelphia Zoological Garden, Philadelphia, Pa.

Assay. The Abbott Ausab solid-phase RIA was used throughout. All test samples were compared with normal human serum which was supplied by the manufacturers of the assay. Ratios were expressed as counts per minute of test sample divided by the mean counts per minute of the normal human serum. A ratio of 2.1 or greater was considered positive.

Hemagglutination. Influenza virus (obtained from the Research Resources Branch of the National Institutes of Health, Bethesda, Md.) was serially diluted (twofold dilutions in a volume of 0.05 ml) in a microtiter plate with physiological saline as diluent. To each well 0.05 ml of butanol-extracted, alcohol-precipitated bovine HBBS and 0.1 ml of a 2% suspension of human type A (D−) erythrocytes were added. Physiological saline was substituted for HBBS as a control. The plate was incubated at room temperature for 2 h.

Enzyme susceptibility. Abbott Ausab beads were each reacted with 0.2 ml of HBBS. (HBBS used for these assays was derived from bovine serum [Microbiological Associates, Bethesda, Md.]). It was precipitated with 50% saturated $(NH_4)_2SO_4$ and passed through a Sephadex G200 column. The pooled first-peak material was concentrated to original volume by Amicon P30 pressure filtration. (This fraction contained 100% of the original binding activity.) Ausab beads were aspirated and washed with distilled water, 14 ml per bead, with the Abbott Pentawash apparatus. Enzymes were added, 0.2 ml each, and incubated at 37°C for 2 h. Ausab beads were washed, and 0.2 ml of ^{125}I-labeled HBsAg was added to each. The Ausab beads were incubated for 4 h at room temperature, washed, and counted in a gamma counter. Controls

consisted of (i) Ausab beads incubated first with saline instead of HBBS and treated with enzymes, (ii) Ausab beads incubated with HBBS and no enzymes, and (iii) Ausab beads incubated with anti-HBs (Abbott's positive control serum). The results of treating Ausab beads with enzymes (control 1) indicated no alteration of the bound HBsAg. This was determined by comparing the radioactivity of control 1 with that of control 3. Alteration of HBBS bound to HBsAg on the Ausab bead was determined by the following formula: [(counts per minute of enzyme-treated HBBS-coated Ausab bead) − (counts per minute of enzyme-treated Ausab bead)/(counts per minute of HBBS-coated Ausab beads)] × 100.

Enzymes. The enzymes used were as follows: Pronase (Calbiochem), 0.1% in distilled water; trypsin, crystallized (Sigma), 0.25% in 0.01 M phosphate-buffered saline, pH 7.0 (PBS); pepsin, 2× crystallized (Sigma), 0.25% in 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 6.0; chymotrypsin (Worthington Biochemicals), 0.125% in PBS; papain (Worthington), 0.25% in PBS; α-amylase (Worthington), 10 mg/ml in PBS; muramidase (Nutritional Biochemicals), 10 mg/ml in PBS; wheat germ lipase (Worthington), 1.0 mg/ml in PBS; collagenase (Nutritional Biochemicals), 10 mg/ml in 0.05 M Tris-hydrochloride buffer (pH 7.5) plus 0.3 mM $CaCl_2$; phospholipase C (Worthington), 1.0 mg/ml in PBS; β-galactosidase (Worthington), 1.0 mg/ml in PBS; neuraminidase (Worthington), 10 U/ml in 0.1 M acetate buffer (pH 5.2).

Bacterial fractions. *Escherichia coli* (strain F⁻W1-3) type 1 pili were prepared by a slight modification of the Brinton procedure (3). The bacteria were grown in liquid Lennox medium, and the cells were harvested by low-speed centrifugation. The cell pellet was resuspended in 5 mM *N*-tris(hydroxymethyl)methyl-2-aminomethane-sulfonic acid (TES) adjusted to pH 7.0. The suspension was blended at 14,000 rpm for 5 min in a VirTis "45" blender to break the pili from the cells. The bacteria were then centrifuged down, and the supernatant was saved. Dry $MgCl_2$ was added to the supernatant to a concentration of 0.1 M and allowed to incubate for a few minutes. The pili form macrocyrstals under these conditions and were pelleted by low-speed centrifugation. The pellet of pili was resolubilized in TES buffer and recycled at least three times to remove extraneous bacterial material.

Pili from *Neisseria gonorrhoeae* Pittsburgh strain 3, type 2 colonial form, were prepared by alkaline solubilization (C. C. Brinton et al., *The Proceedings of the Symposium on Sexually Transmitted Diseases*, in press). The bacteria were grown on solid media (Difco GC base medium supplemented according to Kellogg et al. [12]). The cells were scraped from the agar in a pH 10.5, 0.63 M ethanolamine buffer wash (EAB). The wash was centrifuged to pellet the cells. The supernatant was retained and dialyzed overnight against pH 8.0 Tris-saline buffer (0.1 M Tris and 0.29 M NaCl). On the next day the pili were centrifuged into a pellet at low speed. Subsequently the pili were recycled two more times by resolubilization in pH 10.2 EAB, centrifuged at 30,000 × *g* to remove large particulate material, dialyzed against the Tris-saline buffer to aggregate the pili, and pelleted at low speed to remove more soluble contaminants.

Rabbit antiserum to *E. coli* F⁻W1-3 pili was prepared by a series of three subcutaneous injections at 2-week intervals of the antigen incorporated into Freund complete adjuvant. Blood was collected 1 week after the final inoculation.

Pseudomonas aeruginosa fractions were kindly provided by L. Weng of this Institute. A discription of these fractions and fractionation procedures will be published separately.

RESULTS

Distribution. The distribution of HBBS among animal sera is shown in Table 1. The highest prevalence was found in cattle and bison; this group also contained the highest activities expressed as equivalent anti-HBs. Chicken sera, sera from a variety of fish, and several batches of newborn and fetal calf sera (data not shown) were uniformly negative. Of interest is the finding that 1 of 2 dogfish sera and sera from 10 vervets (captured in the field in Uganda and bled immediately) contained high activity. Rodent sera were mainly negative, although sera from 2 of 20 woodchucks contained respectable amounts of HBBS. Of 50 normal human blood donor sera tested, none was found positive.

HBBS characteristics. To characterize the binding component physically and chemically, a batch of bovine serum (Microbiological Associates) which was positive for HBBS by RIA at a 1:64 dilution was fractionated as described above. This fraction was then passed through a Sephadex G200 column a second time with physiological saline as eluent. A quantitative recovery was obtained in the pooled first peak (void volume), provided that the fraction was not repeatedly frozen and thawed during storage. Freezing and thawing resulted in fragmentation because two fractions with HBsAg binding activity could be recovered by Sephadex gel filtration; one fraction was still detected in the void volume, and the second was detected in the peak containing serum proteins of 150,000 molecular weight.

Both the void volume and smaller-molecular-weight fragments were firmly bound by diethylaminoethyl (DEAE)-cellulose (DE 52, Whatman Biochem. Ltd., London, U.K.) in 0.005 M phosphate buffer, pH 8.0. Elution was attempted by washing the column with 100-ml quantities of increasing concentrations of potassium phosphate buffer, from 0.01 to 0.5 M. Immunoglobulin G was eluted with 0.01 M potassium phosphate buffer, pH 8.0. Peaks containing components with absorbance at 280 nm were eluted with increasing salt concentration; only the fraction eluted at 0.5 M contained binding activity.

TABLE 1. *HBsAg binding activity (equivalent anti-HBs) in various animal species*

Species	No. tested	No. negative	No. with HBsAg binding activity at ratio (cpm of test serum/cpm of normal human serum):						
			2.1-5	6-10	11-20	21-30	31-40	41-50	>50
Bos taurus[a] (Jersey cows, lymphosarcoma)	38	0	9	8	9	5	2	1	4
Bos taurus (Jersey cows)	18	1	5	7	3	1	1		
Bos taurus (Holstein cows)	1	1							
Bos taurus (Guernsey cows)	1					1			
Bison bison (Kansas)	11	4	3	1	3				
Gallus domesticus (chicken)	6	6							
Sus scrofa (swine)	12	4	6	2					
Ovis aries (sheep)	1	1							
Canis familiaries (dog)	1				1				
Capra hercus (goat)	1	1							
Ovis aries (lamb)	1	1							
Equus caballus (horse)	1		1						
Oryctolagus cuniculus (rabbit)	2		2						
Marmota monax (woodchuck)	24	20	2	1		1			
Citellus parryii (squirrel)	2	2							
Mus musculus (mouse)	15	14	1						
Rattus exultans (rat)	1	1							
Callorhinus alascanus (seal)	47	43	4						
Mustilus canis (dog fish)	2	1				1			
Hylobates agilis (gibbon)	2	2							
Cercopethecus pygerythrus[b] (vervet)	10				3	6	1		
Macaco rheus (rhesus monkey)	1	1							
Papio porcarius (baboon)	7	4	3						
Miscellaneous[c]	36	36							

[a] University of Pennsylvania, School of Veterinary Medicine.

[b] Uganda vervets captured in the wild and bled immediately.

[c] *Micropogon undulatus* (atlantic croaker), 1; *Paralichthus dentatus* (northern fluke), 1; *Scomberomous maculatus* (spanish mackerel), 1; *Sheroides maculatus* (puffer), 3; *Opsanus tan* (toad fish), 1; *Dasyatis centrura* (sting ray), 2; *Carcharia littoralis* (sand shark), 1; *Acipensei cxrhynchus* (sturgeon), 1; *Prionotus scitulus* (sea robin), 1; *Squatina squatina* (monk fish), 1; *Lutjamis vaigiensis* (snapper), 1; *Cymnosarda nuda* (tuna), 1; *Birgus latro* (cocoanut crab), 1; *Oncorhynchus keta* (Kuskakwin chums), 5; *Rachycentron canados* (cobia), 2; *Elaphe absoleta* (black chicken snake), 1; *Oncorhynchus tshawytscha* (Nushigak chinook), 6; *Oncorhynchus tshawytscha* (Spring Creek chinook), 6.

Replicate experiments with potassium phosphate buffer (pH 6.0) and increasing concentrations of NaCl gave similar results.

A 4-ml amount of HBBS (the first peak from the Sephadex G200 column) was dialyzed against distilled water and then mixed with 3.0 ml of normal butyl alcohol in a ground-glass-stoppered centrifuge tube. The mixture was then shaken vigorously for several minutes and held at 0°C for 1 h. The mixture was next centrifuged in a table top clinical centrifuge with a swinging-bucket rotor for 10 min to separate the water from the butanol phase. The water phase was carefully separated and dialyzed against distilled water at 2 to 5°C. A 3.5-ml amount of material was recovered. Four volumes of cold absolute ethanol was added slowly to this in an ice bath. A flocculent white precipitate was pelleted by centrifugation at 5,000 rpm for 20 min in a Beckman J21B centrifuge with a JA21 rotor. The supernatant was discarded, and the pellet was dissolved in 3.5 ml of distilled water. The butanol extract contained 59% and the alcohol precipitate contained 54% of the original HBBS activity.

HBBS could be quantitatively removed by mixing it with serum containing HBsAg or with purified HBsAg. An HBsAg-positive serum was diluted with 1% normal human albumin 1/1, 1/5, 1/10, 1/20, 1/40, and 1/80. An equal volume of bovine butanol-extracted, alcohol-precipitated HBBS was added to each and incubated at 37°C for 1 h. The mixtures, as assayed by RIA, showed no difference in HBsAg binding activity; that is, they still reacted wtih Ausab beads. When assayed for antigen, however (Ausria II), the 1/1 HBBS-HBsAg mixture showed a drop in radioactive count from 1,500 to 460 cpm. The 1/5 HBBS-HBsAg mixture produced a count equivalent to that of our negative antigen control sera.

None of the mixtures produced a precipitin band against human or animal anti-HBs when tested by immunodiffusion or counterelectrophoresis. Thus, HBBS molecules bind or coat HBsAg particles, but they probably have a single binding site because they do not precipitate the antigen. The coated HBsAg still reacts with uncoated HBsAg (Ausab positive) but not with anti-HBs (Ausria II negative).

Bovine HBBS interferes with influenza virus (Type A-1) hemagglutination of erythrocytes. In the presence of HBBS, there was complete inhibition of influenza virus hemagglutination. The control virus hemagglutination titer was 1/128. Whereas bovine HBBS did agglutinate sheep and human type A (D−) erythrocytes, this agglutination could be distinguished from that produced by influenza virus. The influenza virus produced an uneven pattern, whereas that produced by HBBS appeared as an even button which did not flow when the hemagglutination plate was tilted. The hemagglutination titer of bovine HBBS alone with either sheep or human erythrocytes was 1/64.

Attempts to purify HBBS by affinity chromatography. Approximately 100 polystyrene beads (Ausab, Abbott) were stirred with 10.0 ml of bovine HBBS (from the first peak of Sephadex G200) for 2 h at 37°C. The beads were packed in a small column (12 by 0.75 inches [ca. 30.48 by 1.9 cm]), and elution of the active component was attempted with a variety of phosphate and Tris buffers from 0.1 to 0.5 M in a range of pH from 6 to 9.0. No material absorbing at 280 nm was eluted. A peak of 280-nm-absorbing material could be eluted with 0.1% sodium dodecyl sulfate. The pooled fractions comprising this peak were concentrated on an Amicon P30 filter to one-tenth the original volume of material reacted with the beads. Assay for HBsAg binding activity proved negative; the eluted beads still retained HBsAg as determined by reactivity with ^{125}I-labeled anti-HBs.

Enzyme susceptibility. Table 2 shows the results of RIA assay of HBBS-coated beads after various enzyme treatments. Preliminary experiments indicated that treatment of the Ausab beads with various enzymes had no effect on HBsAg coated on the polystyrene beads. This was shown by comparing the reactivity of the Ausab beads with ^{125}I-labeled anti-HBs before and after enzyme treatment. The proteolytic enzymes had the greatest degradative effect, because 86 to 100% of binding activity was destroyed. Muramidase and neuraminidase had weak activities, indicating some exposed *N*-acetylglucosamine-muramic acid and *N*-acetylgalactosamine-sialic acid bonds, respectively. α-Amylase and β-galactosidase treatment resulted in unexpected increased binding activity, probably resulting from cleavage of internal α-1,4-glucan and β-D-galactoside linkages. Wheat germ lipase and phospholipase C had no effect on binding activity.

TABLE 2. *Susceptibility of HBBS to enzyme degradation*

Enzymes	% Destruction[a] of HBBS
Pronase	100
Trypsin	86
Pepsin	100
Chymotrypsin	86
Papain	94
α-Amylase	0[b]
Muramidase	11
Lipase (wheat germ)	0
Collagenase (*Clostridium histolyticum*)	39
Phospholipase C	0
β-Galactosidase	0[c]
Neuraminidase	15–30[d]

[a] See text.
[b] Binding activity increased 1.2×.
[c] Binding activity increased 2.6×.
[d] Range of four separate experiments.

Relationship of HBBS to bovine disease, age of animal, and time of bleeding. Because the prevalence of HBBS was high in the serum of cows with lymphosarcoma (Table 1), the question arose whether HBBS could be related to this disease. Table 3 shows the results of comparing sera (aseptically collected) for the presence of HBBS from cows carrying the bovine leukemia virus with sera from segregated negative control cows. The cows carrying the bovine leukemia virus fall into three categories: asymptomatic, persistent lymphocytotic, and leukemic. There appears to be no correlation between the disease state and the presence and/or titer of HBBS. Two additional cows with detectable bovine leukemia virus were bled at different times (Table 4). Again, there was no correlation with the age of the cows or time of bleeding with the presence and/or titer of HBBS. Twenty-three cows, free of leukemia virus, ranging in age from 16 to 41 months at the time of bleeding showed varying amounts of HBBS.

Neutralization of HBBS by antisera. Antisera to HBBS was produced by immunizing DBA/2 mice with bovine HBBS in Freund adjuvant mixture. A specific precipitin band to HBBS could be observed by immunodiffusion after absorption of the antiserum with bovine serum negative for HBBS. The mouse anti-HBBS, when added to Ausab beads coated with

TABLE 3. *Relationship of equivalent anti-HBs ratio to infectious status of cows*

Animal identification no.	BLV infection[a]	Persistent lymphocytosis	Leukemia	Anti-HBs ratio (cpm of test/cpm of NHS)[b]
647	+	+	+	4.8
77	+	+	+	4.5
910	+	+	+	18.0
60	+	+	−	2.3
29	+	+	−	15.0
110	+	+	−	7.3
715	+	−	−	60.0
686	+	−	−	2.84
758	+	−	−	17.36
se 382	−	−	−	2.2
se 354	−	−	−	2.9

[a] BLV, Bovine leukemia virus.

[b] Ratio of 2.1 or greater is considered positive for anti-HBs. NHS, Normal human serum.

TABLE 4. *Serial bleeding: relationship of age of cow, anti-bovine leukemia virus infectivity titer, and equivalent anti-HBs ratio*

Animal identification no.	Bleeding date	IF titer[a]	Age (mo)	Ratio (cpm of test/cpm of NHS)[b]
BF089	11 January 1971	16	106	2.6
	21 June 1971	8	111	5.6
	6 January 1972	8	118	2.2
	14 June 1972	8	123	3.5
	27 March 1973	8	132	2.7
BF099	11 January 1971	2	108	40.6
	12 November 1973	2	142	8.7
	10 June 1974	2	149	15.7
	11 November 1974	2	154	30.7
	9 October 1975	4	165	2.9

[a] IF, Immunofluorescence test for detection of antibodies to the internal antigen of the bovine leukemia virus. Twenty-three IF-negative cows, ranging in age from 16 to 41 months at time of bleeding, had the following serum equivalent anti-HBs ratios: <2.0 (negative), 17.4%; 2.1 to 9 (moderately positive), (65.2%); >10 (strongly positive), (17.4%).

NHS, Normal human serum.

HBBS and incubated for 2 h at 37°C decreased the attachment of ^{125}I-labeled HBsAg (Table 5). Pooled sera from the preimmunized mice did not contain HBBS activity. Rabbit anti-*E. coli* pili decreased HBBS activity to a lesser degree. Preimmunized serum from this rabbit contained no HBBS activity.

Relationship of HBBS with bacteria. Purified pili preparations from *N. gonorrhoeae* (strain 3-2) and *E. coli* (F⁻W1-3) produced results similar to those of the binding glycoproteins isolated from animal sera (Table 1). The results with pili are shown in Table 6. Another known HBsAg binding material is concanavalin A (4). As shown in Table 6, this material did not produce a positive binding (Ausab) reaction but appeared to interfere with HBBS binding (a drop in ratio from 4.3 to 1.9). This indicates that concanavalin A has a single binding site because it does not bridge the bound HBsAg with the ^{125}I-labeled HBsAg.

DISCUSSION

Plant proteins known as lectins are useful in probing the structure of cell membranes. These substances, chemically glycoproteins, are not unique to plants and may be ubiquitous in nature. Concanavalin A, an extract of jack beans (*Conavalia enisformis*), is the only known nat-

TABLE 5. *Neutralization of bovine HBBS[a] by antisera*

Antiserum (0.1 ml)	Normal serum (0.1 ml)	% Decrease in binding[b]
Mouse anti-HBBS	Pooled, DBA/2 sera	0
		85–94
	Preimmunized rabbit serum	0
Rabbit anti-*E. coli* pili		58

[a] A 0.1-ml amount of HBBS was used in each assay.

[b] [(Counts per minute of bovine HBBS + antiserum)/(counts per minute of bovine HBBS + normal serum)] − 100. Mean of three assays.

TABLE 6. *HB_sAg binding activity of lectins*

Lectin (ml)	Ausab bead treated with:[a] HBBS (ml)	0.9% NaCl (ml)	NHS (ml)	Ratio (cpm of test/cpm of control)
0.2 of *N. gonorrhoeae* pili (0.1 mg/ml)				5.1
0.2 of *E. coli* pili (0.1 mg/ml)				17.6
0.1 of ConA (10 mg/ml)		0.1		1.1
0.1 of ConA (10 mg/ml)	0.1			1.87
	0.1	0.1		4.3
		0.2		1.3
		0.1	0.1	1.0[b]

[a] Each Ausab bead was incubated with each mixture for 18 h at room temperature. The beads were washed with distilled water, and ^{125}I-labeled HBsAg was added and incubated as recommended by Abbott. Each ratio represents the mean of three separate experiments. ConA, Concanavalin A (Calbiochem, San Diego, Calif.).

[b] Control.

ural lectin which binds to HBsAg (4). The HBsAg binding substance investigated here has some features in common with lectins.

The role of lectins in recognition processes has intrigued biologists and their importance has recently been the subject of discussion (15a). Viruses which invade the animal host have in most instances a selective affinity for specific tissues which appears to be lost when these same viruses are grown in a tissue culture system. For example, polio and rabies viruses seek out and invade nerve tissue in vivo, whereas in vitro they grow readily in other tissues (monkey kidney and embryonated eggs). The in vivo direction that a virus takes could be explained by the selective affinity of its surface components for molecules on the surface of susceptible tissue cells. The loss of certain virus-binding surface components or the uncovering of recognition sites on tissue cells under in vitro conditions might explain the relative ease with which these viruses can be propagated in a tissue culture environment.

The common surface component of several viruses and mammalian membranes is glycoprotein. Uromucoids (urinary glycoprotein) have been identified as inhibitors of influenza, mumps, and Newcastle disease virus hemagglutination in 1950 by Tamm and Horsfall (18, 19). Hemagglutination by viruses (the Hirst reaction) can be reversible because many viruses contain neuraminidase which reacts with the erythrocyte membrane to liberate sialic acid. Viral glycoproteins contain 20% bound carbohydrate consisting of galactose, mannose, fucose, glucosamine, and a considerable amount of neuraminic acid (8, 13). The uromucoids described by Tamm and Horsfall act as powerful competitors with viruses for hemagglutination. From this competition the common substrate in both the erythrocyte surface and urinary glycoprotein (neuraminic acid) was identified. The property of competition with viruses for erythrocyte surface is shared with other glycoproteins rich in sialic acid. These are serum glycoproteins (1, 7, 14), ovarian cyst mucins and ovomucins (6, 10), and salivary mucins (15) from bovine and ovine origin. These glycoproteins can also compete with specific antibodies for (D+) human red cell surface antigen. The salivary mucins of bovine, ovine, and porcine species have been extensively studied (2, 5, 9, 11, 16, 17). Their molecular weights are about 10^6 and consist of 66% carbohydrate, of which half is sialic acid and the remainder is hexosamine. These glycoproteins are the most potent of the viral hemagglutination inhibitors.

The HBsAg binding substance which we have described appears to have similar physical and chemical properties to those above and at the same time appears unique. It agglutinates sheep and human erythrocytes and interferes with the agglutination of erythrocytes by at least one virus, influenza type A-1. In the latter respect it resembles the uromucoids and ovomucoids. However, neither chicken ovomucoid (Worthington, Freehold, N.J.) nor human glycoprotein fraction VI (Miles Laboratories, Kankakee, Ill.) specifically combines with HBsAg (unpublished data). The distribution in nature of HBsAg binding substance is difficult to explain. No batch of fetal or newborn calf serum that we have tested has been found to specifically react with HBsAg as does the sera from older cattle. The incidence in cows appears to be approximately 40%, and we can find no correlation with age or time of bleeding. Because of the widespread incidence of this HBsAg binding substance among different species, it is tempting to speculate that the source of this material is the normal bacterial flora of the intestinal tract. Both *N. gonorrhoeae* and *E. coli* pili preparations produced reactions with HBsAg-coated beads similar to that found with HBBS of different sources. Furthermore, the antiserum to *E. coli* pili produced in rabbits neutralized the binding activity of HBBS as did that produced by mouse anti-HBBS. Unfortunately, these antisera did not produce precipitin bands of sufficient intensity in agar to be compared for full or partial identity. Antisera presently being prepared in mice, with more purified bovine HBBS, may allow this in the future. Weng et al. (20) reported that the activity of HBsAg was lost after incubation with membrane vesicles of *P. aeruginosa*. Whereas the immunological inactivation was attributed to enzyme activity, the HBsAg particles remained intact as shown by electron microscopy. It is conceivable that the surface reactive groups on HBsAg were covered with *Pseudomonas* lectin. This would imply that lectins produced and secreted by bacteria somehow escape digestion and are absorbed into the blood stream; it is possible that the lectin is present in all animals but at an undetectable level at times. Evidence for this is provided in Table 4, where it can be seen that animal BF099 varied in HBBS level from 2.9 to 40.6 depending on time of bleeding.

It appears reasonable to speculate that the hepatitis B virus has an affinity for the human hepatocyte surface membrane and that some materials capable of binding to HBsAg may have the same determinants as hepatocyte surfaces analogous to but different from antibody. Such determinants may not reside solely on the surface of susceptible cells, but like cross-reacting antigens may be found widely in nature. For example, blood group substances are found in

intestinal mucins, and certain rickettsial agents share antigens with the *Proteus* OX strains of bacteria. It is entirely possible that HBsAg affinity reactants may also be found widely represented in nature. Such reactions could also partly explain the apparent resistance of all but human and certain nonhuman primates to infectivity with the hepatitis B virus. We have never found the HBsAg binding substance in human serum.

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