JOURNAL OF VIROLOGY, Sept. 1975, p. 508-515
Vol. 16, No. 3

Printed in U.S.A.

Production of Antibody to Individual Polypeptides Derived from Purified Hepatitis B Surface Antigen

GORDON R. DREESMAN,* RUBEN CHAIREZ,[1] MONICA SUAREZ,[2] F. BLAINE HOLLINGER, RICHARD J. COURTNEY, AND JOSEPH L. MELNICK

Department of Virology and Epidemiology, Baylor College of Medicine, Houston, Texas 77025

Received for publication 14 April 1975

Purified preparations of hepatitis B surface antigen (HB_s Ag) were solubilized with sodium dodecyl sulfate and urea under reducing conditions and subsequently fractionated by preparative sodium dodecyl sulfate-urea polyacrylamide gel electrophoresis (PAGE). Pools of the individual fractions eluted from the preparative PAGE were concentrated and purified further by analytical PAGE. Five purified polypeptides were isolated from HB_s Ag, types *adw* and *ayw*, with molecular weights of 19,000, 24,000, 27,000, 35,000, and 40,000. Each preparation was emulsified in Freund complete adjuvant and injected into guinea pigs. Antibody to each HB_s Ag type was measured by radioimmunoassay. The 19,000 molecular weight polypeptide derived from *ayw* particles and the 27,000 molecular weight subunit obtained from both types failed to elicit an antibody response. The other three polypeptides derived from the *ayw* particles elicited group-specific antibody responses. Similar group-specific reactivities were observed in the testing of anti-*adw* 35,000 and anti-*adw* 40,000 molecular weight polypeptide sera. However, guinea pigs immunized with the 19,000 and the 24,000 molecular weight polypeptides of the *adw* type produced antibody that reacted preferentially with *adw* particles. This indicates that either these subunits carry predominately *d* determinants or that, because of the low levels of material used for inoculation, no immune response or an undetectable one was elicited to the *a* or *w* components.

The principal antigenic constituent observed in plasma obtained from humans with hepatitis B infections is a pleomorphic spherical particle measuring approximately 22 nm in diameter and referred to as the hepatitis B surface antigen (HB_s Ag). These particles are found in association with tubular forms, which have a diameter of 20 nm with varying lengths, and with a spheroid form measuring about 42 nm in diameter, referred to as the Dane particle, which contains an inner 27-nm core (2, 4, 9, 21). The 22-nm particles are antigenically related to the tubules and the surface components of the Dane particles (1, 19).

Several laboratories have analyzed the subunit structure of purified HB_s Ag (6, 7, 11, 13-15, 20). Although there is a discrepancy in the reported number and size of the component polypeptides, it appears that the particle contains from five to nine subunits ranging in molecular weight from approximately 19,000 to 120,000. Three of these subunits have been characterized as glycoproteins with molecular weights of 24,000, 27,000, and 35,000 (7). Purified preparations of HB_s Ag have a number of distinct antigenic determinants, including a group-specific determinant, *a*, and one member from each of two pairs of mutually exclusive determinants, *d*,*y* and *w*,*r* (3, 22). However, it has not been established that any or all of these determinants are associated with the individual component polypeptides.

In this study we purified several of the major polypeptides derived from reduced HB_s Ag/*adw* and HB_s Ag/*ayw* sodium dodecyl sulfate (SDS)-urea-solubilized particles and prepared antisera to each subunit in guinea pigs. Studies were also carried out to characterize the cell-mediated immune response in guinea pigs to these subunit preparations and will be reported elsewhere (G. A. Cabral, R. Chairez, F. Marciano-Cabral, M. Suarez, G. R. Dreesman, J. L. Melnick, and F. B. Hollinger, Infect. Immun., in press).

MATERIALS AND METHODS

Purification and characterization of HB_s Ag. The methods used to purify spherical, 20 ± 2-nm

[1] Present address: George Mason University, Department of Biology, Fairfax, Va. 22030.

[2] Present address: Department of Virology, University of Chile, Santiago, Chile.

particles containing HB_sAg from human serum have been described previously (10, 17). Briefly, the particles were pelleted for 18 h at 34,000 rpm in a Spinco type 50.1 rotor at 4 C, suspended in saline, and repelleted for 4 h at 40,000 rpm and 4 C in the same rotor. The resulting pellet was acidified to pH 2.5 with 0.05 M potassium phthalate-hydrochloride buffer, incubated for 1 h at room temperature, and then banded twice in CsCl in a Spinco type SW-41 rotor at 4 C and 36,000 rpm for 18 h. The second isopycnic density centrifugation was followed by a rate-zonal centrifugation for 5 h at 36,000 rpm and 4 C in a preformed 5 to 30% (wt/wt) CsCl gradient using a Spinco SW-41 rotor. The CsCl was removed by dialysis against 0.15 M NaCl, and fractions containing HB_sAg were monitored with a complement fixation test using guinea pig anti-HB_s serum (17). The type specificity of each preparation was determined by double diffusion in agar gel with *adw* and *ayw* type-specific guinea pig antisera (17). Protein levels of each purified preparation were measured by the Lowry method (24), with crystalline bovine serum albumin as standard.

Preparation of purified HB_sAg polypeptides. Purified preparations of HB_sAg were solubilized for electrophoresis by heating at 60 C for 10 min in 6 M urea-1% SDS-1% 2-mercaptoethanol. Six to 9 mg of the solubilized HB_sAg preparation was fractionated by preparative polyacrylamide gel electrophoresis (PAGE) with a Buchler Poly-Prep electrophoresis apparatus (Buchler Instruments, Fort Lee, N.J.). The fractionation procedure has been described previously (8). The separating gel consisted of: 15 ml of a buffer (pH 8.9) containing 18.1 g of Tris, 0.12 ml of *N,N,N',N'*-tetramethylenediamine, and 24 ml of 1 N HCl in 100 ml of water; 15 ml of a solution containing 56 g of acrylamide and 1.40 g of *N,N'*-methylene-bis-acrylamide (Bis) in 100 ml of water; 6 ml of 5.0 M urea; and 0.6 ml of 10% SDS. An equal volume of a 0.14% ammonium persulfate solution was mixed with the acrylamide-Bis mixture immediately before it was added to the column. This resulted in a 13.2% separating gel with an acrylamide-to-Bis ratio of 40:1. The stacking gel consisted of the following mixture: 4 ml of a buffer (pH 6.7) containing 5.91 g of Tris, 0.41 ml of *N,N,N',N'*-tetramethylenediamine, and 48 ml of 1 N HCl in 100 ml of water; 4 ml of a solution containing 20 g of acrylamide and 5 g of Bis-acrylamide in 100 ml of water; 4 ml of a solution containing 4 mg of riboflavin in 100 ml of water; 16 ml of sucrose (40%, wt/vol); 2.8 ml of 5 M urea; and 0.28 ml of 10% SDS. This resulted in a 1.3% stacking gel with an acrylamide-to-Bis ratio of 4:1. The stacking gel volume was adjusted to equal that of the sample. The electrode buffer, pH 8.9, consisted of 3.0 g of Tris, 14.4 g of glycine, 100 ml of 5 M urea, and 10 ml of 10% SDS per 1 liter of water. Electrophoresis was performed at a constant current of 60 mA, a constant temperature of 24 C, and with a flow rate of approximately 0.5 ml/min.

Urea, excess SDS, and buffer salts were removed from the eluted material by chromatography in a column (2.5 by 30 cm) of Sephadex G-10. Each column was equilibrated and eluted with distilled water. The fractions containing protein, as monitored at an optical density reading at 280 nm, were pooled and lyophilized. The lyophilized protein was suspended in a small volume of 0.01 M Tris-hydrochloride buffer, pH 8.2 (Tris buffer), containing 6 M urea, 1% SDS, and 1% 2-mercaptoethanol and refractionated by analytical PAGE on 14% gels by a previously described method (7). Electrophoresis was maintained at 4.0 mA per gel until the bromophenol tracking dye migrated at least 8 cm. The following proteins of known molecular weights (29) were used to estimate the relative molecular weight of each subunit: cytochrome *c*, 11,700; chymotrypsinogen, 25,700; ovalbumin, 43,000; and bovine serum albumin, 68,000. The polypeptides were detected either by protein stain with Coomassie brilliant blue or by scanning the unstained gel in a Gilford gel scanner at a fixed wavelength of 280 nm. All gels were scanned, and selected protein bands were cut out of each gel and stored frozen for animal immunization.

Guinea pig immunization. The protein bands prepared as described above were used to immunize guinea pigs. The specific polypeptides were excised from three gels, emulsified with an equal volume of Freund complete adjuvant, and injected into the footpads of adult (400 to 500 g) Hartley strain guinea pigs on days 0, 9, and 17. The protein present in a single gel was used to inoculate each of three animals. With the relative absorbancy of purified bovine serum albumin as standard, it was estimated that 0.05 to 0.1 μg of protein was used per animal per inoculation. Each guinea pig was boosted on day 37 with an intramuscular injection of the same preparation in Freund complete adjuvant. The animals were bled by cardiac puncture on day 0, prior to immunization, on day 17 (referred to as primary antisera), and on day 48 (referred to as booster antisera).

Radioimmunoassay. A modification of a technique (17) described by Figenschau and Ulstrup (12) was used to measure the titers of guinea pig antipolypeptide sera. The separation of the labeled HB_sAg/anti-HB_s complexes from free, unbound ^{125}I-labeled HB_sAg was accomplished by the addition of purified staphylococcal protein A (Sigma Chemical Co., St. Louis, Mo.). This protein binds to the Fc fragment of immunoglobulin molecule classes IgG_1, IgG_2, and IgG_4. Briefly, 0.1 ml of antiserum diluted in phosphate-buffered saline (0.01 M phosphate and 0.15 M NaCl, pH 7.2) containing 16% normal human serum (NHS) was added to 0.2 ml of phosphate-buffered saline. A 0.1-ml volume of ^{125}I-labeled purified HB_sAg containing approximately 20,000 counts/min was added to each tube. The tubes were sealed with Parafilm, and the reactants were mixed and incubated in a water bath at 37 C for 16 to 18 h. A 0.1-ml volume of staphylococcal protein A, containing 1 mg of protein, was added to each tube. The reaction mixtures were incubated at 37 C for an additional 30 min. The resulting precipitates were washed twice with 4.5 ml of cold phosphate-buffered saline by centrifugation at 1,550 $\times$ *g* for 15 min. The supernatants were decanted and the pellets were counted in a Nuclear-Chicago (Chicago, Ill.) autogamma counter. Specimens were considered to be positive if the

precipitated counts per minute exceeded 2.0 times the mean of a panel of preimmune guinea pig sera. Antibody titers were expressed as that end point dilution which bound 10% of the respective ^{125}I-labeled HB_s Ag particles.

Complement fixation test. The guinea pig anti-polypeptide sera were tested for antibody reactivity to NHS components by a two-dimensional complement fixation titration (10, 17). Dilutions of guinea pig antisera ranging from 1:10 through 1:320 were tested against HB_s Ag-negative NHS diluted fourfold from 1:5 through 1:1,280,000. This method has previously been demonstrated to detect low levels of antibodies in antisera prepared against intact HB_s Ag to diluted NHS (10).

RESULTS

Fractionation of solubilized HB_s Ag. The elution profiles of solubilized HB_s Ag fractionated by preparative SDS-PAGE are illustrated in Fig. 1. Nine mg of HB_s Ag/*adw* and 6 mg of purified type *ayw* were electrophoresed. A broad profile of material that absorbed at 280 nm was eluted, and no distinct individual peaks were observed with either fractionated preparation. The 4-ml fractions were pooled in six individual pools as indicated in Fig. 1, and each pool was desalted by gel filtration on Sephadex G-10 columns equilibrated in distilled water.

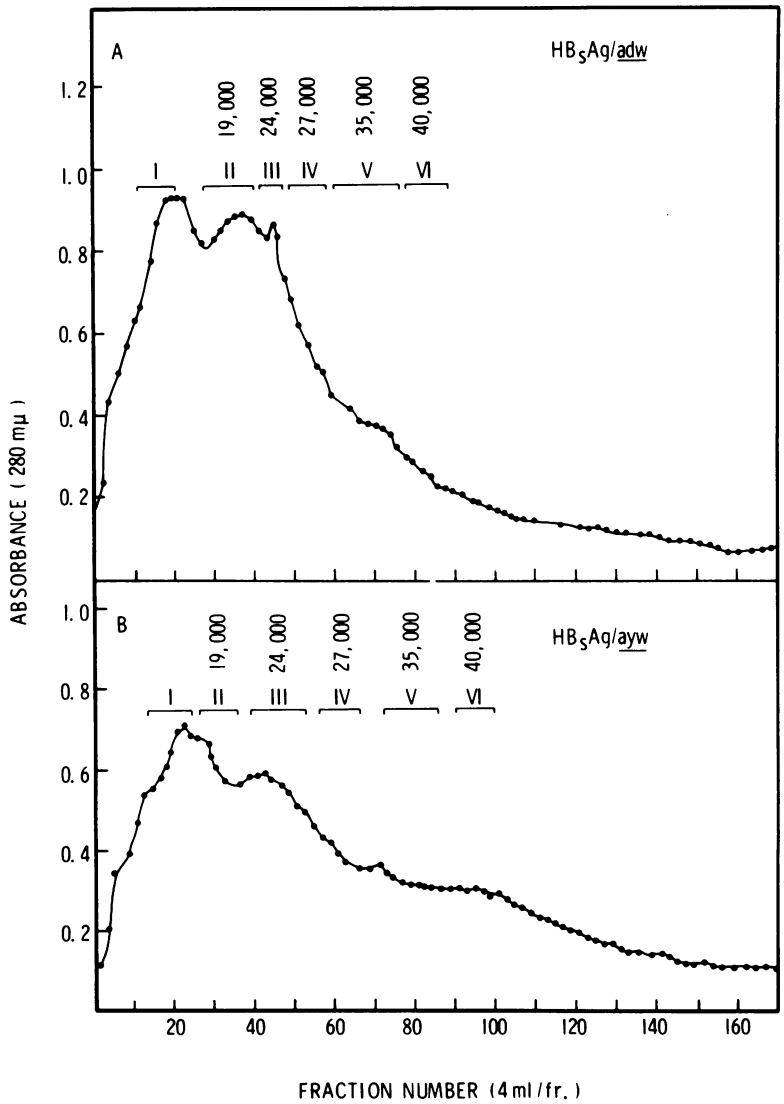

FIG. 1. *Elution profiles of purified (A) HB_s Ag/adw and (B) HB_s Ag/ayw solubilized in SDS and urea from preparative PAGE utilizing 13.2% polyacrylamide gels. Pools of the eluted fractions were made as indicated by Roman numerals, and the molecular weight of the major component in each pool is given.*

The pools were then concentrated by lyophilization, suspended in a minimal volume of Tris buffer, and characterized by analytical urea-SDS-PAGE. Each gel was scanned for protein with a Gilford gel scanner at 280 nm, and representative patterns are illustrated in Fig. 2 and 3. Very similar results were obtained with the purified preparations of *adw* and *ayw*. The molecular weights of selected components in each concentrated pool were: pool II, 19,000, pool III, 24,000; pool IV, 27,000; pool V, 35,000; and pool VI, 40,000. Pool IV yielded a polypeptide with a molecular weight of 27,000, but since no antibody was produced in response to this subunit the elution profile is not shown. It should be noted that no protein was detected in pool I after the concentration procedure.

Antibody titers to intact HB$_s$ Ag particles. Guinea pigs were immunized with each subunit preparation as described above. The antibody titers of both primary and booster antisera were determined by radioimmunoassay

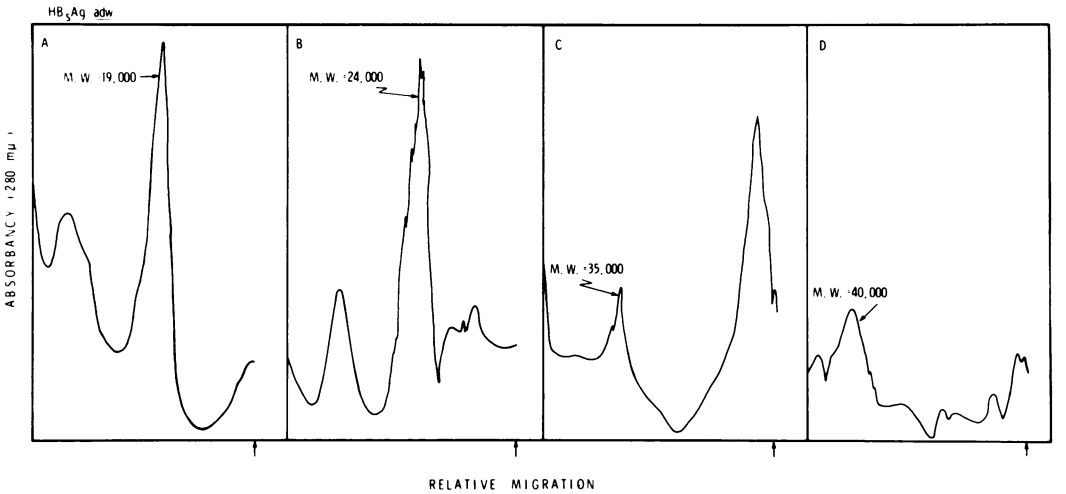

FIG. 2. *Densitometer tracings of HB$_s$ Ag/adw-derived polypeptides by analytical PAGE found in pools eluted from preparative PAGE illustrated in Fig. 1. (A) A 4.1-μg portion of pool II, resolving a 19,000-dalton polypeptide; (B) 3.4 μg of pool III, resolving a 24,000-dalton polypeptide; (C) 4.0 μg of pool V, resolving a 35,000-dalton polypeptide; (D) 2.2 μg of pool VI, resolving a 40,000-dalton polypeptide. Arrows indicate position of dye front.*

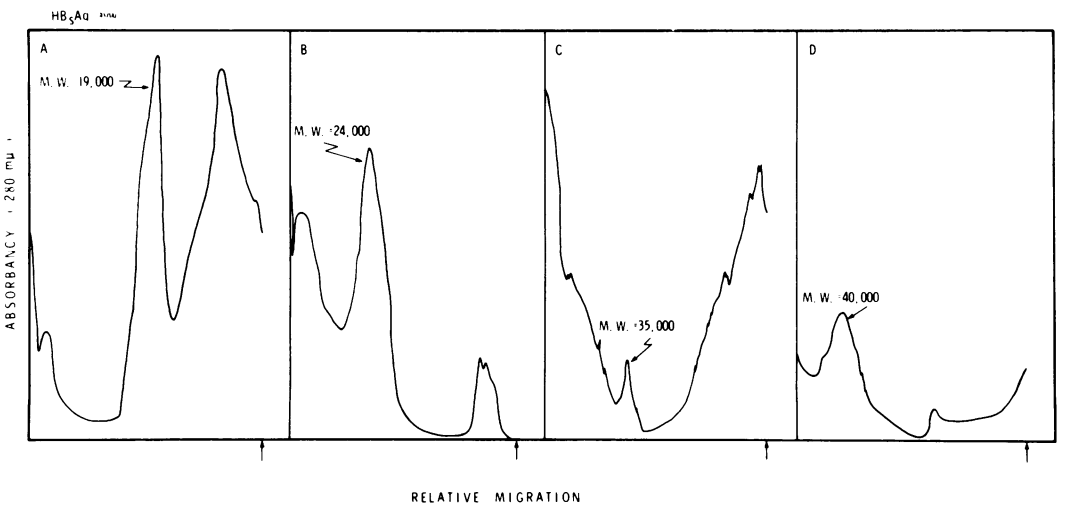

FIG. 3. *Densitometer tracings of polypeptides derived from HB$_s$ Ag/ayw. Materials derived as given in legends to Fig. 1 and 2. (A) A 4.2-μg portion of pool II, resolving a 19,000-dalton polypeptide; (B) 2.2 μg of pool III, resolving a 24,000-dalton polypeptide; (C) 3.6 μg of pool V, resolving a 35,000-dalton polypeptide; (D) 1.8 μg of pool VI, resolving a 40,000-dalton polypeptide. Arrows indicate position of dye front.*

with ^{125}I-labeled intact *adw* and *ayw* particles (Tables 1 and 2). Several points can be made from these results. (i) The 19,000 molecular weight subunit derived from HB_s Ag/*ayw* and the 27,000 molecular weight subunit from both HB_s Ag types failed to elicit a detectable humoral antibody response. (ii) Five of six guinea pigs immunized with the 19,000 and the 24,000 molecular weight polypeptides derived from purified *adw* particles produced antibody that reacted preferentially with intact *adw* particles. (iii) The *adw* 35,000 and 40,000 molecular weight subunits elicited a relatively greater group-specific immunogenic response in that a greater degree of cross-reactivity was noted with these antisera in tests done against intact *adw* and *ayw* particles. (iv) The *y* determinant associated with the 24,000, 35,000, and 40,000 molecular weight polypeptides derived from *ayw* particles elicited a group-specific response. In many instances, animals injected with these *ayw*-derived polypeptides produced antibody that reacted at higher titers with purified *adw*

TABLE 1. *Anti-HB_s response in guinea pigs immunized with polypeptides derived from purified HB_s Ag/adw particles*

Animal no.	Mol wt of immunizing polypeptide ($\times 10^3$)	Antibody titers[a] to intact type-specific HB_s Ag			
		Primary response[b]		Booster response[b]	
		adw	*ayw*	*adw*	*ayw*
R-1	19	<6	<6	3,600	<6
R-2		210	<6	32,000	<6
R-9		290	<6	28,000	3,900
R-6	24	360	<6	900	<6
R-7		740	<6	7,400	<6
R-10		1,250	<6	8,200	8
R-11	27	<6	<6	<6	<6
R-12		<6	<6	<6	<6
R-13		<6	<6	<6	<6
R-14	35	ND[c]	ND	8,400	3,500
R-15		2,000	180	6,000	750
R-16		250	10	5,500	2,800
R-30	40	ND	ND	62,500	5,000
R-31		ND	ND	3,500	1,200
R-32		ND	ND	1,500	40

[a] Reciprocal of serum dilutions as measured by radioimmunoassay.

[b] Animals were injected with hepatitis polypeptides in Freund complete adjuvant on days 0, 9, and 37. Primary response was measured on day 17 and the booster response was measured on day 48.

[c] Not done.

TABLE 2. *Anti-HB_s response in guinea pigs immunized with polypeptides derived from purified HB_s Ag/ayw particles*

Animal no.	Mol wt of immunizing polypeptide ($\times 10^3$)	Antibody titers[a] to intact type-specific HB_s Ag			
		Primary response[b]		Booster response[b]	
		adw	*ayw*	*adw*	*ayw*
R-17	19	<6	<6	<6	<6
R-20		<6	<6	<6	<6
R-21		<6	<6	<6	<6
K	24	100	<6	2,300	280
M		1,100	<6	2,250	550
N		600	<6	120	600
O	27	<6	<6	<6	<6
R		<6	<6	<6	<6
P		<6	<6	<6	<6
S	35	1,200	<6	2,500	<6
U		5,000	15	12,000	620
W		<6	<6	<6	10
Z	40	<6	<6	750	280
X		6	<6	800	2,500
CC		<6	<6	570	220

[a] Reciprocal of serum dilutions as measured by radioimmunoassay.

[b] Animals were injected with hepatitis polypeptide in Freund complete adjuvant on days 0, 9, and 37. Primary response was measured on day 17 and the booster response was measured on day 48.

than they did with *ayw* particles.

All of the antisera listed in Tables 1 and 2 were tested against HB_s Ag-negative NHS by a two-dimensional complement fixation test. No antibody activity to NHS was detected in any of these guinea pig antisera.

DISCUSSION

Data accumulated in this and several other laboratories during the past 5 years have shown that the chemical and antigenic composition of purified HB_s Ag is very complex (1, 3, 6, 7, 11, 13–15, 20, 22). The 17- to 25-nm particles studied contain seven to nine polypeptides, three of which are glycoproteins, as well as cholesterol, polar lipids, and glycolipids. Antigenically, a spectrum of determinants has been reported in which a common cross-reacting group determinant, termed *a*, and two sets of mutually exclusive determinants, known as the *d*,*y* and *w*,*r* determinants, are present. This chemical and antigenic complexity is extraordinary in view of the fact that the probable virus candidate, the Dane particle, contains a relatively

small double-stranded DNA with a molecular weight of 1.6×10^6 (28). In light of these observations, a detailed study of the individual polypeptides should aid in establishing the location of distinct antigenic determinants on each subunit and in determining whether each polypeptide has unique chemical characteristics or whether the polypeptides exist as polymers of smaller polypeptides with similar chemical properties. This study is an initial attempt to isolate the subunits and to shed some knowledge on the association of antigenic determinants with individual polypeptides.

Five purified polypeptides with molecular weights ranging from 19,000 to 40,000 were isolated in this study. Polypeptides derived from *adw* particles with molecular weights of 19,000, 24,000, 35,000, and 40,000 were immunogenic. Although there was some variation in each group of three animals immunized, the 19,000 and 24,000 molecular weight polypeptides from *adw* particles gave a type-specific response in that they reacted predominantly with HB_s Ag/*adw*.

These results indicate that either the subunits derived from *adw* particles carry predominantly *d* determinants or that at the low levels of material used for immunization in this study a detectable immune response was not elicited to the *a* or *w* components. In fact, one animal (guinea pig R-9) did produce antibody that reacted with *ayw* particles. In a brief report by Gerin (14), only group reactivity was observed to several polypeptides used for immunization. Although the conditions of the immunization schedule were not given in his brief report (14), larger levels of protein may have been injected so that antibody was elicited to the *a* or *w* determinants in his animals.

The other two polypeptides obtained from *adw* particles with molecular weights of 35,000 and 40,000 elicited antibodies that reacted with both *adw* and *ayw* particles. However, all six animals reacted preferentially with *adw* particles, indicating that the group determinant α and type-specific determinants were associated with these subunits.

The results obtained with subunits derived from *ayw* particles are difficult to interpret. Antibody was produced in response to immunization with the 24,000, 35,000, and 40,000 molecular weight subunits. However, antisera from two of three animals in each group reacted at higher radioimmunoassay titers with *adw* preparations than with *ayw* preparations. There is some animal-to-animal variation though, since one of three of each group reacted at somewhat higher levels with *ayw* particles (animals N, W, and X: Table 2). This would indicate that all three subunits contain both *a* and *y* determinants. The above findings are similar to those published in a preliminary report by Gerin (14) on the preparation of anti-HB_s subunit sera in guinea pigs.

One possible explanation for the preferential reactivity of anti-*ayw* polypeptide sera for *adw* particles is that the *y* determinants are not accessible on the surface of *ayw* particles. However, this explanation may be ruled out since lines of partial identity are seen in agar-gel precipitin tests between *adw* and *ayw* antigens and type-specific antisera. This would indicate that the *y* determinant is reactive on the intact particle. A second possibility is that the *y* antigenic determinant is a conformational-dependent antigen. It should be noted that these antisera produced in response to reduced SDS-urea-denatured polypeptides were reacted against native intact particles. Since denaturing conditions, such as reduction and urea and SDS treatment, are known to disrupt conformational antigenic determinants in several globular proteins (27), the possibility that the *y* determinant is conformation dependent is a plausible explanation.

No explanation is available for the fact that the 27,000 molecular weight polypeptide derived from both types and the 19,000 molecular weight polypeptide derived from *ayw* did not elicit antibody in guinea pigs. This may be a reflection on the amount of subunit used in the present study in that the levels of these particular subunits may have been too low to induce antibody production.

Glycoproteins have been detected in purified HB_s Ag in several laboratories (5, 7, 14; I. Gordon, personal communication). In addition, Burrel et al. (5) have reported that antigenic activity may be associated with these carbohydrate moieties. We have reported that the 24,000, 27,000, and 35,000 molecular weight subunits are glycoproteins (7). It would be of interest to determine whether any of these carbohydrate moieties are essential for immunogenic activity of these purified subunits.

These antisera, in addition to those produced to the higher-molecular-weight subunits, should provide valuable reagents to determine whether host cell components, either liver cell constituents or NHS components, are present in the purified particle. It is controversial as to whether or not NHS components are an integral component of HB_s Ag. Two groups have reported that human serum proteins are associated with purified HB_s Ag (25, 26). However, Goudeau et al. (16), using a method similar to

that used by Neurath et al. (26), were unable to detect these serum components in HB_s Ag. There is evidence that there may be an immune response to liver-specific lipoprotein in patients with hepatitis B infection (23, 30).

No antibody to NHS components was detected in any of the guinea pig antisera prepared in the present study. However, higher levels of protein than were used in the present study may be necessary to elicit anti-NHS component antibody. It should be pointed out that the cellular immune response may be more sensitive for measuring such a cross-contaminating component. A study from our laboratory, which is being reported elsewhere (Cabral et al., Infect. Immun., in press) also failed to demonstrate anti-NHS activity in antibodies produced in guinea pigs to an *adw*-derived 24,000 molecular weight polypeptide. However, a cellular immune response, as measured by a macrophage inhibition test, was detected when peritoneal exudate cells from guinea pigs immunized with NHS were challenged with the 24,000 molecular weight polypeptide.

It has been established that HB_s Ag shares antigenic determinants with the envelope of the Dane particle (1, 19). Persons manifesting titers of anti-HB_s are protected against hepatitis type B infection (18). Therefore, HB_s Ag subunits which appear to be free of antigenically cross-reactive host cell components and which do not contain infectious viral nucleic acid may yield an effective hepatitis B vaccine.

ACKNOWLEDGMENTS

This investigation was supported by research contract DADA 17-73C-3074 from the U.S. Army Medical Research and Development Command and by Public Health Service research grant HL-17269-01 from the National Heart and Lung Institute.

LITERATURE CITED

1. Almeida, J. D., D. Rubenstein, and E. J. Stott. 1971. New antigen-antibody system in Australia-antigen-positive hepatitis. Lancet **2**:1225-1227.
2. Almeida, J. D., and A. P. Waterson. 1969. Immune complexes in hepatitis. Lancet **2**:983-986.
3. 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**:420-425.
4. Bayer, M. E., B. S. Blumberg, and B. Werner. 1968. Particles associated with Australia antigen in the sera of patients with leukaemia, Down's syndrome and hepatitis. Nature (London) **218**:1057-1059.
5. Burrel, C. J., E. Proudfoot, G. A. Keen, and B. P. Marmion. 1973. Carbohydrates in hepatitis B antigen. Nature (London) New Biol. **243**:260-262.
6. Chairez, R., F. B. Hollinger, J. P. Brunschwig, and G. R. Dreesman. 1975. Comparative biophysical studies of hepatitis B antigen, subtypes *adw* and *ayw*. J. Virol. **15**:182-190.
7. Chairez, R., S. Steiner, J. L. Melnick, and G. R. Dreesman. 1973. Glycoproteins associated with hepatitis B antigen. Intervirology **1**:224-228.
8. Courtney, R. J., and M. Benyesh-Melnick. 1974. Isolation and characterization of a large molecular weight polypeptide of herpes simplex virus type 1. Virology **62**:539-551.
9. Dane, D. S., C. H. Cameron, and M. Briggs. 1970. Virus-like particles in serum of patients with Australia-antigen-associated hepatitis. Lancet **1**:695-698.
10. Dreesman, G. R., F. B. Hollinger, R. M. McCombs, and J. L. Melnick. 1972. Production of potent anti-Australia antigen sera of high specificity and sensitivity in goats. Infect. Immun. **5**:213-221.
11. Dreesman, G. R., F. B. Hollinger, J. R. Suriano, R. S. Fujioka, J. P. Brunschwig, and J. L. Melnick. 1972. Biophysical and biochemical heterogeneity of purified hepatitis B antigen. J. Virol. **10**:469-476.
12. Figenschau, K. J., and J. C. Ulstrup. 1974. Staphylococcal RIA for hepatitis B antigen and antibody. Acta Pathol. Microbiol. Scand. Sect. B **82**:422-428.
13. Gerin, J. L. 1972. Isolation and physicochemical characteristics of HB Ag, p. 205-219. *In* G. N. Vyas, H. A. Perkins, and R. Schmid (ed.), Hepatitis and blood transfusion. Grune & Stratton, New York.
14. Gerin, J. L. 1974. Structure of hepatitis B antigen (HB Ag), p. 215-223. *In* W. S. Robinson and C. F. Fox (ed.), Mechanisms of virus disease, vol. 1. W. A. Benjamin, Inc., Menlo Park, Calif.
15. Gerlich, W., and G. May. 1973. Hepatitis-associated antigen: purification and properties. Zentralbl. Bakteriol. Parasitenkde. Infektionskr. Hyg. Abt. 1: Orig. Reihe A **224**:49-60.
16. Goudeau, A., B. Houwen, and J. Dankert. 1974. Cross-reaction of human serum-proteins with HB_s Ag. Lancet **2**:1325.
17. Hollinger, F. B. 1974. Hepatitis viruses, p. 819-833. *In* E. H. Lennette, E. H. Spaulding, and J. P. Truant (ed.), Manual of clinical microbiology, 2nd ed. American Society for Microbiology, Washington, D.C.
18. Hollinger, F. B., J. Werch, and J. L. Melnick. 1974. A prospective study indicating that double-antibody radioimmunoassay reduces the incidence of post-transfusion hepatitis B. N. Engl. J. Med. **290**:1104-1109.
19. Hoofnagle, J. H., R. J. Gerety, and L. F. Barker. 1973. Antibody to hepatitis B virus core in man. Lancet **2**:869-873.
20. Howard, C. R., and A. J. Zuckerman. 1974. Characterization of hepatitis B antigen polypeptides. Intervirology **4**:31-44.
21. Jokelainen, P. T., K. Krohn, A. M. Prince, and N. D. C. Finlayson. 1970. Electron microscopic observations on virus-like particles associated with SH antigen. J. Virol. **6**:685-689.
22. Le Bouvier, G. L. 1971. The heterogeneity of Australia antigen. J. Infect. Dis. **123**:671-675.
23. Lee, W. M., W. D. Reed, C. G. Mitchell, R. M. Galbraith, A. L. W. F. Eddleston, A. J. Zuckerman, and R.Williams. 1975. Cellular and humoral immunity to hepatitis-B surface antigen in active chronic hepatitis. Br. Med. J. **1**:705-708.
24. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
25. Millman, I., H. Hutanen, F. Merino, M. E. Bayer, and B. S. Blumberg. 1971. Australia antigen: physical and chemical properties. Res. Commun. Chem. Pathol. Pharmacol. **2**:667-686.
26. Neurath, A. R., A. M. Prince, and A. Lippin. 1974. Hepatitis B antigen: antigenic sites related to human serum proteins revealed by affinity chromatography.

Proc. Natl. Acad. Sci. U.S.A. **71**:2663-2667.
27. Reichlin, M. 1975. Amino acid substitution and the antigenicity of globular proteins, p. 71-123. *In* F. J. Dixon and H. G. Kunkel (ed.), Advances in immunology, vol. 20. Academic Press Inc., New York.
28. Robinson, W. S., D. A. Clayton, and R. L. Greenman. 1974. DNA of a human hepatitis B virus candidate. J. Virol. **14**:384-391.
29. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**:4406-4412.
30. Zuckerman, A. J. 1975. Hepatitis vaccine: a note of caution. Nature (London) **255**:104-105.