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CONTROL OF HEPATITIS VIRUS INFECTIONS BY NEW METHODS

ANNUAL PROGRESS REPORT

by

Joseph L. Melnick, Ph.D.

January 31, 1983

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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DD Form 1473, Item 20: Abstract

During the past contract year, we have continued our investigations with an HBsAg synthetic peptide (SP1) which contains amino acid residues 122-137 associated with the ayw subtype sequences of the major envelope polypeptide, P25. Several important immunochemical parameters associated with SP1 were established: (1) Conjugation of SP1 to a tetanus toxoid (TT) carrier or aggregation of SP1 in micelle form yielded material that produced a good booster response in mice. In fact, similar titers were noted in mice injected with 2 doses of alum-precipitated material of either SP1 conjugated to TT or purified HBsAg 22-nm particles. (2) Immunization of a chimpanzee with preexisting anti-HBs with 2 inoculations of SP1-TT in alum produced a significant booster antibody response. (3) SP1 contains a conformational a epitope and a sequential y epitope.

We have also produced an anti-idiotype reagent in rabbits that recognizes a common idiotypic determinant on human anti-HBs molecules. This reaction between the human idiotype and the rabbit anti-idiotype antibody can be inhibited at the same level with the 3 different HBsAg subtypes (adw, adr and ayw), with purified P25-GP30 polypeptide, and with SP1. Reduction and alkylation of native polypeptides (P25-GP30) or of SP1 abolished their ability to react with the human idiotype. This demonstrated the idiotypic determinant was associated with the antigen-combining site that was specific for the conformation-dependent group a HBsAg reactivity. In addition, it was demonstrated that the common human idiotypic determinant was expressed on anti-HBs molecules derived from several other mammalian species.

Finally, we have demonstrated that mice injected with specifically purified anti-idiotype antibody produced to human anti-HBs produce immune cells that secrete antibody which reacts specifically with HBsAg. We are currently investigating the potential of this reagent for the specific modulation of the immune response.

Foreword:

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences-National Research Council.

TABLE OF CONTENTS

A. Immune Response to HBsAg Synthetic Peptides

As reported previously, two cyclic disulfides have been synthesized: the first (referred to as synthetic peptide 1, SP1) contains amino acid residues 122-137 and the second (referred to as SP2) contains amino acid residues 117-137. Both synthetic peptides were incorporated into different adjuvants (Freund's complete adjuvant, aluminum gel and liposomes) without being linked to any protein carrier and were inoculated (50 µg of peptide per dose) into groups of 6 BALB/c mice (1). The animals were bled 21 days after the primary inoculation, boosted on day 22, and bled 10 days after the booster inoculation. Both synthetic peptides induced anti-HBs antibodies in mice. Liposomes were the most efficient adjuvant vehicle and aluminum gel was the poorest. 50% of the animals in the group inoculated with the peptide-liposome preparations responded after one inoculation, and the titers increased significantly after the booster. The unexpectedly high immunogenicity of the cyclic peptides may be due to the presence of a major immunogenic determinant or to the locking of the secondary structure of a potent immunogen by the disulfide cyclization.

We tried to enhance the immunogenicity of SP1 by procedures that would be acceptable for future field trials in humans (2). Peptide micelles were prepared through a procedure similar to that described previously for virus protein. These aggregates were smaller (40-80 nm in diameter) and had a lower density (1.10 g/cm^3 in sucrose) than micelles prepared from solubilized HBsAg particles. In addition, a conjugate was obtained by covalent coupling of SP1 to a protein carrier – tetanus toxoid – using carbodiimide as crosslinker. Both the synthetic peptide micelles and the synthetic peptide-tetanus toxoid conjugate (SP1-TT) were tested for immunogenicity in mice, either as an aqueous suspension or as an alum gel precipitate. The relative immunogenicity of the 4 preparations, illustrated by the number of mice developing an anti-HBs response and by the anti-HBs levels, is summarized in Table 1. After the primary inoculation, the response appeared to be no better than after liposome-entrapped, uncoupled synthetic peptides. However, after a booster inoculation, 80-100% of the animals responded to synthetic peptide micelles precipitated on alum gel and to both formulations of SP1-TT conjugate. The mice immunized with SP1-TT responded with high titers, the peptide micelles precipitated on alum gel induced relatively low levels of anti-HBs, and the peptide micelles in saline were a weak immunogen.

The geometric mean anti-HBs titers in mice immunized with the SP1-TT conjugate precipitated on alum gel were compared to the titers induced by intact HBsAg particles, SDS-denatured P25 + GP30 polypeptides, and P25 + GP30 micelles (Table 2) (3). The P25 + GP30 micelle preparation derived under non-denaturing conditions from HBsAg particles was clearly the superior immunogen. It was noteworthy that after one booster inoculation the geometric mean titer obtained with the SP1-TT conjugate was comparable to that induced with intact HBsAg particles and showed only a small decrease (Table 2). We are presently optimizing our SP1-TT conjugate by testing different crosslinkers as well as different SP1-TT conjugation ratios.

B. Anti-HBs Response in an HBV-Immune Chimpanzee to a Synthetic HBsAg Peptide

Two chimpanzees, previously infected with HBV and whose sera contained anti-HBs, were inoculated with 100 µg of peptide 1 conjugated to tetanus toxoid and alum-adsorbed. Both animals were boostered once 8 weeks later. Blood specimens were collected weekly and examined for liver enzyme abnormalities (ALT/AST). These remained within the normal range. Three punch liver biopsies were obtained during the study for histological evaluation. Chimpanzee No. 90 (Phoebe) failed to respond to the initial injection of

synthetic peptide, although a slight rise may have occurred during the sixth and seventh weeks. Conversely, a significant rise in anti-HBs occurred following the second injection, with anti-HBs levels going from a baseline of 2905 ± 132 mIU/ml to a peak of 16,246 mIU/ml 3 weeks later (Figure 1). The second chimpanzee's sera contained high levels of anti-HBs prior to inoculation (44,018 mIU/ml) and failed to demonstrate an anamnestic response to the two injections of synthetic peptide. Such a response was not entirely unexpected since booster responses are usually depressed or nonexistent in animals or humans already maximally stimulated. The response in the one chimpanzee indicates that (i) receptors on lymphocytes previously primed to recognize native HBsAg responded to the synthetic peptide or (ii) that a new set of lymphocytes was primed to elicit antibodies that bind to HBsAg. Further studies are in progress to elucidate these hypotheses.

C. Epitopes Associated with Synthetic Peptide 1

A major aspect in selecting a synthetic peptide to use as a potential HBV vaccine is to ascertain the specificity of the antigenic determinants, or epitopes, present on that peptide. It is known that the cross-reacting group antigenic determinant a of HBsAg is of prime importance in conferring immunity, since anti-a antibodies induced by immunization with one serotype confer protection against reinfection with the other HBV serotypes. Therefore, a synthetic peptide suitable as an HBV vaccine candidate should contain the a epitope. To determine which HBsAg antigenic determinants are present on SP1, we assessed the ability of the peptide to react with a panel of anti-HBs monoclonal antibodies of known specificity, produced and characterized in our laboratory (4). Cyclic SP1 reacted with 5 of 13 anti-a monoclonal antibodies and failed to react with the remaining 8 (Table 3) (5). This indicated that SP1 contains an a epitope and also that the a specificity associated with HBsAg has one or more antigenic determinants, unrelated to that expressed on SP1. After reduction and alkylation of cyclic SP1, the resulting linear peptide no longer reacted with the 5 anti-a monoclonal antibodies. This demonstrated that the disulfide bond is critical in conferring the a specificity and that the a epitope present on cyclic SP1 is conformation-dependent. SP1 also reacted with 3 of 3 anti-y monoclonal antibodies (Table 3). Reduction of the intrachain disulfide bond and alkylation of free thiol groups did not affect this reactivity, indicating that SP1 contains a sequential y epitope. SP1 did not react with 1 monoclonal antibody with anti-w specificity. The presence of a y epitope on SP1 was not unexpected. Two differences in the amino acid residues of P25 subtype ayw compared to subtype adw were reported by Peterson in the 122-137 region (6). The ayw contains threonine and tyrosine, while adw contains asparganine and phenylalanine in positions 131 and 134, respectively. These amino acid substitutions were thought to reflect the subtype y and d activities. This was confirmed by our detection of an epitope with y specificity on SP1 (amino acid residues 122-137).

D. Common Idiotype Associated with Human Anti-HBs

The impetus to study idiotypes associated with anti-HBs came from the knowledge that anti-HBs, but not anti-HBc, was protective against HBV infection. We initially generated four rabbit anti-idiotype antisera against affinity purified anti-HBs from two different individuals. Each of the four anti-idiotype reagents detected a common anti-HBs idiotype (Table 4) (7). A single anti-idiotype antiserum was selected for further study. The common human idiotype was detected in purified anti-HBs from three individuals and also in anti-HBs-positive sera obtained from six hemophilic patients. The ability of both HBsAg and a virus-derived HBsAg native polypeptide to inhibit the idiotype-anti-idiotype reaction suggested the anti-HBs idiotype was associated with the antibody-combining site (Table 5) (7). Idiotype determinants were detected because the anti-idiotype antisera did not interact effectively with IgG preparations from: (i) the

idiotype donor after removal of anti-HBs; (ii) a pool of human sera negative for anti-HBs; and (iii) an individual with a high level of antibody to herpes simplex virus. Attesting further to the idiotypic specificity was the inability of the anti-idiotype to bind either HBsAg or the native HBsAg-derived polypeptide. These data indicated that we were detecting an antibody-combining site associated common human anti-HBs idiotype (7).

E. Common Human Anti-HBs Idiotype Is Associated with the Group a Conformation-Dependent Antigenic Determinant

Further characterization of the common idiotype revealed that it was induced by the group a determinant because three HBsAg preparations purified from three pools of human plasma positive for HBsAg adw, ayw or adr subtypes inhibited the idiotype-anti-idiotype reaction equally on a weight basis (Figure 2) (8). We also tested the ability of HBsAg-derived polypeptides to inhibit the idiotype-anti-idiotype reaction and found that sodium dodecyl sulfate-denatured HBsAg viral polypeptides virtually lost their capacity to inhibit when compared to the native polypeptides (Table 5) (8). Also, reduction of the disulfide bonds and alkylation of free thiol groups destroyed the ability of the native HBsAg-derived polypeptide to inhibit the idiotype-anti-idiotype reaction. These data suggested that the common anti-HBs idiotype was directed against a conformation-dependent group-specific a epitope, confirming our studies in which SP1 was analyzed with monoclonal antibodies (see above).

F. Inhibition of the Common Anti-HBs Idiotype by a Cyclic Synthetic Peptide

We have also tested the ability of a cyclic synthetic peptide that contained amino acid residues 122-137 analogous to P25 to inhibit the common idiotype-anti-idiotype reaction. On a molar basis this peptide was 10^3-fold less efficient than intact HBsAg in inhibiting the idiotype-anti-idiotype reaction (Table 5) (9). The inability of peptide 1 to compete equally on a molar basis with HBsAg indicates that this peptide does not represent the complete a determinant and suggests other amino acid sequences are also important in defining the complete a epitope. The inhibition of the idiotype-anti-idiotype reaction by cyclic peptide 122-137 suggests that this sequence is related to antigenic determinants responsible for eliciting a population of human anti-HBs expressing a common idiotype. The importance of conformation was again demonstrated by reducing the disulfide bond in peptide 1 and alkylating the free thiol groups. This destroyed the ability of peptide 1 to inhibit the idiotype-anti-idiotype (Table 5) (9).

G. Detection of an Interspecies Idiotype Cross-Reaction Associated with Anti-HBs

We have recently characterized the expression of the common idiotype on anti-HBs produced in rabbits, guinea pigs, swine, goats, chimpanzees, and BALB/c mice that had been immunized with HBsAg. Expression of the idiotype in sera from other species was associated with anti-HBs molecules (Table 6) (10). We also determined that anti-HBs from chickens successfully immunized with HBsAg failed to express the common idiotype (10). It is interesting that the murine humoral immune response to the a epitope(s) of HBsAg is under the control of the H-2 complex. BALB/c mice, which possess the $H\text{-}2^d$ haplotype, are high anti-a responders and express the common anti-HBs idiotype. Analyses of other high-responding anti-a inbred mouse strains with different H-2 haplotypes than BALB/c for the expression of the common idiotype have not been performed.

H. Enhancement of the Anti-HBs Immune Response by Prior Injection of Anti-Idiotype Antibodies

We have studied the modulating effects of in vivo administration of anti-idiotype reagents that recognize the common anti-HBs idiotype prior to antigenic challenge with HBsAg at the cellular level. The injection of anti-idiotype antibodies prior to HBsAg resulted in an increased number of IgM anti-HBs secreting cells (Table 7). Anti-HBs secreting cells were also induced by administration of anti-idiotype antibodies without antigen exposure. Although we have not looked for anti-HBs in the mouse serum, these findings do indicate that the immune response to HBsAg in mice is regulated through an idiotype-anti-idiotype network (11).

The above series of experiments indicates to date that studies involving idiotype networks associated with HBV infections have been fruitful. It also suggests that inhibition of this common anti-HBs idiotype-anti-idiotype reaction by synthetic HBsAg peptides is a useful procedure for screening prospective peptide vaccine candidates prior to safety testing in chimpanzees. Since the idiotype was induced by the a epitope of HBsAg and is also expressed on anti-HBs from humans that have undergone a natural HBV infection, a synthetic peptide that inhibited the idiotype-anti-idiotype reaction on a molar basis equally well when compared to HBsAg would be the best synthetic vaccine candidate for animal testing. With the cost of safety testing new vaccine preparations in chimpanzees increasing and the number of HBV-free chimpanzees decreasing, the inhibition of the idiotype-anti-idiotype reaction may be a means to monitor various putative vaccine candidates prior to the expense of animal testing. Finally, because anti-idiotype reagents can enhance the anti-HBs response, such reagents may be useful in priming individuals prior to administration of an HBsAg vaccine, thus decreasing the number of injections necessary to elicit an anti-HBs response. Also, anti-idiotype reagents may have profound effects on HBV carriers by manipulating any idiotype networks that allow the persistence of the carrier state.

I. Hepatitis B Polypeptide Vaccine

1. Preparation of Polypeptides

During the past year, we have evaluated a number of important parameters leading toward the development of a polypeptide vaccine for human use. Our initial intention was to prepare solubilized, SDS/urea/2-mercaptoethanol subunits from 22-nm HBsAg/ayw monodispersed particles similar to those used in our earlier chimpanzee studies (12, 13). However, we found that this treatment rendered the polypeptides less immunogenic on a molar basis than native HBsAg particles. More recently, Howard et al. (14) and Sanchez et al. (15) have shown that solubilization of HBsAg with the nonionic detergent Triton X-100 to create P25/GP30 polypeptides followed by their incorporation into micelles produces a potent immunogen. Recovery of total protein has been quite satisfactory (30-50%), making this an attractive alternative for large-scale production of a hepatitis polypeptide vaccine.

The revised protocol for preparing this vaccine is found in Appendix A. The detailed nature of the protocol is designed to maintain sterility throughout the procedure, particularly after step 3.0.0. The buffers have been prepared and tested for anaerobic and aerobic bacteria, fungi, mycoplasma and endotoxins. Additional experiments have been performed to answer the following questions:

(1) Will the filter sterilization step in section 3.0.0 using the Millex-GV filter result in significant protein loss?

a. 10 mg of HBsAg was purified and solubilized with 2% Triton X-100 and passed through a Sepharose-4B lentil-lectin column which binds glycoproteins. 40% of the disrupted HBsAg was bound as a P25/GP30 glycoprotein complex. After elution with α-methyl mannoside, 85% was recovered. The sample was filter-sterilized through a Millex-GV 0.22-μm filter, pretreated with Tris-HCl buffer containing 2% Triton X-100, pH 7.2. Aliquots were tested before and after filtration. The total computed S/N ratio was 7482 before filtration and 7710 after filtration, indicating no nonspecific adsorption onto the membrane.

(2) Can a PBS buffer be substituted for the Tris-HCl buffer (buffer E) in section 5? This would be preferable since we ultimately end up with the vaccine in phosphate buffer.

a. The P25/GP30 Triton X-100 preparation described in (1)a above was divided equally and one portion was dialyzed for 15-18 hours against 0.15 M saline containing 0.01 M total phosphate ion, pH 7.2. The other portion was dialyzed against 0.10 M saline containing 0.05 M Tris-HCl, pH 7.3. Sucrose gradients (10-50%, w/w) were prepared in the same buffers and the corresponding sample was layered over each and centrifuged at 36,000 rpm for 24 hr. Fractions (0.6 ml) were collected and analyzed for HBsAg by RIA. The results (Table 8) indicate that a more compact micellar complex formed in the Tris-HCl/saline buffer. Conversely, the micelle P25/GP30 complex was more heterogeneous when centrifuged through PBS.

We have recently discovered that sucrose solutions (section 5.2.1) contain a significant amount of endotoxin. Since the buffer is free of endotoxin, we assume that the sucrose (BRL, lot 20601, 99.9% ultra-pure reagent containing no DNase or RNase, < 5 ppm heavy metals, < 0.0001% arsenic) must be the culprit. We plan to look at other lots of sucrose from BRL, Mallinkrodt and Schwarz-Mann and to attempt removal of the endotoxin (a lipopolysaccharide) using Pellicon PT series ultrafilters (Millipore). These are made of a polysulfone polymer and are compatible with aqueous solution. They can rapidly depyrogenate a variety of solutions without changing levels of effective elements, e.g., glucose concentration. These steps are being planned at this time to ensure safety of the final product.

2. Screening of Potential Candidates

To date, we have screened 78 individuals for the detergent-solubilized polypeptide vaccine. 26 (33.3%) were rejected for the following reasons. Nine subjects (11.5%) were excluded because they had clinical problems that could potentially interfere with our evaluation of the vaccine during the reactogenicity part of the trial. Previous hepatitis B infection was documented serologically in 6.4% of the candidates while anti-HBs only (consistent with a "vaccine-type" response) was detected in another 11.5% (median anti-HBs = 2.5 mIU/ml). Abnormal liver function tests were observed in 3.8%. The percentage of subjects excluded is similar to that found in our other vaccine trials. Only 3.8% of the volunteers had serologic evidence of a previous hepatitis A infection. Assuming that no complications arise, we plan to begin the immunization trial by April 1983.

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TABLE 1

Immunogenicity in Mice of SP1 in Micelle Form or Conjugated to Tetanus Toxoid

	SP1 micelles, saline	SP1 micelles, alum	SP1-TT, saline	SP1-TT, alum
32 days after primary inoculation	0/6[a]	2/6	3/6	1/6
32 days after booster inoculation	1/6 (2.7)[b]	4/5 (7.5-76)	4/5 (3.4-153)	6/6 (2.1-123)

[a] Number of mice responding/number of mice inoculated. All sera were tested at a 1:6 dilution by the AUSAB test (Abbott Laboratories, North Chicago, Ill.). Sera with S/N values $\leq$ 2.1 were considered negative.

[b] Numbers in parentheses represent the range of S/N values in the responding animals.

TABLE 2

Mean Anti-HBs Titers in Mice[a] Inoculated with Alum-Adsorbed HBsAg Vaccine Preparations

HBsAg preparation	Days after primary inoculation		Days after booster inoculation	
	14	25	14	35
Intact 22-nm HBsAg particles	3,655[b]	6,250	53,000	6,250
SDS-denatured P25+GP30 pool	< 10	< 10	85	17
Nondenatured P25+GP30 micelles	6,250	8,137	1,300,000	270,000
SP1-tetanus toxoid conjugate	ND[c]	ND	6,250	5,500

[a] Groups of 6 mice were inoculated with each designated preparation on days 0 and 35.

[b] Reciprocal of the geometric mean titer of the 6 mice in each group, determined by micro-SPRIA. 5-fold antiserum dilutions were added to HBsAg/adw-coated wells. Affinity purified ^{125}I-labeled goat IgG anti-mouse IgG was used as detecting probe.

[c] ND = not done.

TABLE 3

Analysis of SP1 for HBsAg Group and Subtype Antigenic Determinants Utilizing a Panel of Monoclonal Antibodies

Monoclonal antibody	Reactivity of the monoclonal antibody with:				
	HBsAg[a]			SP1[b]	
	adw	ayw	adr	cyclic	linear
Anti-a (5)[c]	+	+	+	+	-
Anti-a (8)	+	+	+	-	-
Anti-y (3)	-	+	± [d]	+	+
Anti-w (1)	+	+	-	-	-

[a] Determined by micro-SPRIA. Wells of a polyvinyl microtiter plate were coated with purified 22-nm HBsAg particles of the designated subtype, and monoclonal antibody was then added; affinity purified ^{125}I-labeled goat anti-mouse IgG was used as detecting probe.

[b] Determined by the ability of SP1 to inhibit the reaction of the monoclonal antibody with the HBsAg on the solid phase. An optimal predetermined dilution of each monoclonal antibody was first incubated with 80 µg of either cyclic or linear SP1. The mixture was then tested for residual anti-HBs activity by micro-SPRIA, as described under "a".

[c] Number in parentheses represents the number of individual monoclonal antibodies tested.

[d] Two of the 3 monoclonal antibodies characterized as having anti-y specificity also reacted with the adr subtype. Preincubation of these antibodies with cyclic or linear peptide inhibited the reaction with HBsAg subtype ayw but did not affect the reactivity with adr subtype.

TABLE 4

Inhibition of Binding Anti-Idiotype Antiserum with ^{125}I-Labeled Idiotype by Whole Sera

Serum source[a]	Anti-HBs[b]	Inhibition (%)[c]
RDH	18	9
RL	12	5
DB	120	26
H no. 1	34,820	32
H no. 2	1,800	26
H no. 3	2,370	31
H no. 4	2,370	30
H no. 5	2,370	28
H no. 6	6,400	38
Negative[d]	—	0-5[e]

[a] Sera inhibitor used at a 1:10 dilution. "H" denotes hemophilic patients.

[b] Reciprocal dilution of antisera that gave a P/N ratio of 2.1.

[c] Mean of triplicate values.

[d] Eight sera obtained from different individuals who were negative for anti-HBs.

[e] Range of values.

TABLE 5

Percent Inhibition of the Common Anti-HBs Idiotype Binding Its Anti-Idiotype Antiserum by Different Concentrations of Various Inhibitors

Inhibitor	Concentration (µg)	Inhibition[a]
Native HBsAg-derived polypeptide[b]	7.5	63
	3.75	45
	0.75	21
	0.375	18
Native HBsAg-derived polypeptide, reduced and alkylated[c]	7.5	2
	0.75	0
	0.075	0
SDS-denatured P25 and GP30[d]	25.0	9-13
	2.5	2-5
Synthetic peptide 1	250	63
	25	33
	2.5	30
Synthetic peptide 1, reduced and alkylated	25	1
	2.5	0
Human serum albumin	20.0	0
	5.0	0

[a] The inhibition values represent the mean of triplicate determinations. A solid-phase radioimmunoassay was used to determine the percent inhibition of the idiotype-anti-idiotype reaction.

[b] Aggregate of P25-GP30 isolated as a micelle by lentil lectin affinity chromatography of HBsAg solubilized with 2% Triton X-100 and 0.5 M NaCl.

[c] Native HBsAg-derived polypeptide micelles and synthetic peptide 1 were reduced with a 3-fold molar excess of 2-mercaptoethanol and alkylated with a 10 molar excess of iodoacetamide under N_2 atmosphere.

[d] Isolated by preparative SDS-PAGE under reducing conditions.

TABLE 6

Presence of Common Anti-HBs Idiotype in Sera Obtained from Other Species

Species[a]		Antibody specificity	Anti-HBs[b]	% inhibition[c]
BALB/c mice	(5)	none	-	1-9
BALB/c mice	(5)	anti-HBs	3.10-5.19	21-50
Rabbits	(3)	none	-	0-3
Rabbits	(2)	anti-HSV2	-	2-5
Rabbits	(4)	anti-HBs	7.43-8.82	16-70
Swine	(1)	none	-	0
Swine	(1)	anti-HBs	7.18	40
Goats	(2)	none	-	0-3
Goats	(4)	anti-HBs	5.83-8.03	24-39
Guinea pigs	(1)	none	-	2
Guinea pigs	(13)	anti-HBs	3.10-5.49	22-43
Chimpanzees	(1)	none	-	2
Chimpanzees	(2)	anti-HBs	5.49-6.10	16-28
Chickens	(1)	none	-	0
Chickens	(2)	anti-HBs	5.56-6.86	0

[a] Numbers of animals tested are indicated in parentheses.

[b] The range of $\log_{10}$ serum dilutions that gave an S/N value of 2.1.

[c] The range of values obtained for percent inhibition of the common anti-HBs idiotype binding its anti-idiotype antiserum.

TABLE 7

Anti-HBs Response as PFU Per Spleen[a]

First injection	Second injection	Direct (IgM-secreting cells)	Indirect (IgG-secreting cells)
Anti-idiotype	HBsAg	470 ± 111[b]	180 ± 41
Pre-IgG	HBsAg	225 ± 67.8	133 ± 12.9
Anti-idiotype	anti-idiotype	66.7 ± 47.8	100 ± 29
Pre-IgG	pre-IgG	16.7 ± 16.7	8.3 ± 8.3
HBsAg	HBsAg	167 ± 36	1683 ± 168

[a] Each group of six mice received 40 µg of anti-idiotype or pre-IgG or 5 µg of HBsAg on day 0, followed by the indicated injections on day 7, all by the intraperitoneal route. Spleens were removed on day 21. Cells from each mouse spleen were assayed in duplicate for the presence of both direct and indirect anti-HBs PFU. No hemolytic plaques were obtained with any spleen cells using uncoated or ovalbumin-coated SRBC as control.

[b] Mean ± standard error.

TABLE 8

HBsAg S/N Ratios (RIA)

Gradient fraction	Tris-HCl/NaCl		PBS	
	1:10	1:100	1:10	1:100
15	23.1	4.0	34.8	4.9
16	48.1	8.1	41.6	6.3
17	57.6	11.2	42.6	7.7
18	88.5	29.2	41.5	5.5
19	75.8	22.4	42.3	6.8
20	18.2	3.5	41.1	6.2
21	9.4	1.9		
22	21.9	3.4		
23	18.8	3.3		
24	1.1	1.2		

FIGURE 1

ANTI-HBs RESPONSE (mIU/ml) FOLLOWING INOCULATION OF AN HBV IMMUNE CHIMPANZEE (PHOEBE) WITH A SYNTHETIC HBsAg PEPTIDE

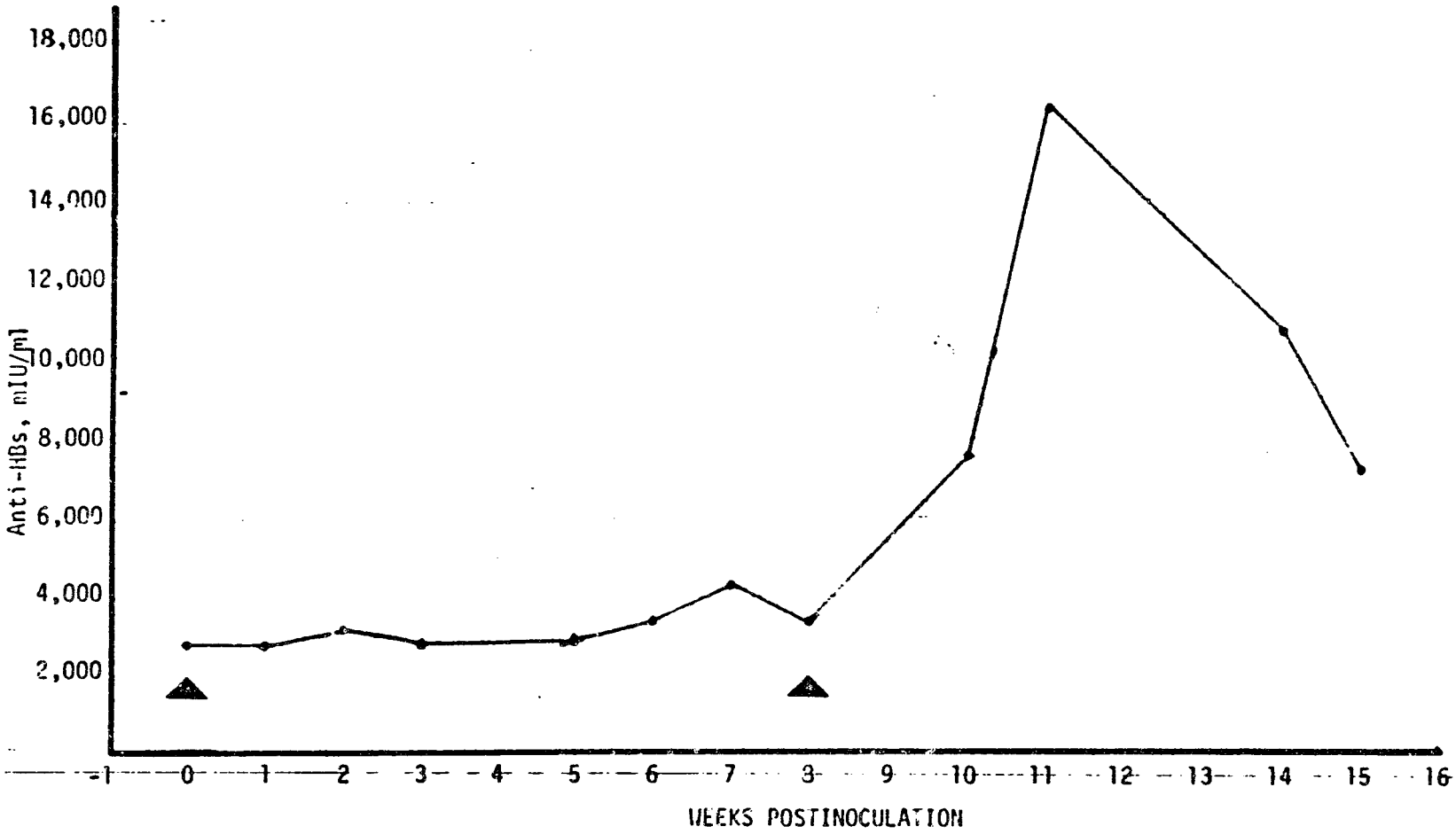
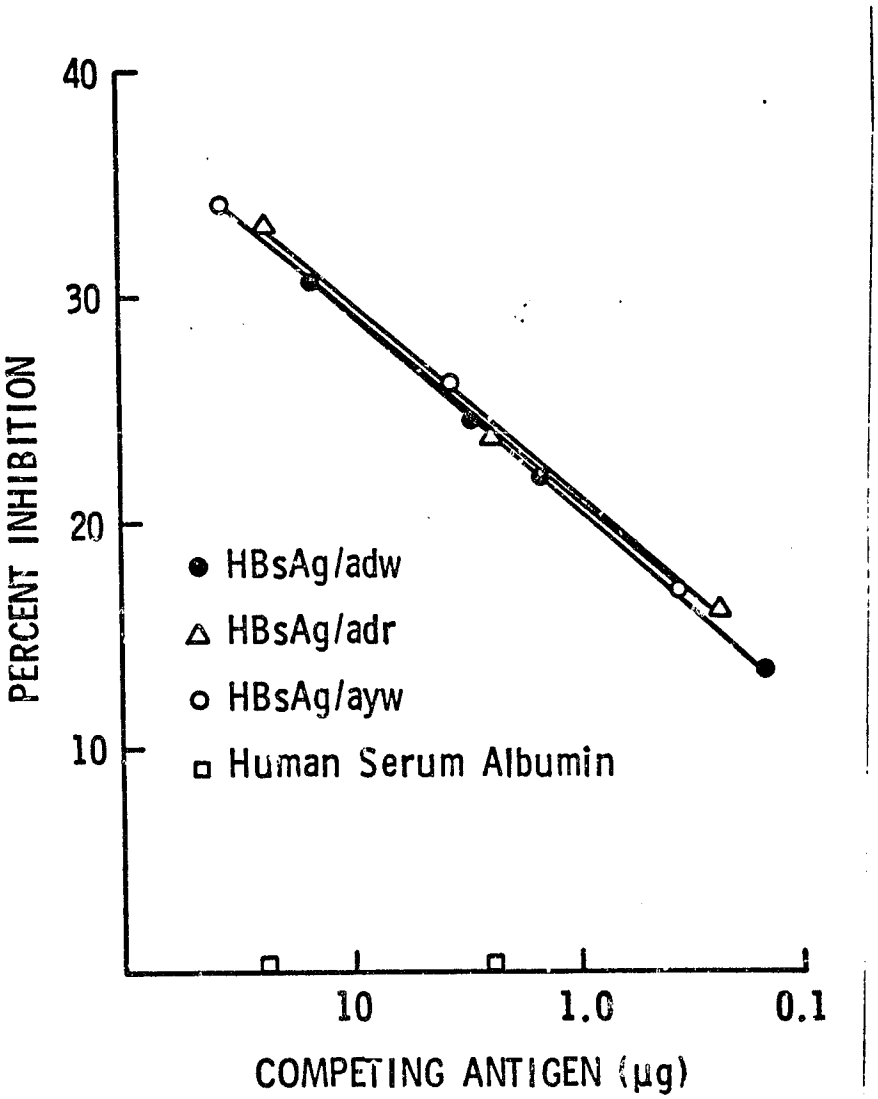


Figure 2

Inhibition of binding ^{125}I-labeled idiotype to its anti-idiotypic antiserum by various intact HBsAg particles.



Publications during the Past Year that Acknowledged Support by This Contract

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Distribution of the Annual Report

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Appendix A

PROTOCOL

1.0.0: Disruption of the NIH HBsAg vaccine, subtype adw, Lot A

1.1.0 Approximately 10 mg HBsAg in ____ PBS will be brought to 80 ml with Buffer F. To this volume will be added 20 ml buffer A (100 µg/ml). Mix and place a 250 ml erlenmeyer flask with a stirring bar. Adjust pH 7.2 with either 0.1 N HCl or 0.1 N NaOH. (pH probe to be decontaminated for 1" in 0.1 N HCl.

1.2.0 After sealing the flask with a sterile cap, the sample will be place in a Precision water bath and incubated at 37 C (± 0.5 C) for 24 hours.

2.0.0: Adsorption and elution of the polypeptide complex (P25 + P30 complex) by affinity chromatography

2.1.0 Preparation of lentel-lectin-Sepharose 4B (LLS43) (Lot No. _____)

2.1.1 Wash 25 ml LLS43, containing approximately 10 gms of gel five times with Buffer B as follows. Remove 15 ml supernate from gel after settling for 15" at 23 C ± 3 C. Replace with 15 ml Buffer B. Place on shaker for 10', remove and allow to settle for 15' at 23 C ± 3 C. Repeat above for a total of three times.

2.1.2. Add washed gel in Buffer B directly to the disrupted HBsAg in 1.1.0. Incubate at 23 ± 3 C for 60 minutes while continuously shaking the mixture.

2.1.3 To a 1 x 30 cm Econocolumn chromatography column (sterilized by ethylene oxide), held in place by extension clamps in a base support, add the slurry prepared in 2.1.2 using a 10 ml siliconized glass pipet.

2.1.4 As the column is packing, eluate is collected in a sterile, siliconized 250 ml Erlenmeyer flask until the fluid level above the gel bed is 2mm above the top of the packed gel. The eluate will be saved at 4 C until the fluid product is analyzed.

.2.2.0 After packing, the column is washed with 150 ml Buffer B or until OD_{280} of 2.5 ml fractions reaches a stable baseline tracing. Fractions will be collected using a Gilson fraction collector and will be monitored at OD_{280} with an ISCO model UA-6. Tubing and flow through cell will be sterilized by ethylene oxide.

2.3.0 Before adding eluting buffer (Buffer C) let buffer B penetrate to surface of gel. Add 5 ml eluting buffer C to column, then connect tope of column via sterile tubing to a 60 ml syringe with plunger replaced by sterile cotton plug. Syringe will be filled with Buffer C as required.

2.3.1 Monitor at OD_{280} and collect 2.5 ml fractions with Gilson fraction collector into siliconized glass tubes (13 x 100 cm)

2.4.0 Pool fractions containing protein activity as monitored by absorbancy at 280 nm into 50 ml siliconized container.

3.0.0 Filter-sterilize using a Millex-GV 25 nm 0.22 um size filter prewashed with 1.0 ml Buffer C. Collect sample into 125 ml siliconized beaker.

4.0.0 Microsolute exchange and macrosolute concentration of HBsAg polypeptide sample using an Amicon ultrafiltration system

4.1.0 Sterilization of equipment

4.1.1 An assembled Amicon Model No.52 and membrane YM10 will be sterilized by ethylene oxide gas sterilization (< 12% gas)

4.2.0 Macrosolute exchange of Buffer C in HBsAg preparation for Buffer D

4.2.1 At least two hours prior to the addition of our sample, 50-75 ml of sterile water will be passed through the stirred cell with YM10 membrane in place at a nitrogen (N_2) pressure of 25 $\pm$ 5 psi. The N_2 will be sterile filtered using an in-line Millex-FG filter, 0.2 um pore size, inserted between the N_2 tank and the stirred cell. Stirring speed will be moderate e.g. the vortex will be no greater than one-fourth the height of the starting volume. After the removal of the glycerine and sodium azide from the YM10 membrane the sample will be added to the filtration cell. The sample volume will be reduced to approximately 20 ml ($\pm$2 ml), then Buffer D will be exchanged for Buffer C by adding 6 volumes (_____ ml) of Buffer D to the Model 52 system by filling up the Model 52 stirred cell and allowing the volume to decrease to 20 ml before adding additional Buffer D until the total volume has been expended.

4.3.1 The sample will be concentrated to 5.0 ± 1.0 ml at a nitrogen pressure of 25 ± psi using a moderate stirring speed as described in 4.2.1. Before removing the retentate, the pressure will be released slowly over 1 minute allowing the stirrer to continue at atmospheric pressure for an additional 15 minutes. The sample will be withdrawn using a 10-ml siliconized pipette and placed in a 2 drain sterile siliconized vial. Up to 2.0 ml of Buffer D will be used to rinse the YM10 membrane and the fluid allowed to stir for an additional 15 minutes before removal and pooling with the initial sample. Until use, the sample will be stored in the refrigerator at 2-8 C.

5.0.0 Micelle-formation of polypeptide complex (p25 ± gp 30) in a preformed linear sucrose gradient

5.1.0 Sterilization of equipment

5.1.1 The Beckman SW41 buckets, caps and nitrocellulose tubes (1 x 3½") bucket holder rack, and the gradient tube holder will be gas sterilized in ethylene oxide.

5.2.0 Preparation of sucrose gradient

5.2.1 A 50% w/w sucrose solution will be prepared by adding 500 ml Buffer E to 500.0 gms of sucrose in a 1 liter beaker containing a 1½" magnetic stirring bar. Solubilize for 23 hrs. at 24 ± 3C with stirring. Check solution in refractometer for refractive index and adjust to 1.42 ± 0.002 with sucrose or Buffer E of value is above or below this range. The sucrose solution will be filter-sterilized using a 0.22 um Sterivex-GS Millipore filter. Concentrations of sucrose will be prepared as follows in 25 ml borosilicate Erhlenmeyer fasks.

CONCENTRATION OF SUCROSE	VOLUME (ml) OF	
	50% sucrose	Buffer E
50%	25	0
40%	20	5
30%	15	10
20%	10	15

To each of six SW41 nitrocellulose tubes, (9/16" x 3½") will be added 2.5 ml of 50%, 40%, 30% and 20% sucrose solutions in that order. The sucrose gradients will be placed in the SW41 buckets, capped and allowed to equilibrate to form a linear gradient at 2-8 C for 15 ± 3 hours.

5.3.0 Isopycnic centrifugation of polypeptide complex.

5.3.1 The concentrate described in section 5.2.1 will be carefully layered over the sucrose gradients. Three, four or six gradients will be prepared based on the final volume of concentrate. Additional Buffer E will be added to raise the liquid meniscus to ¼" from the top of the tube. The buckets will be tightly capped and placed opposite to each other on the SW41 rotor which has been precooled to 2-8 C. The rotor with buckets will be placed into a precooled (0-4 C) Beckman L3-40 ultracentrifuge chamber and the chamber evacuated. At that point the rotor speed will be set at 36,000 rpm and the centrifuge will be activated. The temperature control will be set to maintain the rotor temperature between 2-6 C. Centrifugation will continue for 24 hrs. The centrifuge will be turned off and the rotor allowed to come to a halt without braking. After releasing the vacuum the rotor with buckets will be removed and taken to the hood where the buckets will be placed in the holder rack. After removal of the cap over the sample, the tube will be removed using a sterile hemostat and placed in a sterile gradient tube holder. Visible bands will be recorded diagramatically.

5.4.0 Harvesting of the HBsAg polypeptide micelle preparation

5.4.1 Sucrose will be removed so that the meniscus lies 15-20 mm above the major visible band. Then the tube will be side-punctured 3-5 mm below the band with a 20 gauge regular beveled 1" needle attached to a sterile Glaspak 10 cc syringe and the major band removed. The sample will be place in a 25 ml siliconized sterilized Erhlenmeyer flask. 0.5-1.0 ml will be transferred to a 1 dram siliconized vial for biophysical/biochemical characterization (Lowry, PAGE, EM, HBsAg activity by RIA).

6.0.0 Preparation of alum-adsorbed micelles

6.1.0 Prepare and filter-sterilize 0.2 M $AlK(SO_4)_2 \cdot 12 H_2O$ in distilled water

0.2 M $AlK(SO_4)_2 \cdot 12 H_2O$, (mwt-474.39)	9.49 gm	23.72 gm
H_2O q.s. to make	100.0	250.0 ml

Filter-sterilize with 0.22 u filter.

This preparation contains 5.4 gms Al3+/liter or 5.4 mg/ml

6.2.0 Adsorption of micelle onto hydrated aluminum pltassium sulfate

6.2.1 Perform a Lowry protein determination on the micelle preparation obtained from the sucrose gradient: _____ µg/ml

_____ ml of solution x _____µg/ml = _____µg polypeptide micelle

6.2.2 Determine amount of aluminum salt needed to adsorb the micelle vaccine in a total volume of 10 ml.

_____µg micelle protein x 0.02 mg Al+++/µg micelle protein=_____mg Al3+

_____mg Al3+ 5.4 mg Al3+/ml hydrated salt = _____ ml hydrated salt

10 ml-_____ml hydrated salt+_____ml micelle=_____ml of H_2O to be added while stirring (vortex is less than 1/8 height of fluid in 25 ml in flask). This solution is clear.

6.2.3 Adjust to pH 5.0 with 1.0 n NaOH. The solution becomes opalescent. Adsorb at 24-26 C for 2 hours with continuous stirring. Transfer the suspension to a 50 ml Falcon centrifuge tube. Wash the beaker with 2.0 ml Buffer F (PBS, pH 6.6) and add to the centrifuge tube. Centrifuge tube at 3000 rpm for 10 minutes. Remove supernatant and save for HBsAg determination (_____S/N). Add 25 ml Buffer F, mix by inversion and recentrifuge as before. Remove supernatant. Resuspended in Buffer F to a calculated concentration of 40 µg alum-adsorbed micelle protein/ml of suspension.

_____µg micelle protein (initial) - 40 µg/ml=_____ml of final PBS suspension

Dilution may necessitate adding sample to a larger graduated flask prior to dispensing into vials.

6.3.0 Protein determination of gel suspension

6.3.1 Remove two 0.5 ml aliquots of the alum suspension and place in micro-dialyzing tubes. Dialyze with constant agitation on a one liter multiple-dialyzer unit against 3% sodium citrate for 2 hours at 24-26 C. After the solution clears and the aluminum is dissolved, dialyze against PBS, pH 7.2 (Buffer G) overnight at 4 C with one volume change (one liter) and test retentate for HBsAg by RIA. Dilute and compare with standard reference reagent at 10 ng/ml to determine final concentration.

7.0.0 Distribution of alum-adsorped preparation into 1.0 ml vials.

7.1.0 The alum-adsorbed vaccine is placed in 125 ml siliconized flask containing a magnetic stirrer. The sample is continuously stirred at a speed of less than 100 rpm. The samples are dispensed in 0.7 ml aliquots into 1.5 ml Wheaton "200" glass vials using a 1.0 ml Cornwall automatic pipetter. The vials have a teflon lined cap with a septum opening.

BUFFERS

BUFFER	CONCENTRATES	pH (±0.1)	VOLUME (ml)	CHEMCIAL	WEIGHT OR VOLUME	INITIAL
A	5x	7.3	500	10% Triton X-100	50.0 ml	
				0.05 M Tris-HCl	3.95 gms	
				2.5 M NaCl, USP	73.05 gms	
				Pyrogen-free H_2O	400 ml	
				Titrate to pH 7.3 with 1.0 NaOH		
				H_2O q.s. to make	500 ml	
B	1x	7.3	1000	10% $CaCl_2$	1.11 ml	
				.001 M $MnCl_2$	0.198 g	
				1xA q.s. to make	1000 ml	
C	1x	7.3	500	0.01 M Tris-HCl	0.790 gms	
				5%α-methyl D-mannoside	25.0 gms	
				2% Tritox X-100	10.0 ml	
				Pyrogen-free H_2O	400 ml	
				Titrate to pH 7.3 with 0.1 N NaOH		
				H_2O q.s. to make	500 ml	
D	1x	7.3	1000	0.01 M Tris-HCl	1.58 gms	
				H_2O q.s. to make	1000 ml	
E	1x	7.3	2000	0.05 M Tris-HCl	15.8 gms	
				0.10 M NaCl, USP	11.69 gms	
				H_2O	1900 ml	
				Adjust with 0.1 N NaCl to pH 7.3 q.s. with H_2O to	2000 ml	
F	1x	6.2	~~500~~ 1000	0.00775 M $NaH_2PO_4H_2O$ USP	1.0674 gms	
				0.00225 M $Na_2H.PO_47H_2O$, USP	0.6032 gms	
				0.15 M NaCl, USP	4.38 gms	
				H_2O q.s. to make	1000 ml	