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Humoral and Cellular Immunity to Hepatitis B Virus-Derived Antigens: Comparative Activity of Freund Complete Adjuvant, Alum, and Liposomes

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Complete Freund adjuvant, aluminum gel, and liposomes were compared for their ability to enhance the immunogenicity of an intact 22-nm HBsAg particle vaccine and an HBsAg-derived polypeptide vaccine in guinea pigs. Both humoral and cell-mediated immune responses were evaluated. The greatest immune response was obtained with complete Freund adjuvant, regardless of the antigen preparation. Aluminum gel appeared to be a better adjuvant for 22-nm HBsAg particles, but the liposomes rendered polypeptide preparations more immunogenic. The possibility that various proportions were entrapped in aqueous compartments instead of being inserted into the lipid bilayers of liposomes might account for this difference. The development of both humoral and cellular immunity was dependent upon the use of an adjuvant, because aqueous preparations had poor immunogenicity.

A number of hepatitis B surface antigen (HBsAg) vaccines have been administered to chimpanzees as well as to selected groups of humans to document the interaction between vaccine and host in an attempt to find a safe and efficient preparation. Inactivated HBsAg particle vaccines and HBsAg-derived polypeptide vaccines have been studied (8, 17, 19; G. R. Dreesman, F. B. Hollinger, Y. Sanchez, P. Oefinger, and J. L. Melnick, submitted for publication). Although theoretically free of nucleic acid, the highly purified 22-nm-particle preparations are treated with heat or Formalin as a safety precaution. The polypeptide vaccines are composed of virus-specific polypeptides obtained under reduction conditions from purified 22-nm HBsAg particles. For both types of vaccine, the immunogenicity of some preparations has been lower than the optimal level, and in chimpanzees these preparations do not seem to induce a detectable cell-mediated immune response (20). It is not clear whether this relatively low level of immunogenicity in primates is due to the unique biochemical properties of HBsAg (20) or to an inadequate adjuvant.

In this study, we evaluated humoral and cell-mediated immunity in guinea pigs after immunization with an HBsAg particle vaccine or with an HBsAg-derived polypeptide vaccine. Both vaccine preparations were administered as aqueous solutions, either emulsified in complete Freund adjuvant (CFA), precipitated with aluminum sulfate, or entrapped in liposomes. Liposomes were included because their potential for enhancing immunological responses has been established for other viral antigens and because their use in humans has not been contraindicated (10, 22).

MATERIALS AND METHODS

Isolation and purification of HBsAg. HBsAg particles were purified as previously described (6). Clarified human plasma positive for HBsAg/*adw* was pelleted twice by high-speed centrifugation; the suspended pellet was treated with 0.05 M potassium phthalate-hydrochloride buffer (pH 2.6) for 1 h at room temperature, banded twice on isopycnic CsCl gradients for 24 h each, and then recentrifuged in a rate-zonal linear preformed CsCl gradient for 5 h.

Purification of HBsAg-derived P22 and P25. The two major HBsAg-derived polypeptides were obtained by preparative polyacrylamide gel electrophoresis as previously described (5). Purified 22-nm HBsAg/*adw* particles were solubilized by heating at 100°C for 2 min in 0.5 M urea-1% sodium dodecyl sulfate-1% 2-mercaptoethanol. The polypeptides were fractionated by electrophoresis in cylindrical 10% polyacrylamide gels (14.5 by 122 mm). Protein bands were localized on a small wedge-shaped section cut longitudinally from the gel and stained with 0.25% Coomassie brilliant blue. Segments from the unstained gel corresponding to the P22 and P25 polypeptides were cut out, and the proteins were eluted from the gel slice. Each purified population was concentrated by lyophilization and checked for purity by analytical slab gel electrophoresis. A mixture of equal amounts of P22 and P25 (P22 + P25) was used for immunizing the animals.

Antigen-adjuvant preparation. (i) Emulsifica-

tion in CFA. The necessary amount of protein was diluted with 0.01 M phosphate-buffered saline, pH 7.2, to obtain a concentration of 25 μg in 250 μl. This protein solution was emulsified with an equal volume of CFA (Difco Laboratories, Detroit, Mich.) by the double-hubbed needle method (2). The final suspension, containing 25 μg of protein in 500 μl, was used for immunizing the guinea pigs.

(ii) Precipitation with aluminum potassium sulfate. Protein adsorption to aluminum salt was carried out as described previously (11). The amount of hydrated aluminum potassium sulfate [$AlK(SO_4)_2 \cdot 12H_2O$; Fisher Scientific Co., Fair Lawn, N.J.] was calculated according to the necessary amount of protein to assure a proportion of 0.8 μg of aluminum ion (Al^{3+}) to 100 μg of protein. A 10% (0.21 M) solution of hydrated $AlK(SO_4)_2$ was prepared in 0.01 M phosphate-buffered saline, pH 6.2. The protein solution and the corresponding amount of 10% aluminum salt solution were mixed to a final volume of 10 ml in 0.01 M phosphate-buffered saline, pH 6.2. The pH was then adjusted to 5.0 with 1 N NaOH, and the adsorption proceeded for 2 h at room temperature with gentle stirring. The gel was then pelleted, washed twice with saline, and resuspended in 0.1 M phosphate-buffered saline, pH 7.2. The resuspension was made based on the adsorption of protein to the aluminum gel, the efficiency of which was over 95% (11). The final preparation contained 25 μg of protein adsorbed to 0.2 μg of Al^{3+} in 500 μl. This dose was administered to each animal.

(iii) Incorporation into liposomes. Multilamellar liposomes were prepared from dipalmitoylphosphatidylcholine, cholesterol, and phosphatidic acid (Sigma Chemical Co., St. Louis, Mo.) in molar ratios of 2.0:1.5:0.2, respectively (13). A dried film of the lipid mixture (20 mM with respect to phospholipid) was swollen in a solution containing 200 μg of either 22-nm HBsAg particles or P22 + P25 polypeptide pool per ml. For estimating the efficiency of entrapment, 5×10^4 cpm of ^{125}I-labeled HBsAg (22-nm particles) or of ^{125}I-labeled P22 was added to the unlabeled antigen preparation. To remove free antigen, the liposomes were centrifuged at 18,000 rpm for 30 min and suspended to the original volume in sterile phosphate-buffered saline. Repeated washing did not further reduce the amount of antigen associated with the liposomes. The percentage of antigen incorporated for individual vaccine preparations is shown in Table 1. Depending on the percentage of entrapment, the liposome suspension was adjusted to 25 μg of protein in 500 μl for further use in guinea pigs.

Antisera. Guinea pig antisera were prepared against purified 22-nm HBsAg/*adw* particles and against a mixture of equal amounts of P22 and P25 HBsAg/*adw*-derived polypeptides, further referred to as the particle and the polypeptide vaccines, respectively. The following preparations were used for each of the two tested vaccines: (i) 25 μg of protein suspended in 500 μl of sterile 0.85% saline solution; (ii) 25 μg of protein emulsified with CFA in 500 μl; (iii) 25 μg of alum-precipitated protein suspended in 500 μl; and (iv) 25 μg of protein entrapped in liposomes and suspended in 500 μl. Three to five adult (400 to 500 g) male Hartley guinea pigs were inoculated with each preparation and then given boosters three times at 2-week intervals. The animals were sacrificed and evaluated for humoral and cellular immune responses at 10 days after the third booster.

Iodination. The procedures used to iodinate intact HBsAg particles as well as proteins (P22 + P25) were carried out as previously described (9, 20a). An optimal predetermined proportion of protein and $Na^{125}I$ (New England Nuclear Corp., Boston, Mass.) was reacted in the presence of chloramine-T (Eastman Kodak Chemical Co., Rochester, N.Y.), and the reaction was stopped with sodium metabisulfite (Fisher Scientific Co.). More than 90% of the radioactivity was precipitated with 20% trichloroacetic acid. The specific activity of preparations ranged from 3 to 5 μCi/μg for ^{125}I-labeled HBsAg and from 8 to 16 μCi/μg for ^{125}I-labeled P22 + P25.

Antibody titration. For the antisera titration, a double-antibody radioimmunoprecipitation assay (RIA-DA), previously described in detail, was employed (12, 21). Guinea pig antiserum prepared by inoculation with HBsAg/*adw* particles or with a P22 + P25/*adw* polypeptide pool was used as the primary antibody. Fivefold dilutions were prepared in 10% normal guinea pig serum (Pel-Freeze Biologicals, Inc., Rogers, Ark.), beginning at a 1:10 dilution. Antibody titers for each vaccine group were determined with ^{125}I-labeled HBsAg/*adw* as the antigen. A constant amount (25 ng) of labeled HBsAg/*adw* particles diluted in 1% normal guinea pig serum was added to serial dilutions of the primary antibody. After incubation at 4°C for 18 h, the antigen-antibody complexes were precipitated by the addition of a constant, predetermined amount of rabbit anti-guinea pig immunoglobulin G. The percentage of radioactive HBsAg precipitated by antibody was determined by the formula: percent specific precipitation = 100 × [1 − (counts per minute of sample − counts per minute of trichloroacetic acid)/(counts per minute of normal guinea pig serum − counts per minute of trichloroacetic acid)]. In agreement with our previous studies, the titer of an antiserum was considered as that serum dilution which bound 20% of the ^{125}I-labeled HBsAg,

TABLE 1. *Efficiency of incorporation into liposomes*

Prepn	Incorporation (%)[a]
Subunit HBsAg	53
	31
	38.5
	64.6
P22 + P25	80.9
	56
	40.8
	58.7
	45.5
	84
	24
	76.7

[a] Incorporation of ^{125}I-labeled antigen in individual preparations. The mean percentages of incorporation were as follows: subunit HBsAg, 46.7; P22 + P25, 58.3.

as tested by RIA-DA. Titers were obtained by extrapolation of the titration curves.

Macrophage migration inhibition. Cell-mediated immunity was measured in vitro by the macrophage migration inhibition assay, as described in a previous paper (4). Peritoneal exudate cells harvested from immunized guinea pigs were adjusted to a concentration of 2×10^7 viable leukocytes per ml in Eagle medium with 15% heat-inactivated normal guinea pig serum. Equal volumes (0.2 ml) of cell suspension and HBsAg particles diluted in saline (5, 25, or 50 μg) were incubated for 30 min at 25°C with occasional stirring. As a negative control, 0.85% saline solution was used. For each sample, at least six microcapillary tubes (no. B4195-1; Blu-Tip, full tube volume, 40 μl; Scientific Products, McGaw Park, Ill.) were packed. Each tube was incubated at 37°C in an individual well in migration plates (6-240-5; Cook Laboratory Products, Alexandria, Va.). After 48 h of incubation, the areas of migration were projected with a Nikon profile projector (6C; Nipon Kagaku, KK, Tokyo, Japan), traced on uniform-weight paper, and weighed. The inhibition of macrophage migration was determined by the formula: percent inhibition = 100 × [1 − (mean weight of migration areas with antigen)/(mean weight of migration areas without antigen)]. Based on previous experience, an inhibition of 20% or greater was considered to be significant and an indication of the development of cell-mediated immunity for that particular antigen (4, 5).

RESULTS

Humoral immunity. Titrations of guinea pig anti-HBs and guinea pig anti-P22 + P25 were carried out by RIA-DA with homologous ^{125}I-labeled HBsAg as a ligand protein. All of the adjuvants used in this study greatly enhanced antibody production when coupled with whole HBsAg particles, in comparison with the relatively low titers obtained with the saline solution (Table 2). The best response was obtained with CFA; lower responses were obtained with aluminum gel and liposomes.

The aqueous polypeptide preparation had poor immunogenicity (titers of ≤3). The same preparation, coupled with the tested adjuvants, showed a substantial increase in immunogenicity; the titers ranged from 100 to 2,700 for CFA, from 10 to 80 for alum gel, and from 20 to 480 for liposomes (Table 3).

Cellular immunity. The results of the cell-mediated immunity studies, as measured by the macrophage migration inhibition assay with peritoneal exudate cells obtained from guinea pigs immunized with the particle or the polypeptide vaccine, are summarized in Tables 4 and 5. No significant degree of migration inhibition was observed with macrophages from guinea pigs immunized with aqueous solutions of either particle or polypeptide vaccine. However, both vaccines, when coupled with an adjuvant, were able to induce a vigorous cell-mediated immune response, as indicated by the high percentage of inhibition in the presence of HBsAg and by the greater number of responding animals. Under the test conditions, all three concentrations of HBsAg used to challenge the cells (5, 25, and 50 μg) gave a good inhibition of migration from 24 to 72 h after stimulation. However, optimal conditions were achieved with 25 μg of HBsAg after a 48-h incubation.

DISCUSSION

In this study, we compared three different adjuvants (CFA, aluminum gel, and liposomes) for their ability to enhance the immunogenicity of a 22-nm HBsAg particle vaccine and an HBsAg-derived polypeptide vaccine. The highest antibody titers and the greatest inhibition of the macrophage migration were obtained with

TABLE 3. *Antibody titers in guinea pigs immunized with HBsAg-derived polypeptide vaccine*

Vaccine administered in association with:	Antibody titer in individual guinea pigs[a]					Mean titer
	1	2	3	4	5	
Saline solution	3	2	1			2.0
CFA	2,700	100	920	250	130	820.0
Aluminum gel	10	80	30	22	50	38.4
Liposomes	480	200	18	42	57	159.4

[a] Titers are expressed as the reciprocal of that serum dilution which bound 20% of the ^{125}I-labeled HBsAg, as tested by RIA-DA. Three to five guinea pigs were immunized with each antigen preparation.

TABLE 2. *Antibody titers in guinea pigs immunized with particle HBsAg vaccine*

Vaccine administered in association with:	Antibody titer in individual guinea pigs[a]					Mean titer
	1	2	3	4	5	
Saline solution	250	3,400	470			1,373
CFA	81,000	17,000	250,000	156,000	250,000	150,800
Aluminum gel	4,000	25,000	156,250	18,000	1,250	40,900
Liposomes	2,200	11,000	3,500	11,500	1,250	5,850

[a] Titers are expressed as the reciprocal of that serum dilution which bound 20% of the ^{125}I-labeled HBsAg, as tested by RIA-DA. Three to five guinea pigs were immunized with each antigen preparation.

CFA, regardless of the antigen preparation (Table 6). However, the data obtained for aluminum gel and liposomes are of particular interest, in view of their actual or potential use in humans.

Aluminum gel is widely used in vaccines for humans as an adjuvant for diphtheria and tetanus toxoids. It has also been evaluated as an adjuvant for particle HBsAg vaccines in humans (18) and for a polypeptide vaccine in chimpanzees (11; Dreesman et al., submitted for publication). In both cases, it was shown to improve the immunogenicity of the preparations at the humoral level. No data are available regarding its potential in inducing detectable cell-mediated immunity in primates.

Liposomes can be prepared from lipids which are well known to be completely metabolized in humans. In fact, similar mixtures of lipids are currently used in parenteral hyperalimentation. This makes possible the use of liposomes as an adjuvant for humans (12a, 22). Their adjuvant properties have been established for some viral antigens, such as adenovirus fibers and hexons (13) and influenza virus hemagglutinin and neuraminidase (1). Recent studies showed that, despite their size, intact HBsAg particles are actually incorporated (not just adsorbed) into multilamellar liposomes of variable compositions and that the highest percentage of incorporation is obtained with positively charged liposomes (15). The liposomes increased the humoral and cellular immune responses in guinea pigs immunized with a particle HBsAg vaccine, in comparison with the responses obtained with the same vaccine in aqueous solution (16). However, no other adjuvants were simultaneously tested in those studies.

We quantified the humoral and cell-mediated immune responses in guinea pigs immunized with the particle or the polypeptide vaccine. Both preparations were administered free in saline solution or in conjunction with one of the test adjuvants. When administered in an aqueous form, the polypeptide vaccine induced very low antibody titers whereas the particle vaccine induced titers that were at least 100-fold higher. With the exception of one animal in the polypeptide test group, both aqueous vaccines failed to induce cell-mediated immunity. When the vaccines were coupled with any of the three adjuvants, considerably higher responses for both types of immunity were observed. These results indicate that, at least in guinea pigs, the development of high antibody titers and of cell-mediated response is dependent upon the use of an adjuvant.

It is necessary to point out that no direct comparison can be made between the antibody titers obtained for the two vaccines. One vaccine consists of whole 22-nm HBsAg particles with a very complex structure (3, 7) and a variety of antigenic determinants (14), all of which are capable of inducing a specific antibody response. On the other hand, the P22 + P25 polypeptide pool was obtained under denatured conditions, and it induced antibodies only to a limited num-

TABLE 4. *Percent inhibition of migration of macrophages from guinea pigs immunized with particle HBsAg vaccine*

Vaccine administered in association with:	Stimulating HBsAg (μg)[a]	Inhibition in individual guinea pigs (%)[b]						Positive/total[c]	Mean titer
		1	2	3	4	5	6		
Saline solution	5	13	0	18				0/3	10.3
	25	2	0	0				0/3	0.6
	50	0	0	0				0/3	0
CFA	5	77	100	97	86			4/4	90.0
	25	89	100	98	96			4/4	95.7
	50	ND[d]	ND	97	94			2/2	95.9
Aluminum gel	5	39	46	53	95	66	8	5/6	51.1
	25	95	65	49	92	86	29	6/6	69.3
	50	ND	ND	56	95	69	50	4/4	67.5
Liposomes	5	33	56	0	0			2/4	22.2
	25	46	66	37	7			3/4	39.0
	50	41	53	35	0			3/4	32.2

[a] Macrophages obtained from each individual animal were stimulated with three concentrations of purified HBsAg ranging from 5 to 50 μg.

[b] Three to six guinea pigs were immunized with each antigen preparation.

[c] Ratio of responding animals to the total number of animals tested.

[d] ND, Not done.

TABLE 5. *Percent inhibition of migration of macrophages from guinea pigs immunized with HBsAg-derived polypeptide vaccine*

Vaccine administered in association with:	Stimulating HBsAg (μg)[a]	Inhibition in individual guinea pigs (%)[b]					Positive/ total[c]	Mean titer
		1	2	3	4	5		
Saline solution	5	6	0	28			1/3	11.3
	25	0	13	44			1/3	19.0
	50	0	0	26			1/3	8.6
CFA	5	100	98	88			3/3	95.3
	25	100	95	82			3/3	92.3
	50	ND[d]	ND	75			1/1	75.0
Aluminum gel	5	64	45	39	19	21	4/5	37.6
	25	57	66	62	18	36	4/5	47.8
	50	30	29	57	3	13	3/5	26.4
Liposomes	5	40	39	53	35	55	5/5	44.4
	25	68	58	83	18	77	4/5	60.8
	50	52	62	71	40	57	5/5	56.4

[a] Macrophages obtained from each individual animal were stimulated with three concentrations of purified HBsAg ranging from 5 to 50 μg.
[b] Three to five guinea pigs were immunized with each antigen preparation.
[c] Ratio of responding animals to the total number of animals tested.
[d] ND, Not done.

TABLE 6. *Mean antibody titers and mean percentage of inhibition of migration*

Antigen preparation inoculated in:	Particle HBsAg vaccine		Polypeptide HBsAg vaccine	
	Antibody titer	Migration inhibition (%)[a]	Antibody titer	Migration inhibition (%)[a]
Saline solution	1,373	0.6	2.0	19.0
CFA	150,800	95.7	820.0	92.3
Aluminum gel	40,900	69.3	38.4	47.8
Liposomes	5,890	39.0	159.4	60.8

[a] As obtained with 25 μg of stimulating HBsAg.

ber of virus-specific determinants (20a). Therefore, the use of ^{125}I-labeled HBsAg particles as a ligand antigen in the RIA-DA titrations is expected to detect antibody titers higher for the intact particle than for the isolated polypeptides. The use of ^{125}I-labeled P22 instead of ^{125}I-labeled HBsAg as a ligand in RIA-DA might have been more satisfactory, as only the antivirus specificities would have been quantitated. However, we chose to use ^{125}I-labeled HBsAg particles as a ligand because a more instructive comparison could be made with previous and related studies.

In our study, guinea pigs immunized with the particle vaccine coupled with aluminum gel gave better humoral and cellular responses than did those immunized with the same antigen incorporated into liposomes. However, the situation was reversed for the polypeptide vaccine. The P22 + P25 preparation incorporated into liposomes was more immunogenic at both the humoral and the cellular levels than was the aluminum gel complex. It appears that aluminum gel is a better adjuvant for intact HBsAg but that the liposomes render polypeptide preparations more immunogenic. The contrasting effect of the two adjuvants with the two vaccine preparations is not understood. A poor efficiency of adsorption can be ruled out, as over 95% of the polypeptide is precipitated in the gel. Although the polypeptide vaccine in this study induced a low level of response, a similar polypeptide vaccine adsorbed to aluminum gel gave a vigorous antibody response in chimpanzees after one inoculation (Dreesman et al., submitted for publication).

In this study, we used negatively charged multilamellar liposomes. A considerable proportion of HBsAg particles and P22 + P25 polypeptide pool was associated with liposomes. The mean percentage of entrapment was 47% (31 to 64.6%) in four particle HBsAg preparations, consistent with previously reported data (15). The mean proportion of incorporation was 58.9% (24 to 84%) for eight polypeptide preparations. The difference in incorporation of the two groups of preparations may indicate that various proportions were entrapped in aqueous compartments instead of being inserted into the lipid bilayers. A greater quantity of the polypeptide preparation might have been inserted into the mem-

branes, thus accounting for the increased immunogenicity of the polypeptide vaccine, compared with that of the particle HBsAg preparations. Similar studies in susceptible chimpanzees would be instructive for future decisions regarding the best method to induce immunity in humans.

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