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Human Immunodeficiency Virus Type 1 Major Neutralizing Determinant Exposed on Hepatitis B Surface Antigen Particles Is Highly Immunogenic in Primates

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Hepatitis B surface antigen (HBsAg) produced by recombinant DNA technology is now widely and safely used worldwide for hepatitis B vaccination. We used the HBsAg particle as a carrier molecule for presentation of selected human immunodeficiency virus type 1 (HIV-1) determinants to the immune system. Immunization of rhesus monkeys with an HBsAg chimera carrying the HIV-1 envelope major neutralizing determinant allowed us to generate proliferative T-cell responses and, in some cases, neutralizing antibodies and antibody-dependent cellular cytotoxicity. Since there is an overlap between populations at risk for hepatitis B virus and HIV, HBsAg recombinant particles may be relevant carriers for HIV-1 epitopes and could offer a new approach to the development of an AIDS vaccine.

The outer envelope glycoprotein gp120 of human immunodeficiency virus type 1 (HIV-1) is a logical candidate as a subunit vaccine for this virus. This glycoprotein binds to CD4, the viral receptor (17), and has been shown to be the major target of neutralizing antibodies (25, 27, 29). The epitopes involved in virus neutralization seem to be located in both variable and conserved regions of gp120. Type-specific antibodies appear to be due largely to the presence of an immunodominant epitope in the third hypervariable domain, the so-called V3 region (amino acids 305 to 330) (8, 15, 23, 28). Sera from most HIV-1-infected patients have neutralizing antibodies which primarily recognize the V3 region. However, later in the course of infection, group-specific neutralizing antibodies are detected, indicating that conserved antigenic epitopes are also involved in neutralization (13).

To deal with antigenic competition between epitopes present within the full length of gp120, our strategy in the design of a vaccine that will confer protection against HIV is the presentation of selected epitopes to the immune system. The hepatitis B surface antigen (HBsAg) particle-based hepatitis B vaccine was the first subunit vaccine produced by recombinant DNA technology, and the worldwide usage of hepatitis B vaccination makes the HBsAg particle a suitable and safe carrier of foreign antigenic determinants for human vaccination. We have shown previously that the envelope protein of the hepatitis B virus (HBV) can be used as a carrier for producing recombinant HIV antigens that induce both B-cell and T-cell responses in experimental animals (18, 19). We now report the results of immunogenicity studies with rhesus monkeys (*Macaca mulatta*) of the HIV-1/BRU V3 sequence presented as a particulate fusion protein with HBsAg (V3/HBsAg). Because the efficiency of AIDS vaccines may be limited by the lack of immunostimulatory adjuvants to enhance humoral and cell-mediated immune responses, we have compared two clinically relevant adjuvants, aluminum hydroxide (alum) and threonyl muramyl dipeptide (threonyl-MDP), in order to optimize the immunization regimen.

To produce hybrid HIV/HBsAg particles containing the V3 domain, synthetic oligomers encoding gp120 amino acids 308 to 331 from isolate HIV-1/BRU were inserted into an HBsAg expression vector, the pSV2S plasmid (19). The resulting plasmid (Fig. 1) contains an HIV-HBV fusion gene under the control of the simian virus 40 early promoter. In this gene, two in-frame initiation codons allow the expression of both the major protein of the HBV envelope encoded by the S gene and a hybrid HIV/HBsAg protein encoded by the fusion gene. The polyadenylation signal is provided by untranslated HBV sequences downstream of the S gene. Following transfection in animal cells, hybrid proteins were synthesized and assembled, together with the HBsAg major protein, into 22-nm particles, which were secreted and could be detected in cell culture supernatants by an HBsAg-specific commercial enzyme-linked immunosorbent assay (ELISA) (Monolisa; Diagnostics Pasteur, Paris, France). These particles were purified on a cesium chloride gradient according to their densities by a procedure that has been described previously (20). The presence of the BRU V3 domain on the HBsAg particles was confirmed by using different monoclonal antibodies (MAbs) in a sandwich radioimmunoassay (Table 1). MAb 0.5β, specific for the HIV-1/IIIB V3 domain (15), and MAbs 110-3, 110-4 (14), and III 2-3 (24), which are specific for the HIV-1/BRU V3 domain, recognized the V3/HBsAg hybrid particles but not the native pre-S2 HBsAg particles. MAb I 18-1, specific for the HIV-1/MN V3 domain (Hybridolab Institut Pasteur, Paris, France), was used as a negative control. Differences between specific reactivities may be related either to the affinities of the various MAbs for the particles or to differences between coating wells with purified MAbs and coating with ascitic fluid. Because these MAbs are able to neutralize HIV-1/BRU infectivity in vitro, this shows that the V3 domain is exposed on the hybrid particles as well as on native HIV-1.

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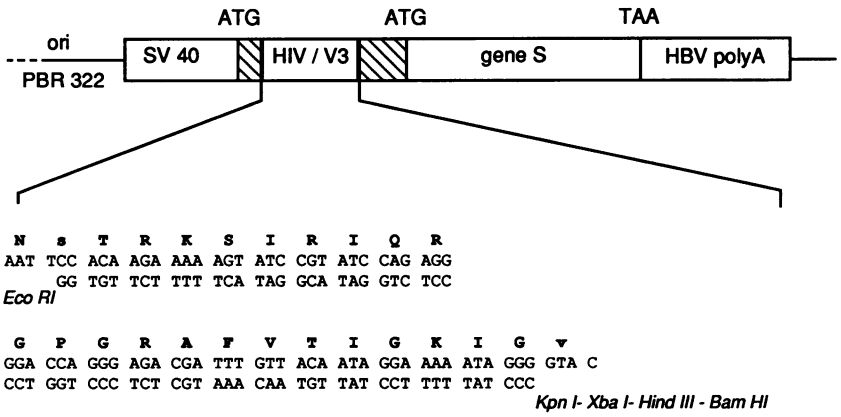

FIG. 1. Structure of the V3/HBsAg expression vector (top) and nucleic acids and predicted amino acid sequences of oligomers encoding the V3 domain of HIV-1 isolate BRU (bottom). SV 40, fragment of simian virus 40, including origin of replication, enhancer, and early promoter; hatched boxes, remaining sequences from the HBV pre-S2 region after insertion of a polylinker (19). The S gene and polyadenylation sequences are from HBV. Restriction sites *Xba*I, *Hin*dIII, and *Bam*HI are the remaining sites from the pSV2S plasmid (19).

To evaluate the immunogenic potential of this antigen, we immunized six rhesus monkeys divided into two adjuvant groups. One animal of each sex was assigned to either the threonyl-MDP group (ZI and CH) or the alum group (JU and KI), and each was immunized with the hybrid V3/HBsAg particles. One control animal (male) in each group received the native pre-S2 HBsAg particles (PA and AL, respectively). They all received two intradermal injections at a 1-month interval and one intradermal boost 8 months later of either antigen (16 μg of HBsAg per dose). Animals were housed according to the official rules and recommendations of the European Economic Community and were monitored for deleterious effects of immunization. Immunization with hybrid or native particles in either adjuvant did not induce any changes in the absolute number of leukocyte subsets per cubic millimeter or in the absolute number of $CD4^+$ and $CD8^+$ T cells over the course of the immunization (data not shown). No adverse reaction was observed at the injection sites. Thus, neither biological nor clinical adverse effects were induced by this immunization regimen.

TABLE 1. Binding of HIV-1-specific MAbs to hybrid V3/HBsAg or native pre-S2 HBsAg particles[a]

Antigen	Amt (ng/ml)	Binding of MAb[b]:				
		0.5β	110-3	110-4	III 2-3	I 18-1
V3/HBsAg	14	2,660	7,401	6,139	4,834	566
	7	2,219	5,282	5,084	4,110	527
	3.5	1,544	ND	ND	ND	ND
	1.75	998	ND	ND	ND	ND
Pre-S2 HBsAg	14	45	400	507	396	379
	7	72	395	420	387	469
	3.5	52	ND	ND	ND	ND
	1.75	53	ND	ND	ND	ND

[a] Flexible microtiter wells (Falcon Microtest III; Falcon, Oxnard, Calif.). were coated with purified MAbs (125 ng of 0.5β or I 18-1) or a 1:1,000 dilution of ascitic fluid (MAbs 110-3, 110-4, and III 2-3). Hybrid V3/HBsAg or native pre-S2 HBsAg titrated by a commercial HBsAg ELISA was added and detected by a rabbit anti-HBsAg antiserum (1:5,000) (Behring, Marburg, Germany). After fixation of ^{125}I-anti-rabbit immunoglobulin (Amersham), wells were separated and counted in a gamma counter.

[b] Radioimmunoassay binding results are expressed as counts per minute. ND, not done.

Sera from macaques immunized with recombinant V3/HBsAg particles were analyzed by a specific ELISA (18). HIV-specific antibodies were quantified by coating on the solid phase either 2.5 μg of heat-inactivated purified HIV-1/BRU virus per ml, 1.5 μg of vaccinia virus (VV)-derived recombinant BRU gp160 per ml, or 1.5 μg of BRU V3 peptide (amino acids IRIQRGPGRAFVTIGK) per ml, as shown in Fig. 2. Antibodies to BRU gp160 were detected for each V3/HBsAg-immunized animal after the first or the second injection, and a complete response to all the HIV antigens tested was observed after the boost. HIV-specific antibody responses in these animals were also confirmed with a commercial immunoblot (data not shown). In contrast, no HIV-specific antibodies were found in the control animals' sera. As expected, all the animals developed an HBsAg-specific response. The kinetics of both HBV and HIV antibody responses were similar. Control animals immunized with native recombinant particles developed the same level of anti-HBsAg antibodies as did the experimental animals (Fig. 2). When the two adjuvant groups were compared, animals with the same weights and ages (i.e., ZI and JU, and CH and KI) had similar patterns of antibody responses. Thus, no significant difference between the two groups could be defined.

The ability of sera from immunized macaques to neutralize HIV-1/BRU infectivity in vitro was monitored by inhibition of HIV-1-specific reverse transcriptase (RT) activity and by inhibition of syncytium formation. Rhesus monkey sera obtained before any injection, at weekly intervals after the second injection, and after the boost were tested in both assays. In the RT inhibition assay, 4×10^3 50% tissue culture infectious doses of an HIV-1/BRU virus stock produced and titrated on CEM cells was incubated at 37°C for 1 h with prebleed and immune macaque sera (1:50 dilution) or MAb 0.5β (1:250 dilution) on microplates. CEM clone 13 cells (10^4 cells in 0.1 ml) in medium containing 2 μg of Polybrene per ml were added to an equal volume of virus-serum mixtures. The assays were carried out in triplicate. RT activity in cell supernatants was measured at days 6 and 9 by an RT microassay described previously (31). No neutralizing activity was detected in the preimmune sera or in the control animals' sera. The sera collected after the second immunization had neutralization titers less than 1:50 (data not shown). However, macaque ZI (threonyl-MDP group) de-

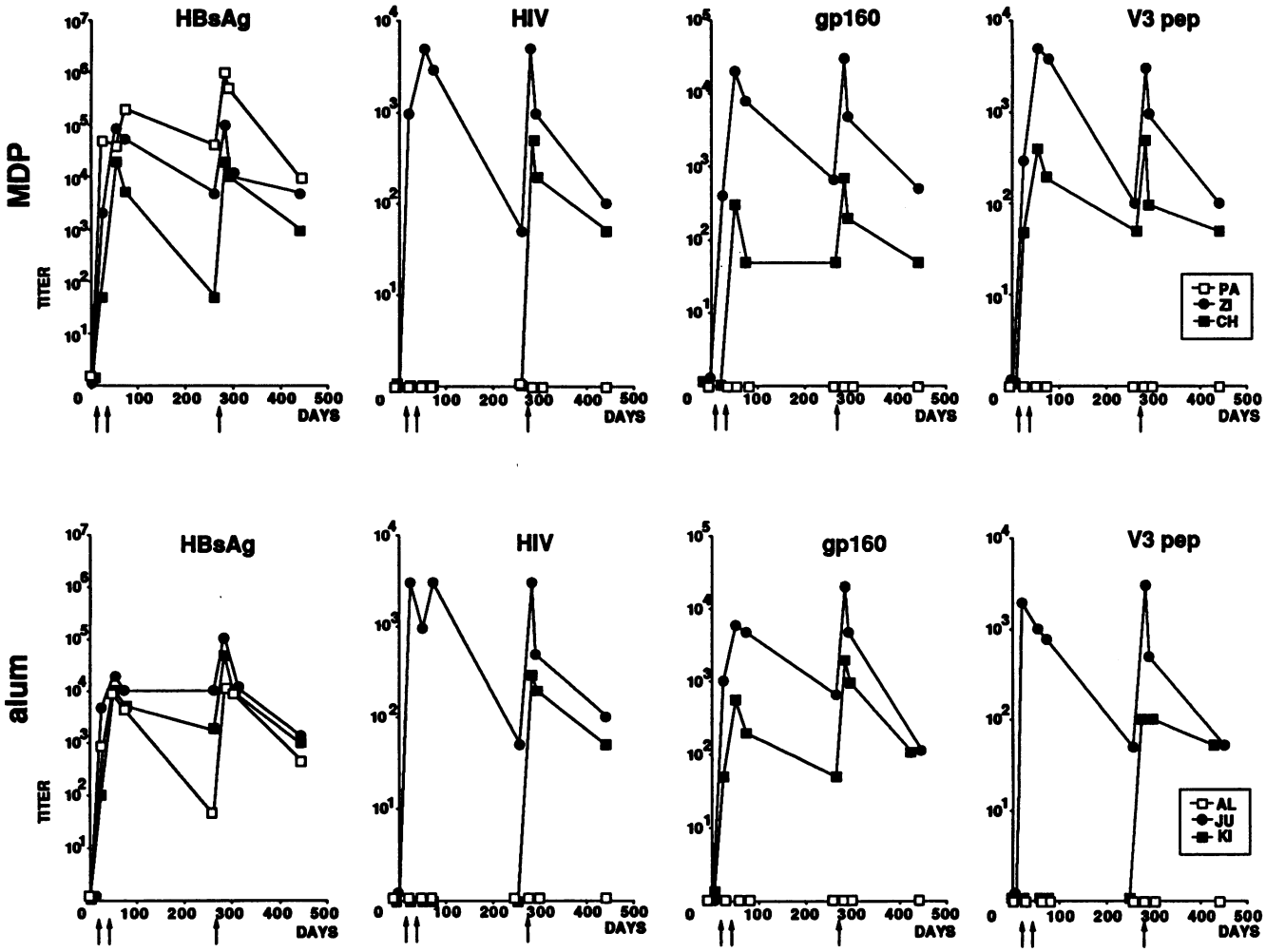

FIG. 2. Antibody titers in macaques immunized with threonyl-MDP (top) or alum (bottom) as the adjuvant. □, control macaques (males) immunized with native pre-S2 HBsAg particles in each group; ● and ■, female and male V3/HBsAg-immunized animals, respectively. Arrows indicate the days on which the animals were immunized. HBsAg, HIV-1/BRU (HIV), BRU gp160, and BRU V3 peptide (pep) are the antigens coated on the solid phase. Fixed antibodies were detected with macaque immunoglobulin conjugated with horseradish peroxidase (1/1,000) (Cappel, Durham, N.C.). The endpoint titer was defined as the highest serum dilution that resulted in an absorbance value three times greater than the absorbance value of the preimmune corresponding macaque serum.

veloped neutralizing antibodies after the boost, as shown in Table 2. A serum sample obtained 2 weeks after the boost was able to completely inhibit RT activity at a serum dilution of 1:50. These experiments were carried out four different times with MAb 0.5β (12 μg/ml), which is specific for the V3 domain, as a positive control. In an endpoint syncytial induction assay, 10 50% tissue culture infectious doses of an HIV-1/BRU stock produced on human peripheral blood mononuclear cells (PBMC) and titrated on MT2 cells were incubated for 1 h at 37°C with serial dilutions of monkey sera on microplates. MT2 cells (2×10^4 per well) were added for 1 h at 37°C. Cells were washed twice with phosphate-buffered saline and resuspended in culture medium. At days 7 and 9, syncytia were quantified with an inverted microscope. A reference serum drawn from an HIV-1-seropositive individual was used as a positive control. As shown in Table 2, three serum samples of the same animal (ZI), collected weekly after the boost (weeks 1, 2, and 3), were able to inhibit syncytium formation at a dilution of 1:252. No neutralizing antibodies were detected in the preimmune sera

TABLE 2. Specific neutralizing antibodies of macaque ZI after V3/HBsAg boosting

Serum of macaque ZI or MAb	Result by inhibition assay	
	RT (%)[a]	Syncytium (dilution)[b]
Preimmune	0	<1:32
Wk 1[c]	86	1:252
Wk 2	95	1:252
Wk 3	79	1:252
Wk 4	67	1:32
MAb 0.5β	98	ND

[a] Input virus (HIV-1/BRU) had an RT activity between 109,000 and 166,000 cpm in the presence of the preimmune serum. The background level of the assay was between 100 and 550 cpm. A culture was considered positive for neutralizing activity if there was more than 90% reduction of counts per minute, relative to the value for the preimmune serum of the same animal.

[b] Neutralization titers were expressed as the last serum dilution in which no syncytia were detectable in all four wells of the quadruplicate at days 7 and 9.

[c] After the booster injection.

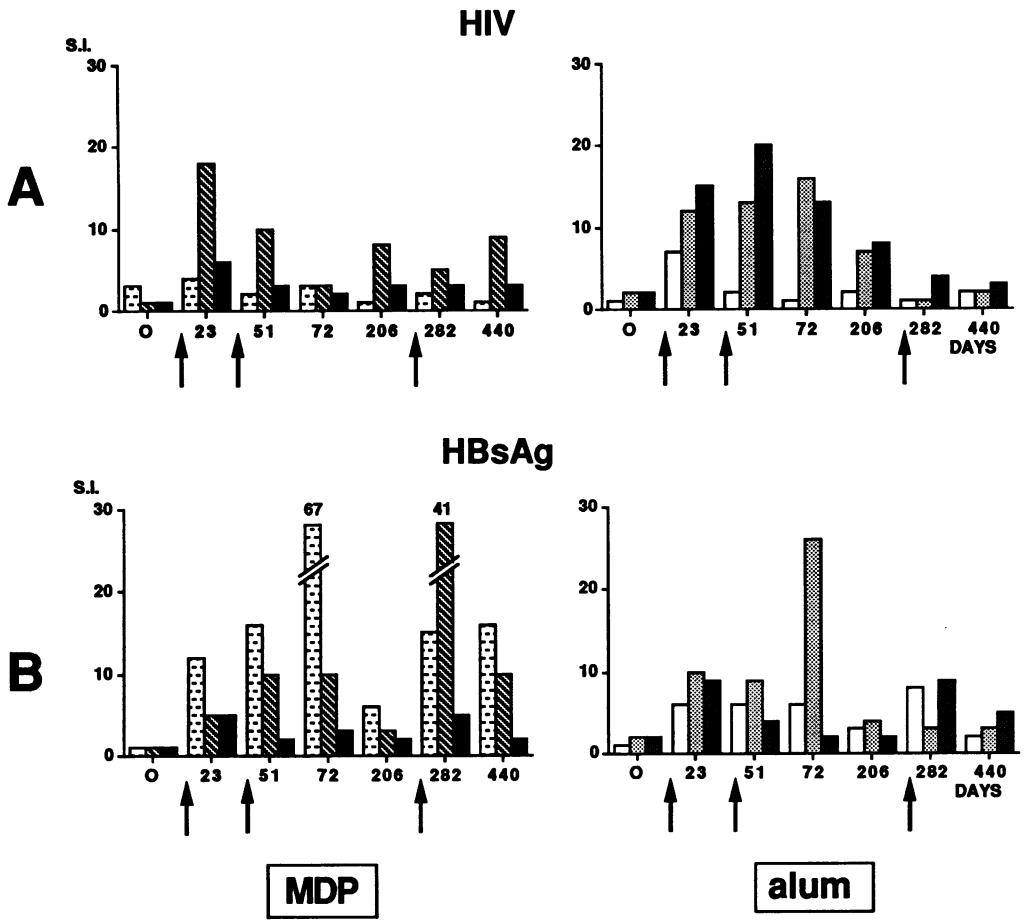

FIG. 3. Kinetics of macaque lymphoproliferative responses after in vitro stimulation with purified HIV-1/BRU (A) or HBsAg (B). Results from the threonyl-MDP and alum adjuvant groups are presented on the left (▨, PA; ▧, ZI; ■, CH) and right (□, AL; ▩, JU; ▦, KI) sides of the figure, respectively. Arrows indicate the days on which the animals were immunized. Results are expressed as stimulation indices (S.I.) and are calculated by dividing the counts per minute of [^{3}H]thymidine incorporated into stimulated cells by the counts per minute incorporated into nonstimulated cells.

or in the sera of the other immunized animals (data not shown). It is noteworthy that the serum of animal ZI was able to block both free-virus infectivity and virus-induced cytopathogenic effect at the same time (i.e., after the boost). This rhesus monkey was the one with the highest titers to gp160 and to V3 peptide, as determined by ELISA. These results are in agreement with other studies, in which high antibody titers are often necessary to obtain neutralizing activity (7). No significant neutralizing titers were found in the sera of other animals. This could be ascribed to several parameters. First, identical amounts of antigen were administered to all monkeys, independent of body weight. Second, it is already known that differences in age and sex may affect the ability of monkeys and humans to mount responses against a given immunogen. Third, the amount of injected antigen was calculated with respect to HBsAg. One should note that the fusion middle protein represents about 20% of the total protein of the particle. In addition, the V3 domain constitutes 24 of the 271 amino acids of the fusion protein and thus represents only 2% of the particle. Thus, it is perhaps not surprising that neutralizing antibodies were not detected in most animals. Indeed, it is remarkable that even with low doses and a reduced immunization regimen, the V3 domain is still able to induce high titers of HIV-specific antibodies. Using a comparable system with a yeast-derived Ty particle as a carrier for the V3 domain, Griffiths et al. also obtained neutralizing antibodies in rabbits, but by using a 500-μg dose of purified antigen (9).

The proliferative responses of PBMC isolated from macaques before and after each immunization were tested against a range of HBsAg and HIV-1/BRU antigens, as described by Michel et al. (18). The results in Fig. 3, expressed as stimulation indices for the optimum dose of antigen (heat-inactivated sucrose gradient-purified HIV-1 antigen at 500 ng/ml and purified HBsAg at 250 ng/ml), show the kinetics of the lymphoproliferative response from immunized animals. No in vitro stimulation of the six animals' T cells was observed with HBsAg or HIV antigens before immunization. In vitro HIV-1-specific proliferative response to killed virus of the four V3/HBsAg-immunized macaques was observed after the first immunization and was maintained several months after the second immunization (Fig. 3A). A weak and delayed specific proliferative response to recombinant BRU gp160 was also observed with PBMC isolated from the same animals, and no proliferation was found in response to V3 peptide stimulation (data not shown). The PBMC from the control animals immunized with the native recombinant particles did not proliferate in

response to any HIV antigens except at day 23, when control animal AL had a stimulation index of 7 for HIV-1. This animal was injected in a previous and unrelated experiment with complete Freund's adjuvant and thus may have had nonspecific T-cell activation. A comparison of the two adjuvant groups (threonyl-MDP and alum) suggested that antigen injected with alum improved the HIV-specific proliferative response. Because the V3 domain contains a helper T-cell epitope, it is likely that the V3 domain exposed on HBsAg particles is processed in a manner similar to that which occurs when this domain is presented by the virus. Figure 3B summarizes the results of PBMC stimulation with HBsAg particles. Since both antigens carry a common HBsAg domain, a specific proliferative response to HBsAg was elicited in the control animals as well as in the animals immunized with the hybrid particles. This response appeared following the first immunization and was enhanced after the boost. Thus, the processing of the hybrid particle did not alter the processing and presentation of the HBsAg-specific T-cell epitopes. These experiments provide evidence that HBsAg carrying the V3 domain is able to induce immunity to and memory of both HBV and HIV at the T-cell level.

One of the major goals of vaccination is to generate immune cells able to recognize and kill infected cells. We examined this possibility by testing the lytic activity of fresh PBMC from immunized animals toward autologous B lymphoblastoid target cells obtained by transformation with herpesvirus papio (26). Target cells were infected with either wild-type VV or two different recombinant VVs encoding an uncleaved *env* gp160 (VV TG 1139) and an *env* precursor without the signal peptide (VV TG 3183), allowing us to discriminate between antibody-dependent cellular cytotoxicity and class I-restricted cytotoxic T lymphocyte activities (16). In a standard chromium release assay, PBMC from CH obtained 1 month after the boost were able to specifically lyse VV TG 1139-infected target cells (17% specific lysis at an effector/target cell ratio of 100 to 1). In the same assay, PBMC from ZI gave a comparable result, whereas PBMC from the control macaque (PA) did not lyse any target cells (data not shown). In the group immunized with alum as the adjuvant, PBMC from JU were able to induce 20% *env*-specific lysis of the VV TG 1139-infected target cells. In both experiments, no specific lysis was observed with wild-type VV- or VV TG 3183-infected target cells. This indicates that macaque cells recognized the HIV envelope only when it was expressed at the cell surface, suggesting that specific lysis of the target cells was due to antibody-dependent cellular cytotoxicity.

In this report, we have shown that immunization of macaques with recombinant HIV/HBsAg particles expressing the BRU V3 domain elicited an HIV-specific proliferative T-cell response in all four macaques tested. Cell-mediated cytotoxicity was detected in three of the four macaques, and in one case, neutralizing antibodies were detected. When we compare the two adjuvant groups, the heterogeneity of both cellular and humoral responses seems more related to individual macaque responses than to the adjuvant used.

There has been a great deal of interest in the HIV-1 V3 domain, not only because it contains the principal neutralizing determinant (11) but also because it contains epitopes for cellular cytotoxicity (33), helper T cells (2, 30), and antibody-dependent cellular cytotoxicity (3). In humans, V3-region antibodies have been shown to play an important role in the beginning stage of infection because of their early and rapid induction and high neutralizing efficacy. Furthermore, immunization with recombinant gp120 (1) or successive immunization with a variety of immunogens, including the V3 domain of gp120 (7), can induce protective immunity in chimpanzees against later challenge with HIV-1/IIIB. Recently, a synthetic peptide corresponding to the HIV-1 V3 epitope was able to induce high titers of neutralizing antibodies in rhesus monkeys when threonyl-MDP or incomplete Freund's adjuvant was used as an adjuvant (10). More direct evidence for the in vivo protective effect of anti-V3 antibodies was provided by experiments of Emini et al., in which a chimpanzee perfused with a V3 MAb was protected against homologous challenge (6).

The role of neutralizing antibodies in protection against AIDS is still controversial. In the chimpanzee model, which has not been shown to develop an AIDS-like disease after HIV infection, challenge experiments using free virus have shown that neutralizing antibodies seem to play a major role in clearing the virus (1, 7). In contrast, challenge experiments with the simian immunodeficiency virus-macaque model showed no relationship between neutralizing antibodies and protection (5, 21, 32). Presumably, other components of the immune response are involved in dealing with both free virus and infected cells; these components include helper T cells, which contribute to the efficient induction of antibody, cytotoxic T lymphocytes, and antibody-dependent cellular cytotoxicity in this system.

Strategies to develop a vaccine against viral disease utilize two types of antigen: soluble antigen and antigen carried by a live vector. The potential immune response induced by each type of antigen could be different, since a live vector stimulates mainly cellular cytotoxic response with minor or no antibody production. On the other hand, soluble antigen induces high-titered antibodies able to clear free virus and also cellular response, such as production of helper T cells and major histocompatibility complex class II (22)- or class I (4, 34)-dependent cytotoxicity. Because we think that a live recombinant vaccine may present risks, especially in an immunodepressed population, we chose to present HIV antigenic determinants on a soluble antigen, HBsAg, as a carrier. In addition, in vaccinated humans, HBsAg is one of the few soluble antigens able to induce T-cell clones capable of recognizing viral antigen processed in the exogenous pathway as well as in the endogenous pathway (12).

HBsAg is a good carrier for presentation of HIV determinants, since it enabled us to obtain a wide spectrum of immune responses, including production of neutralizing antibodies, proliferative T-cell response, and cytolytic activities in immunized primates. This system does not exclude the possibility of combining various epitopes or different sequences of the same epitope, either on the same particle or in a cocktail of particles. Since the safety and efficacy of HBsAg in human vaccination have already been proven, the use of such hybrid particles could be a new approach in multivalent vaccination.

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