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T- and B-Lymphocyte Responses to Human Immunodeficiency Virus (HIV) Type 1 in Macaques Immunized with Hybrid HIV/Hepatitis B Surface Antigen Particles

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Recombinant human immunodeficiency virus (HIV)/hepatitis B surface antigen (HBsAg) subviral particles of dual antigenicity and immunogenicity were obtained by fusing 84 amino acids of the HIV type 1 external envelope glycoprotein within the pre-S2 part of the hepatitis B middle protein (M.-L. Michel, M. Mancini, E. Sobczak, V. Favier, D. Guétard, E.-M. Bahraoui, and P. Tiollais, Proc. Natl. Acad. Sci. USA 85:7957–7961, 1988). We now describe the humoral and cellular immune response of rhesus monkeys immunized with these hybrid particles. Macaque antisera raised by subcutaneous injections of the HIV/HBsAg particles were shown to be specific for HIV in peptide-binding assays. Moreover, we were able to generate in these vaccinated animals a T-cell-proliferative response to both parts of the hybrid particle, i.e., HIV and HBsAg. These results establish the presence of a T-cell epitope in this HIV segment, which has been shown previously (L. A. Lasky, G. Nakamura, D. H. Smith, C. Fennie, C. Shimasaki, E. Patzer, P. Berman, T. Gregory, and D. J. Capon, Cell 50:975–985, 1987) to be an important domain involved in the binding of the virus to its cellular receptor, the CD4 molecule. This work demonstrates the feasibility of using the HBsAg subviral particle as a carrier protein for the presentation of foreign immunogenic epitopes to the immune system.

We recently reported the construction of recombinant human immunodeficiency virus (HIV)/hepatitis B surface antigen (HBsAg) particles that present antigenic determinants on their surface HIV envelope (9). The particles were obtained by fusing 84 amino acids (amino acids 384→467) of the HIV type 1 (HIV-1) external envelope glycoprotein (gp120) within the pre-S2 portion of the HBsAg middle protein. When injected into rabbits, these particles were able to induce neutralizing antibodies. To show that these hybrid particles induce a humoral as well as a T-cell-mediated immune response to HIV in primates, we immunized macaques with either the hybrid or native HBsAg particles.

Two rhesus monkeys (*Macaca mulatta*) (mac H and mac S) were immunized with purified HIV/HBsAg particles by three subcutaneous injections at 1-month intervals and then by booster injections two times 3 and 4 months later. A control macaque (mac A) was immunized with native HBsAg particles in four doses by a similar protocol (Fig. 1). Blood samples were collected from each animal before and after each immunization. Specific serum antibodies were assayed by enzyme-linked immunosorbent assay (Fig. 1) with either native HBsAg particles or HIV homologous synthetic peptides (peptide 6, amino acids 373 to 398; peptide 7, amino acids 421 to 441) bound to the solid phase. Anti-HBsAg titers peaked 3 weeks after the third dose of vaccine. A second rise in titers was observed after the booster immunization. Anti-HIV antibodies were detected with both peptides. The titers reached maximal levels 3 weeks after the second or the third injection and then decreased slightly. As with anti-HBsAg titers, a booster effect resulting from the fourth or the fifth dose was clearly detected in the sera of both macaques. As determined by an assay based on inhibition of the cytopathic effect of HIV in CEM cells (14), these sera were devoid of neutralizing activity (results not shown). The control macaque (mac A), immunized with native HBsAg, had slightly higher anti-HBs antibody titers than mac H and mac S did, but no anti-HIV antibody response was detected.

These studies demonstrate that immunization with three doses of HIV/HBsAg particles seems to be sufficient to establish HIV-specific immunologic memory and that booster doses are needed to maintain high antibody titers. The time course of the anti-HIV antibody response after immunization is similar to that of the anti-HBsAg response, suggesting that the response to one antigen does not suppress a response to the other.

To determine whether T-lymphocyte responses to both parts of the hybrid particle could be detected in these immunized macaques, we measured lymphocyte proliferation in response to stimulation with purified native HBsAg, HIV/HBsAg particles, heat-inactivated HIV, and concanavalin A. Peripheral blood lymphocytes (PBL) of each macaque were isolated before immunization, 21 days after the second and third injections, and before and after boosting. The lymphocytes were cultured in medium alone or were stimulated for 4 days with HIV/HBsAg, HBsAg, or concanavalin A and for 5 days with HIV-1. Typical dose-response curves obtained 21 days after the third immunization are shown in Fig. 2. The PBL of mac H incorporated eight times more [^{3}H]thymidine in response to stimulation with an optimal concentration of HIV/HBsAg particles and six times more in response to stimulation with native HBsAg than did nonstimulated PBL. In response to an optimal concentration of HIV, PBL of mac H incorporated five times more [^{3}H]thymidine than did PBL stimulated with medium alone. In contrast, the PBL of mac A immunized with native HBsAg proliferated in response to stimulation with either

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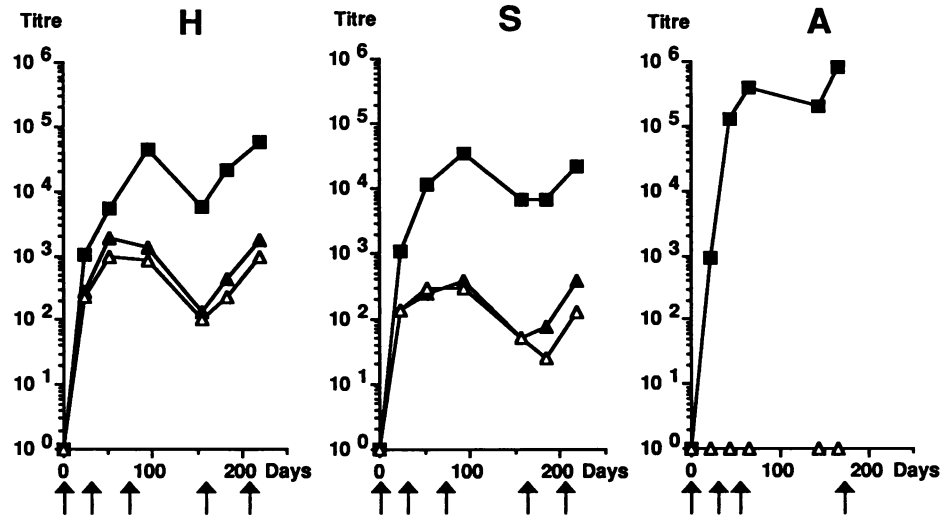

FIG. 1. Antibody titers in macaques immunized with HIV/HBsAg or native HBsAg particles. Two macaques (S and H) were injected subcutaneously with purified HIV/HBsAg (10 and 15 μg, respectively) on days 0, 24, 70, 167, and 203, as indicated by arrows. One macaque (A) was injected with 15 μg of purified plasma-derived HBsAg particles on days 0, 21, 43, and 144. The first dose was given in Freund complete adjuvant, and subsequent doses were given in Freund incomplete adjuvant. Macaques S and H received the fourth dose without adjuvant. Serum samples obtained at different times after injection were serially diluted and tested for HBsAg (■) or HIV-specific antibodies (▲, peptide 6; △, peptide 7) by enzyme-linked immunosorbent assay. Titers are expressed as the reciprocal of the serum dilution that gave an absorbance value three times higher than the value obtained with the preimmune sera. Each point is the mean of at least four determinations.

native or hybrid HBsAg but did not proliferate in response to stimulation with HIV at any concentration.

The proliferation of PBL in response to HBsAg or to HIV was monitored for each animal during the immunization regimen. Figure 3 summarizes the results of lymphocyte proliferation in response to stimulation with HIV/HBsAg, HBsAg, and HIV. The maximal proliferative response to HIV/HBsAg occurred after the second injection and was always greater than the response obtained after stimulation with native HBsAg for both macaques immunized with hybrid particles. Conversely, the PBL from the control animal (mac A) were stimulated more with native HBsAg than with HIV/HBsAg. Although the magnitude of proliferation varied for the two animals immunized with HIV/HBsAg, the kinetics of the responses were similar. In comparison, there was significant proliferation in response to HIV by the lymphocytes of mac H at all time points after the second immunizing dose. In mac S, a response to HIV occurred only after the third injection and a second booster. In the control animal (mac A), stimulation of lymphocytes with HIV did not induce a significant response. Comparison of proliferative responses to the three antigens shows that PBL from mac H were stimulated equally well by HIV or by native HBsAg. Stimulation indices were comparable except at day 52, when the number of HBsAg-specific T cells was significantly higher than the level of HIV-specific T cells.

We have also tested the PBL proliferative response to purified recombinant gp160 (7), to native gp160 assembled in immunosomes (15), and to peptide. The PBL of mac H isolated 3 months after the last immunization with HIV/HBsAg were stimulated equally well by purified HIV-1 and by gp160 immunosomes (Table 1). In addition, there was a proliferative response to peptides 6 and 7 with the PBL of mac H isolated 3 and 6 weeks after the last immunization (stimulation indices, 3.6 and 3, respectively, with 250 ng of peptides per ml). However, recombinant gp160 did not induce a proliferative response. At the concentration used, the antigens were not toxic. These HIV antigens did not induce proliferation of PBL from mac A, which was immunized with HBsAg (data not shown). These results suggest that the processing of gp160 is more efficient with the glycoprotein assembled in the virion or in immunosomes than with the glycoprotein in soluble form and that the fusion protein contains at least one intact T-cell epitope which is processed as efficiently as whole virus.

These experiments demonstrate that immunization of rhesus monkeys with HBsAg particles carrying HIV envelope determinants results in the generation of envelope-

TABLE 1. Proliferative responses of PBL from macaque H, which was immunized with HIV/HBsAg particles, after stimulation with HIV or HIV envelope glycoproteins

Stimulating agent[a]	Concn of stimulating agent (ng/ml)	[^{3}H]thymidine counts per minute	Stimulation index
HIV	0	250	
	50	837	3.3
	100	930	3.7
	500	1,512	6.0
	1,000	1,735	6.9
Immunosomes	0	250	
	25	764	3.0
	50	829	3.3
	250	1,345	5.4
	500	1,545	6.1
gp160	0	250	
	50	209	0.8
	100	308	1.2
	500	328	1.3
	1,000	398	1.6

[a] PBL were stimulated for 5 days as described in the legend to Fig. 2 with sucrose gradient-purified, heat-inactivated HIV, immunosomes containing gp160 (kindly provided by L. Thibodeau, Institut Armand Frappier), or soluble gp160 produced by a recombinant vaccinia virus (kindly provided by M. P. Kieny, Transgène).

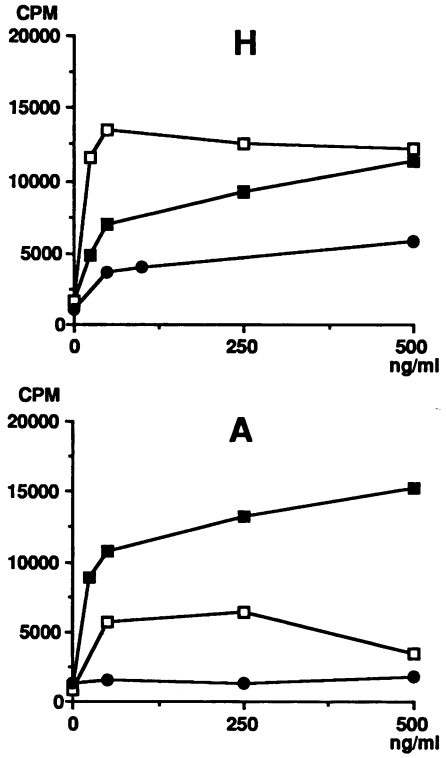

FIG. 2. Lymphocyte-proliferative responses of macaques immunized with HIV/HBsAg (mac H) or with native HBsAg (mac A). After the third immunization (see text and legend to Fig. 1), PBL were isolated by Ficoll-Hypaque centrifugation from heparinized blood. The PBL were suspended at 1.5×10^6 cells per ml in RPMI 1640 medium supplemented with 10% heat-inactivated pooled human AB serum, and 0.1 ml of PBL suspension was placed in triplicate in round-bottom wells of 96-well microtiter plates. The PBL were stimulated with 0.1 ml of one of the following antigens diluted in medium: sucrose gradient-purified HIV-1 antigen (●) (kindly provided by M. Sallé, Diagnostic-Pasteur) or HBsAg particles purified on two successive cesium chloride gradients (■, HBsAg; □, HIV/HBsAg). Each antigen was assayed at different concentrations. Five and four days after stimulation, the cultures were labeled with 1 μCi of [^{3}H]thymidine per well for 6 h and harvested on fiberglass filters, and the [^{3}H]thymidine counts per minute (CPM) were determined by liquid scintillation counting.

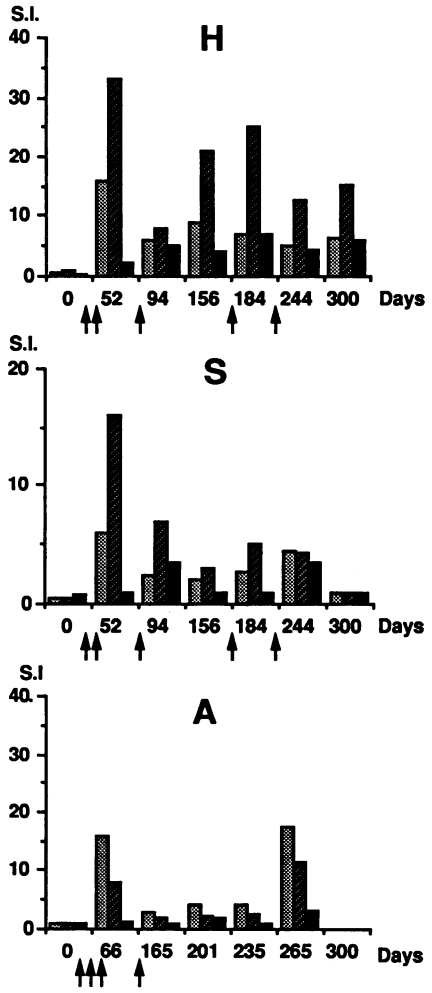

FIG. 3. Kinetics of lymphocyte proliferation in three immunized macaques. Macaques H and S were immunized with HIV/HBsAg, and macaque A was immunized with native HBsAg particles. Lymphocytes were collected before immunization, after the second or third immunization, and after boosting on days indicated in the figure. Arrows indicate the days on which the animals were immunized. [^{3}H]thymidine incorporation in response to exogenous antigen (HIV [■] at 500 ng/ml, HBsAg [▨] at 250 ng/ml, or HIV/HBsAg [▨] at 250 ng/ml) was monitored as described in the legend to Fig. 2. The stimulation index (S.I.) was calculated by dividing the counts per minute of [^{3}H]thymidine incorporated into stimulated cells by the counts per minute incorporated into nonstimulated cells. In these experiments, background counts per minute were between 100 and 2,450, and the stimulation index for concanavalin A was between 5 and 129.

specific antibodies and that the peripheral blood mononuclear cells from such macaques proliferate after in vitro stimulation with HIV-1 and with HIV antigens. Moreover, vaccination with HIV/HBsAg particles elicits a T-cell response to both HIV and HBsAg, and processing of the hybrid protein probably preserves at least one HIV-specific T-cell epitope.

Using hybrid HBsAg particles, others have reported antibody responses to a repetitive epitope of *Plasmodium falciparum* (12) or to a well-characterized poliovirus epitope (4). However, little is known about the T-cell recognition of such hybrid particles. Celis et al. (3) have identified an immunodominant epitope near the amino-terminal end of HBsAg for major histocompatibility complex class II-restricted human T lymphocytes, and Milich and Chisari (10) have mapped recognition sites for murine T cells in the HBsAg major protein. In addition to the predicted cellular response to HBsAg, we were able to generate in vaccinated macaques a T-cell response to HIV. Zarling et al. (17) demonstrated HIV-specific T-cell immunity in macaques infected with a recombinant vaccinia virus expressing the gp120 envelope glycoprotein. Using synthetic peptides and a predictive program for T-cell epitopes, Berzofsky et al. have identified a T-cell epitope in the gp120 molecule recognized both in mice (2) and in vaccinated humans (1). This sequence, called env T1 (amino acids 428 to 443) by Berzofsky et al. (1), is included in the HIV antigenic fragment carried by our HBsAg hybrid particle. Moreover, Lasky et al. (8) have delineated a region in this HIV domain critical for virus binding to its cell receptor, the CD4 molecule. Since this segment of gp120 participates in an indispensable function for the virus, mutation by immune selection may be con-

strained. Thus, this domain is an attractive subject for vaccine design.

Cell-mediated immunity is important in recovery from virus infection (11) and thus may play a role in preventing the progression from HIV infection to acquired immune deficiency syndrome. Humans infected with HIV were found to exhibit limited and inconsistent T-cell-proliferative responses to the whole virus (16). However, proliferation was more clearly shown with short HIV-1 peptides (13). In contrast, the PBL of HIV-infected chimpanzees are able to proliferate in response to intact HIV, to envelope glycoprotein, and to mitogens (6). While susceptible to infection with HIV, the chimpanzee does not typically develop clinical acquired immune deficiency syndrome. Thus, it is not possible to assess the ability of a candidate vaccine to prevent disease in this model.

Since the isolation of simian immunodeficiency viruses (SIV) from nonhuman primates, many studies have demonstrated that SIV is biologically closely related to HIV type 2 (5). Macaques infected with SIVmac have $CD4^+$ T-lymphocyte depletion and symptoms similar to those of human acquired immune deficiency syndrome. This represents a more suitable model for vaccine studies. Testing SIV/HBsAg hybrids in the SIV macaque model will represent the next step of our vaccine approach.

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