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Immunology

Specific cellular immune response and neutralizing antibodies in goats immunized with native or recombinant envelope proteins derived from human T-lymphotropic virus type III_B and in human immunodeficiency virus-infected men

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ABSTRACT **Animals immunized with native or recombinant envelope proteins from the human immunodeficiency virus (HIV, formerly referred to as human T-lymphotropic virus type III) human T-lymphotropic virus type III_B and naturally HIV-infected men were assessed for neutralizing antibodies and cell-mediated immunity toward the virus. Immunization of rabbits or goats with the native external envelope glycoprotein gp120 or with corresponding recombinant proteins elicited strictly type-specific neutralizing antibodies. A broad, group-specific cellular immune response to gp120 and to three different HIV isolates was seen in goats immunized with the native gp120 but not in animals immunized with the nonglycosylated recombinant envelope proteins. In HIV-infected people, no T-cell response was seen, even though their T-cell response toward other foreign antigens was intact. The results show type- and group-specific epitopes on gp120, some of which may be of importance for the development of a vaccine against HIV infection.**

In view of the continuing spread of the acquired immunodeficiency syndrome (AIDS) epidemic (1), there is an urgent need for a vaccine against its etiologic agent, the retrovirus called human immunodeficiency virus [HIV, formerly referred to as human T-lymphotropic virus type III (HTLV-III)] (2). A logical candidate for such a vaccine is the external envelope glycoprotein gp120 or corresponding recombinant or synthetic peptides. gp120 contains conserved and variable regions (3, 4) and is mainly responsible for up to 30% of the variability observed between different HIV isolates. Neutralization assays with sera from HIV-infected individuals (5–7) or from immunized animals (8, 9) suggest that gp120 contains epitopes that elicit antibodies capable of neutralizing HIV infection *in vitro*. As the group-specific neutralizing antibodies in infected individuals have only a limited effect on the progression of the disease (5) and as animal sera obtained by immunization with gp120 or recombinant peptides are strictly type specific, it has been suggested that a vaccine based on gp120 should contain a mixture of recombinant proteins representing selected sequences from the variable regions of different isolates (10).

Protective immunity toward microorganisms is thought to be mediated by humoral and cellular mechanisms (11). In the former, neutralizing antibodies may prevent the binding of the virus to its cellular receptor and subsequent internalization, or complement fixing antibodies may result in virolysis (12). In the latter, cytotoxic T cells, recognizing viral antigens on the cell membrane in association with class I major histocompatibility complex antigens, lyse the infected cells (13). Cytotoxic T cells require help from CD4 antigen-positive helper T cells, possibly by cell-to-cell contact and by mediator molecules, such as interleukin 2 or γ interferon, secreted by activated helper T cells that have recognized viral antigens. Helper T-cell proliferation, therefore, shows T-cell specific epitopes on the antigen used for stimulation and indicates that these epitopes will be recognized by the immune system.

In the present work we describe HIV-specific helper T-cell proliferative responses and neutralizing antibodies in animals immunized with native or recombinant envelope proteins but show the lack of a cellular immune response in HIV-infected individuals.

MATERIALS AND METHODS

HIV Isolates. Three HIV isolates, HTLV-III_B, HTLV-III_{MN}, and HTLV-III_{RF} (2) were cultured in H9 cells in RPMI 1640 medium and 10% (vol/vol) fetal calf serum. These viruses represent the broad spectrum of divergence seen between different HIV isolates (3, 4, 14). For immunoblots and for T-cell assays, virus concentrated by a factor of 5000 was prepared from the culture supernatant by Frederick Cancer Research Facilities (Frederick, MD) or by Electronucleonics (Silver Spring, MD). All three preparations contained the envelope glycoproteins gp120 and gp41, the polymerase proteins p53 and p64, and the core proteins p17, p24, and p55, specific for HIV (15).

Immunogens. Native gp120 was purified from HTLV-III_B-infected H9 cells (2) by immunoaffinity chromatography and polyacrylamide gel electrophoresis, as described (8). A deglycosylated form of gp120 was obtained by treatment with endoglycosidase F, as described (9). Recombinant proteins R10, PB1, and 590 (Fig. 1), representing the external (gp120) and/or the transmembrane glycoprotein (gp41) of HTLV-IIIB (8) were produced in *Escherichia coli*. R10 and 590 from lysed cells by pelleting the membrane fraction by centrifugation at 2000 × *g*, solubilizing the proteins in 8 M urea, and separating the proteins by ion-exchange and gel-filtration chromatography (10). PB1 was purified as described (10).

Immunization. Three rabbits were immunized with recombinant peptides R10, PB1, or 590. Two goats were immunized with PB1, and two goats were immunized with 590. Finally, three goats were immunized with native gp120 and one with its deglycosylated form p58. The immunization schedule has been described (8–10).

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Abbreviations: HIV, human immunodeficiency virus; HTLV-III, human T-lymphotropic virus type III; LIF, leukocyte migration inhibitory factor.

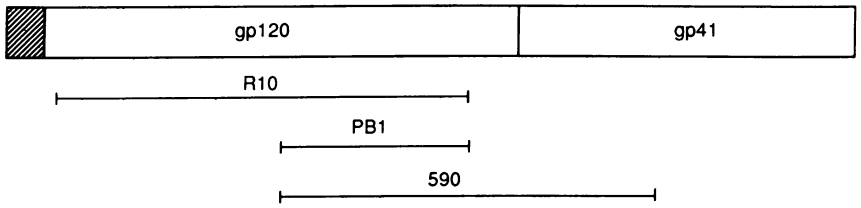

FIG. 1. Schematic representation of the recombinant peptides used for immunization of rabbits and goats.

Immunoblots. The antibody specificity of the sera was tested on immunoblots (14), using proteins from the concentrated HTLV-III$_B$ (Frederick Cancer Research Facilities) as antigen.

Neutralizing Antibody Assay. For assessment of neutralizing antibody activity of the sera, we used a helper T-cell line (ATH8) that is highly susceptible to the cytotoxic activity of HIV (16, 17). The cell line carries the CD4 antigen that is characteristic of helper T cells and thought to be the receptor for HIV (18, 19). The test sera were mixed in different dilutions with 100–500 tissue culture 50% infective dose units of HTLV-III$_B$, HTLV-III$_{MN}$, or HTLV-III$_{RF}$, incubated for 60 min at room temperature, and added to 5×10^3 ATH8 cells, grown in 96-well microtiter plates. The cells were then cultured in RPMI 1640 medium with 10% (vol/vol) fetal calf serum and interleukin 2 at 10%, and fed at 3-day intervals. The cell viability was assessed after 10–14 days of culture by visual inspection. During this time the uninfected cultures had reached a cell density of 10^5 cells per well (see Fig. 3*a*). In contrast, in cultures containing various amounts of virus and no neutralizing antibody, all cells were dead or only a few viable cells were left. Neutralizing titer was defined as the reciprocal of the serum dilution giving 80–100% protection against the cytolytic activity of the virus.

Cell-Mediated Immunity. Peripheral blood mononuclear cells were separated from heparinized blood by Ficoll-Hypaque gradient centrifugation, washed, and cultured in triplicate wells in RPMI 1640 and 10% (vol/vol) autologous plasma. Heat-killed whole HIV virions (56°C for 30 min), native gp120, or the recombinant peptides R10, PB1, or 590 were added to various cultures. Antigen concentrations were 50, 5, and 0.5 μg/ml for the whole virions; 5, 0.5, and 0.05 μg/ml for the recombinant peptides; or 1.0, 0.1, and 0.01 μg/ml for gp120. The cultures were pulse-labeled with [^{3}H]thymidine (0.5 μCi per well; 1 Ci = 37 GBq) on day 5 and harvested on day 6. Incorporated [^{3}H]thymidine was measured in a scintillation counter.

Leukocyte migration inhibitory factor (LIF) activity was assessed at day 5 as described (20), using human polymorphonuclear cells as targets for migration inhibition. An inhibition of migration of 20% or more (LIF index, <80) has been shown to correlate to a positive cellular immune response (20).

RESULTS

Immunoblots. Rabbits or goats immunized with either the native gp120 or with the recombinant peptides R10 or PB1 recognized gp120 in immunoblots (Fig. 2). In addition, some of these animals produced antibodies that reacted with molecules (gp82/86 and gp47) that may represent fragments of the external envelope glycoprotein. Animals immunized with recombinant peptide 590 produced antibodies that reacted with the envelope fragment gp82/86 and faintly with the transmembrane glycoprotein gp41.

Neutralizing Antibodies. In typical experiments (Fig. 3) serum with neutralizing antibodies could be easily distinguished from non-neutralizing serum. All four goat sera obtained by immunization with native gp120 or p58 could neutralize HTLV-III$_B$ infection *in vitro* (Table 1). However, no neutralization was observed with the other two HIV isolates, HTLV-III$_{MN}$ and HTLV-III$_{RF}$. It is noteworthy that goat 508, immunized with a deglycosylated preparation of purified gp120, also had neutralizing antibodies. In this respect our results with ATH8 assay are similar to those obtained earlier (10) with these same goat sera, but using the noncytolytic H9 cell as the target for HIV.

Neutralizing antibodies, although at lower titers, were also observed in the sera of rabbits and goats immunized with recombinant envelope proteins (Table 1). Interestingly, although the rabbit sera against recombinant protein R10 showed high-titer antibodies to gp120 in immunoblots, no neutralizing antibodies were observed. In contrast, sera against recombinant protein PB1 had type-specific neutralizing antibodies. Finally, sera against 590, recognizing the transmembrane glycoprotein gp41, but not gp120, had low-titer neutralizing activity against all three HIV isolates used (Table 1).

Cell-Mediated Immunity. The results of T-cell proliferation assays are shown in Fig. 4 and presented in detail in Table 2. All four goats immunized with the native gp120, showed a broad, group-specific T-cell proliferative response toward all three viral isolates tested. The group-specific nature of the reaction was further demonstrated by using purified gp120 from two different isolates, one being the same and the other one different from the antigen used for immunization (Table 2). It is worth noting that even goat 508, which was immunized with the deglycosylated envelope protein, had a group-specific T-cell response. This response, however, took longer to appear and was demonstrable only after >6 months of immunization. In contrast, goats immunized with two recombinant peptides, PB1 and 590, although having neutralizing antibodies, did not show any T-cell proliferative response toward the viral isolates or the purified envelope glycopro-

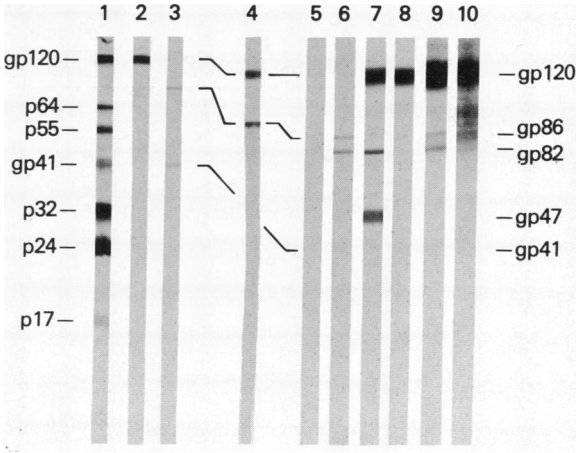

FIG. 2. Immunoblot analysis of the antisera. All strips contain disrupted HTLV-III$_B$, incubated with HIV antibody from an HIV-positive human individual (lane 1), rabbit sera against PB1 (lane 2), 590 (lane 3), or R10 (lane 4); or goat sera against 590 (goats 989 and 991 in lanes 5 and 6, respectively), PB1 (goats 987 and 988 in lanes 7 and 8, respectively), or native gp120 (goats 517 and 2935 in lanes 9 and 10, respectively).

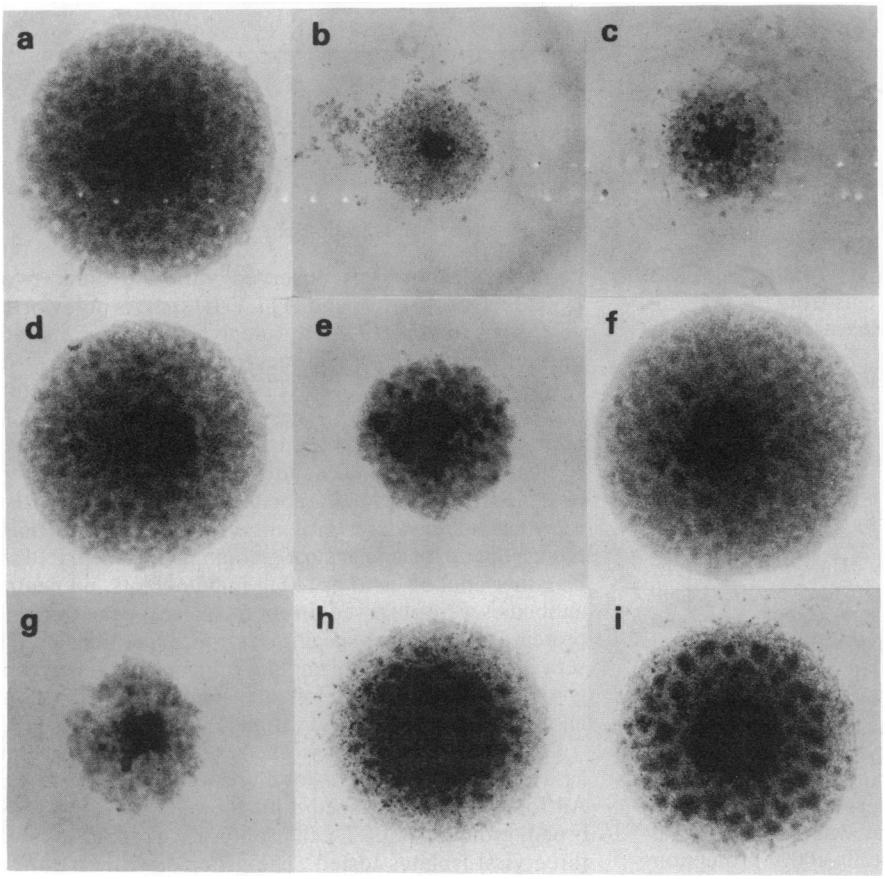

FIG. 3. Representative examples of the neutralization assay. Microtiter wells contain ATH8 cells, cultured for 10 days without (*a*) or with (*b–i*) HTLV-III$_B$, 100 tissue culture 50% infective dose units per well. Wells also contained the following: no serum (*a* and *b*), normal human serum (*c*), antibody positive human serum at 1:80 dilution (*d*), antibody positive human serum at 1:320 dilution (*e*), goat serum 2935 at 1:1280 dilution (*f*), goat serum 2935 at 1:2560 dilution (*g*), goat serum 517 at 1:160 dilution (*h*), and goat 987 serum at 1:80 dilution (*i*).

tein. However, these goats had a good response to the recombinant peptide used for immunization.

The T-cell-specific nature of the proliferative response was identified by measuring the number of B cells (<3%) in the cultures at the end of the culture period and by measuring the amount of LIF, a lymphokine known to be associated with the cellular immune response, in the supernatants of the cultures. All cultures that could incorporate [^{3}H]thymidine produced LIF (Table 2).

Proliferative T-cell responses to HTLV-III$_B$ or to gp120 were assessed in eight HIV-infected individuals with different clinical symptoms. No response toward the virus or purified gp120 was seen (Table 3). Addition of 2′,3′-dideoxyadenosine to the cultures at concentrations shown to

Table 1. Neutralizing antibody titer of serum of animals immunized with native or recombinant HTLV-III$_B$-derived envelope proteins

		Immunoblot		Neutralizing antibody titer†		
Animal	Immunogen	Antigen	Titer*	HTLV-III$_B$	HTLV-III$_{MN}$	HTLV-III$_{RF}$
Goat						
505	gp120‡	gp120,82/86	800	80	0	0
508	p58§	gp120,82/86	800	80	0	0
517	gp120‡	gp120,82/86	1600	160	0	0
2935	gp120‖	gp120,82/86	3600	1280	0	0
987	PB1	gp120,82,47	800	40	0	0
988	PB1	gp120	1600	80	0	0
989	590	−,82	0	0	0	0
991	590	gp41,82/86	400	40	80	20
Rabbit						
1	R10	gp120,86	3600	0	0	0
2	PB1	gp120,82/86	800	80	0	0
3	590	gp41,82/86	800	80	20	20
Control						
goat	None	−	0	0	0	0

*Reciprocal of serum dilution showing immunoblot reactivity to gp120 (to gp41 in animals immunized with peptide 590).
†Reciprocal of serum dilution giving >80% protection for HTLV-III-induced cytolysis of ATH8 cells.
‡PAGE-purified gp120.
§Deglycosylated gp120.
‖gp120 purified with immunoaffinity column chromatography.

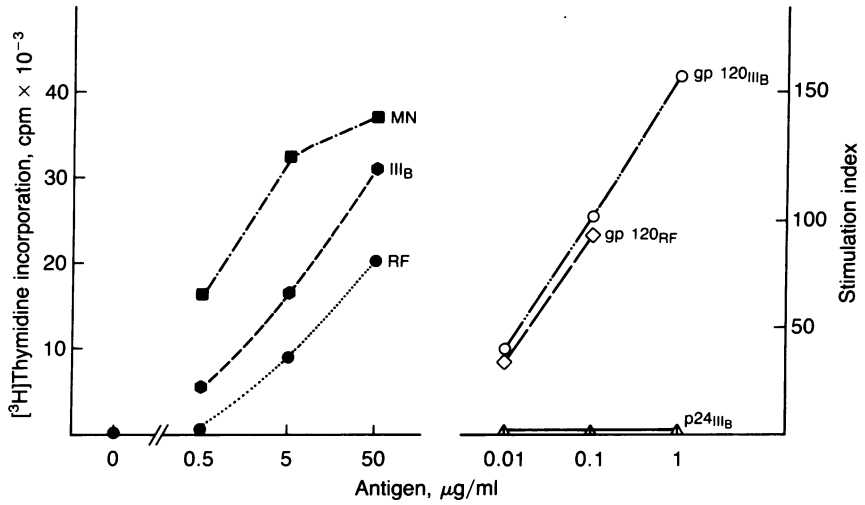

FIG. 4. Blastogenic T-cell responses, indicated both as cpm and as stimulation indices (cpm with antigen/cpm without antigen), in goat 2935 immunized with native gp120. A dose-dependent response toward all three HIV isolates (MN, III_B, or RF; *Left*) and toward purified gp120 of III_B and RF is seen (*Right*).

prevent the expression of HIV (17) did not increase the T-cell responses (data not shown).

DISCUSSION

Analysis of the genomic structure of individual HIV isolates has demonstrated up to 30% variability. Because heterogeneity is greatest in the envelope glycoprotein (3), scepticism has been raised as to the future prospects for a HIV vaccine. The external HIV envelope glycoprotein gp120, which theoretically would be the prime target for a protective immune response toward HIV, contains conserved and variable regions (3, 4). Moreover, computer-assisted analysis predicted highly immunogenic epitopes within the variable and conserved regions of gp120, as well as in the conserved sequences of the transmembrane envelope glycoprotein gp41 (14). Programs to reveal sequences meeting the predicted criteria for T-cell epitopes (21) have also been used for the gp120 molecule (22).

In our present work, neutralizing and non-neutralizing epitopes were demonstrated on gp120. Antisera raised against the recombinant peptide R10, reacting strongly with gp120 in immunoblots, had no neutralizing activity, whereas anti-PB1 had a relatively potent neutralizing effect. The strongest neutralization was seen, however, with the antisera against the native glycosylated envelope protein gp120.

The demonstration of group-specific neutralizing activity in the rabbit and goat sera raised with recombinant peptide 590 is of interest, as this may be due to antibodies against gp41. Earlier studies have indicated that gp41 in fact contains neutralizing epitopes (23), whereas antibodies against one immunogenic region, p121, were not neutralizing. gp41 has three hydrophobic regions (14) and may thus span the viral membrane more than once, forming external structures that could act as targets for neutralizing antibodies. In our work with sera from HIV-infected men (24), neutralizing activity correlated better with antibody activity to gp41 than with activity toward gp120.

The present work shows that gp120 carries also an immunogenic T-cell epitope, which will elicit a group-specific cellular immune response. The importance of glycosylation for this T-cell epitope is still unclear. No T-cell response toward gp120 or toward the whole virion was seen in animals immunized with nonglycosylated recombinant proteins, suggesting that the sugar moieties are necessary for an immune response. Results with the deglycosylated native protein p58 are inconclusive, because the deglycosylation may have been incomplete resulting in an immunogen that contained some glycosylated species. Other alternative explanations for the lack of an HIV-targeted T-cell response in animals immunized with the recombinant peptides 590 or PB1 are differences in the folding of these *env*-derived proteins, interference by nonviral sequences at the ends of these proteins, and the possibility that the epitope is either located outside these peptides or is conformational, requiring the proper folding of extended regions, if not the entire envelope protein.

The nature of the T-cell-specific epitope(s) on gp120 is unknown at present, but it may be related to the "fusion epitope," responsible for binding HIV to its target cell (18, 19), which must also be group specific. Binding of gp120 to CD4 requires proper glycosylation, as the deglycosylated molecule p58 shows only weak binding and the recombinant peptides R10 and PB1 do not bind at all to CD4 (9). The

Table 2. T-cell responses assessed as stimulation indices or LIF indices in goats immunized with recombinant HTLV-III_B envelope proteins

		Stimulation index*								LIF index† for gp120 from III_B
		HTLV-III			gp120		Recombinant			
Goat	Immunogen	B	RF	MN	B	RF	R10	PB1	590	
505	gp120	65	55	75	90	45	110	110	10	45
508	p58	20	35	45	80	35	60	45	5	40
517	gp120	120	100	95	75	55	85	75	15	42
2935	gp120	125	85	140	100	95	85	35	20	60
987	PB1	<2	<2	<2	<2	<2	5	150	<2	98
988	PB1	<2	<2	<2	<2	<2	1	10	<2	112
989	590	<2	<2	<2	<2	<2	1	1	<2	107
991	590	<2	<2	<2	<2	<2	10	50	<2	96
Control	–	<2	<2	<2	<2	<2	<2	<2	<2	102

*Stimulation index = cpm of culture with antigen/cpm of culture without antigen.
†LIF index = (area of migration with antigen/area of migration without antigen) × 100.

Table 3. Proliferative T-cell responses to whole heat-inactivated HIV, to purified gp120, to envelope-related recombinant-proteins, and to purified protein derivative in HIV-infected and control individuals

Individual	HIV antibody	Clinical status	[^{3}H]Thymidine incorporation, cpm					
			Control	HTLV-III$_B$	gp120	PB1	R10	PPD
1	+	LAS	141	164	130	109	101	438
2	+	ARC	160	401	185	147	181	2822
3	+	LAS	140	212	281	189	270	2790
4	+	AIDS	50	94	153	147	117	155
5	+	AIDS	100	100	178	241	253	1291
6	+	ARC	100	250	ND	114	155	1708
7	+	ASX	400	549	ND	432	497	1363
8	+	ASX	1000	646	ND	746	426	5363
9	−	Control	100	456	144	428	284	9374
10	−	Control	150	247	163	545	749	1599

LAS, lymphadenopathy syndrome; ARC, acquired immunodeficiency syndrome-related complex; ASX, asymptomatic; PPD, purified protein derivative.

possibility that the fusion epitope on gp120 is also the main T-cell epitope could explain the lack of T-cell response toward HIV in infected individuals, as observed in the present work and by others (25). Based on blocking studies with monoclonal antibodies against different parts of CD4 (26), we have suggested (27), that the gp120 fusion site three-dimensionally mimics the nonpolymorphic region of the HLA class II molecule that normally binds to CD4. Thus the internal tolerance toward "self" structures would prevent the T-cell response, but not the B-cell response, toward a corresponding structure on gp120. Another possible explanation is the general immune deficiency caused by the infection (28) or viral proteins that could suppress the *in vitro* response (29–31). Our results (Table 3) demonstrate that no T-cell response toward the whole HIV virions was seen in the infected individuals even when their T-cell response toward other foreign antigens, such as tuberculin purified peptide derivative, was intact or only slightly decreased. Moreover, anergy was also observed when purified envelope glycoprotein gp120 or recombinant envelope proteins were used as antigens. These data, therefore, argue against the general immunodeficiency or a suppressive viral protein being the cause for the anergy against HIV in man.

In summary, we have shown potent antigenic epitopes on the envelope glycoprotein of HIV, capable of inducing neutralizing antibodies and a T-cell-specific immune response in immunized animals. Whether the same epitopes are also immunogenic in man and whether they can induce a protective immune response toward subsequent exposure to HIV is a question that will require more direct experiments.

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