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Immunology

Priming of CD4$^+$ T cells specific for conserved regions of human immunodeficiency virus glycoprotein gp120 in humans immunized with a recombinant envelope protein

(AIDS/vaccination)

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ABSTRACT **A nonglycosylated denatured form of human immunodeficiency virus (HIV) 1 glycoprotein gp120 (Env 2-3), which does not bind to CD4, was used with muramyl tripeptide as adjuvant to immunize HIV-seronegative healthy volunteers. In all the volunteers, three 50-μg injections of Env 2-3 induced priming of CD4$^+$ T cells specific for conserved regions of the native glycosylated gp120. Moreover, we found that several major histocompatibility complex class II (*DR*) alleles can function as restriction molecules for presentation of conserved epitopes of gp120 to T cells, implying that a T-cell response to these epitopes can be obtained in a large fraction of the population. The possibility to prime CD4$^+$ T cells specific for conserved epitopes of a HIV protein is particularly important in view of the lack of such cells in HIV-infected individuals and of a possible role that CD4$^+$ T cells may play in the development of protective immunity against AIDS.**

Two major problems in designing a human immunodeficiency virus (HIV) vaccine are the considerable sequence heterogeneity among HIV isolates (1) and the lack of information on protective immunity. Infected individuals develop antibody (2, 3) and CD8$^+$ T-cell (4, 5) responses to HIV but are not protected, since they eventually develop AIDS. Surprisingly, the CD4$^+$ T-cell response to HIV proteins is weak or undetectable, even at early stages of infection when CD4$^+$ T-cell responses to other antigens are still normal (6–9). Thus, the lack of HIV-specific CD4$^+$ T cells is not satisfactorily explained by any of several hypotheses (10–12) that have been put forward to account for the impairment of CD4$^+$ T cells occurring late in HIV infection.

A major goal of the immunization trial herein reported was to prime a CD4$^+$ T-cell response toward parts of the envelope glycoprotein gp120 shared by highly divergent HIV isolates. Since native gp120 might cause immunosuppression because of its high-affinity binding to CD4 (13), a genetically engineered gp120 was produced in yeast using an intracellular expression vector (14). This envelope protein (designated Env 2-3) is nonglycosylated and was purified after denaturation with SDS. Env 2-3 was chosen as immunogen because it does not bind to CD4 and, therefore, avoids possible immunosuppressive or pathological effects caused by the targeting of native glycosylated gp120 to CD4 (10–12).

Env 2-3 from the HIV isolate SF/2 (Env 2-3 SF/2) was used to carry out a phase 1 immunization trial on HIV-seronegative healthy individuals. We report here that volunteers immunized with Env 2-3 developed an effective priming of CD4$^+$ T cells specific for conserved regions of HIV gp120. In addition, several major histocompatibility complex (MHC) class II (*DR*) alleles can present conserved epitopes of gp120 to T cells, indicating that MHC polymorphism is not a major constraint for the priming of such cells.

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MATERIALS AND METHODS

Immunogen. Env 2-3 SF2 is a nonglycosylated 55-kDa polypeptide with an amino acid sequence corresponding to the gp120 envelope glycoprotein of the HIV SF2 isolate. Production and purification of Env 2-3 have been described (14).

Immunization Procedures of Volunteers. As reported in detail elsewhere (J.W., M.J., S.A., D.M., T.S., K.S.S., and A.C., unpublished results), we used three vaccination protocols to immunize healthy seronegative male volunteers aged 20–60 years. Briefly, volunteers received (*i*) three intramuscular injections of either 50 μg or 250 μg of Env 2-3 SF2 or (*ii*) six intramuscular injections of 250 μg of Env 2-3 SF2, all given with 100 μg of lipophilic muramyl tripeptide as adjuvant (15). The data reported here refer to the four volunteers immunized with three injections of the lower Env 2-3 dose (50 μg), since this dose was the more effective dose. In fact, only four out of seven volunteers who received the scheduled injections of 250 μg of Env 2-3 showed a significant T-cell response to gp120 (data not shown).

Antigens Used for *in Vitro* Studies. The native glycosylated 120-kDa gp120 SF2 was produced in CHO cells and purified as described (16). The denatured nonglycosylated envelope proteins Env 2-3 IIIb and Env 2-3 Zaire6 were produced in yeast and purified as described (14, 17). Env 2-3 SF2 [D1–5] is a deletion mutant of Env 2-3 SF2, whose five major hypervariable regions (amino acid positions 131–154, 156–198, 300–332, 388–414, and 456–463) had been deleted by using *in vitro* site-directed mutagenesis. Production in yeast and purification of Env 2-3 SF2 [D1–5] will be reported elsewhere (N.L.H., unpublished work).

Flow Cytometric Analysis. CD4$^+$ T cells (from an established tetanus-toxoid-specific clone) were incubated at 5×10^5 cells per ml for 8 hr at 37°C in the presence either of medium or of various concentrations of gp120 SF2 or Env 2-3 SF2, washed two times, and incubated for 30 min at 4°C with a phycoerythrin-conjugated anti-CD4A monoclonal antibody (mAb; Leu3a; Becton Dickinson) at 2 μg/ml. Cells were then washed twice and fluorescence intensity was determined on a FACStar flow cytometer (Becton Dickinson).

Abbreviations: HIV, human immunodeficiency virus; PBMC, peripheral blood mononuclear cell; APC, antigen-presenting cell; MHC, major histocompatibility complex; EBV-B, Epstein–Barr virus-transformed B cells; mAb, monoclonal antibody.

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Cell Cultures. The medium was RPMI 1640 medium supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 1 mM sodium pyruvate, penicillin (100 units/ml), streptomycin (100 μg/ml), and 5% (vol/vol) human serum (RPMI-HS). For the growth of T-cell clones, RPMI-HS was supplemented with human recombinant interleukin 2 at 100 units/ml (a generous gift of Hoffmann–La Roche).

Isolation of T-Cell Clones and Epstein–Barr Virus-Transformed B (EBV-B) Cells. Ficoll-separated peripheral blood mononuclear cells (PBMCs) were cultured at 8×10^5 cells per ml in RPMI-HS with recombinant native glycosylated gp120 SF2 at 3 μg/ml in 200-μl cultures in flat-bottom microtiter plates. After 7 days, interleukin 2 was added at 30 units/ml and, after an additional 5 days, cultures were cloned by limiting dilution (0.3 cell per well) in the presence of irradiated allogeneic PBMCs, phytohemagglutinin (1 μg/ml; Wellcome), and interleukin 2 (100 units/ml) in 20-μl cultures in Terasaki trays. The T-cell clones obtained were screened for their capacity to proliferate in response to various envelope proteins in the presence of autologous irradiated (6000 R; 1 R = 0.258 mC/kg) EBV-B cells as antigen-presenting cells (APCs). The conditions for maintenance of T-cell clones have been described elsewhere (18). EBV-B cell lines were obtained and maintained as described (18). MHC-homozygous EBV-B cell lines (see Table 2) were obtained from the European Collection of Animal Cell Cultures (ECACC, Salisbury, U.K.).

PBMC Proliferation Assay. Ficoll-separated PBMCs (1×10^5 cells) in 0.2 ml of RPMI-HS were cultured in 96-well flat-bottom microplates alone or in the presence of recombinant HIV-1 envelope proteins at 3 μg/ml. After 6 days 1 μCi of [^{3}H]thymidine (Amersham; specific activity, 5 Ci/mmol; 1 Ci = 37 GBq) was added and the radioactivity incorporated was measured after an additional 16 hr by liquid scintillation counting. The data represent the mean cpm of triplicate cultures.

T-Cell Clone Proliferation Assay. Approximately 2×10^4 T cells with 1.5×10^4 irradiated (6000 R) EBV-B cells in 0.2 ml of RPMI-HS were cultured in 96-well flat-bottom microplates alone or in the presence of recombinant HIV-1 envelope proteins as indicated in table legends. After 2 days 1 μCi of [^{3}H]thymidine was added and the radioactivity incorporated was measured as above.

RESULTS

Denatured Nonglycosylated Envelope Protein (Env 2-3) Does Not Bind to CD4. To assess binding of Env 2-3 to CD4, CD4$^+$ T cells were preincubated with various concentrations of either Env 2-3 or native gp120 and then stained with an anti-CD4A mAb. Preincubation with native gp120 (Fig. 1*a*) reduced in a dose-dependent fashion binding of the anti-CD4A mAb, whereas preincubation with Env 2-3 (Fig. 1*b*), at the same concentrations as native gp120, does not affect binding of the mAb, as shown by the coincidence of the staining curves. This demonstrates that Env 2-3 neither competes with an anti-CD4A mAb for binding to CD4 nor down-regulates CD4. In addition, we exploited the ability of human T cells to process and present only antigens that bind to their own surface molecules (11). We found that native gp120 is presented by activated CD4$^+$ T cells to MHC class II-restricted gp120-specific T cells, whereas Env 2-3 is not (data not shown). Thus these data demonstrate that Env 2-3 avoids two of the major concerns raised on the use of gp120 (i.e., down regulation of CD4 and antigen presentation by T cells) and indicate that Env 2-3 does not bind to CD4.

Immunization with Env 2-3 Primes T Cells Specific for Native gp120. The effect of immunization with Env 2-3 SF2 was first assessed by measuring, at different times after injection, the proliferative response of PBMCs to the immunogen. Fig. 2 shows that four out of four volunteers immunized three times with 50 μg of Env 2-3 SF2 developed a proliferative response to the immunogen. Only one volunteer responded after the first injection, but all responded after the second booster injection, and these responses were still present 6 months after the last injection.

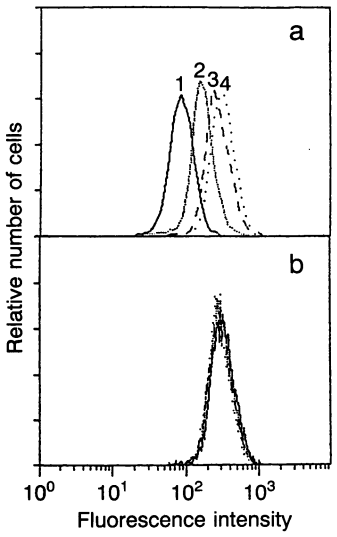

FIG. 1. Denatured nonglycosylated envelope protein (Env 2-3) does not compete with an anti-CD4A mAb for binding to CD4. Human CD4$^+$ T cells were preincubated with medium alone (curve 4) or with native gp120 SF2 at 1 μg/ml (curve 3), 3 μg/ml (curve 2), or 9 μg/ml (curve 1) (*a*) or with denatured Env 2-3 SF2 (*b*). Cells were then stained with a phycoerythrin-conjugated anti-CD4A mAb and analyzed by flow cytometry.

Fig. 2 also shows that the PBMCs from all four volunteers proliferated as well to a recombinant native glycosylated gp120 SF2 produced in mammalian cells. The proliferating cells were CD3$^+$, as detected by immunofluorescent staining of blast cells (data not shown). These results demonstrate that immunization with a denatured nonglycosylated envelope protein can induce effective priming of T cells that recognize epitopes generated by processing of the native glycosylated gp120.

Recognition of Highly Divergent HIV Envelope Proteins. To assess whether primed T cells recognize conserved regions of gp120, we tested the proliferative response to envelope proteins from various HIV isolates. Fig. 3 shows that PBMCs from all four volunteers proliferate not only in response to gp120 from the HIV isolate SF2 but also to envelope proteins from two highly divergent HIV-1 isolates, IIIb and Zaire6 (Zr6), which differ from SF2 in the amino acid sequence by 17% (1) and 28% (17), respectively. In addition, the same T cells proliferate also in response to a truncated envelope protein whose five major hypervariable regions (19) had been deleted (Env 2-3 SF2 [D1–5]). These results indicate that in all four volunteers conserved regions of gp120 can be targets for T-cell recognition.

Isolation of gp120-Specific T-Cell Clones. To better characterize phenotype and specificity of the gp120-specific T cells, we established more than 200 specific T-cell clones from the PBMCs of the vaccinated individuals. All these clones proliferate in response to gp120 SF2 in the presence of irradiated autologous PBMCs or EBV-B cells as APCs. All the clones are CD3$^+$, WT31$^+$, CD4$^+$, and CD8$^-$, and most are HLA-DR-restricted since the proliferative response to gp120 is inhibited by an anti-HLA-DR (clone L-243), but not by an anti-HLA class I (clone W6/32) antibody (data not shown). Although we repeatedly attempted both to stimulate CD8$^+$ cells sorted from PBMCs and to clone the few (5–10%) CD8$^+$

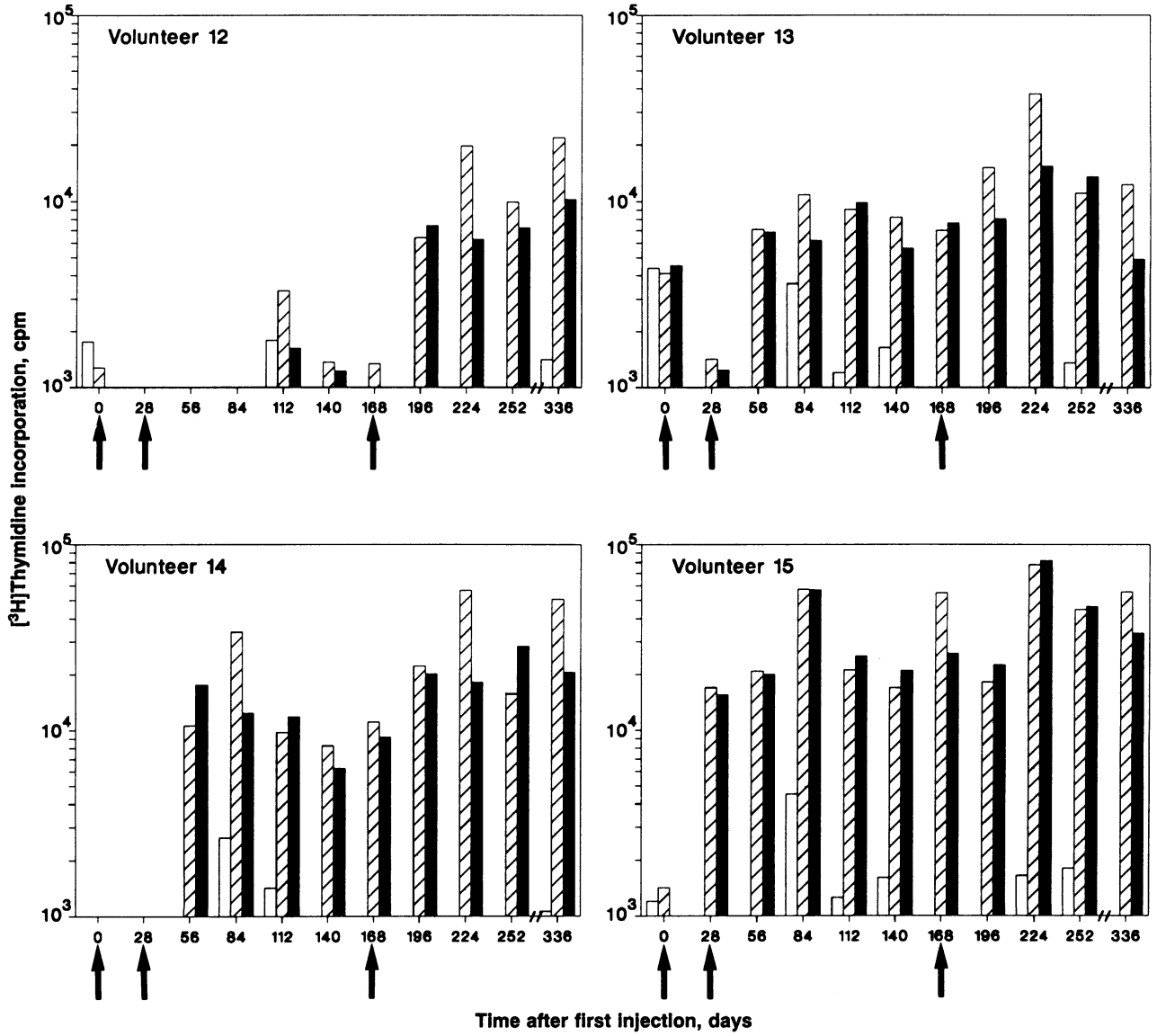

FIG. 2. Volunteers immunized with Env 2-3 develop specific T-cell response to gp 120. Development of PBMC proliferative response to Env 2-3 SF2 (hatched bars), gp120 SF2 (solid bars), or medium alone (open bars) in the volunteers that received three 50-μg injections of Env 2-3. Arrows indicate the time of immunization. Proliferation was assessed at all the days indicated on the x axis. On the days of immunization, proliferation assay was performed on blood samples collected just prior to injection.

T cells sorted from the antigen-expanded cell lines, we always failed, as expected, to isolate $CD8^+$ clones specific for soluble gp120.

The fine specificity of T-cell clones was determined using envelope proteins from three HIV isolates as antigens. Table 1 shows the proliferative response of a few representative clones. Three patterns of antigen specificity can be identified: (*i*) T cells recognizing only one envelope type, indicating that they are specific for variable regions of the envelope. (*ii*) T cells specific for relatively conserved regions of gp120, since they proliferate in response to two of the three envelope proteins. (*iii*) T-cell clones specific for conserved regions of gp120, since they recognize envelope proteins from all three isolates. These results demonstrate that several epitopes of gp120 are immunogenic for $CD4^+$ T cells and that T cells can recognize gp120 epitopes conserved among three highly divergent HIV isolates.

Conserved Epitopes of gp120 Can Be Recognized in Association with Several *DR* Alleles. Since a vaccine ideally should be effective in all individuals, it is important to know whether the MHC polymorphism would be a major constraint to the priming of T-cell response. We, therefore, determined the HLA types of the volunteers and mapped the restriction of some T-cell clones specific for conserved epitopes using allogeneic HLA-homozygous EBV-B cell lines as APCs. The data in Table 2 show that conserved epitopes of gp120 can be presented by all the *DR* alleles present in the four volunteers. Moreover, we found that some T-cell clones recognize gp120 not only in association with a self DR molecule but also with some other *DR* alleles. This "promiscuous" recognition of gp120 by T cells, which has been described also in DR-restricted T-cell responses to other antigens (20), is a further indication that immunogenic peptides of the conserved regions of gp120 can bind to and be recognized in association with several *DR* alleles. We conclude that HLA polymorphism is not a major constraint for the generation of T-cell response to conserved epitopes of gp120.

DISCUSSION

A major goal of our study was to develop a safe vaccine capable of priming, in all individuals, gp120-specific $CD4^+$ T

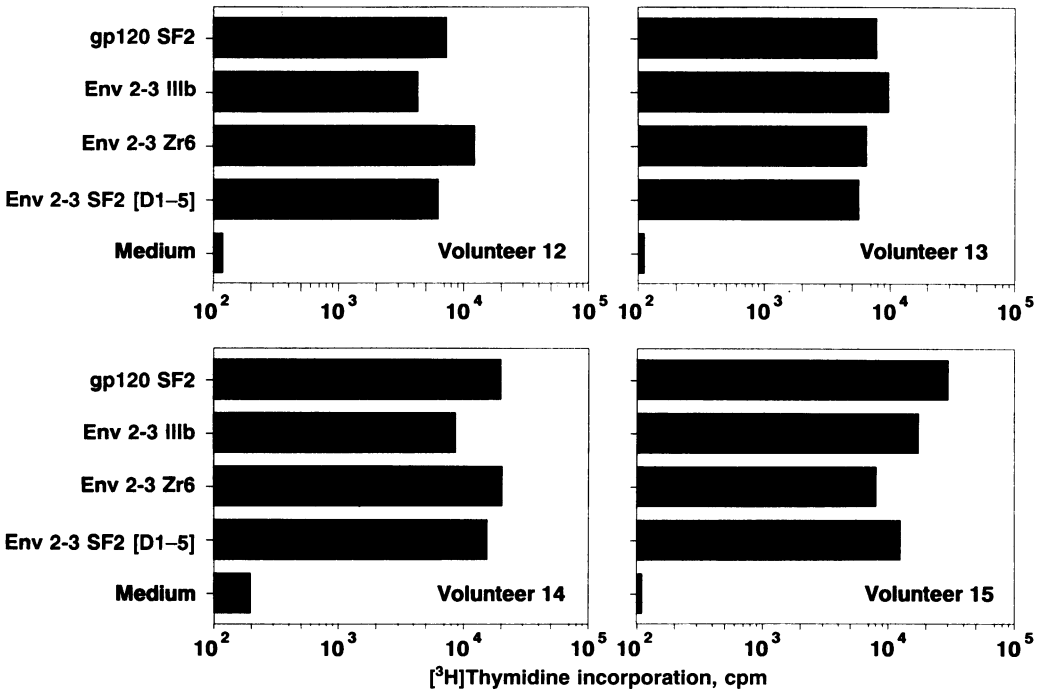

FIG. 3. Immunization with Env 2-3 elicits T-cell responses directed toward conserved regions of gp120. Proliferative response of PBMCs obtained 1 month after the last booster injection (day 196) cultured in the presence of envelope proteins from three highly divergent HIV isolates [SF2, IIIb, and Zaire6 (Zr6)], as well as of an HIV-SF2 envelope protein (Env 2-3 SF2 [D1–5]) whose five major hypervariable regions had been deleted.

cells against gp120 epitopes that are conserved in highly divergent HIV isolates.

Our results show that the recombinant protein Env 2-3 satisfies all these requirements. (*i*) It does not bind to CD4. Thus, it avoids undesirable effects related to the high-affinity binding of native gp120 to CD4 (10–12). (*ii*) It primes gp120-specific CD4$^+$ T cells in all volunteers tested. (*iii*) A fraction of the T cells, in each vaccinated individual, responds to gp120 from three of the most highly divergent HIV-1 isolates and, therefore, can be considered specific for conserved epitopes of gp120. (*iv*) These conserved epitopes are recognized in association with several *DR* alleles, indicating that a large fraction of the population can respond to such epitopes.

Table 1. gp120-specific T-cell clones recognize different epitopes

	[^{3}H]Thymidine incorporation of T-cell clones, cpm × 10^{-3}		
T-cell clone	gp120 SF2	Env 2-3 IIIb	Env 2-3 Zr6
12.a	27	<1	<1
13.a	67	<1	<1
14.a	76	<1	<1
15.a	53	<1	<1
12.b	21	<1	22
13.b	14	19	<1
14.b	23	16	<1
15.b	58	29	<1
12.c	31	17	18
12.c1	20	14	23
13.c	51	39	33
13.c1	26	45	12
14.c	45	56	51
15.c	32	21	27

T-cell clones were cultured in the presence of various recombinant envelope proteins [gp120 SF2, Env 2-3 IIIb, or Env 2-3 Zaire6 (Zr6)] at 3 μg/ml (see Fig. 3) or medium alone and irradiated autologous EBV-B cells as APCs. Thymidine incorporation was measured after 2 days. Thymidine incorporation of T-cell clones in the presence of EBV-B cells and medium alone was always less than 10^3 cpm. The identification number of T-cell clones corresponds to the volunteer's number indicated in Fig. 2.

All T-cell clones isolated are CD4$^+$ and MHC class II-restricted, as expected from immunization with a soluble antigen. These cells exerted helper activity *in vivo*, since gp120-specific antibodies were detected, although at low titers, in sera of vaccinated individuals (J.W., M.J., S.A., D.M., T.S., K.S.S., and A.C., unpublished results).

Priming of gp120-specific CD4$^+$ T cells by immunization contrasts with what is found in HIV-infected individuals. Indeed, several studies have reported that CD4$^+$ T cells specific for HIV proteins were not detectable, even at early stages of HIV infection when CD4$^+$ T-cell responses to other antigens were still normal (6–9). The reason for the apparent absence of gp120-specific CD4$^+$ T cells is not clear. (*i*) Perhaps gp120-specific CD4$^+$ T cells are not primed by HIV infection because of the peculiar mode of antigen presentation (11). This possibility is rather unlikely, since infected individuals have good anti-HIV antibody and CD8$^+$ T-cell responses, both requiring CD4$^+$ T-cell help (for review, see ref. 21). (*ii*) Perhaps CD4$^+$ T cells are primed by HIV infection but recognize only variable regions of gp120. Such T cells would not be detected in proliferation assays, which are usually performed with recombinant proteins from HIV isolates different from the patients' isolates. (*iii*) Perhaps they are infected by HIV present in gp120-presenting macrophages, which results in their selective elimination.

Whatever the reason, the fact remains that CD4$^+$ T cells are the only component of the anti-HIV immune response lacking or defective in HIV-infected subjects. Our data show that CD4$^+$ T cells can be primed *in vivo* using a recombinant envelope protein as a vaccine. We do not know whether the ability to prime CD4$^+$ T cells specific for conserved epitopes of gp120 is a peculiar feature of the denatured nonglycosylated protein Env 2-3. Thus, our data should be compared with those obtained in vaccination trials carried out with recombinant native gp120.

Table 2. HLA restriction of T-cell clones specific for conserved epitopes of HIV gp120

APC	DR phenotype	[^{3}H]Thymidine incorporation, cpm × 10^{-3}					
		12.c	12.c1	13.c	13.c1	14.c	15.c
EDR	1	<1	<1	<1	32	<1	<1
NOL	2[w15]	<1	<1	106	<1	<1	65
AVL	3[w18],w52a	<1	16	<1	<1	<1	<1
BSM	4,w53	<1	<1	<1	<1	<1	<1
ATH	5[w11],w52b	<1	<1	75	<1	<1	<1
APD	6[w13],w52b	25	<1	<1	<1	27	<1
EKR	7,w53	<1	<1	<1	64	<1	<1
LUY	8	<1	<1	<1	<1	<1	<1
DKB	9,w53	<1	<1	<1	<1	<1	<1

HLA-homozygous EBV-B cells were preincubated at 4×10^5 cells per ml with Env 2-3 SF2 (5 μg/ml) for 12 hr at 37°C, washed three times, irradiated, and tested for their capacity to trigger proliferation of various T-cell clones specific for conserved epitopes of gp120 (as shown in Table 1). T-cell clone proliferation to EBV-B cells preincubated with medium alone was always less than 10^3 cpm. The DR phenotype of the volunteers was as follows: volunteer 12, 3,6(w13),w52; volunteer 13, 2(w15),7,w53; volunteer 14, 2(w15),6,w52; volunteer 15, 2(w15),6,w52.

It is tempting to speculate that priming by immunization of $CD4^+$ T cells specific for conserved epitopes of gp120 could result, upon subsequent infection with HIV, in an anamnestic response of T cells that may be critical for the induction of both humoral and cellular protective immunity.

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