JOURNAL OF VIROLOGY, Apr. 1995, p. 2357–2365
0022-538X/95/$04.00+0

Vol. 69, No. 4

Immunogenic Targeting of Recombinant Peptide Vaccines to Human Antigen-Presenting Cells by Chimeric Anti-HLA-DR and Anti-Surface Immunoglobulin D Antibody Fab Fragments In Vitro

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Received 19 October 1994/Accepted 15 January 1995

To increase the inherently weak immunogenicity of synthetic peptide vaccines, we used recombinant DNA techniques to generate chimeras between immunogenic determinants of human immunodeficiency virus type 1 (HIV-1) gp120 and antibody Fab fragments reactive with surface structures displayed specifically on human antigen-presenting cells (APCs), including surface immunoglobulin D (sIgD) and class II major histocompatibility complex (MHC) molecules. Hybridomas producing anti-human MHC class II (HLA-DR) or surface immunoglobulin D monoclonal antibodies (MAbs) that recognize nonpolymorphic determinants were used to clone chimeric Fab gene fragments by employing an established procedure to generate antigen-binding Fab libraries in phagemid vector pComb3. Molecular and immunochemical analysis indicated that the expected chimeric Fab fragments expressing the HIV-1 epitopes were correctly cloned and expressed in *Escherichia coli* and retained the binding specificity of the native (hybridoma-derived) MAb. The chimeric Fab fragments targeted the linked HIV-1-derived antigenic determinants to the surface of human APCs in vitro, as evidenced by fluorescence-activated cell sorter analysis. Furthermore, such recombinant immunotargeted HIV-1 peptide antigens demonstrated improved immunogenicity over equivalent nonimmunotargeted control antigens, as shown by their ability to stimulate interleukin-2 production by $CD4^+$ T-helper cells from human donors exposed to HIV-1 antigens. These data suggest that immunotargeting of recombinant peptide antigens via the attached Fab fragments facilitates uptake by human APCs with subsequent access to the MHC class II processing pathway, thereby validating the immunotargeting concept for such recombinant subunit vaccines in an in vitro human system.

Synthetic peptides constitute one major strategy for the development of a new generation of vaccines against infectious diseases, including AIDS (3–5). Peptides encompassing pathogen-derived T-plus-B-cell epitopes can function as complete immunogens that elicit humoral and cell-mediated immunity (17, 45, 52, 58). The potential advantages offered by such subunit vaccines include easy preparation of a pure immunogen in large quantities, safety, cost effectiveness, stability, and easy storage and delivery. In addition, by selecting defined epitopes that stimulate desirable protective immunity, it may be possible to eliminate pathogen-derived protein sequences that elicit undesirable responses, including general toxicity, immunosuppression, enhancement of antibodies, or autoimmunity, properties ascribed to certain retroviral (including human immunodeficiency virus type 1 [HIV-1]) antigens (8, 11, 12, 34).

One major obstacle to the effective use of synthetic peptide vaccines in humans is inherently weak immunogenicity. Strategies that potentiate the immune response to peptide immunogens are likely to greatly improve the practical value of synthetic peptides as vaccines (4, 17, 52, 58). Since the efficiency of antigen presentation to T cells depends, to a large extent, on the concentration of antigen-major histocompatibility complex (MHC) complexes on the surfaces of antigen-presenting cells (APCs) (29), it can be anticipated that any technique that increases the concentration of immunogenic peptides displayed by APCs will lead to more effective antigen presentation and, hence, successful vaccination. Approaches that have been used to achieve immunotargeting, as evidenced by increased antigen-specific antibody or T-helper (Th) cell responses, include the use of homogeneous antigen-specific B-cell populations as APCs (40, 49) and polyclonal antigen targeting to mouse B cells by using rabbit anti-mouse immunoglobulin (Ig) as an antigen (20, 56).

Another immunotargeting approach involves covalent coupling of protein or synthetic peptide antigens to antibodies specific for determinants displayed on APCs, e.g., surface Ig (sIg) on B cells or anti-MHC class II antibodies on B cells and other APCs such as macrophages or dendritic cells. The feasibility of this approach was established with rodent models by several independent studies. Covalent coupling of protein or short peptide antigens to anti-Ig or anti-MHC antibodies enhanced the specific immune response by 10^1- to 10^3-fold at the level of both antigen-specific T-cell proliferation in vitro (15, 16, 38, 50) and antibody responses in vivo (13, 14, 38, 43). Such antigens were sometimes found to be highly immunogenic in the absence of oil-based adjuvants (13, 14). sIg cross-linking by the antibody was not essential, since monovalent Fab fragments also amplify the immune response, albeit with ~10-fold lower efficiency (15, 16). Several other surface molecules, e.g., class I MHC (16, 50) or a dendritic cell-specific antigen (43), but not CD11/18 (14), CD45, or FcγRII (50), can also serve as targets for antigen focusing.

With covalent coupling, it is nearly impossible to control the antigen-to-immunotargeting antibody ratio or coupling site,

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TABLE 1. Oligonucleotide primers and probes used in this study[a]

Designation	Sequence
cDNA consensus primer	
C_{H2} (γ1, γ2a, γ2b, γ3)	5′-ACCAC(C/A)ACACA(T/C)GTGA-3′
Heavy-chain PCR primers:	
VRP_H	5′-AGGT(C/G)(C/A)A(G/A)CT(G/T)CTCGAGTC(T/A)GG-3′
CRP_{H1} (γ1)	5′-TATGCAACTAGTAGATCTACAATCCCTGGGCACAATTTTCTT-3′
CRP_{H1} (γ2a, γ2b, γ3)	5′-ACAGGGACTAGTAGATCTGGGCACTCTGGGCTCAATTTTCTT-3′
Light-chain PCR primers:	
$VRP_κ$	5′-CCAG(A/T)T(G/C)(T/C)GAGCTC(C/G)(A/T)(C/G)(C/A)T(C/G)AC(C/A)CAG(A/T)CTCCA-3′
V3-$CRP_κ$	5′-GCGCCGTCTAGAATTATTTTCCTATTGTAACAAATGCTCTCCCTGGTCCTCTCTGGATACG GGATCCACACTCATTCCTGTTGAAGCTCTTGAC-3′
ANP-$C_κ$	5′-GCGCCGTCTAGAATTATTTTCCTATTGTAACAAA-3′
Internal oligonucleotide hybridization probes:	
$C_κ$-IP	5′-ACTGCCATCAATCTTCCACTTGAC-3′
C_{H1}-IP	5′-GAA(A/G)TA(G/A)CCCTTGACCAGGCATCC(C/T)AG-3′

[a] CRP, constant-region primer; VRP, variable-region primer; ANP, anchor primer. Underlined nucleotides represent restriction enzyme sites used for cloning.

thus complicating the task of optimizing vaccine design with no (or minimal) batch-to-batch variability. To overcome this difficulty, we constructed chimeric genes that encode fusion proteins of antibody fragments expressing short, immunogenic HIV-1 peptides. Construction of such chimeras at the gene level allows coupling of the desired combination of epitopes to the proper antibodies under precise conditions, including choice of coupling sites and epitope-to-antibody ratio. The availability of convenient restriction sites also allows the preparation of multivalent subunit vaccines by replacement or addition of oligonucleotide sequences that specify defined epitope combinations, e.g., a cocktail of variable pathogen sequences which often coincide with immunodominant epitopes with relative ease, enabling the construction of potential multivalent vaccines.

To determine whether the immunotargeting approach, generally studied only with rodents, can be extended to the human system, chimeric anti-human HLA-DR or sIgD antibodies incorporating an immunodominant V3 loop peptide, P18, and a potent Th epitope, EnvT1 (T1), both derived from the gp120 envelope glycoprotein of $HIV\text{-}1_{IIIB}$, were expressed in *Escherichia coli* and purified as monovalent Fab fragments. These chimeric Fabs bound specifically to human APCs displaying the relevant HLA-DR or sIgD molecules and demonstrated improved immunogenicity as measured by increased stimulation of interleukin-2 (IL-2) production in vitro by human $CD4^+$ Th cells from donors exposed to HIV-1 antigens.

MATERIALS AND METHODS

Cell lines and reagents. Murine hybridomas L203 (IgGγ1, Lκ; ATCC HB1710), BP107.2.2 (IgGγ3, Lκ; ATCC TIB154), and IA6-2 (IgGγ2a, Lκ) were used in this study. Human IgD δ chain and $HIV\text{-}1_{IIIB}$ gp120 were purchased from Chemicon Int., Temecula, Calif., and ABT, Cambridge, Mass., respectively. Purified HLA-DR protein was a kind gift from H. Grey, Cytel Corp., La Jolla, Calif.

RNA isolation and cDNA synthesis. Total RNA from hybridoma cell lines was isolated by the RNAzol B method (Cinna/Biotecx, Friendswood, Tex.). In general, 10^6 cells were lysed in 100 μl of RNazol B solution; this was followed by phenol-chloroform extraction and isopropanol precipitation in accordance with the manufacturer's protocol. Two micrograms of total RNA was used as the template for oligo(dT) (Lκ cloning)- or C_{H2} (Fd cloning)-primed first-strand cDNA synthesis with Superscript RNase H^- reverse transcriptase (Gibco-BRL, Gaithersburg, Md.) by the standard method (see Table 1 for the C_{H2} cDNA primer used).

DNA amplification with consensus Ig PCR primers. Fab-encoding cDNAs were amplified in standard PCRs. The various PCR primers and oligonucleotide probes used are listed in Table 1. The PCR mixture included ~0.5 μg of the cDNA template, 0.3 μg of primers, and deoxynucleoside triphosphate 200 μM each in *Taq* polymerase buffer containing 1.5 mM $MgCl_2$ and 5 U of *Taq* polymerase (Promega, Madison, Wis.) in a final volume of 100 μl. PCRs were conducted with a TwinBlock thermal cycler (Ericomp, San Diego, Calif.). The primers used for amplification of mouse heavy-chain sequences (Fd) were VRP_H and the appropriate subclass-specific CRP_{H1}, and the reactions were performed for 30 cycles under the following conditions: 91°C for 1 min, 52°C for 2 min, 72°C for 1 min, and a final elongation for 15 min at 72°C. To amplify and clone chimeric Lκ fragments encoding the HIV-1 V3 loop peptide (P18) in a single step, we designed hybrid primers containing Lκ-plus-P18-specific nucleotide sequences. The primers used for amplification of mouse chimeric light-chain sequences (Lκ) were VRPκ (5′) and a mixture of 3′ primers consisting of hybrid primer V3-CRPκ and the corresponding primer ANP-Cκ at a molar ratio of 9:1. Temperature cycles were as follows: 5 cycles at 91°C for 1 min, 45°C for 2 min, and 72°C for 1 min; 25 cycles at 91°C for 1 min, 55°C for 2 min, and 72°C for 1 min; and a final elongation step for 15 min at 72°C. The identities of these PCR-amplified fragments were confirmed by Southern hybridization with internal oligonucleotide probes Cκ-IP and C_{H1}-IP (Table 1).

Construction of phage display libraries. Surface expression libraries were constructed in two steps. First, amplified Fd fragments were digested at 37°C for 12 h with an excess of restriction enzymes *Xho*I and *Spe*I and size fractionated by agarose gel electrophoresis. DNA fragments were purified and directionally ligated into *Xho*I-*Spe*I-linearized and phosphatase-treated vector pComb3. Following transformation of *E. coli* XL1-Blue, 100 ml of 2× TB medium (6) containing ampicillin (100 μg/ml) was added and cultures were grown overnight at 37°C. Phagemid DNA containing the Fd fragment library was isolated, linearized with *Sac*I-*Spe*I, and phosphatase treated, and the recombinant 4.7-kb vector fragment was purified by agarose gel electrophoresis. Next, amplified chimeric Lκ fragments were digested with an excess of restriction enzymes *Sac*I and *Xba*I at 37°C for 12 h and size fractionated by agarose gel electrophoresis. The Lκ cDNA fragments were then cloned into the Fd-containing, *Sac*I-*Spe*I-linearized, phosphatase-treated vector pComb3. Following transformation of *E. coli* XL1-Blue, recombinant phage were generated as previously described (6).

Panning of the combinatorial library to select antigen binders. Microtiter plates were coated with 1 μg of purified HLA-DR or IgD protein overnight at 4°C. The wells were blocked, incubated with the phage display libraries, washed, and eluted with 100 mM glycine buffer (pH 2.5). The eluted phage were then used to infect *E. coli* XL1-Blue cultures. New phage stocks were prepared and reincubated with antigen in microtiter wells as described above. After three consecutive rounds of panning, bacterial colonies expressing chimeric Fabs with the desired antigen specificity were detected in a filter assay in which colonies were grown for 16 h at 37°C on Durapore master filters in close contact with a second (Immobilon P) membrane coated with anti-mouse Ig antibodies. This was done on top of Luria broth agar dishes containing 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 100 μg of ampicillin per ml. Secreted bacterium-derived Fabs diffusing onto the second membrane were thereby immobilized by the capture antibodies and detected by specific binding to a radiolabeled antigen (31). By this procedure, colonies expressing antibody fragments with the desired specificity were easily identified and isolated for further analysis.

Preparation of soluble Fab fragments containing three HIV-1 peptides. Phagemid DNA from positive clones was isolated, and the *Bgl*II-*Nhe*I fragment encoding phage coat protein pIII was replaced with artificial gene fragments encoding two tandem HIV-1 peptides (P18 and T1) plus a flexible linker se-

quence (Gly-Gly-Gly-Gly-Ser) and convenient flanking restriction sites. The tandem peptide sequence was synthesized as slightly overlapping single-stranded oligonucleotides, kinase treated, annealed, and ligated with *Bgl*II-*Nhe*I-linearized Fab phagemid DNA. The second strand was filled in with the Klenow fragment of DNA polymerase I before bacterial transformation by standard procedures. Plasmid DNAs from positive clones were sequenced by the dideoxy method with Sequenase 2.0 (U.S. Biochemical Corp.) and analyzed with Mac Vector sequence analysis software.

Expression and purification of chimeric Fabs. Bacterial clones were grown at 26°C in 2× TB medium containing 100 μg of ampicillin per ml and 20 mM $MgCl_2$ until an optical density at 600 nm of 0.8 was achieved. IPTG (0.1 mM) was added, and the culture was induced for 24 h at 26°C. Pelleted (4,000 rpm, 30 min, Sorvall GSA rotor) *E. coli* cells were resuspended in phosphate-buffered saline (PBS) containing 2 mM phenylmethylsulfonyl fluoride and lysed by sonication on ice. Following removal of debris by centrifugation (14,000 rpm, 30 min, Sorvall SS-34 rotor), the filtered supernatant was affinity purified by overnight batch absorption at 4°C with a Sepharose-coupled anti-mouse Lκ antibody (Zymed, San Francisco, Calif.). After extensive washing with PBS, chimeric Fabs were eluted with acidified PBS-HCl (pH 2.5), neutralized with 1 M Tris base (pH 9.0), and concentrated by Centriprep-30 (Amicon, Beverly, Mass.) ultrafiltration. Endotoxin contamination was removed by using Detoxi-Gel (Pierce, Rockford, Ill.) as described by the manufacturer. The Fab preparations were dialyzed against PBS, concentrated to ~1 mg/ml, and stored at 4°C. The concentrations of chimeric Fabs were determined by measuring A_{280} with an extinction coefficient of 1.56, and purity was analyzed by nonreducing sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE) and Coomassie blue R-250 staining.

Western blot (immunoblot) analysis. Purified chimeric Fab fragments were separated by nonreducing SDS–10% PAGE and analyzed by immunoblotting with polyclonal rabbit anti-mouse IgG antibodies or with biotin-labeled H902, an anti-gp120 (HIV-1_{IIIB}) monoclonal antibody (MAb) specific for P18 (19) and then a goat anti-rabbit IgG- or streptavidin-peroxidase conjugate, respectively, and a chemiluminescence detection kit (ECL reagent; Amersham Corp., Arlington Heights, Ill.).

IL-2 production. Healthy volunteers aged 18 to 45 years and HIV-1-seropositive, asymptomatic, nonanergic homosexual males (with CD4 counts between 400 and 760 and reactivity to the purified protein derivative test immunogen) were recruited, and their peripheral blood mononuclear cells (PBMC) were purified by centrifugation over Ficoll-Hypaque (Sigma Chemical Co., St. Louis, Mo.). PBMC (2×10^5) were stimulated in 7-day triplicate cultures supplemented with 5% normal, heat-inactivated human AB serum and 2 μg of anti-IL-2 receptor MAb Tac per ml (to prevent consumption of secreted IL-2 by the activated cells), and IL-2 titers in triplicate culture supernatants were determined with CTLL cells as previously described (21, 22, 24, 25, 48). Data are expressed in terms of a stimulation index, which was calculated by dividing the mean counts per minute incorporated in antigen-containing cultures by the mean counts per minute in cultures without antigen (medium control). The datum points shown are for a culture supernatant dilution of 1:4.

RESULTS

Choice of model epitopes and cloning of chimeric Fab. The epitope chosen for initial analysis was P18 (NH_2-RIQRG PGRAFVTIGK-COOH), a 15-residue peptide corresponding to amino acids 315 to 329 in the V3 loop region of HIV-1_{IIIB}-derived envelope glycoprotein gp120. This well-characterized antigen was chosen because it represents the principal neutralization domain (37, 41), as well as an epitope recognized by MHC-restricted cytotoxic T lymphocytes (CTL) (8, 54, 55). The MHC class I-restricted nature of the CTL response to this epitope (54), its high variability among HIV-1 isolates (41), and the lack of sufficient human Th activity provided by it (42, 45) prompted us to clone and express chimeric Fabs displaying, in addition to P18, the T1 peptide, a well-defined Th epitope (18) represented by the sequence NH_2-KQIINMWQEVGKA MYA-COOH (amino acids 428 to 443 in $gp120_{IIIB}$). T1 was found to be immunogenic in the context of several human MHC haplotypes (9, 21, 22, 24, 25, 48) and to provide help for neutralizing antibody (45) or CTL (42) responses specific for the covalently linked V3 loop epitope.

The targeting anti-human MAbs selected were L203 and IA6-2, which recognize monomorphic determinants of the HLA-DR and sIgD molecules expressed by all human haplotypes, respectively. BP107.2.2, an anti-mouse MHC class II MAb which does not bind to human APCs, was used as a control. To facilitate the cloning and expression of multiple antigen-binding Fab fragments, we made use of the well-established procedure for assembling combinatorial, antigen-binding Fab libraries on phage surfaces by using modified phagemid vector pComb3 (6). To amplify and clone chimeric Lκ fragments encoding the HIV-1-derived P18 sequence in a single step, we designed hybrid primers containing Lκ- and P18 peptide-specific nucleotide sequences. A panning procedure was used to select and enrich antigen-binding phage clones by adsorbing the phage display libraries on microtiter plates coated with purified antigens, i.e., human HLA-DR or IgD. After three consecutive rounds of panning, bacterial colonies expressing chimeric Fabs with the desired antigen specificity were detected in a filter assay by specific binding to a radiolabeled antigen (data not shown). To prepare soluble Fabs containing additional HIV-1 peptide sequences, vector pComb3 was modified by replacing the M13 phage coat pIII sequence with an artificial gene fragment that encodes the T1 and P18 peptides in tandem. The resulting pComb3-chimeric Fab expression system, containing two copies of P18 and one copy of T1, is shown schematically in Fig. 1.

Expression and characterization of chimeric Fabs. Chimeric Fabs were expressed in *E. coli* as monovalent Fab fragments to test their biological activities in vitro. Methods used to express and purify them in sufficient quantities were optimized and resulted in production levels of ~50 to 300 μg of chimeric Fab per liter of bacterial culture. Yields were improved by isolating the antibody fragments from the periplasmic space rather than from culture supernatants (data not shown). Therefore, cell lysates were used directly for affinity purification of chimeric Fabs. Purified material was analyzed by SDS-PAGE and Coomassie R-250 staining (Fig. 2A) or immunoblotting with polyclonal rabbit anti-mouse IgG (Fig. 2B) or MAb H902, a V3 loop-specific anti-$gp120_{IIIB}$ MAb (Fig. 2C). Both antibodies reacted specifically with ~58-kDa proteins, confirming the expression of chimeric, HIV-1 peptide-expressing Fab fragments.

Antigen binding and expression of P18 by the chimeric Fab were independently demonstrated in an enzyme-linked immunosorbent assay in which microtiter plates were coated with the appropriate antigen, e.g., purified HLA-DR or IgD molecules, and then incubated with the chimeric Fabs. Fab binding was detected by using anti-P18 MAb H902 as a probe. Recombinant L203 and IA6-2 retained the binding specificity of the native (hybridoma-derived) MAb (Fig. 3), thereby confirming the functional integrity of these antibody Fab fragments. No binding was detected with recombinant chimeric control Fab BP107.2.2 or with L203 and IA6-2 and their nonappropriate antigens. The specificity of chimeric Fab binding to the immobilized antigens, or that of H902 binding to the P18-expressing chimeric Fab, was further indicated by the ability to block both binding reactions by preincubation with their respective ligands, i.e., excess purified HLA-DR or IgD, or free P18, respectively (Fig. 3, groups 4 and 5).

The binding of chimeric Fabs to HLA-DR or sIgD molecules displayed on the surfaces of appropriate target cells was evaluated by immunofluorescence and fluorescence-activated cell sorter analysis with fluorescein isothiocyanate-labeled $F(ab')_2$ anti-mouse Ig antibodies as secondary reagents. Recombinant chimeric Fab fragments retained binding activity comparable to that of native, hybridoma-derived, intact antibodies L203 and IA6-2 or its Fab fragments (data not shown). Moreover, focusing of P18 on the surfaces of human peripheral blood lymphocytes (PBL) by the chimeric L203 or IA6-2 Fab, but not by control Fab BP107.2.2, was verified by immunofluorescent staining with the P18-specific MAb (Fig. 4A). The small fraction of PBL stained by chimeric IA6-2 is compatible with the proportion of $sIgD^+$ B cells in blood (35). As before,

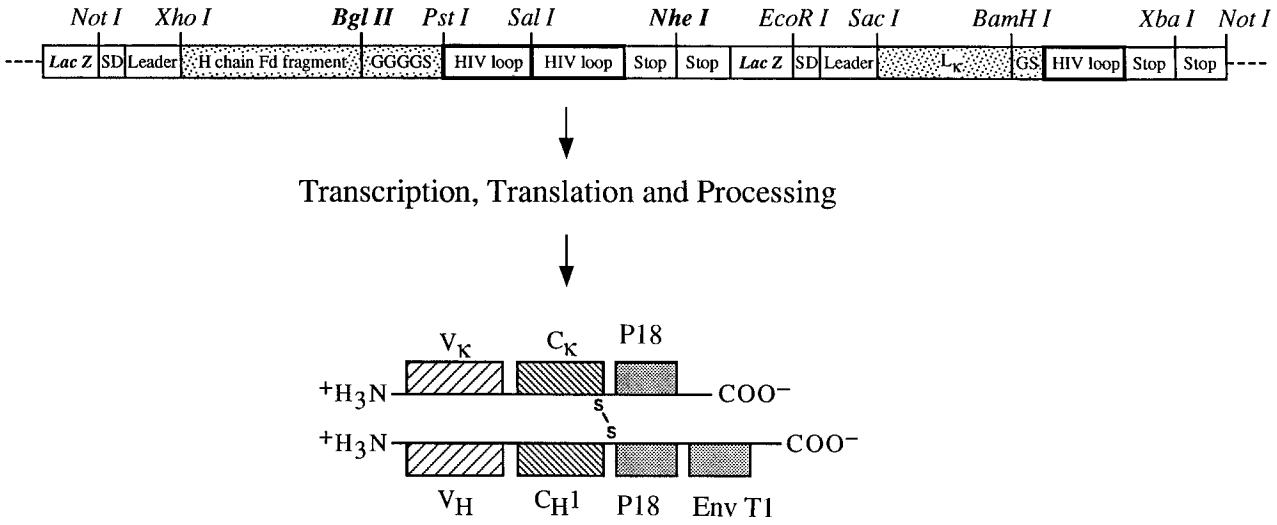

FIG. 1. Structure of the recombinant pComb3 vector system used for expression of chimeric Fab fragments in *E. coli* XL-1 Blue and schematic representation of the chimeric HIV-1 epitope-presenting Fabs. SD, Shine-Dalgarno sequence.

binding was blocked by preincubation of the chimeric Fabs with excess purified HLA-DR or IgD or by pretreating the secondary H902 antibody with free P18 (data not shown). Furthermore, neither L203 nor IA6-2 reacted with murine or rat APCs.

The anti-human HLA-DR chimeric L203 Fab also reacted with spleen cells from a *Cynomolgus* monkey (Fig. 4B). Since L203 recognizes a monomorphic human HLA-DR epitope, this reactivity most likely represents conservation of this epitope between humans and other primates. L203 immunoreactivity (which was blocked by preincubation with excess soluble human HLA-DR) was also observed with baboon lymphoblastoid cell line 26CB-1 (ATCC CRL1495). This interspecies immunological cross-reactivity may eventually allow testing of L203-immunotargeted recombinant peptide vaccines with primate models of HIV infection.

Stimulation of human T cells from HIV-1-exposed donors. To assess the in vitro immunogenicity of the T1- and P18-expressing chimeric Fabs, we used them as antigens to stimulate PBMC from randomly selected HIV-positive, asymptomatic, nonanergic donors or from HIV-negative, age-matched, healthy volunteers. Binding of the L203 and IA6-2 MAbs to monomorphic MHC class II or IgD determinants, respectively, allowed us to use these immunogens with PBMC of different donors irrespective of their MHC (and/or IgD) polymorphisms. In addition to chimeric L203 and IA6-2 and control (nonbinding) BP107.2.2, other, related antigens were used for comparison. These included the free P18 and T1 peptides and a recombinant HIV-1_{IIIB} gp120 protein, as well as tetanus toxoid (TT), which was used as a recall antigen (21, 22, 24, 25, 48). T-cell activation by these antigens was assessed by measuring IL-2 titers in antigen-stimulated and unstimulated culture supernatants. This assay was shown to be considerably more sensitive than proliferation assays based on [^{3}H]thymidine incorporation for detection of virus-specific Th activity among HIV-positive donors (24).

Since only limited amounts of blood from HIV-positive, nonanergic donors were available, preliminary titration experiments were conducted to optimize the conditions for detection of an immunotargeting effect (data not shown). These experiments utilized PBMC from one HIV-seropositive donor who responded well to the corresponding synthetic HIV-1 peptides. Titration of the chimeric Fabs over a range of 25 to 400 μg/ml indicated that maximal differences in stimulation by immunotargeted versus nonimmunotargeted antigens were obtained at a chimeric Fab concentration of 25 μg/ml. This antigen concentration, which is equivalent, on a molar basis, to 1.4 or 0.75 μg of the free P18 or T1 peptide, respectively, per ml, was used in subsequent experiments.

The HIV-1-seropositive Walter Reed stage 1 patients studied were only those whose PBMC generated greater-than-twofold IL-2 responses when stimulated with both TT and the synthetic HIV-1 peptides. PBMC from six of eight HIV-1-positive donors (and all HIV-1-negative donor-derived

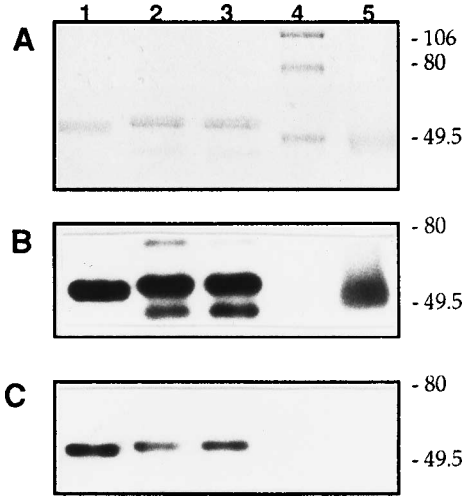

FIG. 2. Coomassie blue R-250 staining (A) and immunoblot analysis of purified chimeric Fabs with polyclonal rabbit anti-mouse IgG antibodies (B) or anti-gp120 MAb H902, which is specific for V3 loop peptide P18 (C). Both antibodies reacted specifically with the expressed Fab fragments, indicating that the corresponding HIV-1 epitope is also correctly expressed. Bands of ~58 kDa represent assembled Fab heterodimers of chimeric L203 (lane 1), IA6-2 (lane 2), or BP107.2.2 (lane 3). The positions of molecular size standards (lane 4) and the native ≈50-kDa Fab fragment prepared by papain cleavage of a purified hybridoma-derived L203 MAb (lane 5) are shown. The numbers on the right are molecular sizes in kilodaltons.

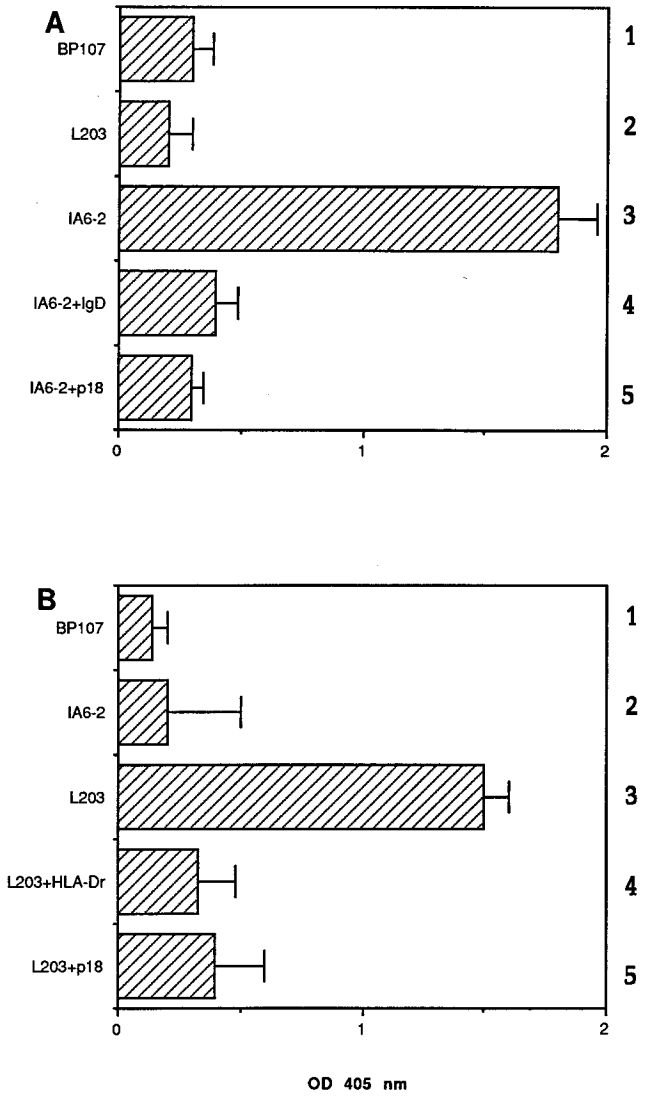

FIG. 3. Enzyme-linked immunosorbent assay analysis of the binding specificity of purified chimeric Fabs. Binding anti-human HLA-DR (L203) and sIgD (IA6-2) and nonbinding anti-mouse MHC class II (BP107.2.2) Fabs were added to wells coated with either purified human sIgD (A) or HLA-DR (B) molecules. In some cases, the chimeric Fab was preincubated in the presence of excess soluble antigen (group 4) or peptide P18 (group 5). Binding was detected by biotinylated anti-P18 MAb H902, followed by an avidin-alkaline phosphatase conjugate and the corresponding color reaction. Chimeric L203 or IA6-2 did not bind to other protein antigens, e.g., mouse I-A^d or IgD, bovine serum albumin, or lysozyme (data not shown). OD, optical density.

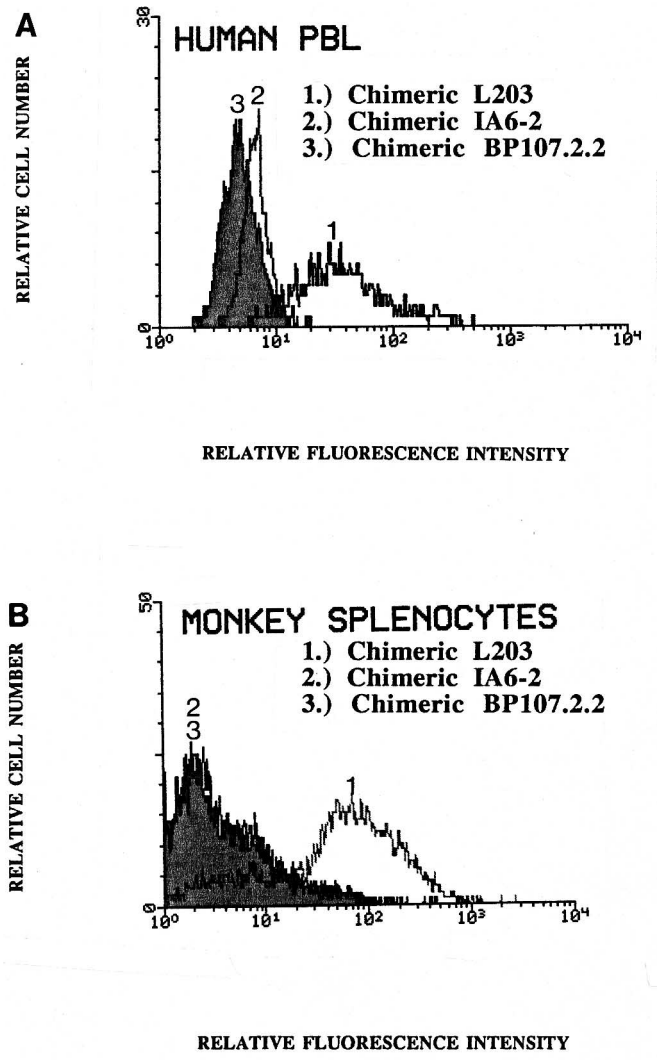

FIG. 4. Focusing of HIV-1-derived loop epitope P18 on the surfaces of human lymphocytes (A) or cryopreserved (>90% viable) *Cynomolgus* monkey spleen cells (B) by chimeric anti-human HLA-DR (L203) or anti-human sIgD (IA6-2) Fabs. Cells were stained with a purified Fab, i.e., L203 or IA6-2 (or nonbinding BP107.2.2 as a negative control), and then exposed to biotinylated H902 and fluorescein isothiocyanate-labeled streptavidin. Positive staining indicates focusing of the HIV-1 epitope to the cells. As shown, this particular anti-human HLA-DR Fab (L203) also binds to primate APCs. Staining with an intact hybridoma-derived MAb and then exposure to fluorescein isothiocyanate-$F(ab')_2$ rabbit anti-mouse IgG produced identical results (data not shown). PBL, peripheral blood lymphocytes.

PBMC) responded to the recall antigen, and PBMC from four of them also responded to the HIV-1_{IIIB} peptide antigens. The IL-2 responses of these four donors, as well as those of two representative healthy controls, are shown in Fig. 5. Whereas all six donors responded to TT, only PBMC from HIV-1-positive donors responded with significant IL-2 production to stimulation with the HIV-1-derived antigens, indicating the antigen-specific nature of the Th response. The two chimeric Fabs that were capable of targeting the attached P18 and T1 epitopes to the surfaces of APCs, i.e., L203 and IA6-2, stimulated a significantly stronger response than did an equivalent concentration of nonbinding chimeric Fab BP107.2.2 or recombinant $gp120_{IIIB}$ (each at 25 μg/ml). Similar IL-2 production induced by free P18 or T1 required high peptide concentrations (~150 μg/ml) that represent a 100- to 200-fold excess over the concentration of the same peptides in the context of the chimeric Fabs. At concentrations equimolar to those of the chimeric Fabs, the free peptides failed to induce detectable IL-2 production (Fig. 6).

Next, we addressed the possibility that the increased immunogenicity of the targeting chimeric Fabs is due to polyclonal activation of antigen-presenting B, or other, cells. Thus, the immunogenicity of the chimeric Fabs was compared with that of a mixture of the corresponding native (nonchimeric) Fabs, prepared by papain cleavage with the free synthetic peptides. In addition, the synthetic peptides alone were tested as immunogens, either at a low concentration (1.5 μg of each per ml as a mixture of P18 and T1) equivalent to their concentration in

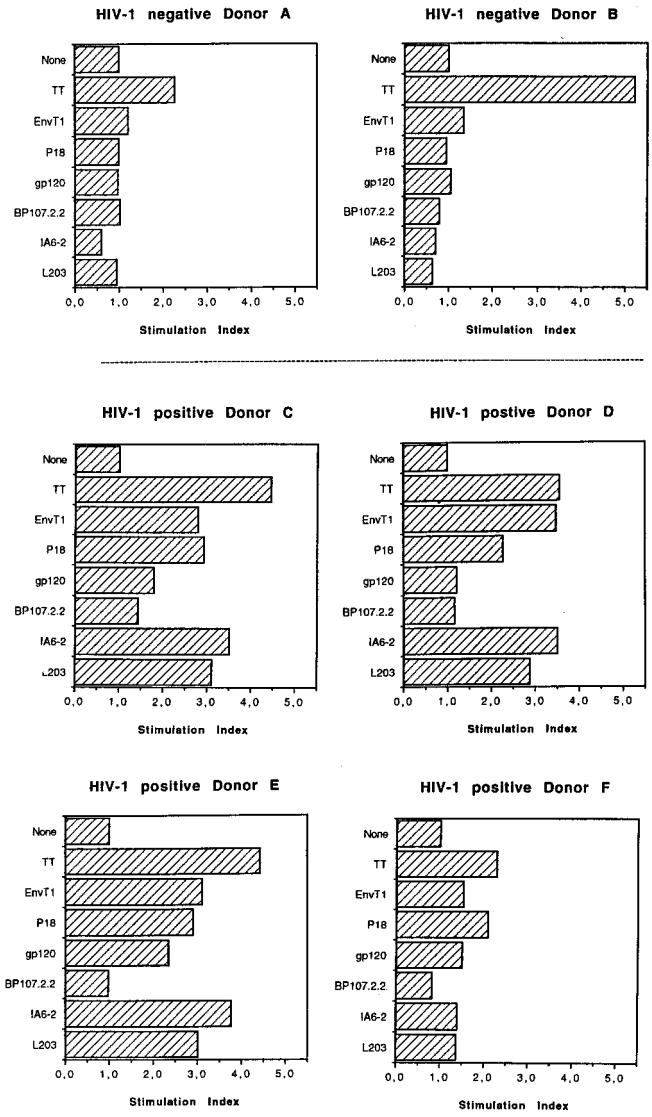

FIG. 5. Enhanced IL-2 production of human T cells by immunotargeted recombinant peptide vaccines. PBMC (2×10^5) were cultured for 7 days in the absence or presence of the antigens indicated. Antigen concentrations were as follows: TT, $gp120_{IIIB}$, or chimeric Fab, 25 μg/ml; free synthetic peptide P18 or T1, 150 μg/ml. IL-2 production was assayed by the ability of culture supernatants to support the growth of CTLL cells as measured by [^{3}H]thymidine incorporation. Data are expressed as stimulation indexes. Responses of HIV-1-seronegative donors A and B to TT were 16,848 and 16,668 cpm, and those of the corresponding medium controls were 7,448 and 3,200 cpm, respectively. Responses of PBMC from the four HIV-1-positive donors in the absence (medium control) and presence of TT (in counts per minute) were as follows: donor C, 2,008 and 8,936; donor D, 1,800 and 6,372; donor E, 1,140 and 5,032; donor F, 1,832 and 4,216, respectively.

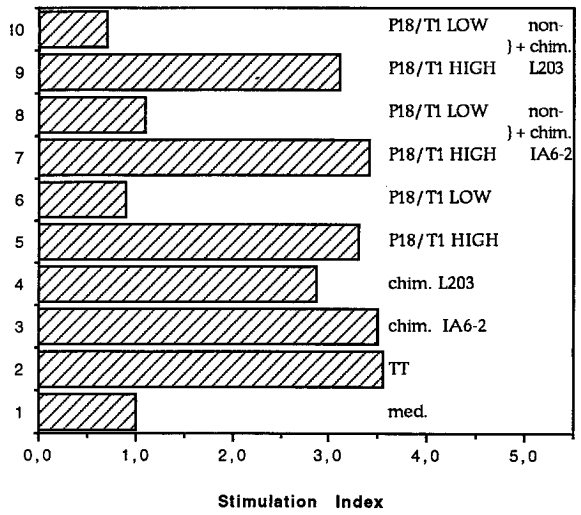

FIG. 6. Lack of polyclonal activation by immunotargeted HIV-1 peptide-expressing chimeric (chim.) Fabs. Purified PBMC (2×10^5) from HIV-1-positive donor D (see Fig. 5) were cultured for 7 days with medium (med.) alone (bar 1); a 25-μg/ml concentration of TT (bar 2), chimeric IA6-2 (bar 3), or chimeric L203 (bar 4); or a mixture of free HIV-1 peptides P18 and T1 in a large dose (each at 150 μg/ml; bars 5, 7, and 9) or a small dose (each at 1.5 μg/ml; bars 6, 8, and 10) added either alone (bars 5 and 6) or together with hybridoma-derived nonchimeric Fabs prepared by papain cleavage of the respective MAb (IA6-2 [bars 7 and 8] or L203 [bars 9 and 10]). IL-2 production was determined as described in the legend to Fig. 5. The response to TT was 6,372 cpm; that of the medium control was 1,800 cpm.

the context of the chimeric Fabs or at a high concentration (150 μg of each per ml). As seen in Fig. 6, the low free-peptide dose, when tested either alone or in combination with the native L203 or IA6-2 Fab, did not stimulate significant responses in a representative HIV-1-positive donor. The high peptide concentration stimulated a significant response which was equivalent to that induced by a 100- to 200-fold lower peptide concentration presented in the context of a chimeric immunotargeting antibody; however, this response was not augmented further by addition of the nonchimeric Fabs. These data indicate that the enhanced immunogenicity of the chimeric Fabs is not due to polyclonal APC activation and further establish the immunotargeting effect of these chimeric proteins.

As shown in Table 2, presentation of either immunotargeted chimeric Fabs or high-dose unconjugated HIV-1 peptides was significantly inhibited by an anti-CD4 MAb (OKT4) but not by an anti-CD8 MAb (OKT8). This result indicates that the peptides generated by processing of the chimeric Fab fragments are recognized in association with MHC class II molecules in a CD4-dependent fashion, similar to the corresponding synthetic peptides.

DISCUSSION

A major objective of AIDS research is development of a safe and effective vaccine capable of providing protection against a broad spectrum of HIV isolates and, possibly, also slowing down disease progression in individuals already infected (30,

TABLE 2. Effects of anti-CD4 or anti-CD8 antibodies on IL-2 production by HIV-1-positive donor-derived PBMC stimulated with chimeric Fab-immunotargeted or free HIV-1 peptides[a]

Antigen(s)	% Inhibition of IL-2 production		
	No MAb	With OKT4	With OKT8
Synthetic P18 + T1	0	78	15
Chimeric IA6-2	0	83	24
Chimeric L203	0	67	13

[a] Purified PBMC cells (2×10^5) from HIV-1-positive donor E (see Fig. 5) were cultured for 7 days with the antigens indicated (150 μg each of P18 and T1 per ml; 25 μg of chimeric Fabs IA6-2 or L203 per ml) in the absence or presence of MAb OKT4 or OKT8 (2.5 μg/ml). IL-2 production was determined as described in the legend to Fig. 5. Data are expressed as percent inhibition relative to groups with no added MAb. The responses of this donor to the corresponding antigens in the absence of an added MAb are given in Fig. 5.

39, 57). However, since HIV infection typically progresses despite the presence of a seemingly strong host immune response consisting of both neutralizing antibodies and virus-specific CTL (30), the immune response to natural infection may be relatively ineffective. The rapid (~10 days), ≤100-fold decrease in peak virus titers following primary HIV-1 infection (27) is also suggestive of a strong, but ineffective, natural immune response. On the basis of these findings, it has been argued that HIV may not follow the paradigm that underlies the successful use of vaccines against other viral infections, i.e., that the ideal vaccine should mimic as closely as possible the immune response to natural infection (26, 48). This argument has led to the suggestion that an artificial HIV subunit vaccine including only selected epitopes may be necessary (8). Many HIV-1-derived epitopes recognized by Th cells, CTL, and neutralizing antibodies have been defined. Inclusion of a Th epitope (T1; 18) and the principal neutralization domain sequence, which contains both neutralizing (37, 41) and CTL (54) epitopes, within the same synthetic peptide increased the magnitude of the HIV-specific neutralizing antibody (45) or CTL (42) response, and T1 priming generated a memory response in rhesus monkeys, evidenced by enhanced antibody production upon subsequent immunization with intact gp160 (36). The extensive strain variability among different HIV isolates presents a difficulty in the development of an effective vaccine, although many HIV isolates that exist worldwide can be grouped into five families (51) and one strain (MN) dominates among seropositive individuals tested in the United States (7). This obstacle could potentially be overcome by using a cocktail of peptides corresponding to the immunogenic but variable HIV-1 epitopes.

The underlying concept of this study is based on the notion that artificial focusing of an immunogenic peptide to APCs by an immunotargeting antibody may allow this peptide to bind at levels much higher than those expected to be achieved by physiological pathways. Thus, "forcing" the binding of a large number of homogeneous exogenous peptides to APCs by linking them to anti-MHC class II (estimated at 0.5×10^5 to 1×10^5 molecules per cell) or sIgD (2×10^5 to 5×10^5 molecules per cell; 16, 50) antibodies may increase the antigen load, thereby enhancing antigen uptake, processing, and presentation and, subsequently, T-cell activation. While the relevant studies that have documented the enhancement of immune responses by this approach (13–16, 38, 43, 47, 50) have not directly established the mechanism underlying the enhanced immune response, they have ruled out several potential mechanisms other than immunofocusing, e.g., polyclonal B-cell activation by anti-Ig antibodies or binding to Fc receptors on APCs. Although the processing pathways of these chimeric antibodies remain to be established, the fact that this experimental approach has worked successfully in a considerable number of independent studies provides a strong rationale for further exploration of its vaccine potential.

On the basis of this concept, we attempted to overcome the inherently weak immunogenicity of peptide vaccines by cloning and expressing chimeric monovalent Fab fragments of anti-human HLA-DR or sIgD MAbs that express copies of immunodominant HIV epitopes, i.e., P18 and T1. Generation of these chimeric antibodies at the gene level offers one potential advantage, i.e., the ability to attach peptide epitopes to antibody fragments under precise conditions with regard to site and copy number, thereby facilitating the preparation of multiple distinct chimeric constructs that can be compared for immunogenicity. Our results indicate that such chimeric antibodies are capable of binding to APCs and displaying the attached HIV-1 epitopes and, furthermore, that they create enhanced and specific immunogenicity by comparison with other forms of the relevant antigen (including a nonbinding chimeric Fab), as determined by their ability to stimulate IL-2 production by PBMC derived from HIV-positive donors. The immunogenicity of these synthetic HIV antigens was also demonstrated by their ability to induce Th and CTL responses in BALB/c mice upon injection into the footpads (data not shown).

Nevertheless, several potential limitations of this immunotargeting vaccine approach need to be addressed. First, bivalent immunotargeting (anti-sIg) $F(ab')_2$ fragments were found to be ~10-fold more potent than the respective monovalent Fab fragments in enhancing T-cell activation (15, 16, 47). This may reflect increased avidity of the bivalent antibody or its ability to cross-link the targeted surface molecules, i.e., MHC class II or sIg, which will, in turn, activate APCs (B cells and/or macrophages). This activation may enhance the APC antigen presentation function by upregulating adhesion molecules that facilitate interactions between T cells and APCs or by inducing secretion of immunopotentiating cytokines (44, 46). Bivalent fragments may also be favored over monovalent Fab fragments because of their longer half-life in serum (10). For these reasons, it might be advantageous to use bivalent versions of the chimeric Fab fragments to achieve improved immunotargeting in vivo. Second, the potential primate anti-mouse Ig response, which may limit the usefulness of this approach, could be addressed by creating humanized antibodies. Third, anti-MHC class II antibodies have been shown to induce immunosuppression (e.g., see reference 1). However, this effect was seen in animals treated with large quantities of antibodies over a long time period but not in successful immunotargeting studies which used single injections of the targeting anti-MHC class II antibodies (47). Thus, because of the immunopotentiating effects, a single injection (or very few injections) of immunotargeting anti-MHC class II, which is unlikely to cause immunosuppression, may be sufficient for effective vaccination.

Additional potential problems are associated with the use of anti-IgD antibodies for immunotargeting. Intravenous injection of a monovalent (but not that of a bivalent) rabbit anti-mouse IgD antibody was found to induce T-cell tolerance in mice (32, 46). Since monovalent, non-cross-linking anti-IgD antibodies failed to activate B cells, this result was interpreted as indicating that antigen presentation by resting (as opposed to activated) B cells induces T-cell anergy. On the other hand, treatment with large doses of bivalent anti-IgD can induce high levels of polyclonal IgG1 and IgE responses, as well as IL-4 production (33, 35), consistent with activation of the Th2 subset of T cells. This, in turn, would inhibit the induction of Th1 (53) cells that is considered essential for the cell-mediated immunity involved in antiviral protection (23), albeit it might potentiate the neutralizing antibody response. This mechanism was proposed to account for the suppression of cell-mediated autoimmune disease in rats treated with an anti-IgD antibody conjugated to an encephalitogenic peptide (28). However, these inhibitory effects on the induction of cell-mediated immunity are usually seen only after intravenous injection of large (100- to 1,000-μg) anti-IgD antibody doses (28, 33, 35), a procedure known to be generally effective for tolerance induction. In contrast, immunotargeting antibodies potentiated cell-mediated immunity when injected by other routes (e.g., subcutaneously) and in much smaller doses (13, 14, 38).

Concluding remarks. Chimeric anti-HLA-DR and anti-sIgD Fab fragments have been shown to act as a specific targeting device that can focus the relevant HIV-1 epitopes on the surfaces of human or primate APCs. This was demonstrated by the enhanced potency of immunotargeted antigens for presen-

tation to primed T cells over equivalent nonimmunotargeted control antigens, presumably via more efficient uptake into APCs with subsequent access to the MHC class II processing pathway, thereby leading to improved antigen presentation. Such immunotargeted chimeric constructs, which are created at the gene level and thereby allow complete control over the attachment site, copy number, and nature of the inserted HIV-1 epitopes (as opposed to the uncontrolled nature of the covalent coupling of immunogenic peptides to immunotargeting antibodies at the protein level), are potentially useful in the design of synthetic, well-characterized, and highly effective, multivalent, recombinant subunit vaccines against HIV-1. The chimeric anti-human-primate HLA-DR L203 Fab may eventually be used in nonhuman primate experiments, e.g., HIV-1 vaccine studies with *Macaca nemestrina* (2). Beyond the realm of AIDS, this approach could rapidly be translated into the generation of similar vaccine constructs targeted to other pathogens.

ACKNOWLEDGMENTS

We thank J. F. Kearney, Department of Microbiology, University of Alabama, and H. Grey for providing hybridoma IA6-2 and purified human HLA-DR, respectively, and N. Hanna (Idec, Inc., San Diego, Calif.) for providing *Cynomolgus* spleen cells. For excellent technical support, we are grateful to Leslie Giampa, Beth Herbert, and David Telford.

This work was supported in part by NIH grant AI28197 and the National Collaborative Vaccine Development Groups, NIH (U01-AI30238).

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