Conversion of an Immunogenic Human Immunodeficiency Virus (HIV) Envelope Synthetic Peptide to a Tolerogen in Chimpanzees by the Fusogenic Domain of HIV gp41 Envelope Protein

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Summary
The fusogenic (F) domain of human immunodeficiency virus (HIV) gp41 envelope (env) protein has sequence similarities to many viruses and mediates the fusion of HIV-infected cells. During a survey of the immunogenicity of HIV env peptides in chimpanzees, we have observed that HIV peptide immunogenicity was dramatically altered by the NH2-terminal synthesis of the gp41 F domain to an otherwise immunogenic peptide. We compared two hybrid peptide types comprised of T helper (Th) and B cell epitopes of HIV gp120 env protein for their immunogenicity in chimpanzees. The Th-B epitope hybrid peptides contained the HIV gp120 Th cell determinant, T1 (amino acids [aa] 428-440) -synthesized NH2 terminal to gp120 V3 loop peptides, which contain B cell epitopes that induce anti-HIV-neutralizing antibodies (SP10IIIB [aa 303-321] and SP10IIIB [A] [aa 303-327]). The F-Th-B peptide contained the HIV gp41 F domain of HIVIIIB gp41 (aa 519-530) -synthesized NH2 terminal to the Th-B peptide. Whereas Th-B peptides were potent immunogens for chimpanzee antibody and T cell-proliferative responses, the F-Th-B peptide induced lower anti-HIV gp120 T and B cell responses. Moreover, immunization of chimpanzees with F-Th-B peptide but not Th-B peptides induced a significant decrease in peripheral blood T lymphocytes (mean decrease during immunization, 52%; p <0.02). Chimpanzees previously immunized with F-Th-B peptide did not respond well to immunization with Th-B peptide with T or B cell responses to HIV peptides, demonstrating that the F-Th-B peptide induced immune hyporesponsiveness to Th and B HIV gp120 env determinants. These observations raise the hypothesis that the HIV gp41 env F domain may be a biologically active immunoregulatory peptide in vivo, and by an as yet uncharacterized mechanism, promotes primed immune system hyporesponsiveness to otherwise immunogenic peptides.

To design immunogens that induced Th and B cell-neutralizing antibody responses against HIV, we have designed synthetic peptides (Th-B) comprised of linear arrays of functional regions of the HIV envelope (env)1 (1-3). The T1 sequence of HIV gp120 (amino acids [aa]428-440) is part of a conformational determinant of the CD4 binding site (4), and is a potent Th epitope (5). The gp120 SP10(A) region (aa 303-327) contains a potent B cell determinant (B) located in the V3 principle neutralizing domain of HIV gp120 env (3, 6, 7), and, as well, contains a CTL epitope for CD8+ cells that is restricted in mice by H2D4 (8) and in humans by HLA-A2 (9). The HIV gp41 env fusogenic (F) domain (aa 519-530) mediates fusion of HIV-infected cells (10, 11). We have previously observed that Th-B and F-Th-B peptides from HIVMN induced high-titered anti-HIV-neutralizing antibodies in goats (M. K. Hart and B. F. Haynes, 1 Abbreviations used in this paper. aa, amino acids, E/C, experimental/control; env, envelope, F, fusogenic
unpublished results), and induced MHC class I–restricted CTL in mice against H2D\(^d\) target cells expressing native HIV gp120 (2).

While the role for the HIV gp41 F domain in mediation of cytopathic effects of HIV is established, an immunoregulatory role for the F domain has not been described. In this study, we found that Th-B peptides were potent immunogens and induced high-titered serum antibody and PBMC-proliferative responses to immunizing peptide and to gp120 protein in chimpanzees. In contrast, the F-Th-B peptide that contained the HIV gp41 F domain did not induce high levels of antipeptide or anti-gp120 T and B cell responses, but rather induced relative peripheral blood T lymphopenia and immune hyporesponsiveness to HIV gp120 env Th and B determinants.

**Materials and Methods**

**Peptides** Peptides used in the study are listed in Table 1. Peptide synthesis was performed using either t-boc or t-moc chemistry with a peptide synthesizer (A431; Applied Biosystems, Inc., Foster City, CA). Peptides were purified using HPLC, and the molecular weight was determined by fast atom bombardment mass spectrometry (R. B. Van Breeman, North Carolina State University, Raleigh, NC) using a double-focusing mass spectrometer (H110HF, Joel Ltd, Tokyo, Japan). For Th-B and F-Th-B peptides (Table 1), expected molecular weight of F-Th-B peptide, F-T1-SP10IIIB(A), was 5,908, observed was 5,907; expected molecular weight of Th-B peptide, T1-SP10IIIB, 4,963, observed was 4,962, and expected and observed molecular weight of Th-B peptide, T1-SP10IIIB(A), was 4,749, and expected and observed molecular weight of Th-B peptide, T1-SP10MN(A), was 4,771. For the peptides used in the study (Table 1) the peptide amounts are gross weights. The percent water by Karl Fischer test (Galbraith Laboratories, Inc., Knoxville, TN) for each peptide was F-T1-SP10IIIB(A), 6%, T1-SP10IIIB(A), 8%, T1-SP10IIIB, 6%, and T1-SP10MN(A), 8%.

**Animals.** Chimpanzees were housed at the New Mexico State University Primate Facility (Alamorgordo, NM) and were studied using protocols approved by the U.S. Department of Health and Human Services and National Institutes of Health primate research committees, and by the New Mexico State University Animal Utilization Committee Chimpanzee nos. 884 (15 yr old) and 1028 (12 yr old) had the same sire, animals 1045 (10 yr old) and 1070 (11 yr old) were unrelated to each other and to animals 884 and 1028. Outbred goats were housed at the Duke University Animal Facilities and studied using experimental protocols approved by the Duke University Animal Use Committee Animal care and study procedures followed American Association of Laboratory Animal Care guidelines.

**Immunizations** For goats, 3 mg of peptide was injected intramuscularly in each gluteal region in CFA (first dose), and then IFA (subsequent doses). For immunization of chimpanzees, varying doses of peptides were injected intramuscularly in IFA in a total volume of 4 cm\(^2\), with 1 cm\(^2\) injected into right and left upper arms and thighs.

**ELISAs.** 2 \(\mu\)g of Th-B peptide, T1-SP10IIIB, or rgp1210IIIB (Repligen Corp., Cambridge, MD) in 15 mM Na\(_2\)CO\(_3\), 35 mM NaHCO\(_3\), pH 9.6) was incubated overnight in each well of a 96-well flat-bottomed plate (3590; Costar, Cambridge, MA). Wells were blocked with CBC buffer supplemented with 3% BSA for at least 2 h and then washed three times with PBS, 0.05% Tween 20. Primary antibodies at various concentrations in serum diluent (95 ml PBS, 0.05% Tween 20, supplemented with 5 g BSA and 2 ml normal serum from same species as secondary antibody) was incubated for 90 min at 20\(^\circ\)C. After washing three times, alkaline phosphatase-conjugated secondary antibody was added to each well (60 min at room temperature), and the plates were washed. Substrate (1 mg/ml p-nitrophenyl phosphate, Sigma Chemical Co., St. Louis, MO) in 0.05 M CBC, 0.002 M MgCl\(_2\) was added to each well, and plates were developed (60 min, 20\(^\circ\)C in the dark and at 405 nm on an ELISA reader (Anthos, Denley Instruments Co., Durham, NC). Endpoint ELISA antibody titers were defined as the serum titer at which the experimental/control (E/C) OD value was \(\geq 3.0\).

**HIV Neutralization Assays.** The ability of chimpanzee or goat serum antibodies to neutralize HIV was determined in syncytium inhibition assay and reverse transcriptase inhibition assay as previously described (1, 3). Sera were heat inactivated (30 min, 56\(^\circ\)C) before each assay.

**PBMC Isolation and In Vitro \(^{3}H\)Thymidine Incorporation Assays.** Chimpanzee or goat PBMC were isolated by standard density centrifugation techniques (1, 12). In vitro assays of \(^{3}H\)thymidine incorporation were performed as described (1, 13). For chimpanzee PBMC assays, in vitro cultures were performed using 10% normal chimpanzee serum. Antigens used in PBMC proliferation were the

| Table 1. Sequences of Synthetic Peptide Constructs Derived from HIVMN and HIVIIIB env gp120 |
|-----------------------------------------------|
| Peptide name | Peptide type | Peptide composition and sequence (epitope type) |
|---------------|--------------|-----------------------------------------------|
| F-T1-SP10IIIB(A) | F-Th-B | AVGIGALFLGLFKQINMWQEVGKAMYACTRNTRNKRKISRQGPRAFVT |
| T1-SP10IIIB | Th-B | KQINMWQEVGKAMYACTRNTRNKRKISRQGPRAFVT |
| T1-SP10IIIB | Th-B | KQINMWQEVGKAMYACTRNTRNKRKISRQGPRAFVT |
| T1-SP10MN(A) | Th-B | KQINMWQEVGKAMYACTPRNYKKRKHIGPRAFYTTK |

Each amino acid is represented by a single-letter code that is the first letter of its name, except for arginine (R), asparagine (N), glutamine (Q), glutamic acid (E), lysine (K), phenylalanine (F), tryptophan (W), tyrosine (Y), and aspartic acid (D). F domain sequence is aa 320-324 from HIVIIIB (27) T1 sequence is aa 428-443 from HIVIIIB (27) SP10MN (A) sequence is aa 301-319 from HIVMN (28) SP10IIIB sequence is aa 303-321 from HIVIIIB (A) sequence is aa 320-324 from HIVMN (28) and aa 322-327 from HIVIIIB (27) Th, Th cell determinant, B cell, B cell–neutralizing antibody determinant A, additional HIV gp120 V3 loop sequences added to the original synthetic peptide (SP10) sequence to add an additional neutralizing and CTL region to the HIV B cell determinant of the hybrid peptide.
Th-B peptides, T1SP10IIIB(A) and T1-SP10MN(A) (Table 1), and Candida albicans antigen (Greet Laboratories, Inc., Lenoir, NC). PHA (Burroughs Wellcome, Research Triangle Park, NC) was used in a wide dose range as a mitogen in 3-d PBMC [3H]thymidine incorporation assays (1, 13). The change in counts per minute was calculated as: Δcpm = experimental cpm - control cpm.

Immuneze Schedule. Because of our previous studies demonstrating the immunogenicity of Th-B peptides in goats and rhesus monkeys (13), the initial comparison of peptide designs when this study began in 1989 was monthly injections of Th-B vs. F-Th-B peptides (Table 1) at a dose of ~0.1 mg/kg (6 mg/animal). When neither peptide design induced neutralizing anti-HIVIIIB antibodies, the peptide doses were increased to ~0.5 mg/kg (30 mg/animal) and the right-hand side neutralizing sequence of HIVIII gp120 V3 loop (the A region) (2, 6) (Table 1) was added to the Th-B peptide to enhance the ability of this peptide to induce anti-HIVIIIB-neutralizing antibodies. After three monthly injections with either ~0.5 mg/kg (30 mg) Th-B or F-Th-B peptide, the animals were rested for 6 mo, and then reimmunized with either F-Th-B or Th-B with sequences from HIVIII, or with the Th-B peptide containing HIV env gp120 V3 sequences from the HVMN isolate.

Flow Cytometry. Chimpanzee PB mononuclear cells were studied by standard flow cytometry methods using a flow cytometer (751, Coulter Electronics, Inc., Hialeah, FL). PBL were identified by the following markers; total T cells, CD3; T cell subunits, CD4 and CD8, B cells, CD19; and NK cells, CD56 and CD16.

Table 2. Time Course of Anti-peptide Antibody Responses in Chimpanzees Immunized with HIV env Synthetic Th-B or F-Th-B Peptides

| Month of study | Immunogen dose | Reciprocal of ELISA titer | Immunogen dose | Reciprocal of ELISA titer |
|----------------|----------------|--------------------------|----------------|--------------------------|
|                | mg             | No 884                   | No 1028        | mg                       | No 1045 | No 1070 |
| 1              | 6              | 0                        | 0              | 0                        |
| 2              | Th-B(IIIB) 6   | 0                        | 0              | F-Th-B(IIIB) 6            | 0       | 0       |
| 3              | Th-B(IIIB) 6   | 51,200                   | 102,400        | F-Th-B(IIIB) 6            | 0       | 0       |
| 4              | Th-B(IIIB) 6   | 25,600                   | 819,200        | F-Th-B(IIIB) 6            | 0       | 800     |
| 5              | Th-B(IIIB) 6   | 25,600                   | 204,800*       | F-Th-B(IIIB) 6            | 1,600   | 200     |
| 6              | Th-B(IIIB) 30  | 51,200                   | 102,400        | F-Th-B(IIIB) 30           | 25,600  | 12,800  |
| 7              | Th-B(IIIB) 30  | 204,800                  | 102,400        | F-Th-B(IIIB) 30           | 25,600  | 12,800  |
| 8              | Th-B(IIIB) 30  | 51,200                   | 25,600         | F-Th-B(IIIB) 30           | 6,400   | 12,800  |
| 9              |                | 51,200                   | 51,200         | 3,200                    | 6,400   |
| 10             | 12,800         | 25,600                   | 800            | 800                      |
| 11             | 25,600         | 25,600                   | 800            | 1,600                    |
| 12             | 51,200         | 25,600                   | 1,600          | 800                      |
| 13             | 25,600         | 25,600                   | 200            | 200                      |
| 14             | Th-B(IIIB) 6   | 51,200                   | 25,600         | F-Th-B(IIIB) 1           | 200     | 400     |
| 15             | 102,400        | 12,800                   | 800            | 800                      |
| 16             | Th-B(MN) 6     | 25,600                   | 12,800         | Th-B(IIIB) 6             | 100     | 0       |
| 17             | Th-B(MN) 6     | 12,800                   | 3,200          | Th-B(MN) 6               | 1,600   | 3,200   |
| 18             | 25,600         | 6,400                    | 6,400          | 25,600                   |
| 19             | Th-B(MN) 6     | 25,600                   | 1,600          | Th-B(MN) 6               | 6,400   | 51,200  |
| 20             | 51,200         | 6,400                    | Th-B(MN) 6     | 51,200                   |

Titer are endpoint ELISA titers (titers at which E/C were $>$3.0) against the Th-B peptide, T1-SP10IIIB
* Animal 1028 did not receive the month 5 injection due to a sterile abscess at the injection site. All injections in animal 1028 after month 5 were in PBS alone.
1 Animal 1070 did not receive the month 20 immunization due to the presence of high levels of anti-HIV-neutralizing antibodies.

For animals 884 and 1028, immunizations at months 2-5 were with T1-SP10IIIB, and months 6, 7, 8, and 14 with T1-SP10IIIB(A). For animals 1045 and 1070, immunization at month 16 was with T1-SP10IIIB(A).
deleted from the immunogen, and remained low throughout the remainder of the immunization period (Table 2). In chimpanzee no. 884, antibody titers rose at month 7 to 1:204,800 after five immunizations with Th-B peptides. Continued immunization of animal 884 with high doses of Th-B peptide (30 mg/dose) resulted in no further increases in antibody titer (Table 2).

In contrast, antipeptide antibody levels were much lower during months 1-10 of immunization of animals 1045 and 1070 with HIVIIIB F-Th-B peptide, with peak antibody levels against immunizing peptide of 1:25,600 and 1:12,800 at month 7 for animals 1028 and 1070, respectively (Table 2). After a 6-mo rest for all four animals, animals 884 and 1028 were immunized at month 14 with 6 mg of Th-B peptide. In chimpanzee no. 884, boosting with Th-B peptide in IFA at month 14 resulted in rise in titer of antipeptide antibody to 1:102,400, while boosting of animal 1028 with peptide in PBS alone led to no antibody rise (Table 2).

In contrast, animals 1045 and 1070 were immunized at month 14 with 1 mg (40.016 mg/kg) of F-Th-B to determine if the prior doses of F-Th-B peptide were excessive and induced high zone tolerance, and if smaller amounts of F-derivatized peptide would be more immunogenic. Immunization of both chimpanzees nos. 1045 and 1070 with 1 mg of F-Th-B peptide after a 6-mo rest resulted in only minimal rises in serum titers of antipeptide antibody to 1:800 (Table 2).

To determine if chimpanzee nos. 1045 and 1070 were tolerant to Th-B peptides, both animals were immunized on month 16 with HIVIIIB Th-B peptide, T1-SP10IIIB(A). Both animals 1045 and 1070 responded minimally to boosting with Th-B peptide with antipeptide antibody responses of 1.1,600 and 1.3,200, respectively, demonstrating that animals 1045 and 1070 were hyporesponsive at month 16 to Th-B HIV env epitopes (Table 2).

Immunization of Animals 1045 and 1070 with HIVMN Th-B Peptide Induced High Levels of Antipeptide Antibodies. Using a previously described strategy of breaking B cell tolerance by immunization with an immunogen that is different from, but structurally related to, the tolerogen (14), we next immunized animals 1045 and 1070 with the HIVMN Th-B peptide. The Th-B peptide from HIVMN contained the same Th (T1) gp120 sequence as the HIVIIIB Th-B peptide, but contained different B cell gp120 V3 B cell epitope sequences than those in the HIVIIIB Th-B peptide (Table 1). After two immunizations with Th-B of HIVMN, beginning at month 17, both chimpanzees nos. 1045 and 1070 had prompt rises in titer of antibodies to HIVIIIB (Table 2) and to HIVMN Th-B peptide (not shown) to antibody levels that were higher than had previously been obtained during the prior 18 mo of study. At month 20, endpoint ELISA titers to the HIVMN Th-B peptide were 1:102,400 for animal 1045 and 1:204,800 for animal 1070.

Chimpanzee B Cell Antibody Responses to Recombinant HIVIIIB gp120 during the 20-mo Immunization Course. Endpoint ELISA antibody titers against recombinant HIVIIIB gp120 were determined for sera from months 4-7 and 16-20 to correlate peak antipeptide antibody levels with anti-gp120 HIV env antibody levels. We found that peak anti-gp120 antibody levels in chimpanzee nos. 884 and 1028 during months 4-7 were both 1:25,600, whereas peak titers to gp120 in animals 1045 and 1070 during the same period were 1:6,400 and 0, respectively. As with antipeptide antibody levels, boosting after a 6-mo rest with peptide in PBS in chimpanzee no. 1028 did not boost anti-gp120 antibodies.

Boosting with F-Th-B peptide at month 14 and with HIVIIIB Th-B at month 16 in animals 1070 and 1045 resulted in minimal rises in anti-gp120 antibody titers by month 17 (to 1:12,800). In contrast, boosting chimpanzee nos. 1045 and 1070 with HIVMN Th-B peptide at month 17 induced high levels of anti-gp120IIIB antibody in both animals (1:102,400 and 1:51,200, respectively) by month 20 that rose coincident with rises in levels of antipeptide antibody.

Induction of Antipeptide and Anti-gp120 PBMC-proliferative Responses by HIV env Peptides. Whereas HIVIIIB Th-B peptides induced high levels (>100,000 Acpm/10^6 cells) of PBMC [3H]thymidine incorporation (animals 884 and 1028) (Fig. 1, A and B) during months 1-8, F-Th-B peptide did not induce levels of [3H]thymidine incorporation >100,000 Acpm/10^6 cells during the same period (Fig. 1, C and D). Immunization of animals 1045 and 1070 with Th-B peptide at month 16 did not induce the presence of circulating PBMC capable of proliferating to Th-B peptide in vitro (Fig. 1, C and D).

Interestingly, Th-B peptides at months 14-18 boosted PBMC-proliferative responses in animal 1028, while antipeptide antibody responses in animal 1028 during this time were not boosted (Fig. 1 B and Table 1).

Next, [3H]thymidine incorporation of chimpanzee PBMC to either recombinant gp120IIIB or to native gp120IIIB was tested. Table 3 shows the peak [3H]thymidine incorporation of chimpanzee PBMC to HIVIIIB gp120 for each animal during months 1-13, and demonstrates that neither chimpanzee no. 1070 nor 1045 (receiving F-Th-B peptide) had PBMC-proliferative responses to gp120 of E/C >2 throughout the first 13 mo of study. In contrast, animals 884 and 1028 (receiving Th-B peptides) did have anti-gp120-proliferative responses during the same period (Table 3).

To determine if PBMC-proliferative responses to mitogenic or antigenic stimuli other than HIV immunogens were normal in the F-Th-B-immunized chimpanzees over the 20 mo of study, we also measured PBMC-proliferative responses to PHA (Fig. 2) and to Candida (Fig. 3). While peak PHA PBMC-proliferative responses were nearly identical in the four chimpanzees, Candida PBMC-proliferative responses varied from animal to animal and from month to month. However, in animals 1045 and 1070, we found that Candida responses were intermittently present during the time of immunization with F-Th-B peptide at levels that were similar to levels present before the immunizations were begun (Fig. 3, C and D).

Characterization of PB Lymphocyte Subsets during Immunization of Chimpanzees with HIV env Peptides. To determine if immunization with either HIV env peptide type had effects on the number of circulating chimpanzee T, B, or NK cell populations, the absolute numbers of these cell types were
Figure 1. Time course of PBMC [3H]thymidine incorporation responses to HIV Th-B peptide, T1-SP10IIIB(A), in chimpanzees immunized with HIV env synthetic peptides. Animals 884 (A) and 1028 (B) received the Th-B peptide, T1-SP10IIIB, initially (months 1–5), then the Th-B peptide, T-SPI0IIIB(A) (months 6–8). After a boost with the Th-B peptide, T1-SP10IIIB(A), at month 14, both animals 884 and 1028 were immunized with the HIVMN Th-B peptide, T1-SP10MN(A). C and D show the responses of animals 1045 (C) and 1070 (D) to the HIVIIIIB F-Th-B peptide (months 1–14), HIVIIIIB Th-B peptide (month 16), and HIVMN Th-B peptide (months 17–19). All immunizations were with the indicated peptide in IFA, except for all immunizations for animal 1028 after month 4, which were with peptides in PBS alone. Solid lines show data for peak proliferative responses (Δcpm) to a wide dose range of HIVIIIIB Th-B peptide. Dotted lines indicate peak proliferative response (Δcpm) to a wide dose range of the HIVMN Th-B peptide.

determined throughout the immunization period (Fig. 4 and Table 4). Whereas preimmunization (before) and postimmunization (during) lymphocyte levels in animals 884 and 1028 were not significantly different (Table 4), animal 1045 became relatively lymphopenic (p <0.001) during the course of immunization with F-Th-B peptide with the lymphocyte count of 650/mm³ at week 12, compared with preimmunization levels of 2,815 and 2,597 lymphocytes/mm³ in months 1 and 2, respectively (Fig. 4 C). Whereas T cell levels significantly dropped an average of 59 and 44% in chimpanzee nos. 1045 (p >0.001) and 1070 (p >0.02), respectively, during the immunization period, T cell levels did not significantly change in animals 884 and 1028 during the same time (p >0.1) (Table 4). B and NK cell levels dropped significantly in animal 1045, but did not change in animals 1070, 884, and 1028 (Table 4). Taken together, these data demonstrated that immunization with the F-derivatized HIV env peptide induced decreases in absolute levels of circulating T cells in both animals 1045 and 1070, and in B and NK cell levels in animal 1045, whereas immunization of chimpanzee nos. 884 and 1028 with HIV Th-B env peptides lacking the F domain did not significantly affect circulating lymphocyte levels.

Table 3. [3H]thymidine Incorporation of PBMC after In Vitro Stimulation with HIV env gp120

| Chimpanzee no. | Immunogen      | Preimmunization | Postimmunization |
|---------------|----------------|-----------------|------------------|
|               | Δcpm / 10⁶ cells |                 |                  |
| 884           | Th-B peptides  | 169             | 39,189 (232)     |
| 1028          | Th-B peptides  | 17,955          | 129,121 (7)      |
| 1045          | F-Th-B peptide | 6,348           | 12,256 (2)       |
| 1070          | F-Th-B peptide | 11,285          | 22,719 (2)       |

Data represent the peak gp120 responses observed during the immunization period of months 1–13. Data for animals 884, 1028, and 1045 represent peak responses using from 2 to 0.5 μg/ml of HIVIIIIB(LAI) recombinant gp120. (Transgene Incorporated, Lyon, France). Data for animal 1070 represent peak responses using from 1 to 0.5 μg/ml of native HIVIIIIB(LAI) gp120 (1). Numbers in parentheses represent post-/preimmunization.

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Ability of HIVIIIB F-Th-B and Th-B Peptides to Induce Anti-HIVIIIB-neutralizing Antibodies in Goats. To determine if the F-Th-B peptide used in the initial phase of the chimpanzee immunization protocol was immunogenic in another species, 3 mg of either F-Th-B or Th-B peptide was used to immunize goats three times over 2 mo and then used to boost goats after an 8-mo rest (Fig. 5). We found that after the fourth immunization, both peptides were capable of inducing serum anti-HIVIIIB-neutralizing antibodies (Fig. 5), and capable of inducing high levels (>500,000 Δcpm/10⁶ cells) of PBMC [3H]thymidine incorporation in vitro to Th-B or F-Th-B peptides (data not shown). In addition, serum end-
point ELISA titers of antibodies to immunizing peptide were the same in Th-B- and F-Th-B-immunized goats (data not shown). Thus, failure of the F-Th-B peptide to induce high levels of antipeptide antibodies and PBMC-proliferative responses in chimpanzees was not due to lack of an inherent immunogenicity of the HIVIIIB F-Th-B peptide, but rather was due to a specific effect of the F-derivatized peptide in chimpanzees.

**HIVMN Th-B env Peptide Induced Anti-HIV-neutralizing Antibody in Chimpanzees.** During the first 17 mo of the immunization trial, serum-neutralizing antibodies against HIVIIIB were always undetectable in syncytium inhibition assay and were <1:45 in reverse transcriptase inhibition assay. However, after immunization of animals 1045 and 1070 at month 17 with HIVMN Th-B peptide, anti-HIV-neutralizing antibodies were seen in syncytium inhibition assay (Table 5).

To determine why antibodies against HIVIIIB Th-B peptides did not neutralize HIVIIIB in vitro during the first 17 mo of immunization, sera from the early peak anti-HIVIIIB peptide antibody responses (month 6) were assayed for reactivity to the individual epitopes of the Th-B peptides. We found that at the time of initial high titers of anti-Th-B peptide responses, most of the antibody reactivity in sera from animals 884 and 1028 was indeed directed to the primary

| Leukocyte subset | No 884 Before | Change | No 1028 Before | Change | No 1045 Before | Change | No 1070 Before | Change |
|------------------|---------------|--------|---------------|--------|---------------|--------|---------------|--------|
|                  | cells/mm³ ± SEM | %   | cells/mm³ ± SEM | %   | cells/mm³ ± SEM | %   | cells/mm³ ± SEM | %   |
| Total lymphocytes| 4,034 ± 452 1,046 ± 249 | -26 | 3,164 ± 396 3,286 ± 660 | +4 | 3,164 ± 397 1,426 ± 116 | -55 | 3,943 ± 885 2,768 ± 296 | -30 |
| T cells          | 2,629 ± 384 2,084 ± 178 | -24 | 2,565 ± 276 2,027 ± 402 | -21 | 2,460 ± 253 1,012 ± 82 | -59 | 3,337 ± 762 1,887 ± 184 | -44 |
| B cells          | 356 ± 47 365 ± 39 | +3 | 411 ± 103 458 ± 47 | +11 | 293 ± 32 175 ± 15 | -40 | 302 ± 53 232 ± 22 | -23 |
| NK cells         | 345 ± 82 317 ± 43 | -9 | 257 ± 25 434 ± 128 | +64 | 112 ± 27 61 ± 7 | -45 | 478 ± 148 306 ± 44 | -36 |

"Before" samples were studied over a 5-mo period before immunization with peptides, n = 5 for lymphocytes, n = 3 for T, B, and NK cells "During" samples were taken from months 2-14 of immunization n = 11 for lymphocytes, T, B, and NK cells. Unless noted, p values for percent change comparing "before" values with "during" values were not significant, with p >0.05 using student's t test

* p >0.001
† p >0.005
‡ p >0.02
amino acid sequence of the neutralizing V3 loop region defined by the peptide (TRKSIRIQRGPGR) (Table 6). These data suggest that antibodies made by chimpanzee nos. 884 and 1028 at 7 mo after immunization with the HIVIIIIB Th-B

Discussion

In this paper we have shown that synthesis of the HIV env gp41 F domain NH2-terminal to synthetic peptides containing Th and B cell epitopes of HIV gp120 conferred on the resulting peptide the ability to induce immune hyporesponsiveness in chimpanzees to Th and B epitopes of HIV gp120 and to induce relative T cell lymphopenia. These observations may have clinical relevance in two areas, the pathophysiology of ineffective anti-HIV host immune responses, and the treatment of pathologic anti-HIV immune responses in HIV infection.

The pathogenesis of ineffective anti-HIV immune responses in HIV-infected humans is thought to be multifactorial, and includes induction of B and T cell defects in antigen responsiveness by HIV, infection of CD4+ APC by HIV, and direct HIV-induced T cell death (reviewed in reference 15). Data in the present study raise the possibility that the HIV gp41 F region may induce immune hyporesponsiveness in HIV-infected hosts to certain domains of the HIV envelope.

Table 5. Neutralization of HIV LAI/IIIB and HIVMN in Syncytium Inhibition Assay in Chimpanzees Immunized with T1-SP10 Peptides

| Animal no | LAI/IIIB | MN | LAI/IIIB | MN | LAI/IIIB | MN |
|-----------|---------|----|---------|----|---------|----|
| 884       | ~       | ~ (20) | ~       | ~ | ~       | ~ (24) |
| 1028      | ~       | ~ | ~       | ~ | ~       | ~ |
| 1045      | ~       | ~ (23) | ± (23) | ~ (23) | ~ (22) | ~ (24) |
| 1070      | ± (92) | ~ (22) | + (100) | + (96) | ± (86) | + (350) |

Data represent the presence of neutralization in syncytium inhibition assay. Numbers in parentheses represent the reciprocal titer in reverse transcriptase inhibition assay. ~, <48% inhibition of syncytia; ±, >49% and <80% inhibition of syncytia; +, >80% inhibition of syncytia, titer 10; + +, >80% inhibition of syncytia, titer 120.

Table 6. Reactivity of Chimpanzee Serum with Truncated Forms of the Th-B Peptide T1-SP10IIIB

| Chimpanzee no | Bleed date | T1-SP10IIIB | T1-flu | SP10C | SP10D | SP10E |
|---------------|------------|-------------|--------|-------|-------|-------|
| 884           | Month 7    | 204,800     | 800    | >102,400* | 51,200 | 3,200 |
| 1028          | Month 7    | 102,400     | 800    | 102,400 | 51,200 | 3,200 |

Data represent endpoint titers >3.0 E/C in ELISA. Peptides used in ELISA were T1-SP10IIIB, KQINMWQEVGKAMYACTRPNNTTRKSIIRQRGPG, T-flu, KQINMWQEVGKAMYATQRTALVGT, SP10C, (C)TRKSIRQGPR(Y), SP10D, (C)IRQRGPR, SP10E, (C)TRPNNTTRKSIIR. ELISA was performed as described in Materials and Methods. Flu sequence (TYQRTRALVGT) is from influenza nucleoprotein, strain A PR/8/34 (29).

* E/C at 1 102,400 = 6.0

724 Tolerance Induction by HIV Fusogenic Peptide
Immunization of chimpanzees with the F-Th-B peptide did not generally immunosuppress the animals, since PBMC PHA and Candida responses remained intact. However, in both animals immunized with F-Th-B (1045 and 1070), a relative T cell lymphopenia developed that was temporally related to immunization with the derivatized peptide (Fig. 4 and Table 4). Thus, we cannot rule out the possibility that F-derivatized peptides induced a more general immunosuppressed state than specific antigen hyporesponsiveness. However, at present, the immune hyporesponsive state induced by F-Th-B peptide most closely resembled classic immune tolerance to specific antigen (14). Immune hyporesponsiveness in animals 1045 and 1070 to HIV env determinants was transient, and was completely reversed by immunization of animals 1045 and 1070 with the Th-B peptide of HIVMN.

Because the observation of F-Th-B peptide-induced hyporesponsiveness was made in only two chimpanzees, consideration should be given to causes of immune tolerance other than immunization with F-derivatized peptides. First, all four animals were studied three to four times before immunization and, as well, were studied monthly throughout the 20 mo of study. The immune hyporesponsiveness seen in animals immunized with fusion domain peptides was a consistent finding throughout the time of immunization, and was temporally related to immunization with F-derivatized peptides. Moreover, PHA and Candida responses were normal throughout the immunization time, while hyporesponsiveness in animals 1070 and 1045 was limited to Th and B determinants on HIV gp120, suggesting specificity of hyporesponsiveness in F peptide–immunized animals. Second, animals were examined thoroughly each month throughout the study and no intercurrent illnesses occurred in animals 1045 and 1070 during the time of immune tolerance induction. Third, all four of the chimpanzees in our study had previously been used in hepatitis A, B, and C trials at the National Institutes of Health and the Centers for Disease Control. All of the animals in the present study were in similar hepatitis trials, and all are clinically healthy now 10–15 yr after the trials. Periodic mild elevations in liver function tests were noted in both Th-B- and F-Th-B-injected animals throughout the study period, a phenomenon frequently seen in animals given general anesthesia. Thus, the careful monitoring of the animals in the study, the consistent findings of hyporesponsiveness during the first 16 mo of immunization only in animals immunized with F-derivatized peptides, and the preimmunization control studies all suggest the immune hyporesponsiveness seen in animals 1070 and 1045 to gp120 Th and B determinants was due to immunization with F-derivatized peptides. However, given the small number of animals studied, we can not conclusively rule out other cofactors that might have contributed to our observations.

Data in the present study may also have relevance to the treatment of pathologic anti-HIV immune responses in HIV infection. One hypothesis to explain immune deficiency in AIDS suggests that the attack of pathologic anti-HIV immune responses of HIV-infected cells may lead to numerous manifestations of AIDS (reviewed in reference 15). For example, regions of the HIV env protein with sequence similarities to MHC class I and II molecules have been described (reviewed in reference 16). Recent data suggest that tissue damage in the lymphocytic pneumonia syndrome associated with HIV (17) and the skin rash associated with acute simian immunodeficiency virus infection in rhesus monkeys (18) are due to antiretroviral CD8+ CTL. Finally, immune-mediated destruction of thymic, bone marrow, and lymph node microenvironments has been postulated to play an etiologic role in end-stage immune dysfunction in AIDS (reviewed in references 15 and 19). Thus, for pathogenic anti-HIV immune responses, what would be needed to treat HIV infection and prevent the development of clinical AIDS would be the induction of specific tolerance to HIV antigens that are targets of pathologic anti-HIV immune responses. Whether the strategy of conjugation of the HIV gp41 F domain to HIV immunogens other than those studied would result in tolerance induction is not known.

The F domain of HIV gp41 has sequence homology to several viruses that mediate cell fusion (10). The HIV F domain inserts obliquely into lipid membranes, and has been postulated to be an amphipathic hydrophobic helix in the context of a lipid bilayer (20). The more hydrophobic amino acids are proposed to be located on one side of the putative helix, with the fusion domain inserting in lipid membranes as a sided insertion helical structure at a 70° angle to promote membrane fusion (20, 21). The observation that F-derivatized HIV env peptides induced immune hyporesponsiveness in chimpanzees, but not in goats or mice (2), suggests that the HIV F domain has specificity for interaction with primate vs. lower species immune cells, although at present, the explanation for F domain tolerance induction in primates remains unknown. It is plausible that the F portion of gp41 in peptide form can be biologically active. HIV gp41 F domain peptides can inhibit HIV-induced cell fusion (22), and can lyse liposomes and insert into planar lipid membranes (23).

Finally, an interesting observation in this study was the lack of requirement for IFA for boosting PBMC proliferative responses, while being required for boost of antibody levels in animal 1028 (Table 1 and Fig. 1). One explanation for the selective induction of proliferative responses by Th-B peptides in PBS would be the induction of Th1-like responses (Th for CTL) by peptides in PBS and the induction of Th2-like (Th for antibody production), as well as Th1-like, responses by peptides in IFA (24). In this regard, we have previously shown anti-HIV CTL generation by HIV env peptides in PBS in mice (M. K. Hart and B. F. Haynes, unpublished results). It has been suggested by some investigators that antiviral T cell responses may be required for protective anti-HIV immunity (reviewed in reference 15), while others have suggested that anti-HIV-neutralizing antibody responses are sufficient for protection from HIV challenges (25, 26).

Thus, the immune responses to peptides seen in this study, i.e., induction of T and B cell anti-HIV responses with IFA, the selective boost of anti-HIV epitope PBMC proliferative responses with peptide with no adjuvant, and immune hyporesponsiveness to HIV env epitopes with HIV gp41 F
domain-derivatized peptide, provide several new ways to modulate anti-HIV immune responses. Moreover, if it is shown that conjugation of the HIV F domain to non-HIV peptides is also tolerogenic, then the F derivatization of peptide epitopes of antigens that induce autoimmune or allergic immune responses may have potential for the treatment of human non-HIV-related diseases.

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Note added in proof It has recently been reported that a 23-aa peptide from the NH2 terminus of gp41 (aa 519–541) that contains the F peptide used in our study (aa 519–530) is capable of lysing human erythrocytes and CD4+ Hut 78 cells in vitro (30). Structural studies of peptide aa 519–541 in erythrocyte membranes have prompted the hypothesis that the NH2-terminal gp41 F domain may bind to cell membranes at either lipid or protein sites (31)

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