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Brucella abortus Conjugated with a gp120 or V3 Loop Peptide Derived from Human Immunodeficiency Virus (HIV) Type 1 Induces Neutralizing Anti-HIV Antibodies, and the V3-*B. abortus* Conjugate Is Effective Even after CD4$^+$ T-Cell Depletion

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Human immunodeficiency virus type 1 (HIV-1) infection is associated with loss of function and numbers of CD4$^+$ T-helper cells. In order to bypass the requirement for CD4$^+$ cells in antibody responses, we have utilized heat-inactivated *Brucella abortus* as a carrier. In this study we coupled a 14-mer V3 loop peptide (V3), which is homologous to 9 of 11 amino acids from the V3 loop of HIV-1 MN, and gp120 from HIV-1 SF2 to *B. abortus* [gp120(SF2)-*B. abortus*]. Our results showed that specific antibody responses, dominated by immunoglobulin G2a in BALB/c mice, were induced by these conjugates. Sera from the immunized mice bound native gp120 expressed on the surfaces of cells infected with a recombinant vaccinia virus gp160 vector (VPE16). Sera from mice immunized with gp120(SF2)-*B. abortus* inhibited binding of soluble CD4 to gp120, whereas sera from mice immunized with V3-*B. abortus* were ineffective. Sera from mice immunized with either conjugate were capable of blocking syncytium formation between CD4$^+$ CEM cells and H9 cells chronically infected with the homologous virus. Sera from mice immunized with gp120(SF2)-*B. abortus* were more potent than sera from mice immunized with V3-*B. abortus* in inhibiting syncytia from heterologous HIV-1 laboratory strains. Importantly, in primary and secondary responses, V3-*B. abortus* evoked anti-HIV MN antibodies in mice depleted of CD4$^+$ cells, and sera from these mice were able to inhibit syncytia. These findings indicate that *B. abortus* can provide carrier function for peptides and proteins from HIV-1 and suggest that they could be used for immunization of individuals with compromised CD4$^+$ T-cell function.

Human immunodeficiency virus type 1 (HIV-1) infection is characterized by an early impairment in T-helper (Th) cell function which becomes progressively worse because of loss of CD4$^+$ T cells (15). Consequently, antibody responses to protein antigens (as components of viruses or other infectious agents) are diminished in such individuals, since such responses are generally Th cell dependent (20). To circumvent the requirement for Th cells in these responses, we reasoned that carriers which stimulate B cells in the relative absence of Th cells may be beneficial. *Brucella abortus* is such a carrier and has been shown to enable trinitrophenyl (TNP) conjugates (TNP-*B. abortus*) to elicit immunoglobulin M (IgM) anti-TNP responses in athymic mice (20).

The potential use of *B. abortus* as a carrier in human vaccines is supported by several murine studies (cited above) and human studies. We demonstrated that TNP-*B. abortus* can elicit anti-TNP IgM responses from human B cells in vitro in the relative absence of T cells (6). In contrast, TNP-keyhole limpet hemocyanin (KLH) responses were abrogated following T-cell removal (6). Furthermore, TNP-*B. abortus* can stimulate human neonatal B cells to differentiate into anti-TNP-producing cells (8).

The finding that *B. abortus* conjugates can elicit IgM antibody responses in a T-cell-independent manner does not imply that T cells or their factors cannot be invoked by *B. abortus*. T cells and their factors increase B-cell responses and are required for switching from IgM to other isotypes. IgG2a levels (total and specific) increase in mice immunized with *B. abortus* or *B. abortus* conjugates, and this effect is abrogated in the presence of antibody against gamma interferon (IFN-γ) (4). IFN-γ is a product of Th type 1 (Th1) cells as well as CD8$^+$ and natural killer (NK) cells. A higher frequency of Th1 cells than Th2 cells was found among spleen cells from mice receiving *B. abortus* in vivo (22). Recently, we showed that *B. abortus* can stimulate human CD4$^+$ and CD8$^+$ T cells to secrete IFN-γ (1). The latter cytokine may be important in protection against viruses.

The critical difference between *B. abortus* and protein carriers (e.g., KLH) is that they activate T and B cells via different pathways. In the case of proteins, they have to be processed and their peptides are presented to T-cell receptors of CD4$^+$ T cells in association with major histocompatibility complex class II molecules. T-cell-B-cell collaboration involves cognate interaction between T-cell receptors on CD4$^+$ T cells and major histocompatibility complex class II-associated peptides on B cells or other antigen-presenting cells. As a consequence, an-

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tibody responses to protein antigens are strictly $CD4^+$ T cell dependent. In contrast, *B. abortus* can activate B cells directly, in the absence of $CD4^+$ T cells. The response can be increased by adding T-cell factors (17), i.e., physical contact between T and B cells is not required. Additionally, *B. abortus* induces antibody responses in the absence of antigen-presenting cells, so that major histocompatibility complex class II presentation of peptide is probably not involved (6). Taken together, these findings indicate that *B. abortus* stimulation of B cells occurs via a different, less restrictive, pathway than that invoked by protein antigens. Furthermore, unlike protein antigens, *B. abortus* can stimulate both $CD4^+$ and $CD8^+$ cells directly, in the absence of antigen-presenting cells, to secrete IFN-γ, which is necessary for B-cell isotype switching from IgM to IgG2a (1, 25). This type of T-cell help was termed noncognate cell interaction. These distinctive features of *B. abortus* suggested to us that it would be a valuable carrier in situations in which $CD4^+$ T-cell help is limiting, such as after HIV-1 infection.

Although *B. abortus* had been shown to provide carrier function for small haptens such as TNP, it was not known whether the same would be true for proteins or peptides coupled to *B. abortus*. Recently, we demonstrated that inactivated HIV-1 virions covalently linked to *B. abortus* (HIV-*B. abortus*) elicited neutralizing anti-HIV-1 antibodies in normal mice and in mice depleted of Th cells by repeated treatment with anti-CD4 antibodies (7). In this report we examine whether *B. abortus* can be used as a carrier for a subunit vaccine against HIV-1 infection. *B. abortus* was conjugated with recombinant gp120 (HIV-1 envelope protein) or with a synthetic peptide derived from the third variable region of gp120 (V3) and tested for immunogenicity in intact mice and in mice depleted of $CD4^+$ cells.

MATERIALS AND METHODS

Materials. Heat-inactivated *B. abortus* 1119.3 was obtained from the U.S. Department of Agriculture, Ames, Iowa. The following peptide, designated V3, was synthesized and purified (80%) in the Biological Resources Branch of the National Institute of Allergy and Infectious Diseases: H-Cys-Gly-**Arg**-Ala-Ala-**Ile-Gly-Pro-Gly-Arg-Ala-Phe-Tyr**-Gly-OH. The amino acids in boldface type are from the hairpin bend of the third variable loop of HIV-1 MN envelope protein. The other amino acids served as spacers, except for the cysteine which was used in coupling the peptide to carriers (see below). Alanines were substituted for histidine and isoleucine in positions 308 and 309 of the MN sequence, because in the presence of these amino acids peptide yield was low and the product was poorly soluble in water.

KLH and Tris-carboxyethyl phosphine hydrochloride (TCEP) were purchased from Pierce Chemical Co., Rockford, Ill. Recombinant gp120 was originally derived from HIV-SF2, expressed in CHO cells, and was kindly donated by Chiron Corporation, Emeryville, Calif. (lots MGC022 and MHC867). *N,N*-Dimethylformamide was obtained from Aldrich Chemical Co., Milwaukee, Wis. 3-Carboxy-4-nitrophenyl disulfide (Ellman's reagent) was supplied by Sigma or Fluka. HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) was furnished by ICN Biomedicals, Inc., and EDTA was purchased from Fisher Scientific Co. Bovine serum albumin (BSA), crystalline, was obtained from Amresco Inc., Solon, Ohio. Iodoacetic acid *N*-hydroxysuccinimide ester was obtained from Sigma or prepared as described by Krutzsch and Inman (14).

V3 peptide conjugates. A portion (2.3 mg) of the peptide was lightly radiolabeled with ^{125}I by using Iodobeads (Pierce Chemical Co.) and was added (~1,000 cpm) to the conjugation mixtures for coupling to *B. abortus*, KLH, and BSA (see below).

B. abortus was coupled to the peptide by iodoacetylating *B. abortus* and linking the two by thioether bonds, with the cysteine of the peptide. In preparation for iodoacetylation, pelleted *B. abortus* (0.43 g) was washed repeatedly after resuspension in >5.0 ml of buffer containing 0.15 M HEPES, 0.074 M NaOH, and 1.0 mM EDTA by centrifugation for 20 min at 8,000 × *g*. The pellet was then resuspended in 4 ml of this buffer, and 75 mg of iodoacetic *N*-hydroxysuccinimide ester, freshly dissolved in 0.7 ml of dimethylformamide, was added. The mixture was placed on a rocker and mixed at room temperature overnight. The modified *B. abortus* was washed four times as described above.

Purified V3 (24.4 mg) was dissolved in 1 ml of buffer containing 0.03 M HEPES, 0.0148 M NaOH, and 0.2 mM EDTA. Approximately 11,000 cpm of ^{125}I-V3 was added, and the mixture was chromatographed on a Bio-Gel P-2 column (0.7 by 19 cm). The first peak fractions (A_{280}) were pooled and placed in 2.2 mM TCEP for 0.5 h, to reduce any disulfide-linked peptides (dimers), and then mixed with the iodoacetylated *B. abortus*. The mixture was placed on a rocker for 4 days and then centrifuged for 20 min at 8,000 × *g*. The pellet was washed three times and resuspended in phosphate-buffered saline (PBS; 10% [wt/vol]). The degree of coupling was determined from the ^{125}I counts per minute and revealed that 1.78 mg (1.28 μmol) of V3 per ml of the final *B. abortus* suspension (~10^{10} *B. abortus* cells per ml) was present. That is 7.7×10^7 peptide molecules per bacillus.

Similar to the *B. abortus* KLH was coupled to the peptide by iodoacetylating KLH and linking the two by thioether groups, with the cysteine of the peptide. In order to couple the peptide to KLH, KLH was first iodoacetylated. Twenty milligrams of KLH was dissolved in 2.0 ml of water, to which 0.1 ml of 0.75 M HEPES buffer was added; next was added 50 μl of dimethylformamide containing 9.9 mg of iodoacetic acid *N*-hydroxysuccinimide ester in a freshly prepared solution. After standing for 2 h at room temperature, the mixture was centrifuged for 3 min at 5,000 × *g*. The supernatant was passed through a column (1.5 by 16 cm) of Bio-Gel P-6DG, with HEPES buffer (0.03 M HEPES, 0.0148 M NaOH, 0.2 mM EDTA). Fractions from the first peak (A_{280}) were pooled and contained 16 mg of iodoacetylated KLH in 3.08 ml.

V3 peptide (6.2 mg) was dissolved in 0.6 ml of HEPES buffer and spiked with ^{125}I-V3 (1,795 cpm) to yield 623 cpm/μmol of V3. TCEP (3.3 mg) was added, and the mixture was allowed to stand for 35 min. This mixture was then added to the 3.08 ml of iodoacetylated KLH. The pH was immediately adjusted to 8.6 with 1.0 M sodium carbonate, and the mixture was allowed to stand overnight at room temperature. The slightly turbid solution was centrifuged for 3 min at 5,000 × *g* to remove a small precipitate and passed through a Bio-Gel P-6DG column (1.5 by 25 cm) with HEPES buffer. Four 1.5-ml fractions were pooled from the breakthrough peak. The pool was clarified with a 0.45-μm-pore-size filter. The protein content was estimated as 14.73 mg, by use of the A_{280} and assuming that the peptide has an extinction coefficient similar to that of the KLH. The amount of peptide (as the Cl^- salt) was 2.43 mg, as determined from the total net counts per minute in the filtrate, this being bound to 14.73 − 2.43 = 12.3 mg of KLH. The density of the bound peptide was calculated as 13.2 peptides per 100,000 Da of KLH.

The peptide was also linked to BSA by a method similar to that used for KLH. The resulting conjugate contained 5.85 mg of peptide linked to 44 mg of BSA, i.e., 6.5 peptides per BSA molecule. The V3-BSA was used to coat enzyme-linked immunosorbent assay (ELISA) plates at 10 μg/ml.

gp120-*B. abortus* conjugates. gp120 from HIV-SF2 was linked to *B. abortus* by first thiolating carbohydrate groups on gp120 and linking those to iodoacetylated *B. abortus* via thioether groups. In preparation for thiolating the gp120, *N*-[3-(3-carboxy-4-nitrophenyldithio)propionyl]-β-alanine hydrazide (CPAH) was synthesized briefly as follows (11a). Both carboxyl groups of 3,3′-dithiodipropionic acid were activated as isobutylcarbonate-mixed anhydrides and coupled to β-alanine *t*-Boc-hydrazide. The latter intermediate was obtained from the *N*α-benzyloxycarbonyl derivative by hydrogenation over palladium on charcoal. The resulting 3,3′-dithiodipropionyl-β-alanine di-*t*-Boc-hydrazide was converted to 3-mercaptopropionyl-β-alanine *t*-Boc-hydrazide by reduction with dithiothreitol (DTT) in dimethylformamide under alkaline conditions. The resulting material was purified, crystallized from hot water, dried, dissolved in methanol, and added to an excess of 3-carboxy-4-nitrophenyl disulfide (Ellman's reagent) in methanol. The product was recovered by multiple extractions, dried, and treated with trifluoroacetic acid to remove the Boc group. The resulting CPAH (as an acid salt of the hydrazide) was treated in a vacuum to form a very hygroscopic solid that was stored at −20°C as a solution (83 mM) in methanol. Its concentration was determined from the A_{409} of a diluted aliquot in 20 mM DTT in 0.075 M HEPES buffer, pH 7.5 (ε_M = 14,700).

Prior to conjugation a fraction of gp120 was radiolabeled. [2,3-^3H]-*N*-succinimidyl propionate (2 μCi; 94.3 Ci/mM; Amersham) was treated with a gentle stream of dry nitrogen to remove the toluene solvent. Approximately 0.8 mg of gp120 in 0.5 to 0.8 ml of PBS was then immediately added and mixed carefully. After standing at room temperature for 2 h and at 4°C overnight, the reaction mixture was passed through a column (1 by 19 cm) of Bio-Gel P-6DG with 0.2 M NaCl. The breakthrough peak, containing ^3H-labeled gp120, was concentrated to a volume of 0.24 to 0.33 ml with a Centricon-30 (Amicon) concentrator and stored frozen (−20°C) or at 4°C with 0.1% sodium azide present.

The gp120 was thiolated by combining CPAH with aldehyde groups generated by limited periodate oxidation of carbohydrate moieties of the glycoprotein. The hydrazide function formed a stable hydrazone linkage, and the 3-carboxy-4-nitrophenylsulfenyl protecting/reporter groups were subsequently removed with DTT; 36 μl of acetate buffer (1.0 M sodium acetate, 0.224 M acetic acid) and 36 μl of 150 mM sodium periodate (freshly prepared in water) were added to the ^3H-labeled gp120. The mixture was allowed to stand in the dark at room temperature for 30 min and was then passed through a column (1 by 19 cm) of Bio-Gel P-6DG with the above acetate buffer diluted 10-fold. The breakthrough peak (monitored by A_{280}) was collected as a pool of 0.5-ml fractions. CPAH was immediately added (10 μl of an 83 mM concentration), and the solution was concentrated to 0.3 ml (Centricon-30). An additional 7.2 μl of CPAH solution was added. After standing in the dark overnight, the reaction mixture was applied

to a P-6DG column (1 by 19 cm) packed and run with 0.15 M HEPES–0.074 M NaOH–1.0 mM EDTA. DTT was added to the leading peak at a final concentration of 2 mM (the A_{409} value was then recorded); the solution was concentrated to approximately 0.6 ml (Centricon-30) and passed through the P-6DG column again. The absorbance units (AU) at 280 nm in the breakthrough peak pool was determined as $A_{280} \times$ volume (milliliters).

The thiolated gp120 was then coupled to iodoacetylated *B. abortus*. *B. abortus* insolubles corresponding to 1.1 to 1.2 g of pellet were washed two or three times by being mixed in HEPES buffer (0.075 M HEPES, 0.037 M NaOH, 0.5 mM EDTA) and centrifuged for 20 min at 8,000 $\times$ *g*. The final pellet was resuspended in 5.4 ml of HEPES buffer for each g of pellet. A solution of 56 mg of iodoacetic acid *N*-hydroxysuccinimide ester in 0.5 ml of dimethylformamide was added for each g of pellet. The suspension was mixed on a rocker overnight at room temperature. The pellet was recovered by centrifugation for 20 min at 8,000 $\times$ *g* and washed three times with 7-ml portions of 0.1 M NaCl and two times with HEPES buffer. Iodoacetylated *B. abortus* pellet was measured out in the proportion of 1.8 g per AU of thiolated gp120 and suspended in the thiolated gp120 (see above). Sodium azide (0.1%) was added as a preservative, and the suspension was gently mixed for 3 to 4 days at room temperature on a rocker. The mixture was then centrifuged and washed four times with PBS in the manner described above. Supernatants were saved for ^{3}H counting.

The presence of bound blue marker dye in the *B. abortus* preparations precluded direct counting of ^{3}H in samples of the conjugate. Also, since scintillation counting of precipitates bearing ^{3}H generally poses problems in determining counting efficiency, the estimation of the amounts of ^{3}H-gp120 bound to *B. abortus* was made by subtracting counts per minute found in the final supernatant and washes from the total counts per minute added to the coupling reaction. Supernatant and wash counts per minute were corrected for a small amount of quenching due to leached dye by recounting samples after the addition of an internal standard ([^{3}H]hexadecane). Specific activities of [^{3}H]gp120 were determined after labeling by estimating the concentration of polypeptide as $A_{280}/1.4$. $E_{1mg/ml}$ at 280 nm was arbitrarily taken as 1.4, the same as for immunoglobulin (16). The concentration of glycoprotein was taken as 2.20 times this value (Chiron data sheet on lot MHC867). On the basis of these calculations, the gp120-*B. abortus* contained 30 μg of gp120 polypeptide and 66 μg of glycoprotein per ml of 10% suspension ($\sim 10^{10}$ *B. abortus* organisms per ml), that is, 3×10^4 gp120 molecules per *B. abortus* organism.

Immunizations. BALB/c mice, aged 6 to 8 weeks, were obtained from the Jackson Laboratories, Bar Harbor, Maine. They were divided into groups of five and immunized intraperitoneally every 2 weeks with 0.1 ml of the following: (i) PBS; (ii) *B. abortus*, 10^9 organisms; (iii) V3, 50 μg, mixed with *B. abortus* 10^9 organisms; (iv) V3, 50 μg, plus KLH, 50 μg; (v) V3-*B. abortus*, 178 μg of V3 per 10^9 *B. abortus* organisms; (vi) V3-KLH, 9.9 μg of V3 and 50 μg of KLH; and (vii) gp120(SF2)-*B. abortus*, 6.6 μg of gp120 per 10^9 *B. abortus* organisms. Mice were bled 5 days after each immunization, and sera were collected. Antibody titers peaked after the third immunization. For memory responses mice were first injected with V3-KLH (50 μg of KLH), rested for 20 weeks, and then boosted with PBS, V3-KLH (50 μg of KLH), or V3-*B. abortus* (10^9 *B. abortus* organisms). The mice were bled 5 days after the second immunization.

Anti-L3T4 treatment. Rat anti-mouse L3T4 (CD4) monoclonal antibody (GK1.5) was partially purified from ascites by ammonium sulfate precipitation and dialyzed against PBS, as previously described (6). The batch used in this study killed >70% mouse thymocytes at a 10^{-4} dilution in the presence of rabbit complement (Low-Tox-M; Cedarlane, Ontario, Canada), and contained 2.2 mg of rat IgG2b per ml by radial immunodiffusion assay (ICN, Costa Mesa, Calif.). In order to deplete mice of $CD4^+$ cells, they were given 0.5 ml of GK1.5 (rat anti-mouse CD4) intraperitoneally daily for 3 sequential days and then once a week for the remainder of the experiment. Flow cytometry showed that these mice had <1% $CD4^+$ splenic cells. This was not due to masking of CD4 by the in vivo treatment with GK1.5, since cells stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-rat antibody were not increased in mice depleted of CD4 cells compared with that in untreated mice.

ELISA. Immunolon 4 (Dynatech, Chantilly, Va.) 96-well plates were coated with 100 μl of V3-BSA, 10 μg/ml, in carbonate buffer, pH 9.6, for 2 h at 37°C. The plates were then blocked by adding BSA in 0.1% azide overnight at 4°C. Sera from individual mice were added to wells, starting at a 1/100 dilution and then serially diluted two- or fivefold, for 1 h at 37°C. Alkaline phosphatase-linked anti-mouse antibodies (obtained from Southern Biotechnology Associates, Birmingham, Ala.) specific for class or subclass (μ, γ, γ1, γ2a, γ2b, and 3) were diluted 1/500 in PBS-Tween, and then 100 μl was added to each well for 1 h at 37°C. After each of the above steps, the plates were washed three times with PBS-Tween. Phosphatase substrate (Kirkegaard & Perry, Gaithersburg, Md.), in diethanolamine buffer, was then added for 1 h at room temperature, and the optical density at 405 nm was read with a V_{max} reader from Molecular Devices Corp. (Palo Alto, Calif.). The specificity of the antibodies used in the isotype analysis was tested as described previously (7). Titers represent the intercept on the *x* axis made by the linear portion of the serum titration curve, expressed as reciprocals of the dilution.

Syncytium inhibition assay. Cell lines, chronically infected with different HIV-1 strains (H9 cells infected with MN, IIIB, or RF and HUT 78 cells infected with SF2 [obtained from the AIDS Reagent Repository, Rockville, Md.]) were preincubated with serial dilutions of control or immune sera for 2 h at 37°C. These pretreated cells were then mixed with the uninfected $CD4^+$ T-cell line CEM at a ratio of 1:3 and cultured in 96-well plates at 10^5 cells per well in triplicate. The numbers of syncytia were counted 3 to 4 h later. All the chronically infected cells were previously characterized by flow cytometry and shown to express similar levels of surface envelopes. Furthermore, the numbers of syncytia formed with the effector CEM T cells (in the absence of sera) were all in the range of 200 to 250 syncytia per culture. Two human sera derived from HIV-1-infected individuals were kindly provided by Luba Vujcic (AIDS Research and Reference Reagent Program, Rockville, Md.). These sera have been tested by several assays for their abilities to neutralize the four HIV-1 laboratory strains used in our study (24) and were used as positive controls in our syncytium inhibition assay. Using these sera, we obtained the same rank order of syncytium inhibition with the four chronically infected cell lines as was found in other assays of viral neutralization. The inhibitory titers were generally lower (see Fig. 3) than those obtained in cell-free virus neutralization assays. This finding is common to other syncytium-based or focus-based assays (9).

TABLE 1. Comparison of amino acid sequences in the V3 loop region of the envelope of different HIV-1 strains

Source	Sequence													
V3 peptide[a]	C	G	R	A	A	I	G	P	G	R	A	F	Y	G
HIV strains[b]														
MN			R	I	H	I	G	P	G	R	A	F	Y	T
IIIB			R	I	Q	R	G	P	G	R	A	F	V	T
SF2			S	I	Y	I	G	P	G	R	A	F	H	T
RF			K	S	I	K	G	P	G	R	V	I	Y	A

[a] V3 was the peptide synthesized and coupled to *B. abortus*. The glycine and two alanines at the NH2 end were inserted as spacers, and the NH2-terminal cysteine was included to facilitate conjugation to *B. abortus* and proteins.

[b] Different HIV-1 strains that were used to infect cells in the syncytium inhibition assay.

Binding of immune sera to cells expressing HIV-1 envelope. The ability of immune sera to bind to HIV-1 envelope expressed on T cells was determined by flow cytometry as previously described (2, 3). Briefly, $CD4^-$ 12E1 T cells were infected with gp160-recombinant (HIV-IIIB) vaccinia virus VPE16. Infected cells were first incubated with control or immune sera (1:100 dilution) at 4°C for 1 h and then with FITC-conjugated goat anti-mouse serum for an additional 1 h (2, 3). The abilities of mouse sera to inhibit the CD4-gp120 interaction were assessed by preincubating gp120/41-expressing cells with control or immune sera at 4°C for 1 h. The cells were then incubated with soluble CD4 (sCD4; ABT, Cambridge, Mass.) at 10 μg/ml for 30 min at 37°C (conditions which are optimal for maximal sCD4 binding without significant shedding of gp120 [3]) and then stained with FITC-OKT4 (Ortho Diagnostics, Raritan, N.J.), which binds CD4 at a site distal from its gp120-binding region. Stained cells were analyzed with an Epics Profile (Coulter Counter, Hialeah, Fla.) to assess fluorescence. Mean fluorescence channel values were converted to fluorescence units with a standard curve generated with beads of increasing fluorescence intensities (Flow Cytometry Standards, Research Triangle Park, N.C.). This approach allowed subtraction of background fluorescence and calculation of the relative binding of a given serum to cells expressing HIV-1 envelope and the relative blocking by serum of sCD4 binding to gp120 expressed on cells. This quantitative assay was described in detail before and was used to measure the kinetics of sCD4 association and dissociation from gp120/41-expressing cells as well as the kinetics of sCD4-induced gp120 shedding (2, 3).

RESULTS

V3-*B. abortus* and gp120-*B. abortus* conjugates elicit antibody responses in BALB/c mice. To determine whether peptides or proteins from HIV-1 remained immunogenic when coupled to *B. abortus*, gp120 from HIV-1 SF2 and a synthetic 14-mer, derived from the third variable region (V3 loop) of the envelope of HIV-1, were covalently linked to *B. abortus* with heteroligation and thioether bonds. The peptide used was based on the MN sequence and has 9 of 11 amino acids identical to MN, i.e., RAAIGPGRAFY instead of RIHIGPGR AFY. It differs from the sequences of other laboratory strains (SF2, IIIB, and RF) by one or two additional amino acids, as shown in Table 1. Sera from mice immunized with V3(MN)-*B. abortus* exhibited higher titers of anti-V3 antibodies than sera from mice immunized with gp120(SF2)-*B. abortus* ($P < 0.01$ for IgM, IgG1, and IgG3) (Table 2). In contrast, gp120-*B.*

TABLE 2. Log antibody titers to *B. abortus* conjugates[a]

Antigen and *B. abortus* conjugate	Titer				
	IgM	IgG1	IgG2a	IgG2b	IgG3
Anti-V3					
PBS	<2.0	<2.0	<2.0	<2.0	2.7
B. abortus	2.7	2.0	2.7	2.7	2.0
V3 + *B. abortus*	2.9	<2.0	<2.0	<2.0	<2.0
V3-KLH	2.7	4.8	4.1	4.1	<2.0
V3-*B. abortus*	3.4	4.1	4.5	4.5	4.8
gp120-*B. abortus*	2.0	2.0	3.4	3.4	3.4
Anti-gp120(SF2)					
PBS	<2.0	<2.0	<2.0	<2.0	2.0
B. abortus	2.0	2.0	2.0	2.7	2.7
V3-*B. abortus*	2.0	2.7	2.7	2.7	3.4
gp120-*B. abortus*	2.0	4.1	5.2	4.1	2.7

[a] Antibody responses of BALB/c mice immunized three times at 2-week intervals. Serum from each mouse was tested for antipeptide or anti-gp120 antibody by ELISA. The results are expressed as log titers of the mean. Standard errors were 15% or less of the means.

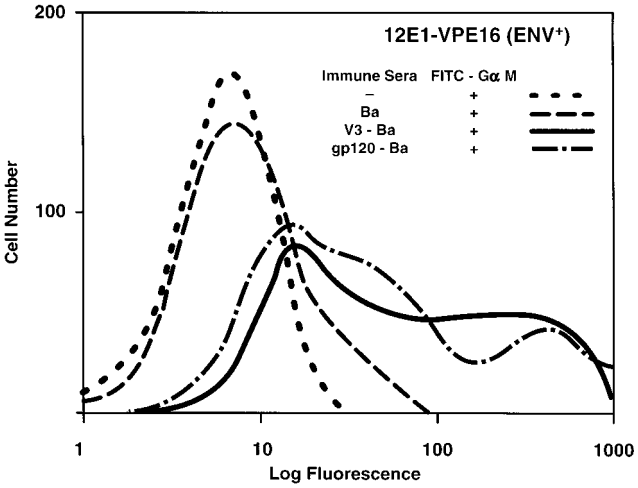

FIG. 1. Sera from mice immunized with V3(MN)-*B. abortus* (Ba) and gp120(SF2) recognize native gp120. Flow cytometry showed that serum antibodies from mice immunized with V3(MN)-*B. abortus* and gp120(SF2)-*B. abortus*, but not from mice immunized with *B. abortus* alone, bound to CEM cells infected with 12E1-VPE16 (Env^+). Surface binding was detected with FITC-conjugated goat anti-mouse antibodies (FITC- Gα M).

abortus induced higher anti-gp120(SF2) titers of certain isotypes ($P < 0.01$ for IgG1, IgG2a, and IgG2b;) than V3-*B. abortus*. These differences in titer probably reflect the disparities in the V3 loop region between MN and SF2, as depicted in Table 1. In addition, the gp120-*B. abortus* conjugates could elicit antibodies to other epitopes besides the V3 loop (see below). There was a tendency for V3-*B. abortus* and gp120(SF2)-*B. abortus* immunizations (Table 2) to trigger anti-V3 and anti-gp120 antibodies, respectively, such that IgG2a > IgG1. In contrast, V3-KLH responses tended to favor IgG1 (Table 2). Although these differences in IgG1 and IgG2a did not reach statistical significance in these experiments ($P > 0.05$), they did achieve significance in subsequent experiments (see Fig. 5 and Table 3). Mixtures of *B. abortus* or KLH with peptide did not elicit antibodies against the V3 peptide (Table 2, and data not shown; see Fig. 3), indicating that covalent bonding between the V3 peptide and the carrier was required to elicit peptide-specific antibody responses. Furthermore, the type of IgG evoked was analogous to what was seen when a small hapten, TNP, was conjugated to these carriers (20) or when HIV-1 proteins were conjugated to *B. abortus* (7).

The difference in isotype patterns observed when *B. abortus* or KLH was used as carriers suggests that these carriers stimulate different cytokines in the immunized mice, probably as a consequence of stimulating different subsets of Th cells. Activation of Th2 cells results in interleukin-4 (IL-4) secretion which promotes switching to IgG1, and triggering of Th1 cells leads to IFN-γ release, which favors switching to IgG2a (19).

Anti-V3 and anti-gp120 antibodies recognize native gp120. To determine whether the antibodies elicited by V3-*B. abortus* and gp120(SF2)-*B. abortus* were able to bind the native protein, sera from immunized mice were incubated with cells expressing gp120/gp41 on their surfaces. Binding of antibodies was detected by FITC-labeled goat anti-mouse antibodies and subsequent flow cytometry. Figure 1 shows that mice immunized with V3-*B. abortus* or gp120(SF2)-*B. abortus* produced antibodies which bound cells expressing gp120/gp41. The broad peaks seen on flow cytometry reflect the heterogeneity of gp120/gp41 present on the surfaces of cells infected with vaccinia virus gp160, as previously described (2). These results indicate that coupling either the linear V3 peptide or a recombinant form of gp120, derived from CHO cells, to *B. abortus* produced conjugates which elicited antibodies capable of recognizing not only the peptide or protein immunogens but also native envelope glycoproteins expressed by infected cells.

It was important to determine whether mice immunized with gp120(SF2)-*B. abortus* generated antibodies which could interfere with the CD4-gp120 interaction. As can be seen in Fig. 2, binding of sCD4 to cells expressing surface gp120/41 could be monitored by flow cytometry with FITC-OKT4. The conditions used in this experiment allowed maximum binding of sCD4 without shedding of the gp120 as previously described (2, 3). Preincubation of the cells with sera from mice immunized with gp120-*B. abortus*-immunized mice, but not sera from mice immunized with V3-*B. abortus*, shifted the curve to the left. The

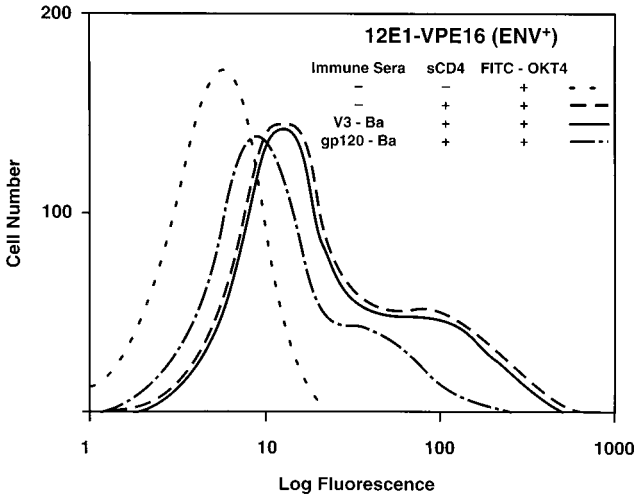

FIG. 2. Sera from mice immunized with gp120(SF2), but not V3(MN)-*B. abortus* (Ba), inhibit binding of sCD4 to gp120. CEM cells were infected with 12E1-VPE16 (Env^+); they were then incubated with sCD4 in the presence or absence of sera from immunized mice. Binding of sCD4 to the cells was assessed by flow cytometry after FITC-OKT4 was added. The addition of sera from mice immunized with gp120(SF2), but not with V3(MN)-*B. abortus*, shifted the curve (generated in the absence of immune sera) to the left. The shift was calculated to represent 50% inhibition of sCD4 binding.

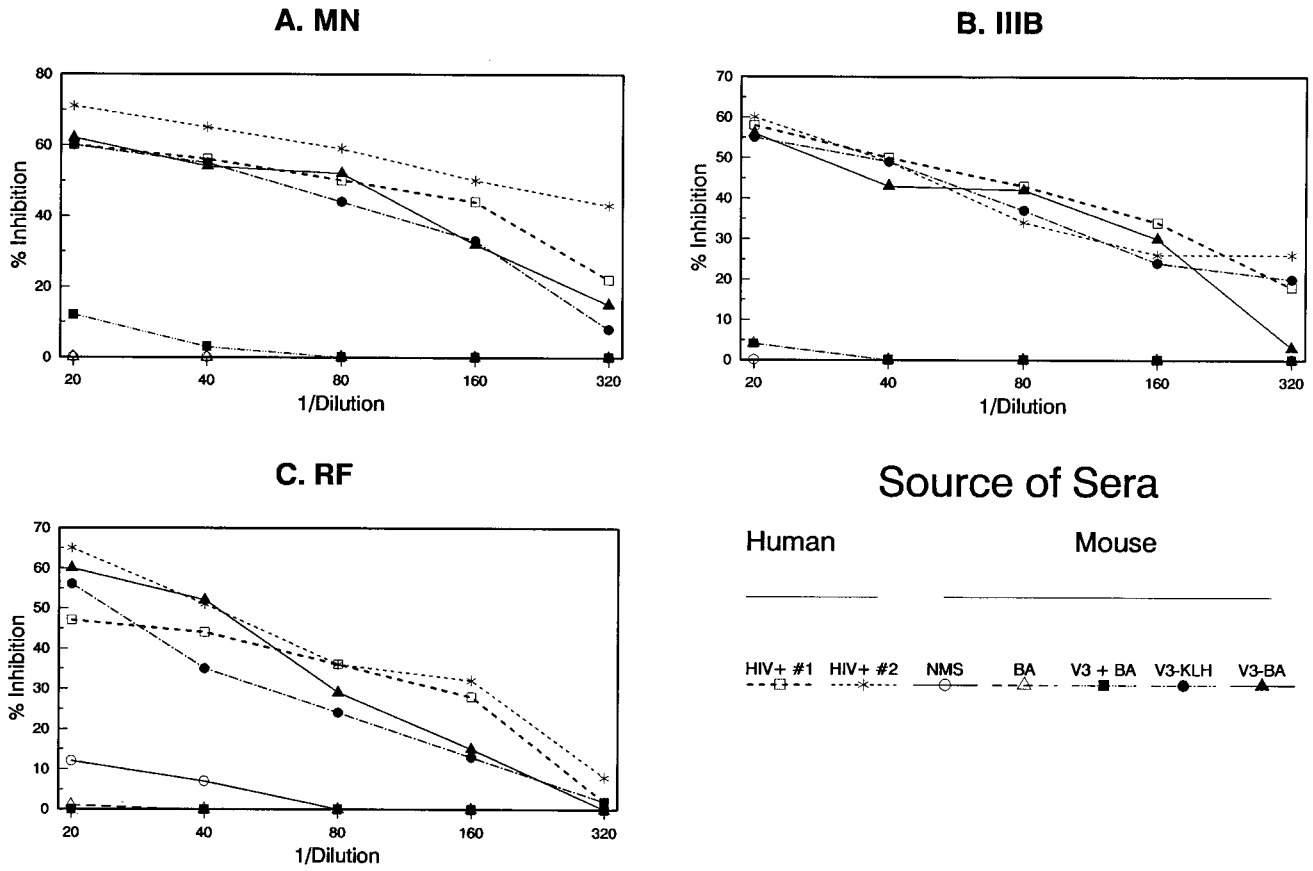

FIG. 3. Syncytium inhibition by HIV$^+$ human reference sera 1 and 2 and by mouse immune sera (normal mouse serum [NMS]), *B. abortus* (BA), V3 + *B. abortus*, V3-KLH, and V3-*B. abortus*. H9 cells (2.5×10^4), chronically infected with HIV-1 MN (A), IIIB (B), or RF (C), were preincubated for 1 h with human HIV$^+$ reference sera or mouse immune sera at 37°C. Uninfected CEM cells were added (7.5×10^4 per well, in triplicate). Syncytia were counted 3 h later. The numbers of syncytia in the absence of sera were similar for all three strains (200 to 250 syncytia per well). The results are presented as percent inhibition of the control cultures (no serum added). Each point represent the mean of three cultures. The standard errors were ≤10% of the means.

shift represented a 50% inhibition of sCD4 binding (on the basis of calculations described in Material and Methods and references 2 and 3). These findings were as expected, since the region on gp120 from which the V3 peptide is derived is not involved in binding to CD4, whereas the CHO-derived gp120 used in the immunization does contain the CD4-binding region. The finding that gp120-*B. abortus* elicited antibodies against this region indicates that linkage of gp120 to *B. abortus* resulted in antibody responses to a conformational determinant, which may be important in protective immunity. Thus, the chemistry used in coupling the gp120 to *B. abortus* did not interfere with this conformational determinant.

Inhibition of syncytia mediated by cell lines chronically infected with different HIV-1 strains by sera from mice immunized with V3 and gp120(SF2). The biological activity of the immune sera was tested by the syncytium inhibition assay. We used a panel of cell lines chronically infected with different laboratory strains (MN, IIIB, SF2, and RF). They all express similar levels of envelope glycoproteins, and following coculture with CD4$^+$ uninfected T-cell lines (e.g., CEM), they form multinucleated syncytia within 2 to 3 h. Blocking of syncytium formation by antibody is more difficult to achieve than neutralization of cell-free virus (9). Nevertheless, since syncytium formation probably resembles cell-to-cell transfer of virus in vivo, it was important to determine whether the antisera we generated could inhibit. In order to validate our assay, we obtained two HIV$^+$ human sera (and one negative serum) from the NIH AIDS Research and Reference Reagent Program (produced by Luba Vujcic, Center for Biologics Evaluation and Research, Food and Drug Administration [see Materials and Methods]) (24). Reference serum 2 was found by various laboratories to have a higher neutralization titer than reference serum 1. Both sera neutralized MN more efficiently than other laboratory strains (MN > SF2 > IIIB > RF). We tested these reference sera along with mouse immune sera in our syncytium inhibition assay. As depicted in Fig. 3, the two human sera blocked syncytium formation between H9 (MN), H9 (IIIB), and H9 (RF) with relative efficiencies similar to those previously established. Reference serum 2 showed a higher neutralization titer than reference serum 1. Interestingly, even at the highest serum concentration used (1:20), the two human sera did not block syncytium formation by 100%. In the same experiment the immune sera derived from V3-*B. abortus*- or V3-KLH-primed mice exhibited syncytium inhibition patterns similar to those of the human reference 1. Inhibition of H9 (MN) syncytia was more efficient than that for IIIB and RF. Importantly, immune sera from mice immunized with a mixture of V3 and *B. abortus* did not contain significant levels of syncytium-blocking antibodies.

Compared with the other HIV-1 strains used in the syncytium assay, the V3 peptide resembles MN most, whereas gp120 was derived from the SF2 strain. As seen in Table 1, the V3

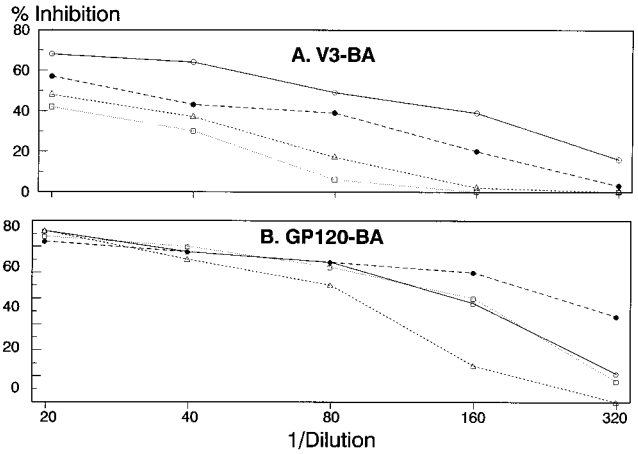

FIG. 4. Syncytium inhibition by sera from mice immunized with gp120(SF2) and V3(MN)-*B. abortus* (BA). Syncytium inhibition of cells infected with HIV-1 MN (○), IIIB (△), RF (□), and SF2 (●) by sera from mice immunized with V3(MN)-*B. abortus* (A) and gp120(SF2)-*B. abortus* (B). Individual sera (see footnote *a* to Table 2) were incubated for 1 h at 37°C, at different dilutions, with infected cells, prior to the addition of uninfected CEM $CD4^+$ T cells. Syncytia were counted 3 to 4 h later. The results are expressed as the percent inhibition of syncytia, i.e., (number of syncytia in the absence of serum/number of syncytia in the presence of serum) × 100. Each point represents the mean for five mice. The standard errors were 10% or less of the means.

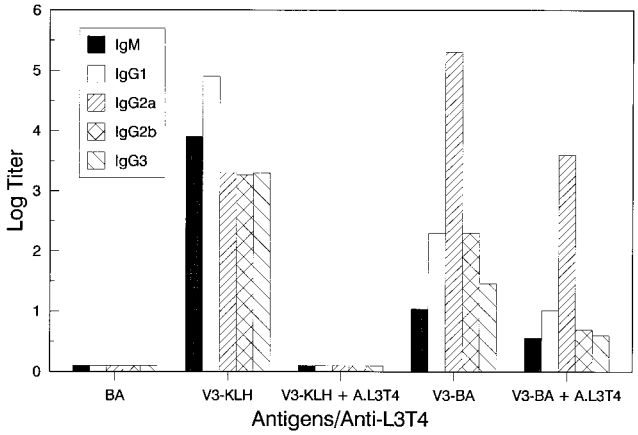

FIG. 5. Mice immunized with V3(MN)-*B. abortus* (BA), but not V3(MN)-KLH, produce anti-V3 antibodies after $CD4^+$ T-cell depletion. Sera from mice immunized with V3(MN)-KLH or V3(MN)-*B. abortus*, in the presence or absence of anti-L3T4 treatment (A.L3T4), were collected 5 days after the third immunization. Log anti-V3 antibody titers were determined by ELISA from endpoint titrations with antibodies specific for mouse μ- and γ-subclass chains. The top of each bar represents the mean log titer for five mice. The standard errors were 15% or less of the means.

peptide contains the GPGRAF motif which is identical in MN, IIIB, and SF2 but different in RF in two positions (valine and isoleucine in place of alanine and phenylalanine). Strain IIIB differs from MN and the V3 peptide at the flanking amino end, with an arginine in place of an isoleucine, and at the carboxy end, with a valine instead of a tyrosine. SF2 differs from MN and the V3 peptide at the carboxy end, with a histidine residue in place of tyrosine. RF differs from MN and the V3 peptide at the amino end, with a lysine instead of isoleucine.

The results in Fig. 4 also demonstrate that sera from mice immunized with V3-*B. abortus* are most efficient in inhibiting syncytia between H9 (MN) and CEM cells. The same sera were less effective in inhibiting the syncytia of the other laboratory strains. The extent of inhibition appeared to correlate with the degree of homology in the V3 region between the V3 peptide used as the immunogen and the strain of virus used in the assay. Serum titers from mice immunized with V3-*B. abortus* that caused 50% inhibition of syncytia (IT_{50}) were calculated from regression analyses of the inhibition curves as 79 for MN, 30 for IIIB, 20 for SF2, and 15 for RF (Fig. 3A). Sera from mice immunized with gp120(SF2)-*B. abortus* (Fig. 4B) exhibited more cross-reactivity, as evidenced by their greater ability to inhibit syncytia induced by T cells chronically infected with nonhomologous strains. Thus, the latter sera inhibited syncytia most effectively when the cells were chronically infected with SF2 (IT_{50} = 91), were less effective in inhibiting syncytia in cells infected with RF (IT_{50} = 77) and MN (IT_{50} = 67), and were least effective in inhibiting syncytia induced by cells infected with IIIB (IT_{50} = 47). These results indicate that the two alanine substitutions we made in the MN peptide did not change the fine specificity of the response. However, differences in the GPGRAF motif (as seen in RF) and, to a lesser extent, differences in flanking amino acids (as exhibited by IIIB and SF2), did diminish the ability of sera to inhibit syncytia.

Sera from mice immunized with gp120(SF2)-*B. abortus* but not sera from mice immunized with V3-*B. abortus* contain antibodies which react with the CD4-binding region of gp120, as illustrated in Fig. 2. The presence of these antibodies may explain the greater ability of sera from gp120(SF2)-*B. abortus*-immunized mice than sera from mice immunized with V3-*B. abortus* to inhibit syncytia formed by heterologous HIV-1 strains.

Mice depleted of CD4 T cells retain the ability to produce neutralizing anti-V3 antibodies following immunization with V3-*B. abortus*. In order to mimic the Th cell defect of HIV-1 infection, mice were rigorously treated with the anti-CD4 monoclonal antibody GK1.5. Previously, we showed that mice receiving anti-CD4 antibody on 3 consecutive days followed by weekly injections of GK1.5 were depleted of $CD4^+$ T cells and lacked Th cell function for T-dependent antibody responses (7). This regimen was utilized in this study, and immunizations were started after the first three consecutive treatments with anti-CD4 antibody. The mice were boosted on weeks 3 and 5. Flow cytometry of splenic cells from these mice confirmed that fewer than 1% $CD4^+$ cells remained.

V3-KLH responses were completely abrogated in mice depleted of $CD4^+$ cells (≥3 log reduction in antibody binding titers), indicating that these mice were functionally Th cell deficient. In comparison, immunization with V3-*B. abortus* resulted in production of anti-V3 antibodies. Although the overall titers were reduced in the mice treated with anti-CD4 (1 to 2 log reduction), the isotype profile was similar to that of intact mice with a predominance of the IgG2a subclass (Fig. 5). As noted before (Table 2) (20), when KLH was used as a carrier, the profile was IgG1 > IgG2a ($P < 0.05$), and when *B. abortus* was the carrier, this profile was reversed, i.e., IgG2a > IgG1 ($P < 0.005$).

These results are in agreement with previous experiments which showed that $CD4^+$ Th cells are not absolutely required for responses evoked when *B. abortus* is used as a carrier but do enhance these responses, especially in the IgG class (7, 17). It is possible that in the absence of $CD4^+$ T cells, IFN-γ, which is required for switching to IgG2a, could be provided by $CD8^+$ or NK cells. Recently, we showed that *B. abortus* can activate purified human $CD4^+$ and $CD8^+$ T cells, but not enriched NK cells, to secrete IFN-γ (25). Thus, in $CD4^+$-depleted mice, *B. abortus* as a carrier is capable of generating noncognate help for B cells in the form of IFN-γ derived from $CD8^+$ T cells.

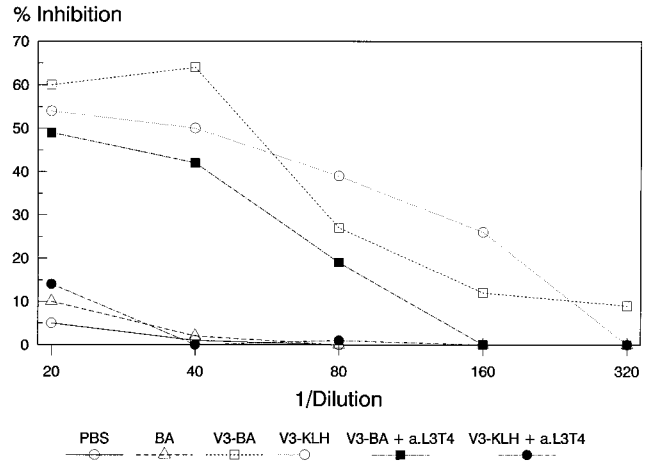

FIG. 6. Syncytium inhibition of HIV-1 (MN) by sera from CD4-depleted mice. Sera from mice immunized with different antigens (as indicated in the figure) in the presence or absence of anti-L3T4 (a.L3T4) treatment were collected 5 days after the third immunization. H9 cells, chronically infected with HIV-1 MN, were incubated with individual sera at different dilutions prior to adding uninfected CEM $CD4^+$ T cells. Syncytia were counted 3 to 4 h later. Each point represents the mean for five mice. The standard errors were 10% or less of the means. BA, *B. abortus.*

Sera from the mice depleted of $CD4^+$ T cells and immunized with V3-*B. abortus* were tested for their abilities to inhibit syncytia. As can be seen in Fig. 6, these sera were able to inhibit syncytia mediated by infection of T cells with HIV-1 MN. The potency of these sera was not much less than that of mice immunized with V3-*B. abortus* in the absence of anti-CD4 treatment. Titers causing 50% inhibition of syncytia were calculated as 21 and 50 for mice immunized with V3-*B. abortus* in the presence and absence of anti-CD4 treatment, respectively. Since IgG2a was the predominant isotype elicited by V3-*B. abortus* in CD4-depleted mice (Fig. 5), these data suggest that IgG2a antibodies are particularly effective in preventing HIV-1 from forming syncytia.

These results demonstrate that *B. abortus* can provide carrier function in mice for HIV-1-derived peptides even when $CD4^+$ Th cell function is limited. This implies that *B. abortus*-peptide conjugates may be beneficial in immunodeficient patients, such as those infected with HIV-1 with impaired $CD4^+$ Th cell function.

Memory responses can be boosted following $CD4^+$ T-cell depletion in mice immunized with V3-*B. abortus* but not V3-KLH. To determine whether V3-*B. abortus* was capable of eliciting secondary antibody responses in mice depleted of $CD4^+$ T cells, mice were first immunized with V3-KLH and 20 weeks later received booster injections with V3-KLH or V3-*B. abortus* in the presence or absence of treatment with anti-L3T4 (Table 3). Compared with naive animals, anti-V3 antibody levels were elevated in mice that received a primary immunization with V3-KLH and were then sham immunized with PBS 20 weeks later. The latter mice exhibited an isotype pattern characteristic of KLH as a carrier, i.e., IgG1 > IgG2a ($P < 0.005$), as seen in Tables 2 and 3 and Fig. 5. Mice receiving a secondary boost with V3-KLH increased their anti-V3 antibody titers such that the IgG1 > IgG2a pattern was maintained ($P < 0.005$). However, the V3-KLH boost was ineffective in increasing anti-V3 antibody titers in mice that had been treated with anti-L3T4 antibody to delete $CD4^+$ T cells. This was the case for both IgM and IgG subclass antibodies, except for a slight increase in the IgG3 titer ($P < 0.01$). IgG3 was

TABLE 3. Log anti-V3 antibody titers[a]

Booster	Anti-L3T4 treatment	Titer				
		IgM	IgG1	IgG2a	IgG2b	IgG3
PBS	−	2.3	4.0	3.2	2.8	2.3
V3-KLH	−	3.6	5.4	4.1	4.1	3.8
V3-KLH	+	2.5	3.9	2.7	2.9	2.9
V3-*B. abortus*	−	4.1	5.3	4.3	4.6	4.8
V3-*B. abortus*	+	3.4	4.8	3.8	4.3	3.9

[a] Titers were determined for the sera of mice immunized with V3-KLH and boosted with V3-KLH or V3-*B. abortus* in the presence or absence of anti-L3T4 treatment at the time of boosting. Endpoint titrations are expressed as the means from groups of five BALB/c mice. Standard errors were 10% or less of the means. Individual sera were collected 5 days after the booster injection and assayed by ELISA on plates coated with V3-BSA.

previously associated with T-independent responses (20). In contrast, boosting with V3-*B. abortus* resulted in increased antibody titers even after $CD4^+$ T-cell depletion. These increases occurred for IgM and all IgG subclasses ($P < 0.05$ compared with mice receiving PBS). Interestingly, the isotype pattern seen in mice boosted with V3-*B. abortus* (with or without anti-L3T4 treatment) was similar to that for the mice receiving a secondary immunization with V3-KLH (not treated with anti-L3T4), in that IgG1 > IgG2a ($P < 0.005$). Thus, the carrier used in the primary response, in this case KLH, appears to determine the isotype profile seen in secondary responses; even if the carrier used in the secondary response is *B. abortus*, which induced an IgG2a > IgG1 pattern if given in both primary and secondary responses (Table 2 and Fig. 5). These data suggest that V3-*B. abortus* conjugates can trigger memory B cells that have switched to IgG1.

The same sera were tested for the ability to inhibit syncytia mediated by HIV-1 MN (Fig. 7). Mice immunized with V3-KLH that received a secondary boost of V3-KLH in the absence of anti-L3T4 were able to inhibit syncytia ($IT_{50} = 24$), but treatment with anti-L3T4 abrogated these responses. In contrast, sera from mice immunized with V3-*B. abortus* in the

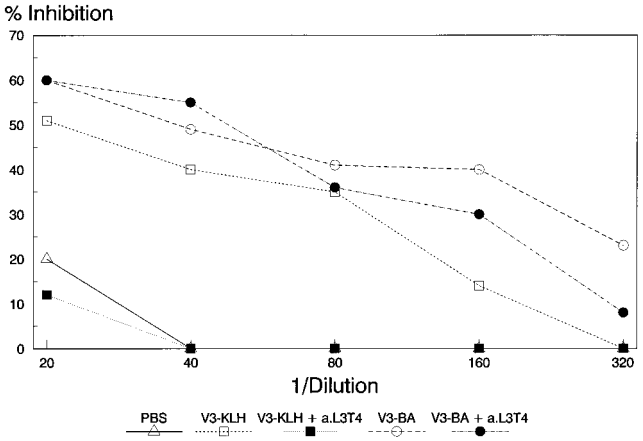

FIG. 7. Syncytium inhibition of HIV-1 (MN) by sera from mice immunized with V3-KLH and boosted with V3-KLH or V3-*B. abortus* (BA) under conditions of CD4 depletion. Mice were immunized with V3-KLH and boosted 20 weeks later with V3-KLH or V3-*B. abortus* in the absence or presence of anti-L3T4 antibody (a.L3T4). Sera were collected 5 days after the third immunization. H9 cells, chronically infected with HIV-1 MN, were incubated with individual sera at different dilutions prior to the addition of uninfected CEM $CD4^+$ T cells. Syncytia were counted 3 to 4 h later. Each point represents the mean for 5 mice. The standard errors were 10% or less of the means.

absence or presence of anti-L3T4 were capable of inhibiting syncytia (IT_{50} = 45 and 41, respectively).

This shows that prior exposure to a T-dependent form of HIV-1 envelope peptide (V3-KLH) can be boosted in a secondary response, even after Th cell depletion (a consequence of infection with HIV-1). This occurs if the second immunization is performed with the peptide conjugated to a T-independent type 1 carrier, namely, *B. abortus*, rather than a T-dependent one, such as KLH.

DISCUSSION

In this report we show that *B. abortus* can provide carrier function for gp120 and a 14-mer peptide containing a segment (RAAIGPGRAFY) with 9 of 11 amino acids identical to a region in the V3 loop region of the HIV-1 MN envelope (RIHIGPGRAFY). High-titer anti-peptide responses that were predominantly of the IgG2a isotype were induced by V3-*B. abortus*. Moreover, these antibodies could bind native gp120/41 expressed on cells infected with vaccinia virus gp160. Viral neutralization of different isolates by sera from V3-*B. abortus*-immunized mice, as assessed by syncytium inhibition, correlated with the degree of amino acid sequence similarities between the laboratory strains in this region of the V3 loop and the MN sequence. The least inhibition was observed with the most disparate strain, RF. Compared with sera from V3-*B. abortus*-immunized mice, sera from mice immunized with gp120(SF2)-*B. abortus* were more cross-reactive as determined by their abilities to inhibit syncytia from heterologous strains at a higher titer. This was probably due to the presence of other neutralizing epitopes, particularly the CD4-binding domain, which is more conserved among strains.

The predominance of specific IgG2a antibodies elicited by the peptide, V3, and the HIV-1 envelope, gp120, when conjugated to *B. abortus* is in accordance with the observation that *B. abortus* stimulates T cells belonging to the $CD4^+$ Th1 subset as well as $CD8^+$ T cells to secrete IFN-γ (1, 25). This cytokine is required for switching of B cells from IgM to IgG2a secretion (4, 20). IgG2a is the preferred isotype in protection from viruses because of its superior ability, compared with that of other murine IgG subclasses, to activate complement (23) and to bind Fc receptors (13). Induction of Th1 cells is beneficial in intracellular parasitic infections (11) and may be important in HIV-1 infection in humans.

The major reason for conjugating peptides and proteins derived from HIV-1 to *B. abortus* is related to the possibility that these conjugates would elicit antibody responses in situations in which T-cell help is limiting. Small haptens coupled to *B. abortus* can trigger anti-hapten antibodies in athymic mice (20), and HIV-1 proteins linked to *B. abortus* were able to stimulate anti-HIV-1 antibodies even in CD4-depleted mice (7). In this report we show that a 14-mer peptide derived from the V3 loop of HIV-1 MN can elicit antibodies which recognize the peptide, as well as native gp120 expressed on the surface of T cells, and which can inhibit syncytia induced by HIV-1. Furthermore, CD4-depleted mice retain antibody responsiveness to V3-*B. abortus* but lose responsiveness to V3-KLH. The latter was seen in primary and secondary responses. After infection with HIV-1, one would expect that later viral reactivation would boost the immune response to the virus. However, this is probably limited by impaired $CD4^+$ Th-cell function. The ability of V3-*B. abortus* to induce a secondary response following T-cell depletion of mice suggests that this type of conjugate may boost anti-HIV-1 responses in individuals infected with this virus even if their Th cells are impaired.

The antipeptide titers attained following $CD4^+$ T-cell depletion were reduced compared with those obtained in the absence of $CD4^+$ T-cell depletion. This is in keeping with our understanding that *B. abortus* as a carrier is relatively T cell independent, in that it can stimulate IgM antibody responses in the absence of $CD4^+$ T-cell help but that removal of $CD4^+$ T cells results in suboptimal responses due to a loss of T-cell factors (17). $CD8^+$ T cells can probably provide IFN-γ under these conditions, so that switching from IgM to IgG2a occurs (1). This is very different from the KLH response which is completely abrogated by $CD4^+$ T-cell depletion, because in addition to T-cell factors, this response requires cognate interaction between $CD4^+$ T and B cells.

The antibodies elicited by V3-*B. abortus* were mainly of the IgG2a subclass in normal mice and mice depleted of $CD4^+$ T cells. Since switching to IgG2a requires IFN-γ (4, 21), the source of IFN-γ in the latter mice may have been from $CD8^+$ T cells, since *B. abortus* can evoke IFN-γ secretion from human $CD8^+$ T cells, but not from NK cells, in vitro (1).

It should be noted that, in the syncytium inhibition assay, the titers of sera from mice immunized with V3-*B. abortus* and treated with anti-CD4 were similar to those from mice immunized in the same way but not receiving anti-CD4 (IT_{50}s were calculated as 21 and 50, respectively). However, when comparing the sera from the same mice in terms of binding to peptide (ELISA), the titers of sera from anti-CD4-treated mice sera were approximately 10- to 100-fold lower than those from mice not receiving anti-CD4 (Fig. 4). This discrepancy can possibly be explained on the basis that IgG2a is the most efficient isotype in syncytium inhibition and that the presence of other isotypes to a greater extent in mice not receiving anti-CD4 treatment reduces the ability of IgG2a to inhibit syncytia.

Splenic T cells from mice injected with *B. abortus* are mainly of the Th1 phenotype, as evidenced by their secretion of IL-2 and IFN-γ, under limiting dilution conditions (22). Th1 cells appear to be desirable in protection against certain infectious conditions in mice (11) and in humans (19), especially if the infectious agent is intracellular. This probably relates to the fact that Th1 responses involve both the cellular and the humoral arms, whereas Th2 responses provide help mainly for antibody responses (18). Because of its potential as a carrier in human vaccines, we have also studied the ability of *B. abortus* to stimulate cytokine production from human T cells and found that it elicits IFN-γ, but not IL-4, in terms of both protein and mRNA. IL-2 mRNA, but not protein, was also detected (1, 25, 26). In addition, *B. abortus* induced human $CD8^+$ T cells, depleted of $CD4^+$ cells, and T cells from individuals infected with HIV-1 (1) to secrete IFN-γ. These findings suggest that *B. abortus* is effective in stimulating immune responses in HIV-infected persons, including those who have impaired Th-cell function.

The ability of *B. abortus* to provide carrier function for proteins and small peptides suggests that this approach could be used for other infectious agents, especially in situations in which immunization with inactivated or attenuated microorganisms is not effective and/or safe. Conjugation to *B. abortus* obviates the need for adjuvants, since high-titer responses occur in their absence. We are currently studying the toxic effects of *B. abortus*. The lipopolysaccharide (LPS) purified from this bacillus is 10,000-fold-less toxic than LPS from *Escherichia coli* in terms of pyrogenicity in rabbits, 300-fold-less lethal in mice, and less potent in releasing IL-1 (300-fold) and tumor necrosis factor alpha (1,400-fold) from human monocytes in vitro (10). Attenuated *B. abortus* has been used for vaccination of humans, but data from those studies are limited (5).

In summary, the findings of this report indicate that *B. abortus* can be an effective carrier for proteins and peptides and

that peptide-*B. abortus* conjugates can induce antipeptide antibody responses even after rigorous depletion of $CD4^+$ T cells. This, together with previous work showing that *B. abortus* stimulates Th1-like cytokines in mice and humans (1, 22), activates human B cells in the relative absence of T cells (6), and has an LPS component which is relatively nontoxic (10), enhances it as a potential carrier in the development of subunit vaccines for human use.

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