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Brucella abortus Conjugated with a Peptide Derived from the V3 Loop of Human Immunodeficiency Virus (HIV) Type 1 Induces HIV-Specific Cytotoxic T-Cell Responses in Normal and in CD4$^+$ Cell-Depleted BALB/c Mice

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We have previously shown that immunization of mice with human immunodeficiency virus (HIV)-derived proteins or peptides conjugated to inactivated *Brucella abortus* induces the secretion of virus-neutralizing antibodies, predominantly of the immunoglobulin G2a (IgG2a) isotype. In addition, *B. abortus* activates human CD4$^+$ and CD8$^+$ cells to secrete gamma interferon. Since these are both characteristics of a Th1-type immune response, which is associated with the development of cell-mediated immunity, it was important to determine if *B. abortus* conjugates would also act as a carrier to induce a cytotoxic T-lymphocyte (CTL) response. To test this hypothesis, we conjugated an 18-amino-acid peptide from the V3 loop of the MN strain of HIV-1 gp120 that contains both B- and cytotoxic T-cell epitopes to *B. abortus* (*B. abortus*–MN 18-mer). A 10-amino-acid fragment of this peptide has been shown to be the minimal CTL determinant presented by murine *H-2D^d*. It was found that two in vivo immunizations with 10^8 organisms of *B. abortus*–MN 18-mer followed by in vitro stimulation with peptide induced a virus-specific CTL response. Conjugation to *B. abortus* was required for in vivo priming, since there was no induction of memory CTLs when *B. abortus* was only mixed with peptide. Targets pulsed with peptide as well as those infected with a vaccinia virus encoding HIV gp160 were killed, demonstrating recognition of naturally processed envelope. Also, major histocompatibility complex-incompatible L cells which were infected with vaccinia viruses that encoded *H-2D^d*, but not *H-2K^d*, and pulsed with peptide were lysed. This demonstrated the appropriate major histocompatibility complex class I restriction. Treatment of the mice with anti-L3T4 prior to immunization caused a severe depletion of CD4$^+$ lymphocytes, yet it did not decrease the CTL priming. Thus, inactivated *B. abortus* can induce non-CD4$^+$ cells to produce the cytokines required for CTL induction. We conclude that *B. abortus* stimulates a cellular as well as a humoral immune response, even in the relative absence of CD4$^+$ helper cells. It may be a particularly useful vaccine carrier in HIV-1-infected individuals or others with impaired CD4$^+$ T-cell function.

The induction of CTL activity is an essential component of an antiviral response. CTLs have been shown to play a critical role in the clearance of many virus infections (23, 32). Although a role for CTLs in the protection against AIDS has not been definitively shown, there is evidence to suggest that it is important. CTLs directed against many HIV proteins can be isolated from infected individuals (23, 26). The rapid decline in viremia during primary HIV infection correlates with an increase in the number of anti-HIV CTL precursors, while a neutralizing antibody response does not develop until later (15). Aside from being directly cytotoxic, CD8$^+$ lymphocytes from HIV-infected individuals secrete a soluble factor that inhibits HIV viral transcription in vitro. This antiviral activity decreases with disease progression (20, 21, 33, 34). In addition, CTL escape mutants in HIV-1-infected persons have been described (14, 22). Such escape mutations resulted in peptides that would no longer bind to major histocompatibility complex (MHC) class I molecules or resulted in the formation of altered peptide ligands that bound to MHC class I molecules but antagonized a normal immune response by interfering with signaling from T-cell receptors. CTLs inhibited by these altered peptide ligands were found in the same individuals from whom the mutated HIV viruses were isolated. These mutations may have resulted from selective pressure placed on the virus by the CTLs, and this suggests an important role in keeping virus replication under control (5). Indirect evidence therefore suggests that an anti-HIV vaccine strategy should include the induction of a CTL response as well as a strong antibody response and has compelled investigators to try a variety of vaccine strategies to induce CTL activity against HIV proteins (23).

Previous studies have shown that immunization of mice with heat-inactivated *B. abortus* conjugated to HIV-derived proteins or peptides induced neutralizing antibodies against HIV, mainly of the IgG2a isotype (9, 10). This response occurred even in mice depleted of CD4 lymphocytes.

Switching of the antibody response from IgM to IgG2a has been shown to be dependent on gamma interferon (IFN-γ), and it has subsequently been shown that *B. abortus* activates purified human CD4$^+$ and CD8$^+$ cells to secrete IFN-γ (30, 36). This is characteristic of Th1-Tc1 immune responses, which have also been shown to favor the induction of CTL activity (4,

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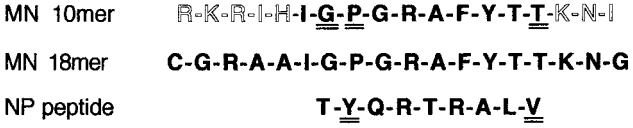

FIG. 1. Peptide sequences used in this study. The MN peptide binds to *H-2D*^{d}, and the NP peptide binds to *H-2K*^{d}. The double underlines indicate anchor residues.

25). In addition, *B. abortus* was recently found to induce the secretion of interleukin 12 (IL-12) from human monocytes (37). IL-12 has been reported to be directly involved in the differentiation of CTL precursors into antigen-specific effectors (3, 6, 24). It therefore seemed likely that *B. abortus* conjugates could induce CTL activity in addition to a humoral response.

In this study, we examined the ability of *B. abortus*, covalently linked with an HIV-1-derived peptide, to induce an MHC class I-restricted CTL response. To create a model antigen, we conjugated an 18-amino-acid peptide based on the V3 loop of HIV-1 MN gp120 that contains both B- and CTL (but not Th) cell epitopes to *B. abortus* (*B. abortus*–MN 18-mer). A 10-amino-acid fragment of this peptide has been shown to be the minimal CTL determinant presented by *H-2D*^{d} (2). It was found that the *B. abortus*–MN 18-mer induced a CTL response in normal as well as in CD4$^+$ T-cell-depleted BALB/c mice.

MATERIALS AND METHODS

Synthetic peptides used in the study. Figure 1 contains the sequences of the peptides synthesized for the study. The top line depicts the V3 (MN) sequence containing the minimal *H-2D*^{d}-restricted CTL epitope (shown in boldface type). The 18-mer peptide synthesized for conjugation (middle line) was made up of the 10-amino-acid minimal CTL determinant flanked by linker residues on both sides that contained three other residues from the MN gp120 V3 sequence. Changes were made in the other amino acids to improve solubility, to facilitate synthesis of the peptide, and to allow linkage of the peptide to *B. abortus*. A control 9-amino-acid peptide containing the *H-2K*^{d}-restricted minimal CTL determinant of influenza virus nucleoprotein (NP) was synthesized as a specificity control (bottom line).

Conjugation of peptides to *B. abortus*. Heat-inactivated *B. abortus*, strain 1119.3, was obtained from the U.S. Department of Agriculture, Ames, Iowa. *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 (18). Peptides were synthesized in the Biological Resources Branch of the Laboratory of Molecular Structure at the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md.

The conjugation of peptides to *B. abortus* has been previously described (10). A portion of the MN 18-mer peptide was lightly radiolabeled with ^{125}I, using Iodobeads (Pierce Chemical Co.), and was added (approximately 11,000 cpm) to the conjugation mixtures for coupling to *B. abortus*. *B. abortus* was coupled to the peptide by iodoacetylating *B. abortus* and linking the two by thioether bonds, using the cysteine of the peptide. In preparation for iodoacetylation, pelleted *B. abortus* 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 centrifuging 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 MN 18-mer was dissolved in 1 ml of buffer containing 0.03 M HEPES, 0.0148 M NaOH, and 0.2 mM EDTA. ^{125}I–MN 18-mer 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 Tris (2-carboxyethyl)-phosphinehydrochloride 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 fraction of bound ^{125}I peptide and calculated to be 7.7×10^7 molecules per bacillus.

In vivo priming. Six- to eight-week-old BALB/c female mice received two to three intraperitoneal immunizations 2 weeks apart with 10^7 to 10^9 *B. abortus* organisms in 100 μl of PBS. The mice received either unconjugated *B. abortus*, *B. abortus* covalently conjugated to MN 18-mer peptide (27.6 μg/10^8 organisms), or *B. abortus* mixed with 27.6 μg of MN 18-mer peptide. A separate positive control group was injected with live recombinant vaccinia virus (10^7 PFU per mouse) expressing the entire MN gp160 envelope (MN Vac, a generous gift from Bernard Moss and Pat Earl, National Institute of Allergy and Infectious Diseases, National Institutes of Health). Mice were also immunized with a recombinant vaccinia virus encoding influenza virus NP (NP Vac, generously provided by Jack Bennink and Jonathan Yewdell, National Institute of Allergy and Infectious Diseases, National Institutes of Health) to verify that CTL responses generated by MN Vac were directed against the MN protein and not the vaccinia virus itself.

Enzyme-linked immunosorbent assay (ELISA). Immunolon 4 (Dynatech, Chantilly, Va.) 96-well plates were coated with 100 μl of V3-BSA per well (10 μg/ml), in carbonate buffer, pH 9.6, for 2 h at 37°C. The plates were then blocked by adding 5% BSA in Dulbecco's phosphate-buffered saline with 0.05% Tween 20 and 0.1% azide overnight at 4°C. Twofold dilutions (from 1:100) of sera from individual mice were added to wells and incubated for 1 h at 37°C. Alkaline phosphatase-linked anti-mouse IgG antibodies (obtained from Southern Biotechnology Associates, Birmingham, Ala.) were diluted 1:500 in PBS-Tween 20 and were added (100 μl per well) for 1 h at 37°C. After each of the above steps, the plates were washed three times with PBS-Tween 20. Phosphatase substrate (Kirkegaard & Perry, Gaithersburg, Md.) in diethanolamine buffer was then added for 1 h at room temperature, and the optical density was read at 405 nm with a V_{max} reader from Molecular Devices Corp. (Palo Alto, Calif.). Titers represent the intercept on the *x* axis made by the linear portion of the serum titration curve, expressed as reciprocals of the dilution.

In vitro CTL expansion cultures. At least 2 weeks after the second immunization, splenocyte suspensions were prepared from mice, and approximately 6×10^7 cells were cocultured with 3×10^7 autologous, MN 18-mer-pulsed splenocytes for 6 days at 37°C. The cells were grown in Iscove's modified Dulbecco's modified Eagle's medium (IMDM) supplemented with 10% fetal bovine serum, 10% rat concanavalin A supernatant (Rat T stim; Collaborative Research), and 10 μg of gentamicin per ml. Cells were pelleted and suspended in IMDM supplemented with 10% fetal bovine serum.

Cytotoxicity assays. P815 (*H-2D*^{d}) targets were prepared in one of several ways. To infect targets with vaccinia virus recombinants encoding the gp160 from the MN strain of HIV-1 or influenza virus NP, P815 cells were washed twice with 0.1% BSA in Hanks' balanced salt solution, suspended at a concentration of 10^7 cells per ml, and added to an equal volume of vaccinia virus suspended at a concentration of 10^8 PFU/ml. After 1 h, 5 volumes of RPMI medium containing 10% fetal bovine serum were added, and the cells were incubated for 4 more h. To sensitize with or without peptide, P815 cells were suspended in 0.4 ml of medium containing 0.2 M MN 18-mer or 0.4 ml of medium alone for 1 to 2 h. L929 cell targets were infected with recombinant vaccinia viruses encoding *H-2K*^{d} or *H-2D*^{d} as described above (5×10^7 PFU of each virus). Peptide-pulsed or vaccinia virus-infected cells were pelleted and suspended in 100 to 200 μCi of $Na^{51}CrO_4$ in PBS and incubated for 1 h at 37°C. The cells were pelleted, washed, and suspended at a concentration of 10^5 cells per ml in IMDM supplemented with 10% fetal bovine serum. Targets were added in 100 μl to round-bottom, 96-well polystyrene microtiter plates containing CTL effectors in 100 μl of IMDM at 4 different effector-to-target ratios. Targets were also added to 100 μl of medium alone for the determination of spontaneous release or to 100 μl of 15% (wt/vol) BRIJ 35 solution for the determination of maximum release. Following a 4-h incubation, supernatants were harvested by using the Skatron system, and the amount of ^{51}Cr released was determined by gamma counting. The percent specific release was defined as (experimental cpm − spontaneous cpm)/(total cpm − spontaneous cpm) × 100. All assays were performed in triplicate.

Anti-L3T4 treatment. Rat anti-mouse L3T4 (CD4) monoclonal antibody (GK1.5) was partially purified from ascites fluid by ammonium sulfate precipitation and dialyzed against PBS, as previously described (10). The batch used in this study killed >70% of 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 as determined by radial immunodiffusion assay (ICN, Costa Mesa, Calif.). In order to deplete mice of CD4$^+$ cells, they were injected with 0.5 ml of GK1.5 (rat anti-mouse CD4) intraperitoneally daily for 3 sequential days prior to the first immunization 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 the numbers of cells stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-rat antibody were not increased in mice depleted of CD4$^+$ cells compared with untreated mice. Furthermore, a parallel group of mice, similarly treated, were shown to be incapable of generating T-cell help for trinitrophenol-keyhole limpet hemocyanin or HIV-1 peptide–keyhole limpet hemocyanin antibody responses (10) (data not shown), confirming the absence of CD4$^+$ T-helper function in the anti-L3T4-treated mice.

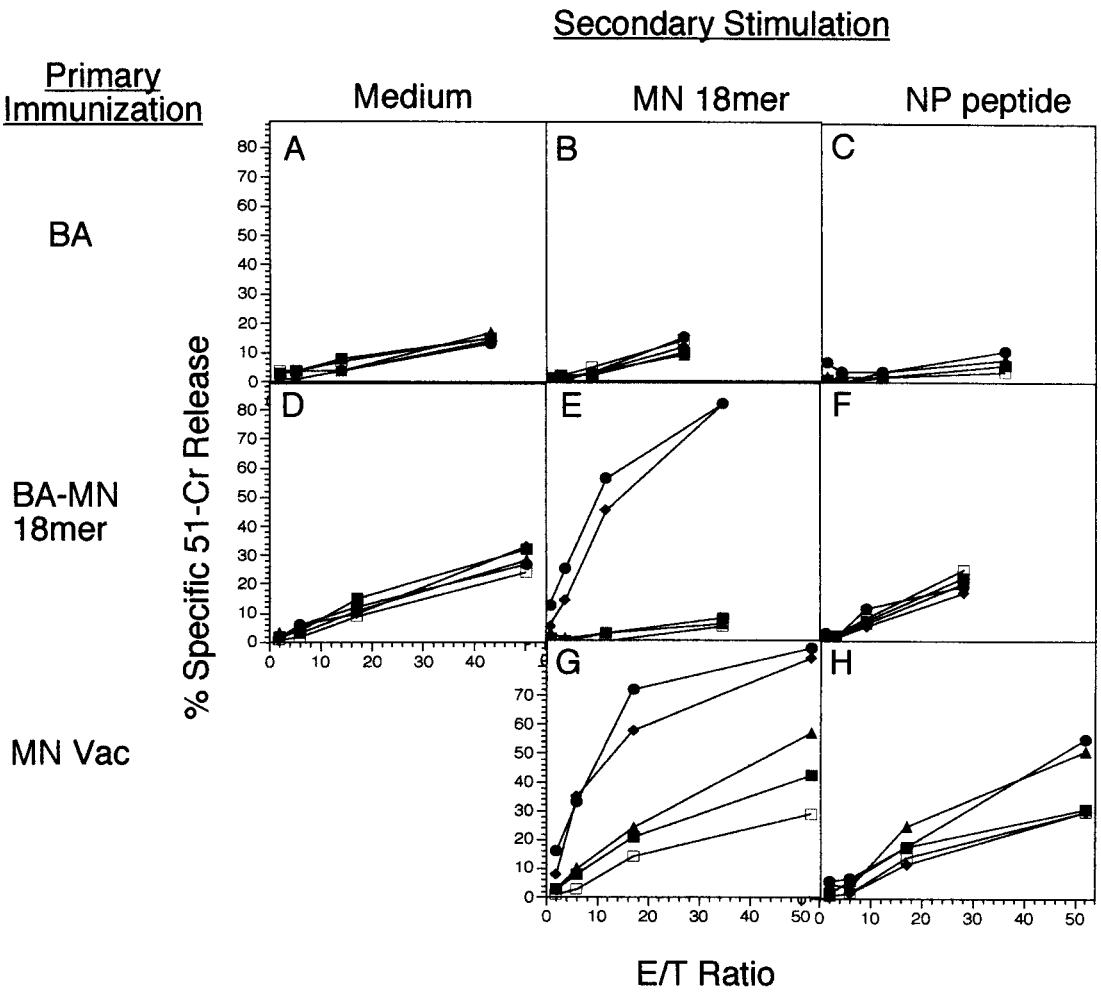

FIG. 2. *B. abortus*-conjugated MN 18-mer induces a specific CTL response. Mice were immunized with *B. abortus* (A, B, and C), *B. abortus*–MN 18-mer (D, E, and F), or MN Vac (G and H). Spleen cell suspensions were stimulated in vitro with medium (A and D), autologous cells plus MN 18-mer peptide (B, E, and G), or NP peptide (C, F, and H). These cells were used as effectors in a ^{51}Cr release assay against P815 cells (■), P815 cells infected with MN, Vac (●), P815 cells infected with NP Vac (▲), P815 cells pulsed with MN 18-mer (◆), and P815 cells pulsed with NP peptide (□). E/T, effector-to-target ratio.

Subpopulation studies. Specific cell populations were depleted by using magnetic bead selection after in vitro cell culture. Between 2×10^7 and 5×10^7 cells were suspended in 250 μl of medium. FITC-conjugated (anti-CD4 or anti-CD8) antibodies were added and incubated on ice in the dark for 30 min. The cells were washed once and suspended in 5 ml of medium. Anti-FITC magnetic beads (Advanced Magnetics) were washed once with medium and suspended in 5 ml of medium (approximately 150 beads per cell). The cells were added to the beads and rocked in the cold for 30 min. To remove cells that were bound to the beads, a magnet was attached to the side of the tube for 15 min. The supernatant was carefully removed, and the cells were used as effectors in a CTL assay. To control for the effect of magnetic bead separation on CTL activity, an antibody against human Ig was used. The effective removal of the unwanted cell population was assessed by flow cytometric analysis. The cells were found to be greater than 98% pure.

RESULTS

CTL induction by *B. abortus*–MN 18-mer conjugate. As *B. abortus* was shown to stimulate a Th1 cytokine response from human and murine T cells (36), we determined whether conjugation of an HIV peptide to *B. abortus* would induce MHC class I-restricted CTL activity. Mice were immunized with *B. abortus* alone or with *B. abortus* conjugated to an 18-amino-acid peptide (Fig. 1) that contained the minimal *H-2D*^{d}-binding peptide from the HIV-1 (MN) V3 region of gp120 (*B. abortus*–MN 18-mer). The mice were immunized twice with 10^9 organisms. Since vaccinia virus has been shown to be a potent inducer of MHC class I-restricted CTL responses (1), a group of mice were immunized with a vaccinia virus recombinant expressing the entire gp160 envelope of HIV-1 MN as a positive control group.

Single-cell suspensions were prepared from spleens of primed mice and were cultured in vitro either in medium alone or in the presence of autologous cells pulsed with the MN 18-mer or with an irrelevant peptide derived from the influenza virus NP that has been shown to bind to *H-2K*^{d} (Fig. 1). The effectors generated in these cultures were tested for their ability to kill P815 targets that were either pulsed with the MN 18-mer or NP peptides, infected with recombinant vaccinia virus expressing HIV-1 (MN) envelope or influenza virus NP, or incubated in medium alone. As can be seen in Fig. 2, mice immunized with *B. abortus* alone generated no CTL effectors, regardless of the in vitro stimulus (Fig. 2A, B, and C). Spleen cells from mice primed with *B. abortus*–MN 18-mer but cultured in vitro with medium alone or autologous cells pulsed with NP 18-mer peptide also exhibited no MN-specific cytotoxic activity (Fig. 2D and F). In contrast, spleen cells from mice primed with *B. abortus*–MN 18-mer and restimulated in vitro with autologous cells pulsed with the MN 18-mer gener-

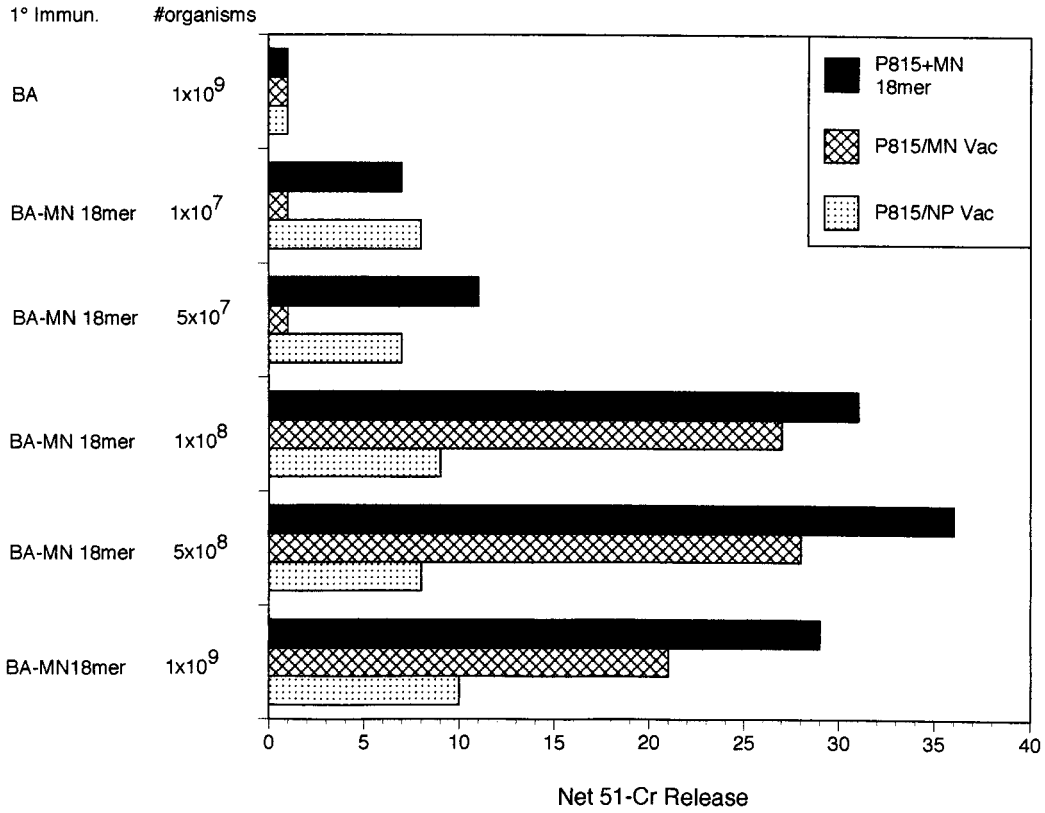

FIG. 3. Dose response of CTL induction by *B. abortus*–MN 18-mer. Mice were immunized twice with the designated number of *B. abortus*–MN 18-mer organisms. As a positive control, some mice were immunized with MN Vac. Spleen cell suspensions were stimulated in vitro with autologous cells plus MN 18-mer peptide and used as effectors in a ^{51}Cr release assay. To calculate the net ^{51}Cr release, the percent specific release against P815 cells was subtracted from the specific release of ^{51}Cr from the following targets: P815 cells pulsed with MN 18-mer, P815 cells infected with MN Vac, P815 cells infected with NP Vac. The data shown represent the net ^{51}Cr release at an effector-to-target ratio of 60/1.

ated significant cytotoxic activity against P815 targets pulsed with the homologous, but not with irrelevant NP-derived, peptide (Fig. 2E). Importantly, the cytotoxic cells generated by in vivo priming with *B. abortus*–MN 18-mer followed by in vitro expansion with the MN 18-mer peptide could also kill P815 cells infected with recombinant vaccinia virus that expressed MN envelope but not control target cells infected with recombinant vaccinia virus that encodes influenza virus NP protein (Fig. 2G). Thus, they could recognize epitopes within the MN gp160 envelope protein which is endogenously expressed and processed. This is more similar to the processing and presentation of envelope protein in HIV-1-infected cells than the addition of exogenous peptide. As expected, cells from mice immunized with MN Vac specifically lysed targets that were pulsed with MN 18-mer or that were infected with MN Vac (Fig. 2G and H). The elevated background cytotoxic activity most likely represents the activity of vaccinia virus-specific CTLs.

Before further characterization of the CTL response, the optimal dose for in vivo priming was determined. Mice were immunized twice with different doses of the *B. abortus*–MN 18-mer conjugate, starting with 10^7 organisms. The optimal CTL response was measured in spleen cells from mice immunized with at least 10^8 organisms (Fig. 3). Interestingly, this dose is within the range which induced optimal antibody responses. The antibody titers observed in response to different numbers of *B. abortus*–MN 18-mer organisms are as follows. When 1×10^9, 5×10^8, and 1×10^8 organisms were used, the titer was 12,800 in each case. When 5×10^7 organisms were used, the titer was 6,400. When 1×10^7 organisms were used, the titer was 400. In these experiments, blood was drawn from mice 1 week after the second immunization with *B. abortus*–MN 18-mer, and anti-MN 18-mer titers were measured by ELISA as described in Materials and Methods. In subsequent experiments, 10^8 peptide-conjugated organisms were used for immunization.

Requirement for conjugation of peptide to *B. abortus* for CTL induction. The experiments described above did not define whether conjugation of peptide to *B. abortus* was required for precursor CTL induction. To determine if *B. abortus* had to be physically linked to the peptide, mice were immunized with *B. abortus* alone, *B. abortus* conjugated to peptide, or *B. abortus* mixed with peptide (in an amount equivalent to the *B. abortus*-conjugated peptide). It was found that cells from mice that were immunized with *B. abortus* alone or *B. abortus* mixed with peptide did not generate CTLs capable of lysing MN Vac-infected or peptide-pulsed P815 targets following in vitro stimulation with peptide (Fig. 4A and B). However, in vivo priming with *B. abortus*–MN 18-mer conjugates generated MN envelope-specific CTLs that were efficiently expanded in vitro with autologous MN 18-mer-pulsed cells (Fig. 4C). These results demonstrate that conjugation was required for CTL induction. These findings suggest that in addition to the favorable cytokine microenvironment induced by the *B. abortus*, the physical linkage of peptide to *B. abortus* is necessary and probably

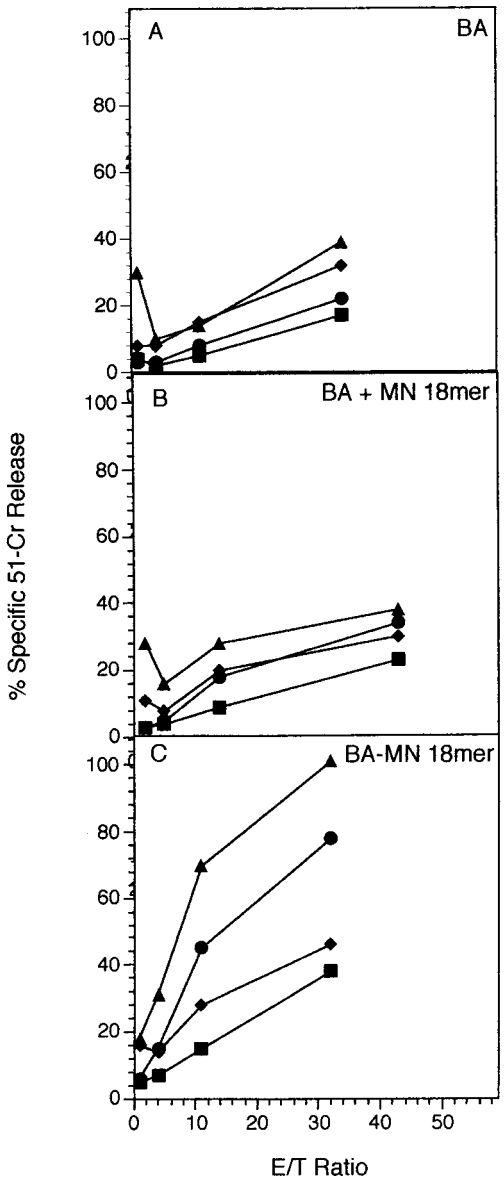

FIG. 4. Requirement for conjugation of MN 18-mer to *B. abortus* for CTL induction. Mice were immunized with *B. abortus* (A), *B. abortus* plus MN 18-mer (B), or *B. abortus* covalently linked to MN 18-mer (C). After in vitro stimulation with MN 18-mer-pulsed autologous cells, splenocytes were used as effectors against P815 cells (■), P815 cells pulsed with MN 18-mer (●), P815 cells infected with MN Vac (▲), or P815 cells infected with NP Vac (◆). E/T, effector-to-target ratio.

results in more efficient processing and presentation to CTL in vivo, perhaps by reducing the peptide clearance rate.

MHC class I restriction of MN 18-mer presentation. The 18-mer used in these experiments includes a CTL epitope known to bind *H-2D*^{d}. In order to verify that the addition of spacer amino acids and the conjugation to *B. abortus* did not alter the MHC restriction, we measured lysis of L929 fibroblast cells (*H-2*^{k}) infected with recombinant vaccinia viruses expressing either *H-2D*^{d} or *H-2K*^{d} molecules by CTLs generated following *B. abortus*–MN 18-mer immunization (Fig. 5). The expression of these MHC class I molecules in the infected L cells was shown by flow cytometric analysis (data not shown). The data in Fig. 5 demonstrate that P815 cells (*H-2D*^{d}) or L929 cells infected with *H-2D*^{d} vaccinia virus followed by peptide pulsing were lysed by MN-specific CTLs (*B. abortus*–MN 18-mer priming followed by in vitro peptide stimulation). However, peptide-pulsed *H-2K*^{d} vaccinia virus-infected L929 targets were not recognized by the same effectors. These results suggested that the CTL response generated by the *B. abortus*–MN 18-mer conjugate was mainly *H-2D*^{d} restricted.

To determine the phenotype of the CTL effectors, CD4$^+$ or CD8$^+$ cells were negatively selected from the effector cell population at the end of culture prior to their addition to the ^{51}Cr-labeled targets. Depletion of the appropriate populations (≥90%) was verified by flow cytometric analysis (data not shown). In addition, nonspecific antibody against human Ig was used as a negative control to determine that the negative selection with magnetic beads did not have an adverse effect on the CTL assay. As seen in Fig. 6, depletion of CD8$^+$ cells completely removed the cytotoxic activity, while depletion of CD4$^+$ cells resulted in enhanced killing of the peptide-pulsed targets. This enhancement may reflect the increased frequency of CD8$^+$ effectors. The use of nonspecific antibody in the depletion process had no effect on the level of cytotoxicity. Together, these results suggest that all of the effectors generated in the cultures were CD8$^+$ cells and were restricted by MHC class I molecules.

Induction of long-term memory. A desirable characteristic of a vaccine candidate is the induction of long-lived antigen-specific memory cells. To study the duration of the cytotoxic response generated by the *B. abortus*-conjugated peptide, mice were immunized with *B. abortus*–MN 18-mer and the responses were measured 1 week or 6 months after immunization (Fig. 7). The background killing of unpulsed P815 targets was lower after 6 months, and more importantly, no reduction in the MN-specific killing was seen (Fig. 7). Additional longitudinal studies are under way. These data suggest that *B. abortus*–MN 18-mer is capable of generating long-lived memory CTLs.

Generation of CTLs in neonatal mice. As a potential prophylactic or therapeutic vaccine for individuals with HIV-1 infection, it was important to determine whether immunization of neonates is possible with this vaccine candidate. Newborn mice were immunized 1 to 3 days after birth and received a booster shot once at 2 weeks. Their CTL responses were evaluated 4 weeks later and were compared with CTL responses of adult mice similarly primed with *B. abortus*–MN 18-mer. A modest but significant CTL response was generated in the neonates compared with that in the adult mice (Fig. 8). This result may reflect the immaturity of neonatal T cells in terms of cytokine production as previously described (7, 19). It will be important to determine the ability to boost this response following several months of rest.

Effect of anti-L3T4 treatment on the induction of an anti-MN V3 CTL response. All of the previously described experiments were conducted with immunologically intact BALB/c mice. However, the potential target population for therapeutic HIV-1 vaccine is HIV-1-infected individuals with various degrees of CD4$^+$ T-helper cell dysfunction and/or depletion. The ability of a vaccine to reactivate virus-specific memory CTLs may be hampered by the lack of T-cell help. However, in previous studies with a similar *B. abortus*-peptide conjugate, we showed that it was possible to generate either primary or secondary antibody responses in mice depleted of CD4$^+$ cells by chronic anti-L3T4 antibody treatment (10). Antibody titers were partially reduced in CD4-depleted mice, but the neutralization titers were only modestly reduced. It was important to determine if cytotoxic responses, which can also be dependent on helper cells, could also be induced in CD4-

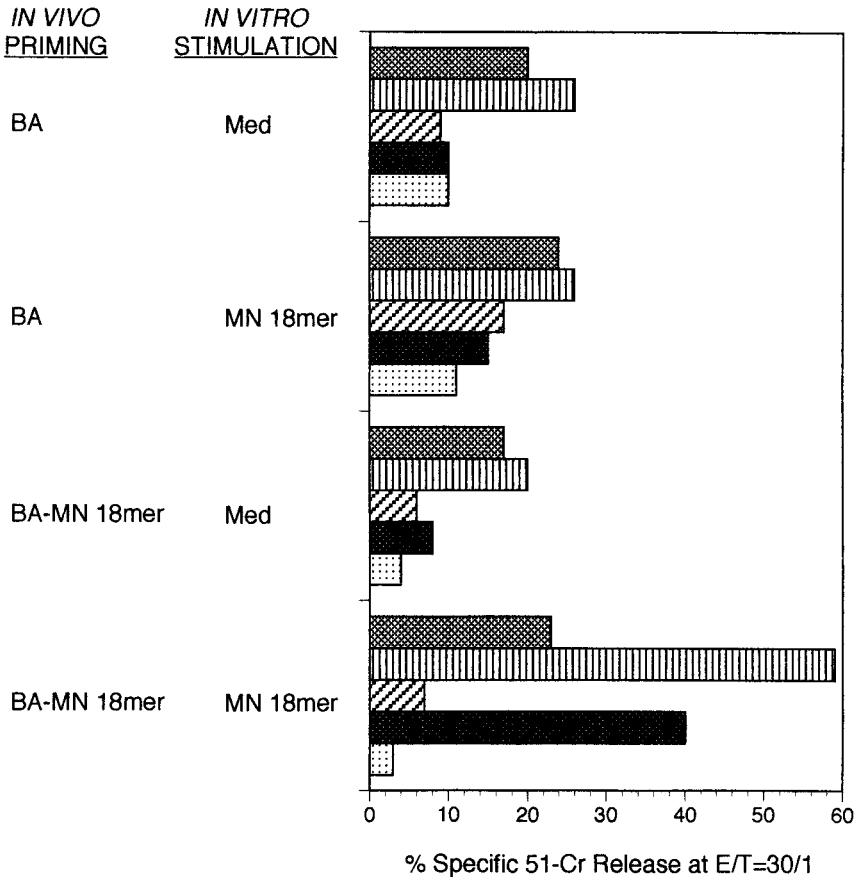

FIG. 5. CTL induction by MN 18-mer is restricted to $H\text{-}2D^d$. Mice were immunized twice with 10^8 *B. abortus* or *B. abortus*–MN 18-mer organisms, and splenocytes were stimulated in vitro with medium or MN 18-mer. The target cells used in the ^{51}Cr release assay are as follows: P815 cells ($H\text{-}2D^d$) (▨), P815 cells pulsed with MN 18-mer (▥), L929 cells ($H\text{-}2K^d$) pulsed with MN 18-mer (▨), or L929 cells infected overnight with vaccinia viruses encoding $H\text{-}2D^d$ or $H\text{-}2K^d$ and pulsed with MN 18-mer (▩ and ▩, respectively). E/T, effector-to-target ratio.

depleted mice by *B. abortus*–MN 18-mer. Mice were treated with GK1.5 (rat anti-L3T4 monoclonal antibody) or with PBS on days −2, −1, and 0 and then once a week thereafter. Flow cytometry demonstrated that the anti-L3T4-treated mice were depleted of splenic $CD4^+$ T cells (data not shown). Furthermore, these mice could not produce antibodies in response to the T-cell-dependent antigens TNP-keyhole limpet hemocyanin and MN 18-mer–keyhole limpet hemocyanin (10) (data not shown). As can be seen in Fig. 9, *B. abortus* conjugated to MN 18-mer induced very similar CTL responses in untreated mice (Fig. 9A) and in mice treated with anti-L3T4 antibodies (Fig. 9B). These data suggest that the *B. abortus*–MN 18-mer is capable of eliciting sufficient help from non-$CD4^+$ cells to support priming of peptide-specific CTLs. These findings support the use of *B. abortus* as a vaccine carrier for target populations with known CD4 T-helper cell immunity.

DISCUSSION

The design of a vaccine candidate for an infectious agent can be greatly aided by identifying the correlates of protection against this agent. In some viral infections (e.g., measles virus, poliovirus, and rubella virus), sterilizing immunity can be achieved by high titers of antibodies. In other infections (e.g., herpes simplex virus types 1 and 2 and cytomegalovirus), antibodies have been shown to provide only limited protection, suggesting a crucial role for cellular immunity in combating these agents. The correlates of protection against HIV infection are still being debated. However, a role for both humoral and cellular effector mechanisms has been demonstrated in studies of infected individuals and simian immunodeficiency virus-infected monkeys (5, 8, 31). Thus, a vaccine capable of generating both humoral and cytotoxic responses against relatively conserved epitopes is likely to be most beneficial as a prophylactic and potentially even as a therapeutic vaccine.

Heat-inactivated *B. abortus* was chosen as a vaccine carrier in our studies because of its unique properties. From a safety standpoint, its lipopolysaccharide (LPS) is structurally different from the LPS of other gram-negative bacteria such as *Escherichia coli*, and *B. abortus* LPS has been shown to be several log units less pyrogenic in rabbits and less lethal in mice (11). In addition, five rhesus macaques were injected with peptide-*B. abortus* conjugates at doses which induced high titers of antipeptide antibodies. The monkeys were monitored for 4 months and did not develop local or systemic adverse reactions (data not shown). Inactivated *B. abortus* and its purified LPS are capable of inducing the production of IFN-γ and IL-2 (but not IL-4 or IL-5) by both $CD4^+$ and $CD8^+$ human T cells (36). Furthermore, in a recent study it was found that *B. abortus* can induce IL-12 production and rapidly upregulate B7.1, B7.2, and ICAM1 expression in human elutriated monocytes (37). Thus, *B. abortus* is likely to generate an environment that favors Th1-Tc1 differentiation. Such an environment

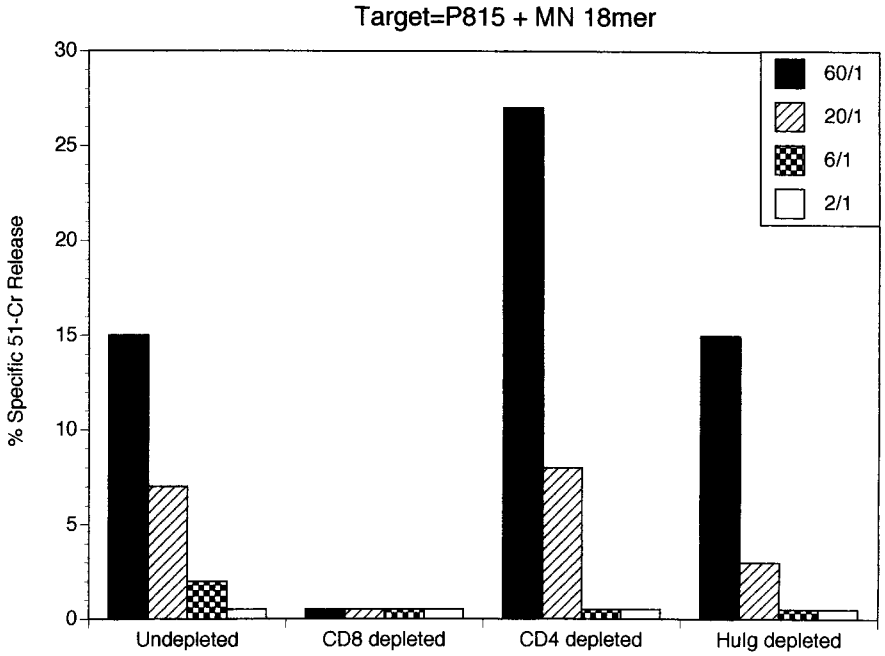

FIG. 6. The CTL effectors induced by *B. abortus*–MN 18-mer are $CD8^+$. Mice were immunized with *B. abortus*–MN 18-mer, and splenocytes were stimulated in vitro with autologous splenocytes pulsed with MN 18-mer. Effector cells were depleted of $CD4^+$ or $CD8^+$ cells by using a magnetic bead separation procedure. Unseparated or purified subsets were added to MN 18-mer-pulsed P815 targets at the indicated effector-to-target ratios. HuIg, control human Ig used in the depletion step.

should favor the development of cellular effector mechanisms. This prediction was tested in the present study.

An 18-amino-acid peptide from the V3 region of gp120 containing a B-cell epitope and a CTL epitope (but no T-helper epitope) was conjugated to inactivated *B. abortus*. This immunogen induced a strong CTL response in BALB/c mice. Targets pulsed with irrelevant peptide were not lysed. The cytotoxic T cells generated were therefore antigen specific. More importantly, they lysed targets that were either pulsed with the priming peptide or with cells that were infected with a recombinant vaccinia virus that expressed the intact HIV-1 (MN) envelope. The lysis of MN Vac-infected targets suggests that the effector CTLs that were induced by the peptide-*B. abortus* priming will recognize the naturally processed endogenous envelope protein generated during in vivo HIV infection. Cell depletion experiments showed the CTL effectors to be $CD8^+$ and not $CD4^+$ lymphocytes. In addition, the use of vaccinia virus recombinants that express different murine MHC class I antigens allowed us to determine that the cytolytic activity was restricted by *H-2D*^{d}. These results indicate that conjugation of a peptide to *B. abortus* can induce an antigen-specific, MHC class I-restricted CTL response. However, these experiments do not rule out the possibility that if the appropriate MHC class II binding peptide were conjugated to *B. abortus*, $CD4^+$ CTLs could also be induced. The mice were also tested for humoral responses. As in our previously published study, high IgG titers were generated with a predominant IgG2a component (data not shown). Our experiments identified an antigen dose (10^8 organisms) that is within the optimal ranges of both CTL and antibody responses.

For antigens to be processed and presented to T lymphocytes in the context of MHC class I molecules, proteins usually must be produced intracellularly (as in the case of viruses) or introduced into the cytosol, where peptides are thought to be produced. However, a few recent studies have shown that

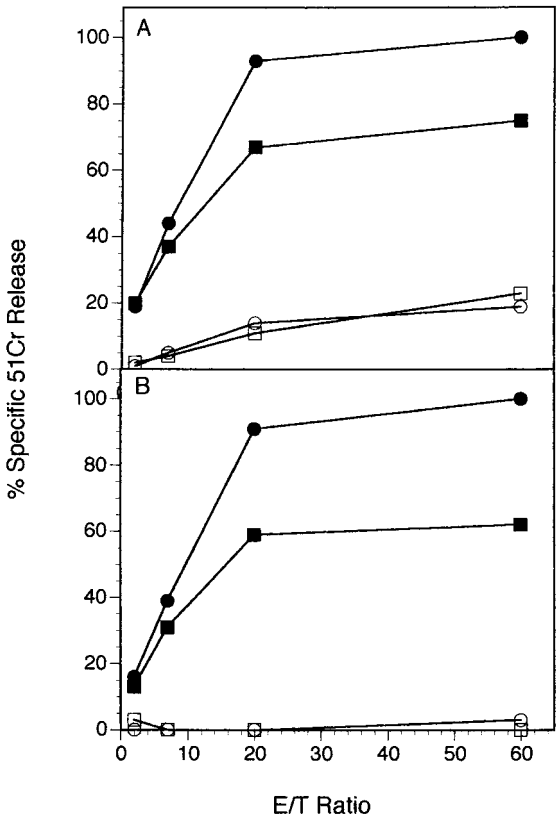

FIG. 7. Long-term CTL activity is induced by *B. abortus*–MN 18-mer. Mice were immunized with *B. abortus*–MN 18-mer, and splenocytes were assayed for CTL activity after 1 week (A) or 6 months (B). Splenocytes were cultured in vitro with MN 18-mer. These were used as effectors in a ^{51}Cr release assay against P815 cells (○), P815 cells pulsed with MN 18-mer (●), P815 cells infected with MN Vac (■), or P815 cells infected with NP Vac (□). E/T, effector-to-target ratio.

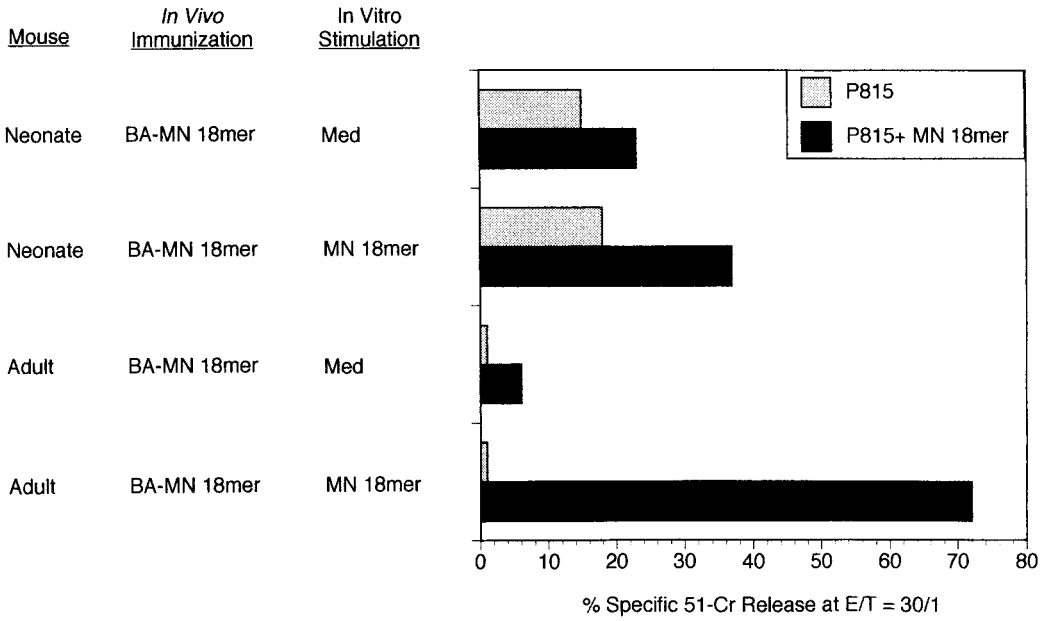

FIG. 8. *B. abortus*-conjugated peptides induce CTL activity in neonates. Neonates were immunized 2 days and 2 weeks after birth with *B. abortus*–MN 18-mer. Adults were immunized as previously described. Splenocytes were stimulated in vitro with the MN 18-mer peptide. The target cells were as follows: P815 cells and P815 cells pulsed with MN 18-mer. Effectors were either from neonates or from adults. E/T, effector-to-target ratio.

macrophages will process external particulate antigens (bacterial antigens, viruses, or proteins bound to silica beads) for presentation by preexisting MHC class I molecules (12, 13, 16, 27). Thus, for the induction of a $CD8^+$ MHC class I-restricted CTL response, conjugation of proteins to a particulate vaccine carrier such as *B. abortus* may obviate the need for endogenous expression of the antigen. On the other hand, extracellular processing of peptide-based antigen by serum proteases such as angiotensin-converting enzyme has been reported (17, 28). However, if extracellular processing does occur, a strict requirement for the chemical conjugation of the peptide to *B. abortus* would need to be explained. Experiments to unravel the pathways involved in the uptake and processing of the *B. abortus*-peptide conjugates that result in the loading of MHC class I molecules are under way.

Importantly, we demonstrated that the induction of $CD8^+$ MHC class I-restricted cytotoxic cells can take place not only in animals with an intact immune system but also in animals with an immature immune system (neonates) and in animals depleted of peripheral $CD4^+$ T cells. Other HIV vaccine candidates which were shown to elicit CTLs either required linkage with a helper epitope (29) or used liposome plus lipid A as a delivery system (35). In the latter study, the requirement for $CD4^+$ T cells was not addressed and the cytokine pattern elicited by this vaccine was not reported. Our findings may be explained in part by the demonstration in an earlier study that *B. abortus* can induce Th1-like lymphokines (IFN-γ and IL-2) in both $CD4^+$ and $CD8^+$ cells (36). In addition, *B. abortus* generates IL-12 from human monocytes and upregulates B7.1, B7.2, and ICAM1 costimulatory molecules on their surface. Thus, *B. abortus* is capable of eliciting all the known required helper factors in the absence of classical $CD4^+$ T-helper cells. This beneficial property of *B. abortus* may also explain our earlier findings that *B. abortus* conjugated either to whole inactivated HIV-1 (IIIB) or to a short peptide derived from the V3 loop of HIV-1 generated strong IgG responses with syncytium-inhibiting activities either in normal BALB/c mice or in

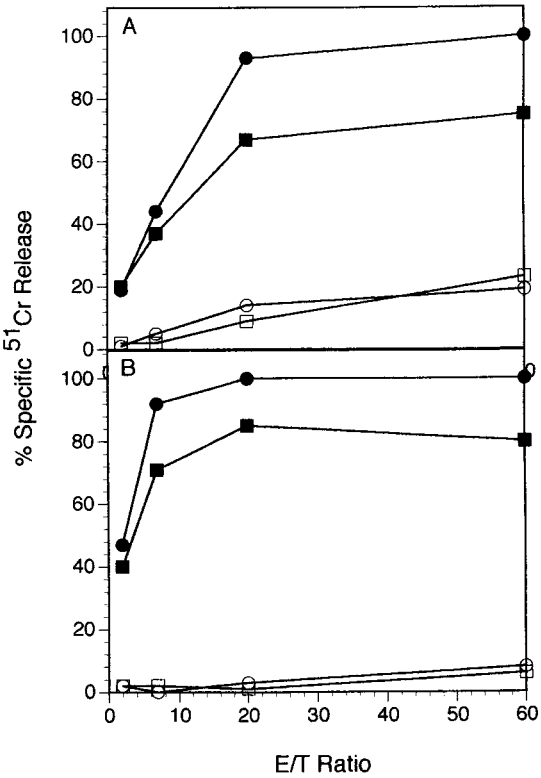

FIG. 9. Anti-L3T4 treatment has little effect on the induction of a CTL response by *B. abortus*–MN 18-mer. Control mice (A) or mice treated with anti-L3T4 (B) were immunized with *B. abortus*–MN 18-mer. After in vitro stimulation with MN 18-mer-pulsed autologous splenocytes, these were tested in a ^{51}Cr release assay against P815 cells (○), P815 cells pulsed with MN 18-mer (●), P815 cells infected with MN Vac (■), or P815 cells infected with NP Vac (□). E/T, effector-to-target ratio.

mice depleted of $CD4^+$ T cells (10). The ability of *B. abortus* conjugates to generate potent antiviral humoral and cytotoxic responses, even under conditions of limited $CD4^+$ T-cell function, makes it a very attractive candidate for a vaccine carrier to treat individuals with T-cell immunodeficiency. Thus, *B. abortus* may be suitable as a carrier in therapeutic vaccines for patients already infected with the HIV-1 and HIV-2 viruses as well as in prophylactic vaccines.

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