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Immunogenicity of a Contiguous T-B Synthetic Epitope of the A/PR/8/34 Influenza Virus

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A contiguously linked T-B synthetic viral epitope (^{110}HA^{120}-^{150}HA^{159},T-B) was investigated for its potency in inducing humoral and cellular immune responses in vivo. The T-cell epitope ^{110}HA^{120} corresponds to the site 1 hemagglutinin (HA) of the A/PR/8/34 (PR8) influenza virus and is recognized by CD4 T cells in association with *I-E*^{d} class II major histocompatibility complex molecules. The ^{150}HA^{159} represents a major B-cell epitope of the HA protein. T-B dipeptide emulsified in Freund's complete adjuvant was able to induce strong antiviral antibody titers and a high frequency of specific T-cell precursors after a single inoculation in BALB/c mice. In contrast, immunization under identical conditions with equimolar mixtures of T and B peptides did not elicit antibody titers or a cellular immune response. As indicated by the isotypes of antiviral antibodies, the T-B dipeptide preferentially induced a Th1-like immune response. Challenge with T-B dipeptide, but not with T or B peptide alone, stimulated peptide-specific T memory cells in mice previously primed with PR8 virus or with T-B dipeptide. As a consequence, 71 and 57% of these mice, respectively, survived infection with two 100% lethal doses of PR8 virus. Our results suggest that, inasmuch as contiguity between T- and B-cell epitopes provides enough signaling capacity to trigger the mechanisms of T-B-cell cooperation in vivo, a T-B contiguous epitope may well represent a minimal built-in subunit vaccine. Aside from their potential bioavailability, the T-B contiguous epitopes may also represent attractive tools for investigating the molecular mechanisms of T-B-cell cooperation responsible for antiviral protection.

Synthetic peptides corresponding to microbial epitopes can mount an immune response in association with major histocompatibility complex (MHC) molecules on the surface of antigen-presenting cells (APCs) after recognition by specific T cells (3). Although these synthetic peptides are able to induce proliferation of immunocompetent cells in vitro, their short serum half-life and poor immunogenicity make them weak candidates for use in vaccination. However, because immunization with peptides lacks the side effects inherent in the use of proteins, the immunogenicity augmentation of the synthetic peptides by various means has become the subject of much investigation. Various chemical, genetic, and enzymatic approaches are currently being used to enhance the in vivo immune response to synthetic microbial epitopes. These approaches are mainly aimed toward extending the serum half-lives of the peptides and/or toward adding intrinsic adjuvanticity to them.

Chemical coupling of synthetic peptides to various protein carriers by using a number of homo- or heterobifunctional cross-linkers is a commonly used technique for increasing the immunogenicity of these peptides in animals (2, 4, 14, 24, 29, 34). Peptide polymerization represents another approach to the problem, through extending the serum half-lives of peptides by increasing their stability in vivo subsequent to increase of their molecular size. In addition, polymerization may reconstitute native conformational epitopes originating from a particular antigen required for recognition by the B cells. Indeed, polymers ranging between 8 and 35 kDa of a cysteinyl peptide corresponding to the amino acid sequence 124 to 147 of the major protein of hepatitis B virus surface antigen are able to reconstitute a conformational native epitope that elicits neutralizing antiviral antibodies (Abs) (23). As an alternative to the polymerization techniques, *N*-bromoacetyl derivatization of peptides enables generation of repetitive peptide subunits (peptomers) or cyclic peptides with enhanced immunogenicity (27). A novel approach referred to as multiple antigen peptide systems consists of building multiple epitopes on branched lysine residues during peptide synthesis (22, 30).

Coupling microbial epitopes to lipoidic moieties such as dipalmitoyl side chains (19) or tripalmitoyl-*S*-glycerol-cysteine (35) allows not only an increase in the molecular masses of synthetic peptides but also the introduction of intrinsic adjuvanticity. Such derivatives induce a significant Ab response and in vivo priming of specific cytotoxic T lymphocytes (CTLs) (13). The possibility of coupling oligosaccharides to synthetic peptides represents another interesting approach (6), but the immunogenic characteristics of such constructs have not been investigated in detail. Embedding minigenes corresponding to microbial peptides in protein carriers represents a genetic approach to enhancing peptide immunogenicity (5). We have demonstrated that a short CD4 viral epitope of the hemagglutinin (HA) of the A/PR/8/34 (PR8) influenza virus grafted into the CDR3 loop of the V_H region of self immunoglobulins (Igs) efficiently stimulates the peptide-specific T cells (36).

Enzymatic coupling of synthetic epitopes to the sugar moieties of proteins has been developed as a recent method for generating immunogens. We have created immunogalactopeptide conjugates made of self Igs and the CD4 immunodominant viral epitope of HA of the PR8 influenza virus. This epitope was enzymatically assembled on the galactose residues of Igs (8) and is able to prime specific T helper cells in vivo, as well as to promote efficient stimulation of specific T-cell precursors (11).

There is now speculation that simultaneous expression of T and B epitopes on the same carrier may enhance the Ab

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response by exploiting T-B-cell cooperation (9, 31, 32). In line with this supposition, the T-cell epitope presented by APCs may activate a T cell, while a neighboring B cell is engaged in producing antiviral Abs. It is known that both T- and B-cell immune responses may offer antiviral protection. Indeed, it has been demonstrated that recombinant DNA repeats made of T- and B-cell epitopes (15, 18), T- and B-cell epitopes assembled as a multiple antigen peptide system (22), or T and B epitopes assembled enzymatically on self Igs (9) are all efficient immunogens in vivo.

Our results demonstrate that (i) in the absence of a carrier molecule, T or B viral synthetic epitopes from HA of the PR8 influenza virus were immunogenic not by themselves but only when assembled as a contiguous dipeptide, and (ii) T-B contiguous epitopes may well represent the minimal built-in subunit vaccine able to trigger T-B-cell cooperation efficiently in vivo.

MATERIALS AND METHODS

Mice. BALB/c mice 7 to 8 weeks old were purchased from Jackson Laboratory (Bar Harbor, Maine). Transgenic BALB/c mice expressing the 14.3.d T-cell receptor (TCR) specific for $^{110}HA^{120}$ of the HA of the PR8 influenza virus on both CD4 and CD8 T-cell subsets (20) were kindly provided by Harald von Boehmer, Institut Necker, Paris, France.

Antigens. PR8 and BLee influenza viruses were prepared in alantoic fluid of embryonated eggs and purified on sucrose gradients according to standard procedures. BLee virus does not have any homology with the HA of the PR8 influenza virus and was used in experiments as a control. The synthetic peptides $^{110}HA^{120}$ (SFERFEIFPKE) and $^{150}HA^{159}$ (WLTEKEGSYP) correspond to T- and B-cell epitopes of the HA of the PR8 influenza virus, respectively (12, 17). To promote greater flexibility in solution between the T and B peptides within the T-B dipeptide, a Gly-Gly spacer was introduced during the synthesis ($^{110}HA^{120}$-Gly-Gly-$^{150}HA^{159}$). The control peptide $^{147}NP^{161}$ (TYQRTRALVRT GMDP) corresponds to a CTL epitope of the nucleoprotein of the PR8 influenza virus. Peptides with or without N-terminal cysteine residues were prepared by 9-fluorenylmethoxycarbonyl technology, purified by reverse-phase high-pressure liquid chromatography on a C_2/C_{18} column (Pharmacia Biotechnology), and analyzed by amino acid sequencing on a gas-phase sequencer (Porton Instruments, Tarzana, Calif.). Bovine serum albumin (BSA) conjugates of cysteinyl-$^{110}HA^{120}$ and cysteinyl-$^{150}HA^{159}$ were prepared as previously described (21).

Abs. Anti-$^{110}HA^{120}$ Abs were obtained by immunization of New Zealand White rabbits with keyhole limpet hemocyanin-$^{110}HA^{120}$ conjugate as previously described (10). The Abs were affinity purified on a BSA-$^{110}HA^{120}$-Sepharose column and then adsorbed on BSA-Sepharose–mouse gamma globulin-Sepharose tandem columns. Rat anti-mouse κ chain monoclonal antibody (MAb) was obtained from the American Type Culture Collection, and B2H1 MAb (immunoglobulin M κ [IgM(κ)]) specific for $^{150}HA^{159}$ peptide was generated in our laboratory by fusion of the SP2/0 cell line with spleen cells from BALB/c mice immunized with keyhole limpet hemocyanin-$^{150}HA^{159}$ conjugate (9). PY102 is an IgG1 mouse anti-PR8 virus MAb fashioned in our laboratory by fusion of the SP2/0 cell line with spleen cells from BALB/c mice immunized with UV-inactivated PR8 virus. B2H1 and PY102 MAbs were affinity purified on a rat anti-mouse κ-chain Sepharose column. PY102 and B2H1 MAbs were also used to construct calibration curves required for the quantitative estimation of the anti-PR8 and anti-B-cell epitope Abs, respectively. Affinity-purified goat anti-rabbit IgG Abs were purchased from Boehringer Mannheim, and the cross-reactivity to mouse Igs was removed by passage through a mouse gamma globulin-Sepharose column. The complete kit containing rabbit anti-mouse isotype Abs was purchased from Bio-Rad Laboratories.

Cells. 2PK3 and M12 B lymphoma cells (*H-2^d* haplotype [American Type Culture Collection]) were used as APCs or target cells in the T-cell activation and cytotoxic T-cell assays, respectively. Cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, and gentamicin. The 14-3-1 T-cell hybridoma (TcH) expressing 14.3.d TCR specific for $^{110}HA^{120}$ peptide was obtained from Klaus Karjalainen (Basel Institute for Immunology, Basel, Switzerland). This hybridoma was grown in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, gentamicin, 50 μM 2-mercaptoethanol, and 0.1 mg of hygromycin B per ml. The 14-3-1 TcH recognizes $^{110}HA^{120}$ peptide in association with *I-E^d* MHC class II molecules. This TcH also contains the *Escherichia coli lacZ* gene under the promoter of the interleukin-2 gene as an early indicator of T-cell activation by the concomitant secretion in cytosol of interleukin-2 and β-galactosidase (26).

Immunization protocol. Groups of seven BALB/c mice were immunized subcutaneously with 100 μg of either UV-inactivated PR8 virus, T-B dipeptide, or a mixture of T and B synthetic peptides (1:1 [wt/wt]) in Freund's complete adjuvant (FCA). Blood samples were collected 0, 7, 14, 21, and 28 days after immunization, and the anti-PR8 virus or anti-$^{150}HA^{159}$ Ab titers as well as their corresponding isotypes were determined by radioimmunoassay (RIA). In parallel experiments aimed at investigating the ability of T-B dipeptide to stimulate memory cells, groups of seven BALB/c mice were primed either intranasally with 10^3 50% tissue culture infective doses ($TCID_{50}$) of PR8 virus in 20 μl of saline, intraperitoneally with 5 μg of PR8 virus in 0.2 ml of saline, or subcutaneously with 100 μg of T-B dipeptide in 0.2 ml of FCA. On day 90, the mice were bled and the inhibition of hemagglutination by anti-PR8 Abs was assessed according to standard procedure. On the same day, mice were challenged with 100 μg of T-B dipeptide, T or B peptides in 0.2 ml of FCA, 2 μg of PR8 virus in 0.2 ml of saline, or no antigen. After 2 weeks, mice were infected with two 100% lethal doses (LD_{100}s) of PR8 virus in aerosols, and after another 10 days, the lung viral titers of the survivors were determined as previously described (33).

RIAs. (i) Capture RIA. The antigenicity of T- and B-cell epitopes of the T-B dipeptide was compared with the antigenicity of T- and B-cell epitopes alone or with that of the HA of the PR8 virus. Briefly, 96-well microtiter plates were coated overnight at 4°C with 50 μg of rabbit anti-$^{110}HA^{120}$ Abs or B2H1 MAb (anti-$^{150}HA^{159}$) per ml in 0.1 M carbonate, pH 9.6, and blocked for 4 h at room temperature with 3% BSA–phosphate-buffered saline (PBS). Antigens (10 μg/ml) in 1% BSA-PBS were added to the microwells overnight at 4°C, and plates were washed with PBS–0.05% Tween 20 and incubated for 2 h at 37°C with 10 μg of either rabbit anti-$^{110}HA^{120}$ Abs or B2H1 MAb per ml. Bound Abs were revealed after 2 h of incubation at room temperature with 10^5 cpm of either ^{125}I-goat anti-rabbit IgG or ^{125}I-rat anti-mouse κ chain MAb per well.

(ii) Competitive inhibition of RIA. In a second set of experiments, the inhibition of RIA was performed under similar conditions as for capture RIA. Briefly, this consisted of the addition of graded amounts of either T-B dipeptide, an equimolar mixture made of T and B peptides, PR8 virus, or BLee virus control to a constant amount of the corresponding antipeptide Abs. Bound Abs were then revealed with ^{125}I-radiolabeled anti-isotype Abs.

To determine the concentration of anti-$^{150}HA^{159}$ Abs in sera of the immunized mice with T-B dipeptide or PR8 virus, sera collected 0, 7, 14, 21, and 28 days after immunization were diluted 1 to 100 in PBS–1% BSA and incubated overnight at 4°C in 96-well microtiter plates coated with 10 μg of either PR8 virus, BLee virus control, BSA-$^{150}HA^{159}$ conjugate, or BSA-$^{147}NP^{161}$ conjugate control per ml. Plates were washed, and bound Abs were revealed after 2 h of incubation at room temperature with ^{125}I-rat anti-mouse κ chain MAb (5×10^4 cpm/well). The amount of anti-$^{150}HA^{159}$ Abs was estimated by using a standard curve constructed with B2H1 MAb against plates coated with 10 μg of BSA-$^{150}HA^{159}$ conjugate per ml after subtracting the background obtained in plates coated with 10 μg of BSA-$^{147}NP^{161}$ conjugate control per ml. The amount of anti-PR8 Abs was estimated by using a standard curve constructed with PY102 MAb against plates coated with 10 μg of PR8 virus per ml after subtracting the background obtained in plates coated with 10 μg of BLee virus control per ml.

Isotyping of the antiviral Abs was performed by RIA, with a kit containing rabbit anti-mouse Ig subclasses (Bio-Rad Laboratories) and ^{125}I-goat anti-rabbit IgG as developing Abs. Mice sera were diluted 1 to 100 in PBS–1% BSA and incubated in plates coated with 10 μg of either BSA-$^{150}HA^{159}$ conjugate or PR8 virus per ml. Bound anti-isotypic Abs were revealed with ^{125}I-goat anti-rabbit IgG Abs at 5×10^4 cpm/well.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Electrophoresis of the peptides was performed as indicated in the Bio-Rad Laboratories instruction kit. Briefly, 20 μg of either T, B, or T-B peptide was electrophoresed for 3 h at 150 V in 4 to 20% polyacrylamide gradient gels with Tris–Tricine–0.1% SDS buffer. Gels were fixed for 10 min with 2% glutaraldehyde and then silver stained.

T-cell activation assay. Activation of 14-3-1 β-galactosidase-positive TcH was performed as previously described (26). Briefly, 10^5 irradiated (5,000 rads) 2PK3 APCs in complete Iscove's modified Dulbecco's medium were incubated for 24 h at 37°C in 3-ml tubes containing 2×10^5 TcHs and graded amounts of either T peptide, T-B dipeptide, or B peptide as control. Cells were exposed to hypotonic shock for 1 min at 37°C by the addition of 2 mM fluorescein di-β-D-galactopyranoside in 100 μl of distilled water (Sigma Chemical Co.). After addition of 3.8 ml of medium per tube, cells were reincubated in the dark for 1 h at 4°C. Cells were pelleted at 3,000 rpm for 15 min and fixed with 1 ml of 1% paraformaldehyde in PBS. The number of β-galactosidase-positive TcHs was scored among 10,000 cells by fluorescence-activated cell sorter (FACS) analysis with the Epics profile analyzer (San Diego, Calif.). A specific window corresponding to 2PK3 APCs was generated to subtract them from the β-galactosidase-positive activated TcHs.

T-cell proliferation assay. The proliferative response of lymph node cells from BALB/c mice immunized with T-B dipeptide was assessed by [^{3}H]thymidine incorporation. Groups of seven mice were immunized at the base of the tail and in the footpads with 100 μg of T peptide, T-B dipeptide, or PR8 virus emulsified in 200 μl of PBS-FCA (1:1 [vol/vol]). After 10 days, the draining lymph nodes were collected and the cells were cultured in flat-bottomed 96-well plates (2×10^5 cells per well) with either concanavalin A (ConA) (2 μg/ml), purified protein derivative (0.1 μg/ml), $^{110}HA^{120}$ synthetic peptide (80 nM), $^{150}HA^{159}$ synthetic peptide (80 nM), PR8 virus (10 μg/ml), BLee virus (10 μg/ml), or no antigen. Cultures were set up in quadruplicate, incubated under standard conditions for 5 days (or 3 days in the case of ConA), and pulsed for the last 18 h with 1 μCi

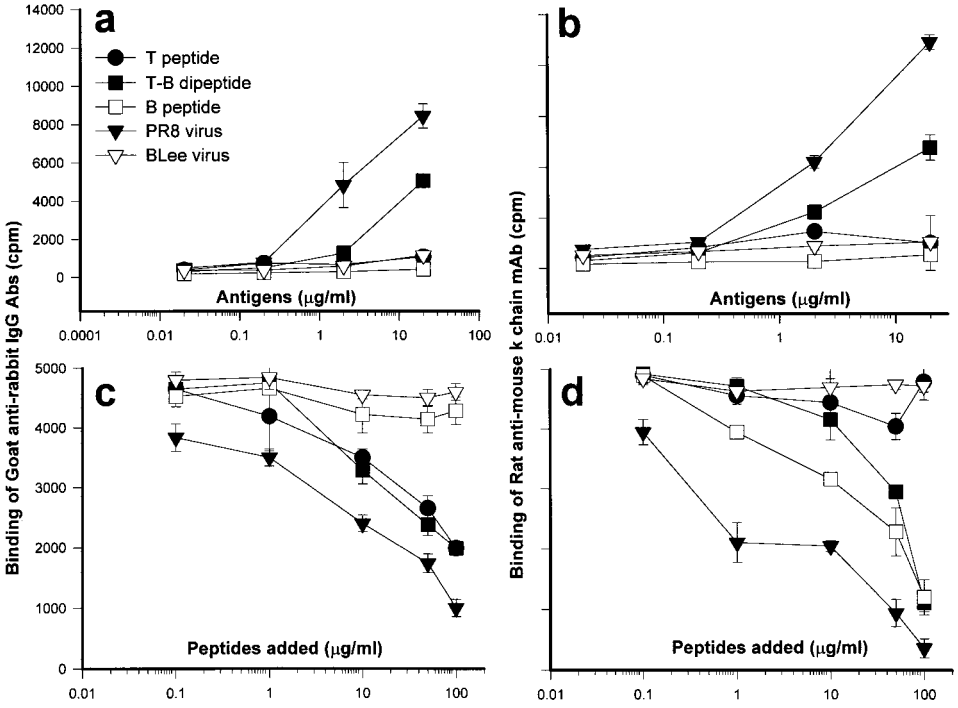

FIG. 1. Antigenicity of T-B peptide. The $^{110}HA^{120}$ and $^{150}HA^{159}$ epitopes of the T-B dipeptide were identified by capture RIA as described in the text. (a) Both T-B dipeptide and PR8 virus were recognized by rabbit anti-$^{110}HA^{120}$ Abs after incubation in plates coated with anti-$^{150}HA^{159}$ MAb B2H1. The $^{110}HA^{120}$ peptide and BLee virus were used as controls. (b) T-B dipeptide as well as PR8 virus was recognized by B2H1 MAb after incubation in plates coated with rabbit anti-$^{110}HA^{120}$ Abs. The $^{150}HA^{159}$ peptide and BLee virus were used as controls. (c) In contrast to $^{150}HA^{159}$ peptide, the T-B peptide as well as $^{110}HA^{120}$ peptide was able to inhibit the binding of rabbit anti-$^{110}HA^{120}$ Abs to the plates coated with BSA-$^{110}HA^{120}$ conjugate. (d) In contrast to $^{110}HA^{120}$ peptide, the T-B dipeptide and PR8 virus were able to inhibit the binding of B2H1 MAb to the plates coated with BSA-$^{150}HA^{159}$ conjugate. Each point represents the mean of triplicate wells ± SD.

of [^{3}H]thymidine per well. Cells were harvested, and the radioactivity was measured in a beta-scintillation chamber (LKB Pharmacia, Uppsala, Sweden).

Determination of frequency of the $^{110}HA^{120}$-specific CTL precursors. Transgenic mice expressing the 14.3.d TCR on the CD8 T-cell subset that recognizes the $^{110}HA^{120}$ peptide in association with *I-E^d*-MHC class II molecules were used to determine the number of specific CTL precursors induced by immunization with T-B dipeptide and to compare it with corresponding numbers for T peptide and PR8 virus. Of 2.5×10^5 spleen cells (responder cells) from the transgenic mice immunized with 100 μg of each of the antigens in FCA, eight serial twofold dilutions were incubated in 96-well tissue culture plates with a constant number (2.5×10^5) of stimulator cells ($^{110}HA^{120}$-coated M12 cells at 15 μg/ml). Plates were then incubated under standard conditions for 3 days in complete RPMI medium.

Individual mixed lymphocyte microcultures were tested in a standard ^{51}Cr release assay with noncoated M12 target cells as control or M12 target cells coated with $^{110}HA^{120}$ peptide at 15 μg/ml. Microcultures showing ^{51}Cr release more than threefold above the standard deviation (SD) of the background were considered positive, and the percentages of cultures corresponding to each dilution step that were negative for specific cytotoxicity were integrated against the number of responder cells per well. The results were computed on a linear regression curve, and the frequency of CTL precursors was calculated according to the formula: $\Im$ (frequency) = 1/(number of responder cells per well corresponding to 37% negative wells). To estimate the absolute number of CTL precursor cells, $\Im$ was also expressed as $1/\Im$.

RESULTS

Preservation of antigenicity. Antigenicity of both T and B peptides contiguously assembled in the T-B dipeptide was analyzed by capture RIA. As illustrated in Fig. 1a and b, respectively, T-B dipeptide was captured either by plates coated with a MAb specific for the B peptide or by a polyclonal Ab specific for the T peptide. The peptidic site of the T-B dipeptide that was unengaged in the interaction with the Ab-coated plate was then recognized by the second antipeptide Ab. The results indicate that the antigenicity of both T and B epitopes of the T-B dipeptide was well preserved after synthesis. This was also confirmed by the ability of T and B peptides to inhibit the binding of Abs directed against the T and B epitopes, respectively, as determined in a standard competitive inhibition RIA (Fig. 1c and d).

A semiempirical conformation of the T, B, and T-B dipeptides was estimated by using Hyperchem II software (Fig. 2). The computation relied on energy optimization with the Polak-Ribiere algorithm. The RMS gradient was set up for 0.01 kcal/mol and 300°K in 2,925 reading cycles. Indeed, the results obtained by gel electrophoresis of the T, B, and T-B peptides suggested not only a greater molecular size for the T-B dipeptide, but also a slightly more globular form compared with the floppy form of both T and B peptides (Fig. 3). Although the electrophoresis was performed in the presence of detergent (0.1% SDS), it is unlikely that the T-B dipeptide was completely unfolded.

Ab response. The antiviral and anti-B-epitope Ab responses elicited by the T-B dipeptide and PR8 virus were studied with BALB/c mice following a single immunization in FCA. Data depicted in Fig. 4a indicate that PR8 virus induced a higher primary antiviral Ab response (60 μg/ml) than did T-B dipeptide (30 μg/ml) on day 14 after immunization. The primary anti-B-epitope Ab response was lower after immunization with PR8 virus (15 μg/ml) than after that with T-B peptide (45 μg/ml [Fig. 4b]). No Ab response was detected after immunization under identical conditions with an equimolar mixture of T and B peptides or with BLee virus control (Fig. 4).

To investigate the homogeneity of T-cell subsets stimulated by the T-B peptide, we analyzed the isotypes of the antiviral and anti-B-epitope Abs at 7, 14, and 21 days after immunization. It is well known that Ig isotype switching is mostly controlled by CD4 T cells (25). The IgG2a anti-PR8 response

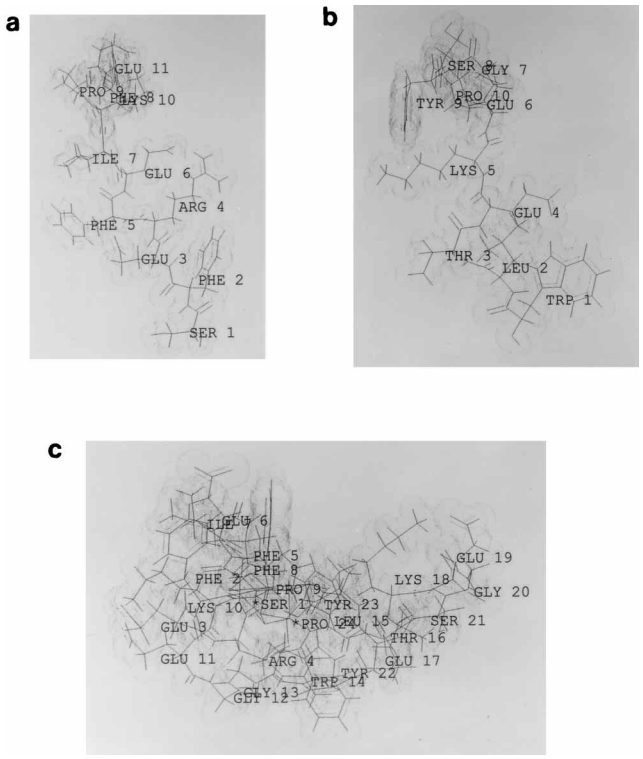

FIG. 2. Computer modeling of T, B, and T-B peptides. The conformation of T, B, and T-B peptides was computed based on the energy optimization test with the Polak-Ribiere algorithm. (a) $^{110}HA^{120}$ peptide; (b) $^{150}HA^{159}$ peptide; (c) $^{110}HA^{120}$-Gly-Gly-$^{150}HA^{159}$ peptide. The hydrogen bonds and the energy fields are also indicated.

represented one of the major Ig subclasses on days 14 and 21 after immunization with either T-B dipeptide (Fig. 5a) or PR8 virus (Fig. 5b). Similar results were obtained for the anti-B-isotypic Abs (not shown). This suggests that, like the PR8 virus, the T-B dipeptide preferentially induces a Th1-like Ab response.

In vitro activation of $^{110}HA^{120}$-specific TcH. To assess the immunogenic capacity of T-B dipeptide versus T peptide

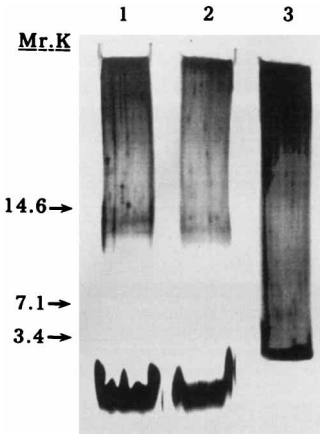

FIG. 3. SDS-polyacrylamide gel electrophoresis analysis of T-B dipeptide. Samples of peptides were analyzed in polyacrylamide gradient gels as described in the text. Lane 1, $^{110}HA^{120}$ peptide; lane 2, $^{150}HA^{159}$ peptide; lane 3, $^{110}HA^{120}$-Gly-Gly-$^{150}HA^{159}$ dipeptide. Peptides were analyzed as a crude preparation after synthesis. Numbers at left are molecular weights in thousands.

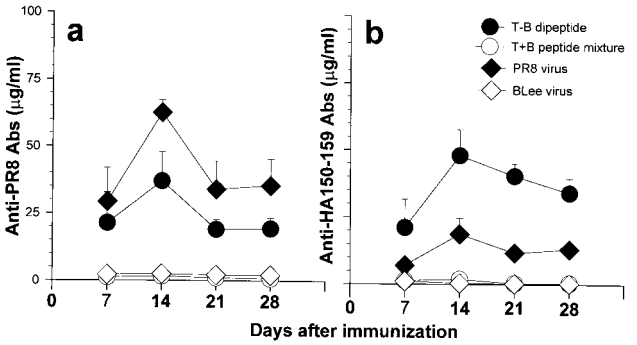

FIG. 4. Ab response. The anti-PR8 and anti-B-cell epitope immune responses were estimated by RIA. (a) The amount of anti-PR8 Abs induced in BALB/c mice immunized with either PR8 virus or T-B dipeptide in FCA is shown. An equimolar mixture of T- and B-cell synthetic epitopes in FCA, as well as the control virus BLee, did not show any significant Ab titers. (b) The amount of anti-B-cell epitope Abs induced in BALB/c mice immunized with either PR8 virus or T-B dipeptide in FCA is shown. The equimolar mixture of T and B peptides and the control virus BLee did not show detectable Ab titers. Neither sera collected from mice immunized with T-B dipeptide nor sera from mice immunized with UV-inactivated virus were able to react with an unrelated peptide, $^{147}NP^{161}$, conjugated to BSA (not shown). Each point represents the mean of duplicate wells ± SD per group of immunized animals.

alone, we studied the in vitro T-cell activation of 14-3-1 TcH by FACS analysis. Figure 6 indicates that, when peptides were added to the cell culture in equimolar concentrations, the T-B dipeptide induced an index of T-cell activation similar to that of the T peptide alone. The activation was dose dependent for both T-B and T peptides, but none was detected for the B peptide alone. This indicates that T-B dipeptide preserved its immunogenicity after synthesis.

In vivo priming of $^{110}HA^{120}$-specific CD4$^+$ T cells. In a previous study, we have shown that immunization of BALB/c mice with the T-cell peptide of the HA of the PR8 influenza virus did not induce a specific proliferative response (11). Herein we assessed the ability of the T-B dipeptide administered in FCA to induce such a response. While immunization with T peptide in FCA did not prime the specific T cells for a proliferative response to T peptide or PR8 virus in vitro (Fig. 7b), immunization with T-B dipeptide under identical conditions did prime the specific T cells (Fig. 7a). Although the proliferative response induced by PR8 virus (Fig. 7c) was stronger than that induced by the T-B dipeptide, these data indicate that only the T-B dipeptide, not the T peptide alone, was able to prime the peptide-specific T cells in vivo.

In vivo stimulation of $^{110}HA^{120}$-specific CD8 T-cell precursors. To study the ability of T-B dipeptide to prime CD8 T cells in vivo, we employed a transgenic mouse strain expressing TCR specific for $^{110}HA^{120}$ peptide on both CD4 and CD8 T-cell subsets. It has previously been shown that, in these particular mice, CD8 T cells expressing the TCR transgene can lyse target cells coated with $^{110}HA^{120}$ peptide in association with the *I-E*^{d} class II molecules (16). Using this system, we investigated the frequency of CTL precursors induced by the T-B dipeptide compared with T peptide alone and with PR8 virus. Interestingly, the frequency of CTL precursors in mice immunized with T-B dipeptide was twofold higher than that for those immunized with PR8 virus (Fig. 8 and Table 1). No significant number of $^{110}HA^{120}$-specific CTL precursors was detected after immunization under identical conditions with $^{110}HA^{120}$ peptide alone or with BLee virus control. Once again, this demonstrates that a T-B contiguous epitope, but not a T-cell epitope alone, may prime specific T cells in vivo.

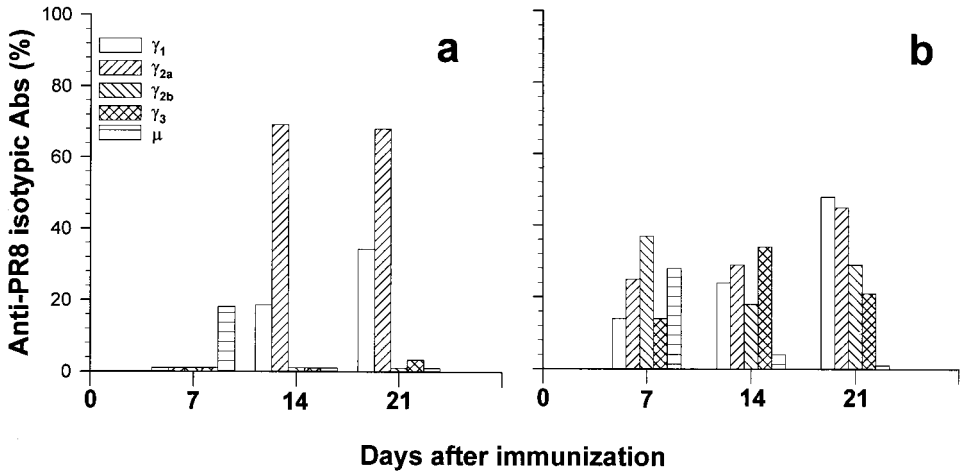

FIG. 5. Isotypic Ab response. The isotypes of anti-PR8 Abs induced in BALB/c mice were determined by RIA on days 7, 14, and 21 after immunization with T-B dipeptide in FCA (a) or UV-inactivated PR8 virus (b). The isotypic antiviral Abs are represented as a percentage of the total amount of Abs, as previously measured by RIA with plates coated with PR8 virus.

Induction of anti-PR8 memory cells. Data depicted in Table 2 show that 57% of the mice primed with T-B dipeptide and challenged 90 days later with T-B dipeptide were protected against PR8 infection, although the level of neutralizing Abs was detected up to a 1/140 serum dilution, and small amounts of the virions ($TCID_{50}$ = 1.7 ± 0.4) were still present in the lungs of the survivors 10 days after infection. Mice primed with PR8 virus developed significant antiviral neutralizing Abs after challenge with T-B dipeptide, but not after challenge with T or B peptide alone. Although the titers of neutralizing Abs developed after challenge with T-B dipeptide were lower than those obtained after challenge with PR8 virus, the rates of survival after PR8 infection were comparable (i.e., 71 versus 42%, respectively, for mice primed intranasally with PR8 virus and 100 versus 71%, respectively, for mice primed intraperitoneally with PR8 virus).

This suggests that not only the neutralizing Abs but also the

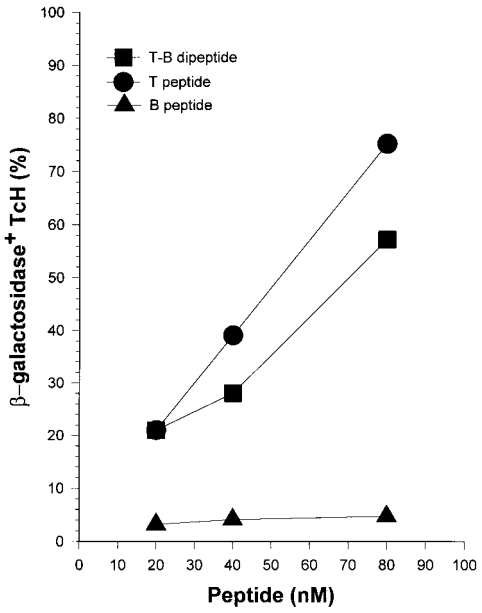

FIG. 6. In vitro activation of $^{110}HA^{120}$-specific TcH. Graded amounts of either T-B dipeptide, or T or B peptide (control) were incubated for 24 h with 2PK3 APCs and 14-3-1 TcH. The percentage of activation (β-galactosidase-positive TcH) corresponds to the equimolar doses of T epitope contained in the T-B dipeptide and to the T peptide itself. Each point represents the percentage of β-galactosidase-positive TcH scored by cytofluorometry among 10,000 cells.

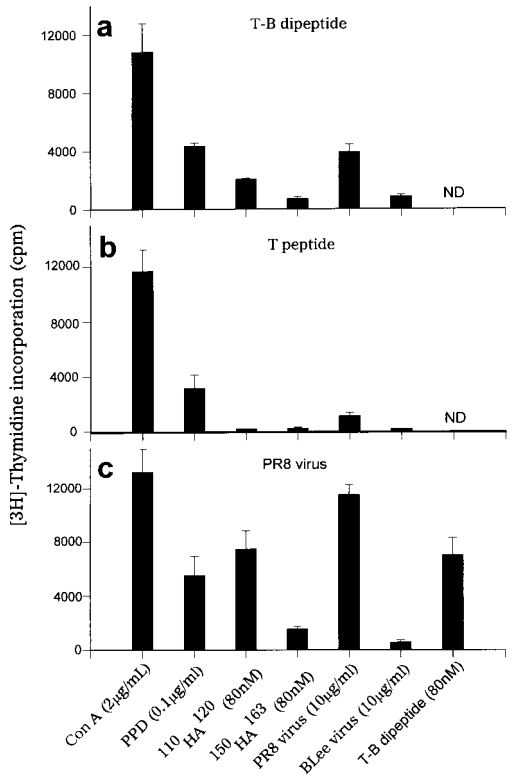

FIG. 7. In vivo priming of $^{110}HA^{120}$-specific CD4 T cells. Groups of seven BALB/c mice were immunized at the base of the tail and in the footpads with T-B dipeptide, T peptide, or PR8 virus in FCA. After 10 days, the draining lymph nodes were collected and the proliferative response in vitro was determined as described in the text. Shown is thymidine incorporation by the lymph node cells from mice immunized with T-B dipeptide, T peptide alone, or PR8 virus. Thymidine incorporation upon in vitro stimulation with ConA (day 3) is also indicated. Each point represents the mean of quadruplicate wells ± SD after subtraction of the counts per minute obtained in the absence of antigen. PPD, purified protein derivative.

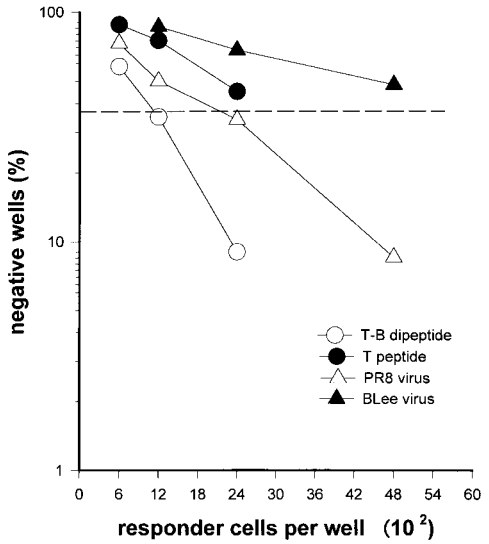

FIG. 8. Estimation of frequency of $^{110}HA^{120}$-specific CTL precursors. Transgenic mice expressing TCR specific for $^{110}HA^{120}$ peptide were immunized with either T-B dipeptide, T peptide, PR8 virus, or BLee virus control as described in the text. Log_2 dilutions of splenocytes from immunized mice were incubated with a constant number of irradiated stimulator cells (2.5×10^5) in the presence of 15 μg of $^{110}HA^{120}$ peptide per ml. After 72 h, each well was tested in a CTL assay with M12 target cells coated with 15 μg of $^{110}HA^{120}$ peptide per ml and the percentage of $^{110}HA^{120}$-specific CD8 T cells was estimated by the intercept at 37% negative wells (dashed line). Each point represents the number of responder cells per well of a particular antigen that intercepts at 37% negative wells.

T-cell compartment makes an important contribution to the immune defense against PR8 infection. All survivors that were previously primed with PR8 virus and subsequently challenged with PR8 virus or T-B dipeptide cleared the virions from their lungs 10 days after infection. In the aggregate, these results demonstrate that a T-B contiguous epitope, but not T or B epitopes alone, may efficiently stimulate T and B memory cells.

DISCUSSION

In the present study, we have investigated the immunogenicity of a contiguous T-B synthetic dipeptide expressing a T- and a B-cell epitope of the HA of the PR8 influenza virus. It has previously been shown that the presence of both T- and B-cell epitopes in immunogenic preparations enhances the antiviral response (9, 31, 32). The T-cell epitope of the T-B dipeptide corresponds to the amino acid sequence 110 to 120 of the HA and is recognized by the CD4 T cells in association with *I-E*^{d} MHC class II molecules (9, 15, 18, 22, 31, 32). The B-cell peptide represents one of the immunodominant epitopes of HA, corresponding to the amino acid sequence 150 to 159 (12). To obtain greater flexibility in solution, the epitopes were linked during synthesis by a spacer made of two glycine residues.

TABLE 1. Frequency of the $^{110}HA^{120}$-specific CTL precursors in transgenic mice immunized with various antigens[a]

Antigen	Frequency	1/frequency	Relative frequency
T peptide	3.3×10^{-4}	2,310	3.3
T-B dipeptide	9.1×10^{-4}	1,100	7.1
PR8 virus	4.8×10^{-4}	2,100	3.7
BLee virus control	1.3×10^{-4}	7,800	1.0

[a] Frequency represents the probability from 0 to 1 to encounter a specific precursor in the spleen cell cultures, 1/frequency represents the number of spleen cells corresponding to a single CTL precursor cell, and the relative frequency is the index of frequency enhancement for CTL precursors after immunization, compared to the controls.

We explored the ability of this minimal built-in T-B subunit to induce antiviral response in vivo and its amplitude per se in comparison with those of the T or B epitopes alone and with those of the UV-inactivated viral particles. At a glance, the computer modeling of the T-B peptide disclosed a compact shape in aqueous solution, compared with the floppier form of the T or B peptide alone. Electrophoretic analysis also suggested that the T-B dipeptide may accommodate more globular shapes than the T or B peptide alone. Although the conformation of the T-B dipeptide was quite different from that of its T and B counterparts, the antigenicity and immunogenicity were fully preserved. These results demonstrated that the contiguous assembly of $^{110}HA^{120}$ and $^{150}HA^{159}$ epitopes of the HA of the PR8 influenza virus in a dipeptide did not alter their individual biological properties.

Not only were mice immunized with T-B dipeptide emulsified in FCA able to develop specific Abs, but the titers on day 14 (35 μg/ml) after a single inoculation were comparable with those induced by immunization with UV-inactivated PR8 virus (60 μg/ml). In contrast, immunization under identical conditions with an equimolar mixture of T and B peptides did not elicit detectable Ab titers. The kinetics of the isotypic antiviral Abs elicited by immunization with T-B dipeptide and PR8 virus were similar. Thus, IgG2a Abs reached their highest titer on days 14 and 21 after immunization with both T-B dipeptide and PR8 virus. This suggests that T-B peptide, like the UV-inactivated PR8 virus, induced a Th1-biased immune response. Whether this represented an innate ability of the T-B dipeptide to drive the immunocompetent cells toward a Th1-like response, or whether this effect was due mainly to the use of FCA, remains to be investigated further.

Interestingly, immunization of BALB/c mice with the T-B dipeptide, but not with T peptide alone, was able to prime specific CD4 T cells. This may account for the better uptake by APCs of the T-B dipeptide, because of its globular structure and bigger molecular size. When transgenic mice expressing a TCR specific for $^{110}HA^{120}$ peptide on both CD4 and CD8 T-cell subsets were immunized with a single dose of T-B peptide in adjuvant, the frequency of $^{110}HA^{120}$-specific CTL precursors was almost twice as high as the frequency of CTL precursors detected in mice immunized with a single dose of PR8 virus (relative frequency, 7.1 and 3.7, respectively). This is because the frequency of $^{110}HA^{120}$-specific CTL precursors induced by PR8 virus was disseminated among the multitude of CTL epitopes expressed by the viral proteins. Altogether, these results demonstrate that T-B dipeptide, but not T peptide alone, was able to prime peptide-specific CD4 T cells and to focus the immune response on the stimulation of resting CD8 T cells specific for the $^{110}HA^{120}$ epitope in transgenic mice. It is noteworthy to mention that both CD4 and CD8 T-cell subsets from these transgenic mice are in a resting state since no activation markers such as CD25 antigen were detected on the cell surface according to FACS analysis.

When mice that had been previously primed with PR8 virus or T-B dipeptide were challenged with T-B dipeptide, their survival after intranasal or intraperitoneal infection with two LD_{100}s of PR8 virus was significantly increased. Although the challenge with PR8 induced a higher rate of survival (71 versus 42% after intranasal priming with PR8 virus and 100 versus 71% after intraperitoneal priming with PR8 virus), all survivors completely cleared the virus from their lungs. These results

TABLE 2. Viral protection induced by immunization with T-B dipeptide[a]

Priming antigen (day 0)	HI titer (day 90)	Challenge antigen (day 90)	HI titer (day 104)	No. of survivors/total no. of infected mice (day 114)	PR8 virus lung titer ($TCID_{50}$) (day 114)[b]
PR8 virus (i.n.)	0	T-B peptide (s.c.)	1/140	3/7	0.0
	0	PR8 virus (i.p.)	1/1,280	5/7	0.0
	0		0	1/7	1.9
PR8 virus (i.p.)	0	T-B peptide (s.c.)	1/320	5/7	0.0
	0	PR8 virus (i.p.)	1/2,560	7/7	0.0
	0		0	2/7	1.4 ± 0.3
	0	T peptide (s.c.)	0	2/7	1.7 ± 0.2
	0	B peptide (s.c.)	0	2/7	1.9 ± 0.2
T-B peptide (s.c.)	0	T-B peptide (s.c.)	1/140	4/7	1.7 ± 0.4
	0		0	1/7	1.9

[a] On day 0, groups of seven BALB/c mice were primed intranasally (i.n.) with 10^3 $TCID_{50}$ of PR8 virus in saline and then immunized intraperitoneally (i.p.) with PR8 virus or subcutaneously (s.c.) with T-B dipeptide as described in the text. On day 90, mice were bled and the hemagglutination inhibitory (HI) titers induced by anti-PR8 neutralizing Abs were assessed. On the same day, mice were challenged with either T-B, T, or B peptide; PR8 virus; or no antigen. Two weeks later, mice were infected with PR8 virus (2 LD_{100}s), and after another 10 days, the survivors were scored.

[b] The viral titers for lungs of the survivors were determined as described in the text. Values represent the $TCID_{50}$ as determined on MDCK cells. For more than one survivor, the mean ± SD was calculated.

demonstrated that the T-B contiguous epitope, but not a T-cell epitope alone, was able to prime peptide-specific T cells and to expand the T and B memory cells in vivo.

Obviously, the contiguity between T- and B-cell epitopes played an important role in triggering efficient T- and B-cell cooperation in vivo. Several explanations may account for the increase in immunopotency of this contiguously linked T-B epitope. First, it may be related to the fact that the immune response elicited by peptide preparations is restricted to relevant epitopes, unlike that elicited by inactivated microbial vaccines carrying a myriad of epitopes, among which only a few are immunogenic. Thus, in the case of synthetic peptides, the pool of immunocompetent responders may be qualitatively restricted to a single epitope. This could explain the higher frequency of CTLs in mice immunized with T-B dipeptide than in those immunized with PR8 virus. In addition, the time required by small peptides to elicit a T-cell response in vitro is shorter than that required by protein carriers. That is because peptides may circumvent some of the lengthy presentation events such as cell internalization and processing. Indeed, in a time chase assay we found that $^{110}HA^{120}$ synthetic peptide, but not the chemically prepared HA of the PR8 virus (BHA) or the viral particles, may activate T cells in vitro as quickly as 15 min (7). Second, the immunopotency of T-B dipeptide in vivo may also account for its failure to induce early down-modulation events on the activated T and/or B cells. It is known that long exposure of immunocompetent cells to antigens, or an intravenous route of immunization, may lead to early apoptotic events mediated by Fas (CD95)-FasL interaction (28). At present, we cannot speculate as to whether the activation of immunocompetent cells by T-B dipeptide may induce significant early apoptotic events compared with the PR8 viral particles.

In the aggregate, our results suggest that a contiguous T-B epitope may represent a minimal built-in subunit vaccine able to induce efficient humoral and cellular immune responses in vivo. The T-B-like subunits may also represent attractive tools for deciphering the intimate mechanisms of T- and B-cell cooperation in vivo.

Several interesting questions still remain to be addressed, specifically with respect to the immunopotency of a contiguous T-B epitope. One might ask whether the contiguous assembly of a T and B epitope may be critical for simultaneous interaction in vivo among APCs, T cells, and B cells. It would also be interesting to know whether repeated administration of a contiguously linked T-B peptide, or coupling to long-life carriers, may produce better immunogens than the T-B dipeptide itself. Ongoing experiments are aimed at investigating the immunopotency of T-B dipeptide in the context of carbohydrate-like carriers. This may open new avenues for designing novel classes of vaccines based on built-in T-B contiguous epitopes.

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