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Immunogenicity of tumor peptides: importance of peptide length and stability of peptide/MHC class II complex

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Abstract Nonameric P815AB, a cytotoxic-T-lymphocyte-defined minimal core peptide encoded by the murine mastocytoma gene *P1A*, fails to initiate $CD4^+$ cell-dependent reactivity in vivo to class-I-restricted epitopes when mice are administered peptide-pulsed dendritic cells. Effective immunization requires T helper effects, such as those mediated by coimmunization with class-II-restricted (helper) peptides or by the use of recombinant interleukin-12 (rIL-12). Although P815AB does possess class-II-restricted epitopes, they are likely suboptimal, resulting in poor affinity and/or stability of MHC/P815AB complexes and inadequate activation of the antigen-presenting cell function of dendritic cells. The present study has examined a series of longer, P815AB-centered peptides (11–14 amino acids in length, all *P1A*-encoded) for their ability to initiate $CD4^+$ and $CD8^+$ cell-mediated responses to the nonamer in vivo, their ability to bind class II MHC in vitro, and their ability to assemble class II molecules stably. By means of a class-I-restricted skin test assay in mice receiving peptide-pulsed dendritic cells, we found that a 12-mer and a 13-mer effectively immunized against the core P815AB peptide, and that this correlated with IL-2 production in vitro by $CD4^+$ cells in response to the nonamer. In vitro studies, involving affinity-purified class II molecules, showed that the capacity to assemble class II molecules stably, more than the affinity for class II MHC, correlated with the ability of the different P815AB peptides to prime the host to the core peptide seen by the T cells.

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Introduction

Cell-mediated immunity involving $CD8^+$ lymphocytes is effective in mediating rejection of murine mastocytoma cells bearing P815AB, a tumor-associated antigen showing similarities to tumor-specific shared antigens in humans [2, 18]. Although this antigen may act as an efficient target for class-I-restricted responses in suitably immunized mice, a synthetic nonapeptide encompassing the relevant class-I-restricted epitopes [10, 19] will not activate unprimed $CD8^+$ cells for in vivo reactivity, which is dependent on the recruitment of $CD4^+$ lymphocytes to the early response [5]. By using an assay system for measuring the class-I-restricted delayed-type hypersensitivity (DTH) response to P815AB in mice administered P815AB-pulsed dendritic cells [14], we have previously shown that either the addition of T helper peptides (in a physical mixture during cell pulsing) or the use of recombinant interleukin-12 (rIL-12) may compensate for the poor immunogenicity of P815AB [1, 5]. Effective induction of DTH responses by either maneuver was accompanied by antigen-specific production of IL-2 by $CD4^+$ cells in vitro [6]. This suggested an important role for IL-12-dependent activation of $CD4^+$ cells in supporting $CD8^+$ cell priming to P815AB [6, 9]. In fact, P815AB possesses I-A^d-restricted epitopes but such epitopes appear to be suboptimal, resulting in the limited persistence of I-A^d/P815AB complexes [6]. Because, in general, both the length and the sequence of a peptide contribute to the stability of binding to class II molecules [12], we have examined a series of P815AB-centered peptides, ranging in length from 11 to 14 amino acids, for their ability to prime the host for activation in vivo of the T cell response to nonameric P815AB in the absence of any added adjuvant activity. We also examined the different P815AB peptides for

their affinity for class II MHC and their ability to stabilize purified I-A^d molecules.

Materials and methods

Mice

DBA/2J (H-2^d) mice were obtained from Charles River Breeding Laboratories (Calco, Milan, Italy). Male mice were used at the age of 2–4 months.

Peptides

The single-letter code sequences of the peptides used in this study are shown in Table 1. All P815AB peptides were derived from the known sequence of gene *P1A* of murine mastocytoma P815 [10]. The T helper peptides used in this study were tetanus toxin (tt) peptide and *Plasmodium berghei* circumsporozoite multiple-antigen (PbCS) peptide [5]. Peptides were synthesized as described [5, 14], purified by means of reverse-phase HPLC and characterized by amino acid analysis.

Cytokines and antibodies

Murine recombinant interferon γ (rIFNγ) and rIL-2 were obtained from Genzyme Corp. (Boston, Mass.). Murine rIL-12 was a generous gift from B. Hubbard (Genetics Institute Inc., Cambridge, Mass.). IL-12, purified from the supernatant of Chinese hamster ovary cells transfected with the p35 and p40 cDNA plasmids, was 98.8% pure, as assessed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE), and endotoxin contamination was less than 0.9 EU/mg on *Limulus* amebocyte assay. The specific activity of the purified rIL-12 preparation, measured as the ability to stimulate proliferation in human phytohemoagglutinin-activated blasts, was 3.1×10^6 U/mg. Endotoxin was removed from all solutions containing IL-12 with Detoxi-gel (Pierce Chemical Co., Rockford, Ill.).

Anti-(mouse IFNγ) mAb R4–6A2 and XMG1.2, and anti-(mouse IL-2) mAb JES6–1A12 and JES6–5H4 were from Pharmingen (San Diego, Calif.). Affinity-purified sheep anti-(mouse IL-12) polyclonal antibody was generously provided by the Immunology Department of Genetics Institute Inc., and the control antibody (sheep IgG) was purchased from Pierce Chemical Co. (Rockford, Ill.). The source and characteristics of monoclonal rat-mouse hybrid cell lines producing GK1.5 (IgG2b anti-CD4) and 53.6.72 (IgG2a anti-CD8) mAb were as described [5], and antibodies were purified by means of affinity chromatography from hybridoma culture supernatants. For in vivo depletion experiments, the 53.6.72 hybridoma line was instead grown in pristane-primed athymic mice. Anti-I-A^d (MK-D6, mouse IgG2a) and anti-I-E^d (14.4.4, mouse IgG2a) mAb were purified by means of affinity chromatography from hybridoma (American Type Culture Collection, Rockville, Md.) culture supernatants.

Table 1 Peptides used in this study

Peptide	Abbreviation	Sequence
P815AB.35–43	P815AB.9	LPYLGWLVF
P815AB.33–43	P815AB.11	EILPYLGWLVF
P815AB.33–44	P815AB.12	EILPYLGWLVFA
P815AB.33–45	P815AB.13	EILPYLGWLVFAV
P815AB.33–46	P815AB.14	EILPYLGWLVFAVV
tt-(947–967)	tt	FNNFTVSFWLRVPK VSASHLE
OVA-(323–339)	OVA	ISQAVHAAHAE INEAGR
λrep-(12–26)	λrep	LEDARRLKAIYEKKK
PbCS-(20–39)	PbCS	PGYGQNKSIQAQRNL NELCY

Dendritic cell preparation

Dendritic cells were prepared from collagenase-treated spleens (collagenase type IV, Sigma Chemical Co., St. Louis, Mo.), as described [8]. Briefly, total spleen cells were suspended in dense bovine serum albumin ($P = 1.080$), overlaid with 1 ml RPMI medium, and centrifuged in a swing-out-bucket rotor at 7500 rpm for 20 min at 4 °C. The low-density fraction at the interface was collected and washed several times. The recovered cells were resuspended in RPMI medium supplemented with 1% normal mouse serum and allowed to adhere for 2 h, and this was followed by an additional 18-h incubation to allow the dendritic cells to detach. For IL-12 treatment of the cells in vitro, the 18-h incubation was conducted in the presence of 100 ng/ml rIL-12 [1, 6]. Contaminating B cells were further removed by one round of panning on polyvalent goat anti-(mouse Ig) (Sigma Chemical Co.). The recovered cells were routinely more than 96% $N418^+$.

Immunization and skin test assay

For in vivo priming, 5×10^6 dendritic cells were pulsed with a single peptide or a mixture of two peptides (P815AB.9 + tt), each at a final 5 μM concentration, at 37 °C for 2 h. Cells were then irradiated (30 Gy) and washed, and each mouse received an i.v. injection of 3×10^5 peptide-pulsed dendritic cells. To study the effect of IL-12 depletion, mice were given i.p. injections of 100 μg IL-12-neutralizing or control antibodies. Mice were treated 1 day before dendritic cell transfer and again on days 1, 3, 7, and 9. In studying the effect of T cell depletion on the afferent induction of DTH, mice received a single i.v. injection of 1.5 mg purified anti-CD4 mAb or 1 ml 53.6.72 ascitic fluid 2 weeks before immunization (to minimize the effect of cell depletion on the efferent response). A single dose of 1 mg anti-CD4 mAb or 1 ml 53.6.72 ascitic fluid was administered 24 h before intrafootpad challenge to deplete T cells in the efferent response. In selected experiments, control groups for mAb treatment received equal amounts of affinity-purified rat IgG (Zymed Laboratories, San Francisco, Calif.) to detect possible nonspecific effects of antibody infusion, and none was found. The efficiency of T cell depletion was assessed as described, by cytofluorometric analysis of spleen cells harvested at the time of sensitization or footpad challenge [1, 5, 14]. Serotherapy consistently resulted in a reduction in $CD8^+$ cells of at least 95% (at the time of DTH assessment, 2 days after mAb treatment). Following anti-CD4 treatment, the percentage reduction in $CD4^+$ cells was more than 98% at 2 days, approximately 85% at 2 weeks, and about 10% at 4 weeks. In the spleens of naive mice the percentages of $CD4^+$ and $CD8^+$ cells were approximately 30% and 10% respectively.

An assay system for measuring the delayed-type hypersensitivity (DTH) response was employed in which 50 μg P815AB.9 peptide, in 30 μl 6% dimethylsulfoxide (DMSO) in saline, was inoculated into the left hind footpads of mice administered dendritic cells 2 weeks earlier [1, 5, 6, 14]. The right hind footpad received the same volume of vehicle. The DTH reaction was recorded 24 h later; when the animals were sacrificed, their hind feet were cut off at the hair line and the weights recorded as a measure of swelling, edema and cellular infiltration. Results were expressed as the increase in footpad weight over that of the vehicle-injected counterpart. Data are the means ± SD for at least six mice per group.

Selection of $CD4^+$ or $CD8^+$ lymphocytes, production of culture supernatants containing cytokines, and cytokine assays

On the same day as the DTH assessment, parallel groups of mice were used as a source of spleen cells from which $CD4^+$ and $CD8^+$

lymphocytes (more than 98% pure) were purified on a positive selective column with either CD4 or CD8 MicroBeads (Miltenyi Biotec GmbH) respectively. For cytokine production in vitro, 5×10^5 lymphocytes were cultured with 5 μM P815AB.9 peptide and 5×10^6 irradiated (20 Gy) syngeneic spleen cells, as antigen-presenting cells, in 24-well plates as described [6]. After incubation at 37 °C for 48 h in 8% CO_2, culture supernatants were harvested by centrifugation and assayed for IFNγ ($CD8^+$ cells) or IL-2 ($CD4^+$ cells) contents. Cell supernatants were tested for their concentration of IFNγ by two-site enzyme-linked immunosorbent assay using mAb R4–6A2 as the primary reagent and biotinylated monoclonal XMG1.2 as the secondary reagent. IL-2 measurements involved the use of mAb JES6–1A12 and biotinylated JES6–5H4. Cytokine titers (means ± SD of replicate samples) were expressed as picograms or nanograms per milliliter, calculated by reference to standard curves constructed with known amounts of rIFNγ or rIL-2. The sensitivity limit was approximately 20 pg/ml for both cytokines.

PBL stimulation in vitro and chromium-release assay

Stimulation of blood lymphocytes was performed by mixing 3×10^5 Fycoll-separated peripheral blood lymphocytes with 1.5×10^5 irradiated P1 cells (a tumorigenic clone of P815 [10]) in the presence of 2×10^6 irradiated syngeneic spleen cells. The cells were incubated in 48-well plates in a 0.8-ml final volume and cytotoxic T lymphocyte (CTL) activity was measured 7 days later.

The chromium-release assay, involving P1 cells as a target, was performed according to standard conditions. One lytic unit (LU) was defined as the number of effector cells that would lyse 50% of the target cells (10^4) in 4 h. This number was derived by regression analysis from the specific release obtained at three different effector-to-target cell ratios in the linear portion of the curve.

Purification of immune-region-associated antigen (Ia) molecules

$I\text{-}A^d$ and $I\text{-}E^d$ molecules were purified from A20 cell lysates as described [17], using either mAb MKD6 ($I\text{-}A^d$-specific) or mAb 14.4.4 ($I\text{-}E^d$-specific) coupled to Sepharose 4B beads. Lysates were filtered through 0.8-μm and 0.4-μm filters and then passed over the appropriate anti-Ia columns, which were then washed with 15 column volumes of 0.5% NP-40/0.1% SDS and 2 column volumes of phosphate-buffered saline (PBS) containing 1% *n*-octylglucoside. Finally, the Ia was eluted with 0.05 M diethylamine in 0.15 M NaCl containing 1% *n*-octylglucoside, pH 10.5. A 1/20 volume of 1.0 M TRIS, 1.5 M NaCl, pH 6.8, was added to the eluate to reduce the pH to approximately 7.5, and the Ia was then concentrated by centrifugation in Centricon tubes (Amicon Inc. Beverly, Mass.).

Assay of peptide binding to class II molecules

The peptide binding assay was carried out essentially as described [17]. Purified class II molecules (5 μM) were incubated with 1–2 μM ^{125}I-radiolabeled peptides for 48 h in PBS containing 5% DMSO in the presence of a protease inhibitor mixture. Purified peptides were iodinated by the chloramine T method. The final concentrations of protease inhibitors were: 1 mM phenylmethylsulfonyl fluoride, 1.3 mM 1.10 phenanthroline, 73 μM pepstatin A, 8 mM EDTA, 6 mM *N*-ethylmaleimide, and 200 μM *Nα-p*-tosyl-L-lysine chloromethyl ketone. The final detergent concentration in the incubation mixture was 2.6% digitonin ($I\text{-}A^d$) or 0.05% NP-40 ($I\text{-}E^d$). Class-II–peptide complexes were separated from free peptide by gel filtration on Superdex 75 (Pharmacia Biotech, Uppsala, Sweden), and the fraction of peptide bound was calculated as described [17]. The percentage of total offered radiolabelled peptide bound to Ia was approximately 10% for OVA ($I\text{-}A^d$) and 3% for λrep ($I\text{-}E^d$). Competitor peptides were typically tested at concentrations ranging from 200 μM to 2 nM. Competition binding curves were plotted and the affinity of the peptide for $I\text{-}A^d$ or $I\text{-}E^d$ was expressed as the 50% inhibitory concentration (IC_{50}), the concentration of peptide required to inhibit the binding of radiolabelled reference peptide to Ia molecules by 50%. Each competitor peptide was tested in at least five independent experiments.

Stability of class II molecules by assay with SDS-PAGE

Stable class II complexes were examined by SDS-PAGE under reducing conditions without boiling the samples as described [3, 12]. Briefly, affinity-purified $I\text{-}A^d$ molecules (5 μg) were incubated with 100 μM peptides at 37 °C in a final volume of 10 μl buffer containing 6.7 mM citric phosphate, pH 7.0, with 0.15 M NaCl, 2% NP-40, 2 mM EDTA, and the protease inhibitors used in the peptide-binding assay. After 72 h, samples were diluted in reducing SDS sample buffer, incubated for 1 h at room temperature, and subsequently separated by electrophoresis on a 12% SDS-PAGE gel. The gel was stained with Coomassie blue R-250. For densitometric analysis of the gel, the software program NIH image 1.64 (supplied by the National Institutes of Health, Bethesda, Md.) was used.

Statistical analysis

The statistical analysis for the DTH assay was performed according to paired Student's *t*-test by comparing the mean weight of experimental footpads with that of control, vehicle-injected counterparts, which served as an internal control for each individual animal [1, 5, 6, 14]. The data reported in this paper are from representative experiments.

Results

DTH responsiveness to P815AB.9 and cytokine production after transfer of peptide-pulsed dendritic cells with or without added adjuvant activity

Unlike DC pulsed with P815AB.9 alone, cells pulsed with a combination of P815AB.9 and tt peptides confer $CD8^+$ cell-mediated reactivity on prospective recipients of an intrafootpad challenge with the tumor peptide. This is accompanied by the production in vitro of IFNγ and IL-2 by $CD8^+$ and $CD4^+$ cells, respectively, at the time of DTH assessment. rIL-12, added externally to dendritic cell cultures before pulsing, can replace the helper peptide in the adjuvant effect [1, 6, 7, 9]. Figure 1 shows the effect of sensitization with P815AB.9, using singly or doubly pulsed dendritic cells, or dendritic cells exposed sequentially to IL-12 and P815AB.9 before transfer into recipient hosts. After 2 weeks, mice were assayed for DTH reactivity and also served as a source of splenic $CD4^+$ and $CD8^+$ cells to be cultured in vitro. After restimulation with P815AB.9 in the presence of antigen-presenting cells, cytokine levels were measured in culture supernatants. Consistent with our previous results [1, 6], P815AB.9-specific reactivity and high antigen-specific cytokine production in vitro were observed only in mice receiving either doubly pulsed dendritic cells or cells exposed to rIL-12 before P815AB.9 pulsing. Besides confirming the detection of activated $CD4^+$ cells in an effective priming to P815AB.9, these data reinforced the notion that the recently described helper epitopes of

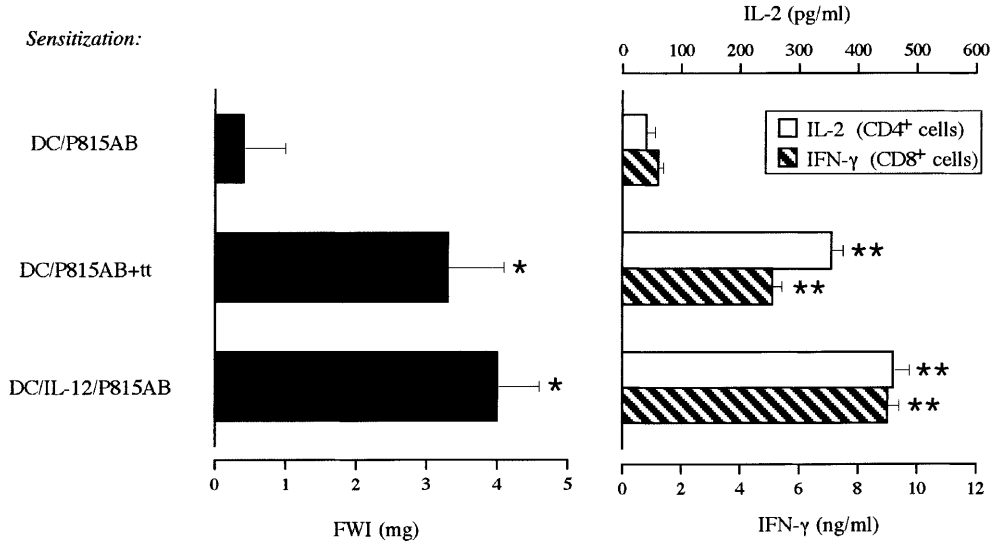

Fig. 1 Induction of T cell responses in vivo to P815AB.9 by host transfer with P815AB.9-pulsed dendritic cells (*DC*). Mice received cells exposed to the tumor peptide, either alone (*DC/P815AB.9*) or in combination with tetanus toxin peptide (*tt*) (*DC/P815AB.9 + tt*). Alternatively, mice received dendritic cells exposed to interleukin-2 (*IL-12*) pulsed with P815AB.9 alone (*DC/IL-12/P815AB.9*). Delayed-type hypersensitivity (DTH) was measured after 2 weeks, and values were expressed as the mean footpad weight increase (*FWI*) ± SD. Mice assayed for DTH also served as donors of purified $CD4^+$ and $CD8^+$ cells that were tested for cytokine production after restimulation in vitro with P815AB.9 and accessory cells. $*P < 0.001$ (experimental versus control footpads). $**P < 0.001$ (cytokine production in effectively sensitized versus DC/P815AB.9-treated mice). Specificity controls (not shown in the figure) involved the use of an antigenically unrelated peptide for both footpad challenge and restimulation in vitro of $CD4^+$ and $CD8^+$ cells. *IFN* interferon

P815AB.9 [6] do not result in substantial activation of the helper T cells necessary to $CD8^+$ cell priming [7, 9].

DTH responsiveness, cytokine production, and CTL generation to P815AB.9 after immunization of mice with dendritic cells pulsed with different P815AB peptides

Because, in general, class II molecules best accommodate and/or present natural ligands of a proper length (usually exceeding 11 amino acids) [15], we became interested in analyzing a series of longer, P815AB-centered peptides for possible adjuvant activity in T cell priming to P815AB.9. All of these peptides, derived from the known sequence of the gene *P1A*, have been previously characterized in detail and overlap with P815AB.9. The latter peptide contains the two CTL-recognized epitopes responsible for in vitro reactivity to the tumor-rejection antigen P815AB, and represents the minimal core peptide for CTL recognition [10, 19]. Dendritic cells were pulsed only with P815AB.9, P815AB.11, P815AB.12, P815AB.13, or P815AB.14 and transferred into recipient mice that were assayed for DTH to P815AB.9 at 2 weeks. Purified $CD4^+$ and $CD8^+$ cells, recovered at the time of footpad challenge, were also assayed for cytokine production after restimulation in vitro with P815AB.9 and accessory cells. Figure 2 shows that significant DTH reactivity to P815AB.9 was only induced by sensitization with P815AB.12 or P815AB.13. This was paralleled by high production of IL-2 by $CD4^+$ cells and of IFNγ by $CD8^+$ cells in response to P815AB.9 restimulation. It is of interest that P815AB.14 initiated a strong $CD4^+$ cell response to P815AB.9, as measured by IL-2 production in vitro, yet neither DTH in vivo nor $CD8^+$ cell production of IFNγ in vitro could be observed in mice primed to P815AB.14. Because a physical mixture of P815AB.9 and P815AB.14 would instead trigger both the in vivo and the in vitro response of $CD8^+$ cells to P815AB.9 in mice treated with doubly pulsed dendritic cells (data not shown), these data argued for the requirement of an optimal peptide length in order to prime the host simultaneously to the class-I- and class II-restricted epitopes of P815AB peptides with a defined core.

IFNγ production is one of the effector functions of CTL. It seemed, therefore, of interest to study the recognition and pattern of CTL lysis of P815 cells endogenously expressing the relevant antigen P815AB. Using PBL from individual mice (ten per group) primed with dendritic cells pulsed with P815AB.9, P815AB.12, or P815AB.13, it was shown that only the 12-mer and the 13-mer resulted in the generation of strong cytotoxic activity to the target cells as measured by calculation of lytic units/10^6 PBL (Fig. 3).

Requirement for production of endogenous IL-12 and for $CD4^+$ cells in triggering class-I-restricted reactivity to P815AB.9

Having demonstrated previously that the P815AB.9-specific T cell response induced by the use of doubly (P815AB.9 + tt) pulsed dendritic cells is dependent on the production of endogenous IL-12 [1], we investigated a possibly similar requirement in the adjuvant activity of P815AB.12 and P815AB.13. Recipients of P815AB.12-

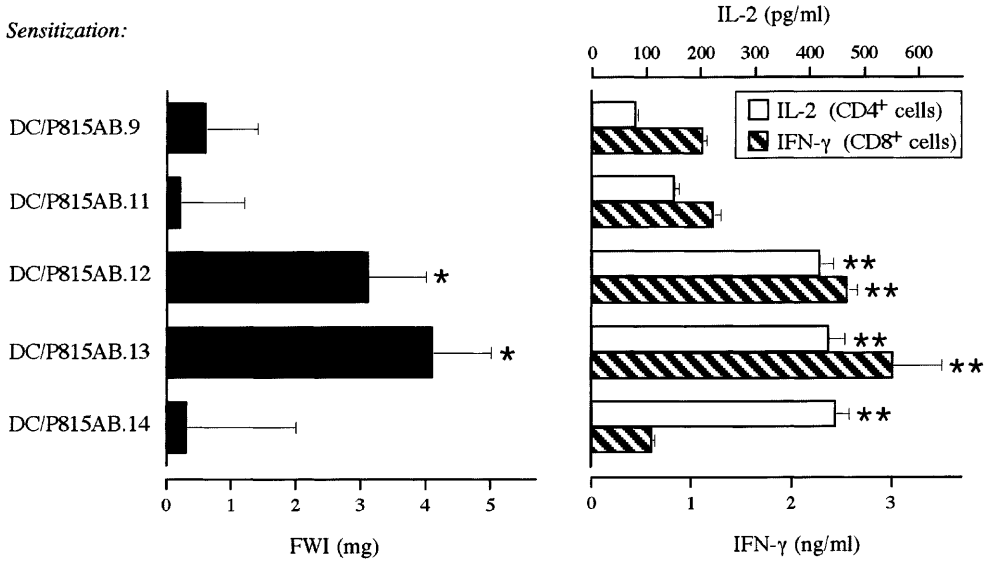

Fig. 2 Differential ability of P815AB peptides of different length to prime the host for T cell reactivity to P815AB.9. Dendritic cells were singly pulsed with the different peptides (indicated) prior to transfer into mice, assayed at 2 weeks for footpad reactivity to P815AB.9. Mice assayed for DTH also served as donors of purified $CD4^+$ and $CD8^+$ cells that were tested for IL-2 and IFNγ production, respectively, after restimulation with P815AB.9 and accessory cells. $*P < 0.01$ (experimental versus control footpads). $**P < 0.001$ (significant difference in cytokine production with respect to mice receiving P815AB.9-pulsed dendritic cells)

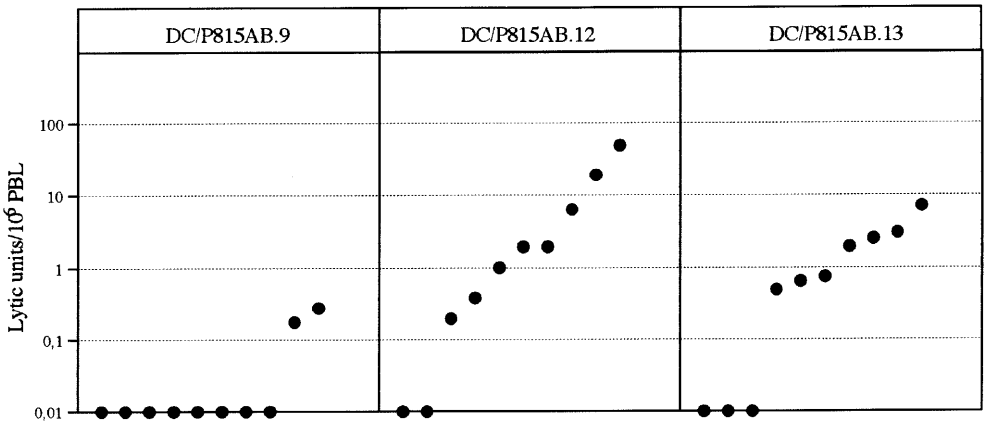

Fig. 3 Differential ability of P815AB peptides of different length to prime the host for cytotoxic T lymphocyte (CTL) activity to P815AB-expressing cells. Dendritic cells were pulsed with P815AB.9, P815AB.12, or P815AB.13 prior to transfer into mice that served as a source of CTL. Two weeks after transfer, peripheral blood lymphocytes (*PBL*) were restimulated in vitro for 7 days with P815AB-expressing cells prior to measurement of their lytic activity with the same target. Each point represents the lytic activity of PBL from individual mice

or P815AB.13-pulsed dendritic cells were treated with polyclonal sheep anti-IL-12 antibody near the time of cell transfer. At 2 weeks after immunization, mice were assayed for DTH and cytokine production in response to P815AB.9. Strong footpad reactivity and elevated cytokine production could only be detected in control-antibody-treated mice. Both the in vivo and the in vitro responses were abolished by IL-12 depletion (Fig. 4). Also, similar to what was observed in the double immunization with P815AB.9 and tt [5], serological ablation of $CD4^+$ cells at the time of dendritic cell transfer or of $CD8^+$ cells completely abolished the skin test reactivity to P815AB.9 in mice primed with P815AB.12-pulsed dendritic cells (Fig. 5).

Binding affinity of P815AB peptides for class II molecules

In principle, the differential ability of P815AB peptides to prime to the core peptide seen by the T cells could be due to structural features of the peptides that affect distinct properties of the class II/peptide complex formation, including binding capacity and ability to assemble class II molecules stably [3, 12]. In the first set of experiments, we examined the different P815AB peptides for strength of $I\text{-}A^d$ and $I\text{-}E^d$ binding, as measured by a peptide-binding assay using purified class II molecules and radiolabelled reference peptides [17]. Table 2 shows the results of competition between ^{125}I-labeled OVA (for $I\text{-}A^d$) or λrep (for $I\text{-}E^d$) and unlabeled peptides (i. e., OVA or λrep and P815AB peptides) in the binding to purified class II molecules. On examining the concentration of a peptide required to inhibit binding of the reference peptide by 50% (IC_{50}), we found that the 9-mer had a more than tenfold lower binding affinity for $I\text{-}A^d$ than did OVA (IC_{50} values of 30 μM and 2 μM respectively). However, no major differences in binding capacity for $I\text{-}A^d$ were found among the different P815AB peptides, with the 12-, 13-, and 14-mer displaying the lowest IC_{50} values (in the range 23–26 μM). In the competition assay between radiolabelled λrep and unlabeled peptides for binding to $I\text{-}E^d$, we found that none of the P815AB peptides, with the exception of

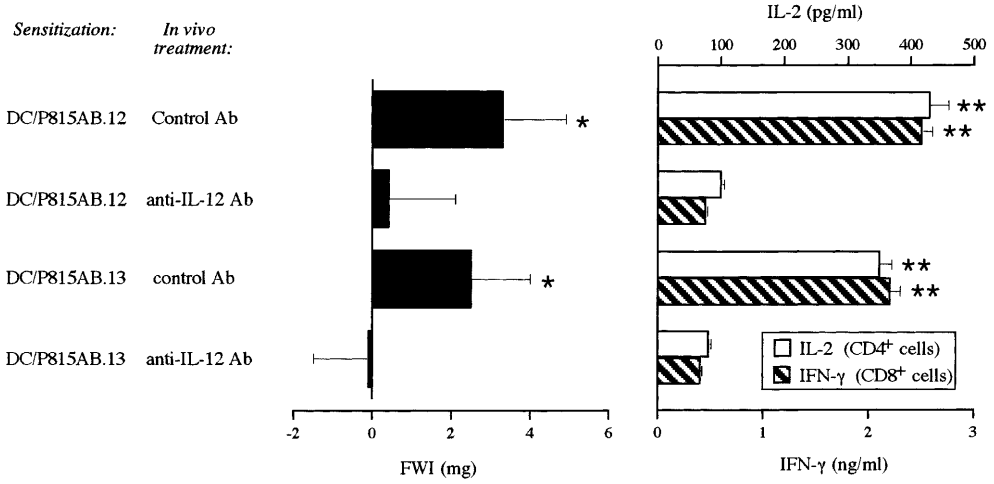

Fig. 4 Effect of IL-12 neutralization on induction of P815AB.9-specific reactivity after immunization with P815AB.12- or P815AB.13-pulsed dendritic cells. Concurrent with cell transfer, mice received control or anti-IL-12 antibodies. DTH reactivity and IL-2 (by $CD4^+$ cells) or IFNγ (by $CD8^+$ cells) were measured at 2 weeks. $*P < 0.01$ (experimental versus control footpads). $**P < 0.001$ (significant difference in cytokine production with respect to mice receiving control antibody)

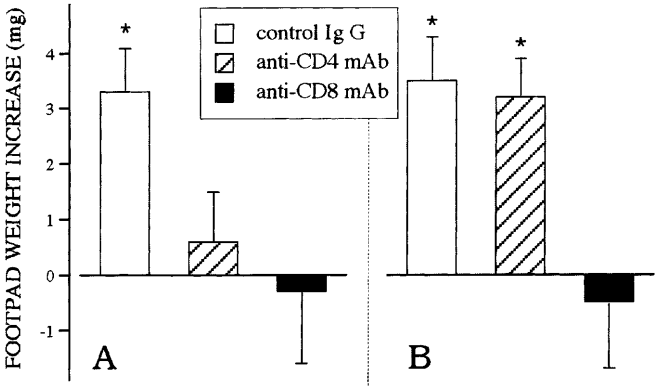

Fig. 5A,B Effect of $CD4^+$ or $CD8^+$ cell subset depletion on induction and detection of DTH to P815AB.9. Anti-CD4 or anti-CD8 mAb were injected into mice 2 weeks before transfer with P815AB.12-pulsed dendritic cells (**A**); alternatively, cell depletion was performed at the time of DTH elicitation (**B**). $*P < 0.001$ (experimental versus vehicle-treated footpads)

Table 2 Competition assay between radiolabeled OVA or λrep peptides and unlabeled P815AB peptides for binding to purified $I\text{-}A^d$ or $I\text{-}E^d$ molecules respectively. $I\text{-}A^d$ or $I\text{-}E^d$ at 5 μM was incubated with 2 μM or 1 μM radiolabeled OVA or λrep, respectively, and a range of doses of each inhibitory peptide for 2 days at room temperature. The degree of inhibition of binding of labeled peptides to the antigen was determined by gel filtration. Results are expressed as the mean inhibitory concentration (IC_{50}) of unlabeled (reference or test) peptides as computed by regression analysis. Data are representative of at least five independent experiments

Unlabeled peptide	Concentration (μM) of unlabeled peptide required for 50% inhibition of binding of	
	^{125}I-OVA to $I\text{-}A^d$	^{125}I-λrep to $I\text{-}E^d$
OVA	2	>200
λrep	20	1
P815AB.9	30	>200
P815AB.11	34	>200
P815AB.12	23	45
P815AB.13	25	>200
P815AB.14	26	>200

P815AB.12, displayed measurable affinity for $I\text{-}E^d$. Though somewhat unexpected, these data could be taken to indicate that affinity *per se* for class II molecules is not the crucial determinant underlying the differential effects on $CD4^+$ cell activation by the various P815AB peptides.

Assay of SDS-stable and -unstable class II molecules using purified $I\text{-}A^d$

We next examined the various P815AB peptides for possible differential effects on the prevailing conformation of MHC class II molecules. These molecules exist in two conformations, which can be distinguished on the basis of their stability in SDS [3, 12]. Upon immunoprecipitation and SDS-PAGE analysis, class II molecules have been shown to yield three distinct bands under at temperatures below boiling: a 55-kDa α/β dimer (stable dimer) and the separated α and β chains of 32 kDa and 29 kDa respectively (unstable dimer). It is also known that there is usually no correlation between the binding capacity of a peptide and its ability to induce the SDS-stable conformation [20]. We assayed the different P815AB peptides for their ability to assemble purified $I\text{-}A^d$ molecules stably. The affinity-purified $I\text{-}A^d$ molecules were incubated with the P815AB or control peptides at 37 °C for 72 h. The binding mixtures were analyzed by SDS-PAGE without boiling under reducing conditions. Two immunogenic helper peptides, namely tt and PbCS, were used as controls, as both had been shown to exert adjuvant activity in dendritic cell priming with P815AB.9 [5]. Figure 6 shows that the resistance to α/β-chain dissociation induced by SDS in the MHC/peptide complexes varied among the different peptides, being the highest for PbCS, tt, and P815AB.13 (more than 65% stable dimers) and the lowest for the 9- and 11-mer (approximately 50% stable dimers, a percentage similar to that observed in the absence of any peptide). This suggested a direct relationship between the proportion of stably assembled class II molecules and the

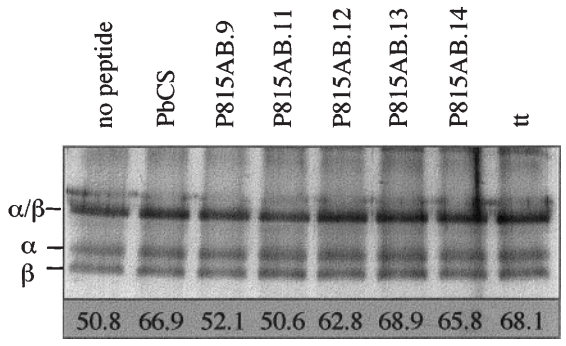

Fig. 6 Relative ability of P815AB peptides to induce formation of sodium-dodecyl-sulfate-stable complexes with class II molecules. Affinity-purified I-A^d molecules (5 μg) were incubated with 100 μM different P815AB peptides (indicated) using tt and PbCS as controls. After 72 h at 37 °C, the binding mixtures were analyzed by SDS-PAGE without boiling under reducing conditions. After electrophoresis, the proteins were detected by Coomassie blue R-250 staining. Numbers corresponding to individual lanes were obtained by densitometric analysis of the gel (see Materials and methods) and are expressed as percentage of stable α/β dimers with respect to total associated and dissociated α and β chains

ability to trigger T cell reactivity to peptides encompassing the minimal CTL-defined core peptide of P815AB.

Discussion

Recent studies in human and murine systems suggest a central role for $CD4^+$ cells in initiating, effecting, and maintaining antitumor immunity [13]. Together with the characterization of tumor-associated antigens recognized by $CD8^+$ cells, a more thorough understanding of $CD4^+$ cell-directed immunoregulation may eventually be critical to the success of peptide-based vaccination strategies. However, the development of broadly applicable and effective therapies targeting MHC-class-II-restricted tumor epitopes will require the identification of these antigens, as well as an increased understanding of the properties of peptides associated with the class II MHC complex. Recent studies have shown that there are structural features of peptides that change the stability of the class II molecule and are independent of the "core" peptide seen by the T cells [12].

P815AB.9 represents a minimal core peptide recognized by CTL in vitro [10] and is part of a protein encoded by gene *P1A* which, silent in most normal tissues except testis and placenta, is instead expressed by murine mastocytoma cells [2, 18]. We have previously shown that nonameric P815AB fails to initiate class-I-restricted reactivity in vivo, presumably as a result of a poor ability to recruit $CD4^+$ cells to the afferent induction of the response initiated by host transfer with P815AB.9-pulsed dendritic cells. However, the poor immunogenicity of P815AB.9 under such priming conditions can be overcome by co-pulsing dendritic cells with helper peptides, such as tt and PbCS, or by pre-exposure of the cells to rIL-12 [1, 5, 6, 8, 9]. This led us to conclude that sequential activation of $CD4^+$ and $CD8^+$ cells is necessary for effective priming to the class-I-restricted P815AB epitopes recognized by $CD8^+$ cells. Subsequent studies have further indicated that P815AB.9 possesses I-A^d-restricted epitopes, yet the resulting complexes with class II molecules on dendritic cells may not be persistent enough to allow for effective $CD4^+$ cell priming [6]. In fact, transfer of mice with DC only pulsed with P815AB.9, in the absence of any added adjuvant activity, results in a state of antigen-specific anergy involving both $CD4^+$ and $CD8^+$ cells [6].

It has been proposed that the core of a peptide, anchored in the class-II-binding groove, generates the antigenic determinant recognized by the T cell receptor, and that the flanking residues at the ends of the peptide make the additional contacts necessary for the stability of the class II structure [12]. The importance of a basic peptide sequence containing the critical class-II-binding and T-cell-contact residues has been stressed in several studies [11, 16]. This is consistent with the notion that both the length and the sequence of a peptide contribute to the stability of binding to class II molecules [12].

In the present study, we have examined a series of *P1A*-encoded peptides of different length (P815AB.11, P815AB.12, P815AB.13, and P815AB.14) for ability to activate the $CD4^+$ cells necessary for $CD8^+$ cell priming to the class-I-restricted epitopes of P815AB.9. Using an experimental model system previously adopted for monitoring the class-I-restricted skin test response to P815AB.9 in mice administered peptide-pulsed dendritic cells [14], we found that the 12- and 13-mer would effectively initiate the T cell response to the nonamer in vivo (Fig. 2). The development of footpad reactivity correlated with $CD8^+$ cell production of IFNγ (Fig. 2) and generation of CTL activity to P815AB-expressing tumor cells (Fig. 3). The skin test response was also dependent on the endogenous production of IL-12 (Fig. 4) and on an intact $CD4^+$ cell compartment in the recipient host, although the effector phase was primarily mediated by $CD8^+$ cells (Fig. 5). It is of interest that P815AB.14 could activate antigen-specific $CD4^+$ cells but was unable to prime the host to the class-I-restricted epitopes of P815AB.9. This suggested that an optimal length may be required for the simultaneous presentation of class-I- and class-II-restricted epitopes of a P815AB peptide by dendritic cells to T cells. We are currently examining P815AB.14 for its ability to prime the host for CTL activity to P815AB-expressing cells and to produce IL-2 and IFNγ. This may allow us to assess the importance of the dual induction of IL-2 and IFNγ for CTL generation. These data may complement previous work assessing the affinity of the induced $CD8^+$ CTL to the different peptides [10]. It was shown that P815AB.9 possesses the highest affinity, which typically decreases in the other peptides as the number of amino acids increases.

In principle, the structural differences allowing P815AB.12 and P815AB.13 to prime the core peptide

effectively could be related to differences in the strength of binding to I-A^d and/or their ability to increase the proportion of stable class II molecules. We therefore examined the different peptides for their ability to bind purified Ia molecules in competition experiments, using I-A^d- or I-E^d-binding peptides (Table 2). Although the affinity of the various P815AB peptides for I-A^d was tenfold lower than that of OVA, no major differences in binding strength were found among the former peptides and, in fact, the 9-mer and 11-mer exhibited the highest IC_{50}. These data are consistent with previous observations in different experimental systems indicating that affinity *per se* of a peptide for class II molecules does not necessarily result in a stable conformation of class II molecules and activation of the T cell response [12, 20].

Peptide binding to MHC class II may influence the biochemical structure of the α/β heterodimer, which permits the formation of functional MHC class II/peptide complexes [3]. The structure of the mature MHC class II α/β heterodimer is very stable, such that purified mature MHC class II molecules migrate as α/β heterodimers in the presence of SDS at room temperature on polyacrylamide gels [21]. Either by more efficiently converting nascent class II molecules into the folded state or by increasing the half-life of the assembled peptide/MHC complex [4, 12], peptides may produce the stable form of MHC class II. We therefore became interested in ascertaining any effect of P815AB peptides on the prevailing conformation of class II molecules. We evaluated the proportion of stable molecules using purified I-A^d in the presence of P815AB peptides. By comparing the relative amounts of SDS-stable and SDS-labile heterodimers, we obtained evidence that the immunogenic P815AB.12 and P815AB.13 resulted in the highest proportion of stably assembled class II MHC (Fig. 6). Therefore, with all P815AB peptides tested, there was no direct relationship between I-A^d binding strength, as measured in the competition experiments, and the ability to assemble class II molecules stably. What is more important, the latter property of the P815AB peptides correlated with their capacity to prime the host in vivo to the minimal core peptide, P815AB.9. We are currently attempting to demonstrate the induction of stable complexes by the longer peptides by means of another approach involving treatment of whole cells with the different peptides rather than using affinity-purified class II molecules. In addition, as an alternative explanation for the effect of the longer peptides, we are exploring the possibility that the number of $CD4^+$ cells recognizing the longer peptides is greater than that of cells recognizing P815AB.9, which may contain fewer T cell epitopes.

In conclusion, our studies indicate that not all P815AB peptides that bind MHC class II molecules confer stability when analyzed by SDS-PAGE. While the P815AB.9 core peptide contains the critical amino acids responsible for recognition by $CD4^+$ cells (as measured by IL-2 production) and $CD8^+$ cells (as measured by the skin test response in vivo and IFNγ production in vitro), it is unable by itself to prevent dissociation of the class II/peptide complex in SDS-PAGE. Nevertheless, P815AB.9 binds class II with an affinity that is not substantially different from that of the immunogenic P815AB peptides. It is likely that effective signaling by the immunogenic peptides through MHC molecules may affect the antigen-presenting function of dendritic cells and increase endogenous IL-12 production. In addition to previous evidence that the dendritic-cell-derived IL-12 may act as both an adjuvant and inhibitor of anergy induction in $CD4^+$ cells [6], we have recently provided direct evidence that the adjuvant activity of IL-12 may involve a primary effect on dendritic cell functions [8]. Whether the ability of immunogenic P815AB peptides to produce the SDS-stable α/β heterodimers derives from more efficient conversion of nascent class II molecules into the folded state, or from increasing the half-life of the assembled peptide/MHC complex, remains unclear. Although the extent to which SDS stability defines a conformation required by T cells for recognition of the peptide/MHC complex is as yet unclear [21], our present data with P815AB peptides of different immunogenicity establish a direct relationship between peptide length and the ability to produce SDS-stable α/β heterodimers.

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