The geometry of synthetic peptide-based immunogens affects the efficiency of T cell stimulation by professional antigen-presenting cells

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Abstract

In the pathway leading to antibody production there are two points at which CD4+ T cells need to be recruited. The first of these is priming of T cells by their interaction with dendritic cells (DC) bearing antigen presented on MHC class II molecules and the second is the collaborative interaction of these primed T cells with B cells presenting the same antigen. We have previously shown that the configuration of T and B cell determinants within synthetic peptide immunogens can greatly influence the amount of immunogen required to produce an antibody response. Here we investigate whether the difference in potency of different immunogens is related to their ability to be presented by either DC or B cells. We show that determinants in a branched configuration, which are the most efficient at eliciting antibody in vivo, are presented to T cell clones by splenic CD8− DC 10-fold more efficiently than the corresponding determinants in a tandem linear arrangement. B cells also showed preferential presentation of branched immunogens to one T cell clone but in contrast to DC, not to a second T cell clone, indicating differences between the two antigen-presenting cell types. We also show that branched immunogens have a greater stability in serum compared to linear peptides, which may further enhance the differences in their in vivo potency.

Introduction

Following administration of a synthetic peptide, a plethora of events must occur in order to generate an antibody response. These include the uptake of antigen by an antigen-presenting cell (APC) and the activation of naive T cells, functions which are carried out most efficiently by dendritic cells (DC). Activated T cells must then engage a B cell to deliver the appropriate signals that initiate B cell differentiation and subsequent antibody production.

A number of reports (1–5) indicate that the orientation of B and T cell epitopes with respect to each other within a synthetic peptide construct affects the ensuing antibody response. Using synthetic peptide-based designer immunogens we have shown that the geometry of assembly of B cell determinants and Th1 cell determinants is also an important factor in the induction of specific antibody (6). Branched synthetic peptide constructs were found to be superior in eliciting antibody when compared to the same determinants assembled in a linear conformation and the copy number of the B cell determinant was also found to affect the level of antibody produced; branched synthetic peptide constructs which contained a Th1 cell determinant and two B cell determinants elicited higher and more consistent antibody titers than the equivalent synthetic peptide constructs containing a single B cell determinant.

The contribution of these physical attributes of a synthetic peptide construct to immunogenicity is often a reflection of the accessibility of amino acids within the epitope that are important binding sites for proteases, antibodies and other receptors encountered throughout the processing pathway. Clearly, such processing and binding events are sequence dependent; however, at another level, the valency, geometry and physical characteristics of an immunogen are also import-
ant. The ability of APC to capture antigen has a major affect on the levels of expression of antigenic determinants on MHC class II molecules (reviewed in 7) and evidence is emerging that different APC display unique combinations of mechanisms for antigen uptake (reviewed in 7,8). These properties must influence downstream mechanisms and even the type of APC that becomes involved in antigen uptake and presentation.

Investigations into the effect that different APC have on the presentation of antigen revealed that the type of APC and also the subtype of APC involved can affect T cell activation. Recent studies using murine DC have revealed that subtypes of freshly isolated splenic DC distinguished by the presence or absence of the cell-surface marker CD8α (9) have very different effects on T cells (10,11). CD8+ DC are much less efficient than CD8− DC at stimulating proliferation of naive allogeneic CD4+ T cells, the result of a regulatory mechanism mediated by the CD8+ DC which results in rapid Fas-mediated cell death of the CD4+ T cell population (10). CD8+ DC can also modulate the proliferation of CD8+ T cells by regulating IL-2 production, which in turn limits T cell expansion (11).

Once DC have activated the appropriate T cell subset, B cells are also required to present the same antigen to the activated T cell to receive the necessary signals to induce antibody production. Antigen is captured by the surface-bound Ig on the extracellular face of the B cell. This B cell receptor (BCR) is the major pathway for the internalization of antigen into the class II processing pathway (8). The increased ability of antigen-specific B cells compared to non-specific B cells in the presentation of antigen to T cells is thought to be not only a result of increased endocytosis of the BCR but also due to post-endocytic sorting events that target the BCR-antigen complex to MHC class II loading compartments (7,12). Although naive B cells are capable of pinocytosis, the kinetics of this process are slow and this is not a major mechanism for antigen uptake. Studies in B cell systems have, however, shown that empty class II MHC molecules are capable of recycling through early endosomes to be re-expressed on the cell surface (13–16), and this pathway is accessible to and often preferred by exogenous antigen (17). Some antigens have been shown to be independent of HLA-DM and invariant chain for peptide loading (13,18) which adds support to a mechanism of recycling class II MHC as an alternate presentation pathway in naive B cells.

In this study we have used the CD8+ DC subpopulation, freshly isolated from splenocytes, together with purified splenic B cells to present synthetic peptide antigens to T cells in experiments aimed at increasing our understanding of the mechanisms responsible for the differences in immunogenicity exhibited by chemically similar but geometrically different peptide constructs.

Methods

Synthetic peptides

The synthesis and purification of the peptide constructs used in this study have been described elsewhere (6). Briefly, all peptides were synthesized using solid-phase peptide chemistry using the free-acid forms of Fmoc-derivitized amino acids. Peptides were purified using reverse-phase FPLC and characterized by analytical HPLC and amino acid analysis. A schematic of the peptide constructs is shown in Fig. 1. Peptide 306-328 has been shown to be capable of eliciting antibody without being coupled to a carrier protein (19), and therefore contains both T cell and B cell epitopes. This peptide is derived from the influenza virus haemagglutinin and contains a T cell determinant PKYVKQNTLKLA (20) and an overlapping B cell determinant TLKLATG (21,22). The T cell determinant ALNRFQIGVELKS is derived from a distal part of the haemagglutinin molecule (23) of influenza virus.

Influenza virus

The virus designated Mem71 is a reassortant containing the haemagglutinin of type A influenza strain A/Memphis/1/71 (subtype H3) and the neuraminidase of A/Bellamy/42 (N1).

Cell lines

All cell lines were maintained by passage in RPMI 1640 supplemented with 10% heat-inactivated (56°C, 30 min) FCS, 2 mM glutamine, 2 mM sodium pyruvate, 0.1 mM 2-mercaptoethanol, 30 μg/ml gentamicin, 100 IU/ml penicillin and 100 μg/ml streptomycin, with or without exogenous cytokines, in six-well culture plates (Nunc, Roskilde, Denmark). A20 cells are derived from a B cell lymphoma cell line expressing H-2d class II MHC (24) and do not require supplements. The cell
Presentation of peptides by APC

line HT2 (25) is cytokine-dependent and requires the presence of IL-2, which was provided in the form of supernatant from concanavalin A-stimulated spleen cells.

**T cell clones**

The derivation, preparation and maintenance of T cell clones 12V1 and 4.51 are described elsewhere (20,23). Briefly, T cell clones were maintained by passage every 5 days with influenza virus Mem71 and γ-irradiated BALB/c spleen cells as a source of APC, alternated with 7 days in medium supplemented with IL-2.

**Animals**

Female BALB/c mice, between 6 and 8 weeks of age, were obtained from the animal facilities of either the Department of Microbiology and Immunology at the University of Melbourne or the Walter and Eliza Hall Institute of Medical Research, Melbourne.

**Isolation of dendritic cells**

DC were isolated as described elsewhere (11). Briefly, spleens from BALB/c mice were digested with collagenase and DNase, DC–T cell complexes dissociated with EDTA, then a density-based separation procedure using Nycodenz (Nyegaard Diagnostics, Oslo, Norway) was performed to separate a low density population of cells. This population was then depleted of T cells, B cells, macrophages, granulocytes and erythrocytes by coating with cell-specific mAb and separating the population with magnetic beads. The remaining cells were sorted into two populations, CD11c⁺CD8α⁺ and CD11c⁻CD8α⁻, using a FACSStar Plus flow cytometer (Becton-Dickinson, San Jose, CA) for immediate use in culture.

**B cell isolation**

Mice were killed by cervical dislocation and their spleens removed. Single-cell suspensions were prepared by passage through a wire sieve and erythrocytes removed by treatment with ammonium thiocyanate–chloride buffer (26). Cells, at a concentration of 10⁶/ml, were then loaded onto a 20 or 30 ml Sephadex G-10 column that had been equilibrated at 37°C in RPMI 1640 supplemented with 5% heat-inactivated (56°C, 30 min) FCS, 10 mM HEPES buffer, 30 µg/ml gentamicin, 100 IU/ml penicillin and 100 µg/ml streptomycin (RF5⁺). This protocol is a modification of the procedure described by Ly and colleagues (27) for the depletion of adherent cells such as macrophages and DC. Cells were then eluted in 1.5 column volumes of RF5⁺ and then adjusted to a concentration of 2–4×10⁷ cells/ml in a solution containing a 1/4 dilution of anti-Thy1.2 mAb (prepared by concentrating supernatant from a culture of the hybridoma 30-H12 using 6000 MW polyethylene glycol). Cells were incubated for 40 min on ice after which time they were washed once to remove excess antibody. Cells were then resuspended at the same concentration in a 1/6 dilution of Low Tox rabbit complement (Cedarlane, Hornby, Ontario, Canada) in RF5⁺. Complement-mediated lysis of T cells was carried out at 37°C for 30 min and the live cells were then separated from dead cells over Isopaque-Ficoll. Each B cell preparation was analyzed by FACS for purity and the resulting B cells used in antigen-presentation assays.

**FACS analysis**

FACS analysis was carried out using a FACSort (Becton Dickinson, San Jose, CA) flow cytometer and data were analyzed using CellQuest software. Each sample was collected ungated with 10,000 events per sample. Isolated B cells were treated with a fluoresceinated sheep anti-mouse Ig antibody (Dako, Copenhagen, Denmark) to detect surface-bound Ig. Cells were incubated on ice with a 1/50 dilution of the antibody in PBS. Labeled cells were compared to untreated B cells and also to untreated splenocyte samples to enable the lymphocyte region to be gated. All cells were then fixed in 1% paraformaldehyde in PBS for 20 min and washed 3 times in cold PBS before analysis. FACS analysis of the B cell populations indicated that the percentage of cells staining with sheep anti-mouse Ig ranged from 87 to 95%.

**T cell proliferation assays**

Cells were cultured in duplicate or triplicate with serial dilutions of peptide antigen in flat-bottom 96-well microtiter trays (Nunc), in a total volume of 250 µl. Cultures contained either nylon wool-enriched T cell preparations (3×10⁵ cells/well) or T cell clones (1×10⁴ cells/well) and, as a source of APC, either B cells (1×10⁴ cells/well), A20 cells (1×10⁵ cells/well) or CD8⁻ DC (50–100 cells/well). Cultures were incubated for 4 days at 37°C in an atmosphere of 5% CO₂. After 24 h, a 50 µl sample of supernatant was taken from each culture and stored at −20°C for cytokine analysis. Proliferation of T cells was determined by addition of 1 µCi/well [³H]thymidine to the cultures 18 h prior to harvest, cells were harvested onto glass fiber filters and the incorporation of [³H]thymidine measured using a Matrix 9600 direct β-counter (Canberra Packard, Victoria, Australia).

**T cell proliferation assays using fixed A20 cells as APC**

Cells were fixed using the protocol described by Harding (28). Briefly cells were resuspended at 5×10⁶ cells/ml in a 1:1 ratio of RPMI 1640 (supplemented with 30 µg/ml gentamicin, 100 IU/ml penicillin and 100 µg/ml streptomycin) and a 2% w/v solution of paraformaldehyde in PBS for 5 min at room temperature. Cells were then washed once with RPMI medium and resuspended in a solution (0.4 M) of lysine in water which had been diluted in an equal volume of RPMI prior to use to give a final wash solution concentration of 0.2 M. Cells were incubated with the lysine wash solution for 25–30 min at room temperature, then washed 3 times with RPMI and diluted to a volume of 1×10⁵ cells/ml (1×10⁵ cells/well) for use in a standard T cell proliferation assay. At 24 h, 50 µl aliquots were collected from each well and the level of IL-2 produced was determined using the HT2 IL-2-dependent cell line.

**Cytokine assays**

Supernatant samples (50 µl) obtained from T cell proliferation assay cultures were added to 4×10³ HT2 cells in 50 µl of medium. The cells were incubated overnight at 37°C and, after addition of 1µCi/well of [³H]thymidine, for a further 4 h. Cells were then harvested onto glass fiber filters and incorporation of [³H]thymidine was measured as above.

**Serum stability assays**

The protocol used was a modification of the method previously described by Hoffman et al. (29). Briefly, peptide solutions
were adjusted to ~2 mg/ml in saline or PBS and 50 µl added to 200 µl of a 1/10 dilution of either normal mouse serum (NMS) or heat inactivated (56°C for 30 min) FCS. At various time intervals each sample was precipitated by the addition of 40 µl of 15% aqueous trichloroacetic acid and left on ice for 30 min. Samples were then centrifuged for 5 min in a benchtop microfuge to sediment denatured high mol. wt material. Supernatants (150 µl) were then injected onto a Phenomenex C4 reverse-phase HPLC column (10 mm×250 mm) installed in a GBC HPLC system (GBC Scientific, Dandenong, Australia). Samples were eluted at a flow rate of 2 ml/min with a linear gradient (0–60%) of acetonitrile in 0.1% aqueous trifluoroacetic acid. Areas under each peak were then integrated using Winchrom software (GBC Scientific) and in this way the percentage of peptide material remaining at any particular time was calculated.

Results

Branch synthetic peptide constructs are presented more efficiently to CD4+ T cells than linear synthetic peptide constructs when CD8+ DC are used as the APC source

CD8+ DC were used to present synthetic peptide constructs of different geometries (Fig. 1) to T cell clones. The relative efficiency of presentation of these synthetic peptide constructs by CD8+ DC was measured by the resulting proliferative response of the clones in cultures containing equimolar amounts of peptide antigen with respect to T cell epitope. The results (Fig. 2) show that there is a marked difference in the ability of CD8+ DC to present synthetic peptide-based constructs of different geometries to T cell clones. The monomeric T cell determinants, PKYVKQNTLKLA (T1) and ALNNRFQIKGVELKS (T2), when presented by DC, stimulated proliferation of clones 4.51 and 12V1 respectively. With T cell clone 12V1 (Fig. 2A) the branched peptide constructs containing either one or two copies of the B cell determinant (T2-K-B or T2-KB-KB) induced ~10-fold higher levels of T cell proliferation than the T2 monomer. With this clone the peptide, T2-B, elicited an intermediate response. A similar pattern was also observed with T cell clone 4.51 (Fig. 2B) the addition of a B cell determinant in a linear tandem arrangement to the T cell epitope (peptide T1-B) did not significantly affect the T cell response; however, when assembled in a branched configuration, with either one or two copies of the B cell determinant (T1-K-B or T1-KB-KB), the presentation efficiency was increased by ~10-fold.

Presentation of branched synthetic peptide constructs by B cells is also more efficient and demonstrates differences in presentation patterns compared to DC as an APC source

Although naive B cells do not contain BCR specific for the B cell determinant and are therefore unable to utilize the BCR for antigen uptake, they may still be expected to present less complex immunogens such as the linear synthetic peptide constructs by virtue of a direct interaction with class II MHC on the B cell surface. We therefore compared the antigen presentation of branched and linear peptide constructs with naive B cells. The results (Fig. 3) show that B cells are capable of presentation of these more complex antigens indicating that perhaps internalization of these antigens by specific interaction with the BCR is not a prerequisite for their presentation to T cells. In a manner similar to that found when DC were used as APC, B cells present the branched peptides T2-K-B and T2-KB-KB to T cell clone 12V1 at ~10-fold lower antigen concentrations than the corresponding linear peptides T2 and T2-B (Fig. 3A). The peptide constructs based on the helper determinant T1 did not show such a clear hierarchy of stimulation when presented to the corresponding T cell clone 4.51 (Fig. 3B), although there was a tendency for the branched synthetic peptide construct T1-KB-KB to effect proliferation at lower doses; the peak of the T cell stimulation to this antigen occurred at 1 pmol, a 10-fold lower dose than required to induce peak proliferation with the T1 determinant alone.

Presentation of peptides by A20 cells mimics the antigen-presentation patterns of freshly isolated splenic B cells

The data presented in Fig. 2 show that DC, at a concentration of only 50–100 cells/well, are able to induce T cell proliferation.
This raises the possibility that very low numbers of DC in the isolated B cell populations may give a false indication of the antigen presentation capability of B cells. The B cell line A20 was therefore used as a source of APC to support the data obtained with freshly isolated B cells. For these experiments, measurement of direct incorporation of thymidine was not used to measure T cell proliferation as the A20 cells continue to proliferate in culture and doses of γ-irradiation needed to suppress this compromise antigen presentation. Instead, supernatants were taken from each culture well and assessed for IL-2 content in a bioassay using the IL-2-dependent cell line HT2.

The proliferation of HT2 cells supported by the IL-2 produced by T cell clones 12V1 and 4.51 in response to peptide constructs presented by the B cell line A20 is shown in Fig. 4(A). Again greater amounts of IL-2 were produced in cultures containing branched synthetic peptide constructs when compared with linear synthetic peptide constructs. Clone 4.51 (Fig. 4B) showed little or no difference between the synthetic peptide constructs in the levels of IL-2 elicited when A20 cells were used as APC. These data parallel the results obtained when naive B cells were used as APC, and are in contrast to those observed with CD8– DC where the greatest
Branched and linear synthetic peptide constructs can be cell determinants were also better presented by B cells patterns observed when B cell populations are used to present production downstream, this appears not to be the only factor branched synthetic peptide constructs to T cell clone 4.51 lular material, which leads to concentration and deposition the B cell epitope is more ef fi

12V1 by non-fi

Fig. 5. IL-2 production by T cell clones 12V1 and 4.51 in response to the presentation of peptide constructs by paraformaldehyde-fixed A20 cells. (A) Presentation of peptides based on the T cell determinant T2 to T cell clone 12V1. (B) Presentation of peptides based on the T cell determinant T1 to T cell clone 4.51. Supernatants from cultures of T cells, A20 cells and antigen were assayed for the presence of IL-2 using a HT2 bioassay; proliferation of HT2 cells was measured by direct incorporation of $[^3H]$thyminedine. Counts are expressed as the mean of duplicate samples; subtraction of background values from these results in some cases resulted in values <0.

differences between linear and branched antigens are observed with T cell clone 4.51. These results imply that contaminating DC are not responsible for the proliferation patterns observed when B cell populations are used to present peptide constructs to T cell clones.

**Branched and linear synthetic peptide constructs can be presented by fixed A20 cells**

The data presented in Fig. 5 show that both branched and linear peptides can be presented to some extent by fixed A20 cells. This is a very inefficient process requiring nanomole rather than picomole doses of antigen to stimulate T cell responses. For presentation of branched antigens to clone 12V1 by non-fixed cells (Fig. 4A) the branched synthetic peptide construct T2-KB-KB, is the most efficient stimulator of T cells. When fixed cells are used as APC (Fig. 5A) the branched synthetic peptide construct with only one copy of the B cell epitope is more efficient.

There is little difference in the presentation of linear and branched synthetic peptide constructs to T cell clone 4.51 by non-fixed A20 cells (Fig. 4). In contrast, in Fig. 5 using fixed A20 as APC the difference observed in the hierarchy of presentation of antigens is that the branched synthetic peptide construct T1-KB-KB is less efficient.

**Branched synthetic peptide constructs are considerably more stable than linear synthetic peptide constructs containing the same peptide sequences after exposure to mouse serum**

There are many factors that can potentially influence the immunogenicity of a peptide construct in vivo amongst which is the relative stability of the immunogens to environmental proteases following injection. We decided, therefore, to investigate the stability of peptides of different geometries in the presence of mouse serum in an attempt to mimic the protease environment to which the immunogens may be exposed when inoculated into mice. This is of particular importance because the longer an antigen can persist in vivo the greater the chances of contacting the relevant immune effector cells.

Serum stability was assessed using HPLC analysis to measure the amount of intact peptide present at different times following exposure to a 10% solution of normal mouse serum (Fig. 6A). The results (Fig. 6B) show that the branched synthetic peptide constructs T1-K-B and T2-K-B were almost twice as resistant to degradation than synthetic peptide constructs containing the same amino acid sequences in a linear conformation. Almost identical results were obtained with branched peptides containing two copies of the B cell determinant (data not shown). In contrast, little or no proteolysis was observed with any of the synthetic peptide constructs following exposure to heat-inactivated FCS suggesting that they would be stable to the in vitro culture conditions used in this study.

**Discussion**

The results of this study show that the enhanced immunogenicity of branched immunogens in the production of antibody demonstrated by our previous studies (6) is paralleled by their more efficient presentation by both DC and B cells, and also by their resistance to degradation by serum proteases. While the increased susceptibility of linear peptides to breakdown in serum could result in their decreased availability for uptake by APC in vivo leading to decreased antibody production downstream, this appears not to be the only factor responsible for the superiority of branched peptides; synthetic peptide constructs with branched configurations of T and B cell determinants were also better presented by B cells and by CD8+ DC to T cells in vitro in the presence of heat-inactivated FCS, which we show has very little, if any, degradative effects on peptide stability. It is likely therefore that the unusual structure of branched immunogens contributes to the increased efficiency of antigen handling by APC and to the enhanced antibody levels observed.

DC were very potent presenters of peptide antigen to secondary T cells with T cell proliferation detected with as few as 50 DC per culture. This contrasts with the B cell system in which 10,000 cells were used as APC to achieve responses of suitable magnitude. Immature DC are capable of constitutive antigen processing through macropinocytosis of extracellular material, which leads to concentration and deposition
Fig. 6. Serum stability of branched and linear peptides. (A) Representative HPLC profile with the peak containing intact peptide (shaded) indicated in each trace. The area under the peak containing intact peptide in each sample is compared to the area under this same peak at time 0 to determine the percentage peptide remaining at various time intervals. The profile shown in (A) is for the branched peptide T2-K-B at time 0 and at the latter time point of 180 min. (B) Relative stability of linear and branched synthetic peptide constructs after exposure to either 10% NMS or 10% heat-inactivated FCS.

of antigen into peptide-loading compartments (30,31). This processing capability decreases as DC mature and the ability to present antigen increases concurrently with the expression of adhesion, co-stimulation and MHC class II molecules (32,33). The splenic DC isolated for this work are relatively mature with high levels of class II molecule expression but may nevertheless still be capable of macropinocytosis; DC isolated directly from spleen have been shown in other studies to be able to process complex antigens within the first 24 h of culture (31). It is likely then that linear and branched peptides used in this study are capable of accessing both macropinocytosis and direct binding to class II molecules on the cell surface for presentation to T cells. Although B cells are also capable of pinocytosis, this is far less efficient than the macropinocytosis of DC and it is likely that the major pathway for peptide uptake in naive B cells is via interaction with class II molecules on the cell surface (17). Perhaps trimming of the more complex structures, to allow their presentation to T cells, occurs during the recycling of these class II molecules through the early endosomes prior to re-expression at the cell surface (13–16).

The results presented here indicate that both DC and B cells exhibit preferences for branched immunogens, although some differences in peptide presentation were observed between the two APC types, e.g. the clear segregation of branched and linear peptides presented to clone 4,51 by DC was not observed with B cells. Similarly, the difference in stimulatory activity of T2-B and T2 observed with DC is not
evident when these peptides are presented by B cells. Because T cell clones were used in this study, the interaction between the class II molecules and the TCR is the same in the two APC types. Any differences observed in DC and B cell presentation of different forms of antigen must presumably be due to an intrinsic interaction between the APC and the antigen itself. The peptides generated from branched peptide constructs that are ultimately presented to T cells may well be different to those derived from linear peptides by virtue of different resistance to proteases or the utilisation of different antigen uptake mechanisms. It might be expected also that the peptides generated from the same antigen may be different for the two APC types. The fact that different processing pathways can be accessed in DC and B cells may influence the efficiency of antigen presentation exhibited by these cells and thereby explain the differences in T cell stimulation observed with these two APC types.

One possibility, consistent with our *in vitro* observations concerning the overall superior efficiency of presentation of branched peptides by DC and B cells, is the increased ability of peptides in this form to survive proteolytic environments during macropinocytosis or in the early endosomes of recycling class II molecules. This hypothesis is made more plausible considering the dramatic difference between the stability of the same peptides in branched or linear form after exposure to attack by serum proteases. The effect of geometry that protects branched peptides may well be important also for survival against deleterious intracellular protease activity.

An alternative possibility is that the peptide constructs do not require processing and simply bind to cell surface MHC class II molecules for direct presentation to T cells with branched peptides binding with higher affinity than linear peptides. As well as failing to provide an explanation for the different patterns of presentation observed with different APC, it is difficult to imagine how a bulky branched peptide bound to a class II molecule could be more efficiently recognized by the TCR because these are presumably more efficient steric inhibitors than linear peptides. Indeed relatively poor presentation of more complex branched immunogens was seen in experiments using fixed APC (Fig. 5). Despite the fact that both linear and branched peptide constructs can be presented to some extent without processing, the hierarchy of antigenicity is not the same as that observed using non-fixed APC. Compared to other synthetic peptide constructs, the antigenicity of trimeric branched peptides is markedly reduced when using fixed APC. This finding suggests that higher affinity of binding of branched peptides to cell-surface class II molecules is not the explanation for their increased ability to stimulate T cells when presented by both B cells and DC. It is therefore likely that additional processing differences which favor branched immunogens are contributing to T cell proliferation with unfixed APC. As proposed above, perhaps the stability of branched constructs in normal mouse serum is reflected in their stability to intracellular proteases during processing preventing destruction of the T cell determinant thereby enabling more efficient presentation to T cells compared to linear constructs that are readily degraded.

Whatever the mechanism, the stimulation of CD4+ T cells is clearly affected by the geometry and stability of the antigen, and also the type of APC involved in antigen uptake. The correlation of increased peptide stability and presentation efficiency of branched immunogens described here with their enhanced ability to induce antibody responses (6) demonstrates that structural aspects of an immunogen are important to consider in the design of synthetic peptide-based vaccines.

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**Abbreviations**

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<th>Abbreviation</th>
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<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
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