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Comparison of antigen presentation by lymph node cells from protein and peptide-primed mice

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SUMMARY

Lymph node cells from mice primed with peptides from the allergens *Der p* I and *Der p* II (the group I and II allergens of *Dermatophagoides pteronyssinus*) were unable to recall responses to the protein antigen when cultured *in vitro* despite being able to mount large responses to the peptides. The T cells could however recall responses to the protein when spleen-adherent cells were added into culture. Treating the spleen accessory cells with the monoclonal antibody (mAb) 33D1 and complement largely abrogated the protein response of peptide-primed T cells which indicates that dendritic cells were mainly responsible for the antigen-presenting function. If mice were primed with two injections of peptide the lymph node cells obtained could respond to both protein and peptides *in vitro* without the need for exogenous accessory cells. Using either negative depletion with the J11D mAb or positive purification, it was found that the presentation of protein antigen to lymph node T cells primed with either protein or peptide was limited to antigen-specific B cells. Peptide antigens could however be presented by both B and non-B populations. In one case the peptide 105-129 from *Der p* II which contains a T-cell epitope could not be shown to induce T-cell responses in the lymph node unless presentation was mediated by spleen-adherent or B-specific cells. These results are important for peptide-based immunomodulation and in interpreting results obtained from lymph node cultures.

INTRODUCTION

There is considerable interest in defining the influence of different types of antigen-presenting cells (APC) on the function of T cells. Dendritic cells have, for example, been identified as the cells with co-stimulatory activity for naive T cells (reviewed in ref. 1), whereas presentation by non-lymphoid cells, for example epithelial cells, and resting B cells can lead to anergy.[2-4] Additionally, it has been reported that differences in the interleukin-1 (IL-1) secretion by macrophages and B cells can selectively stimulate Th1 and Th2 clones.[5] There is, however, still an incomplete knowledge about which cell types participate in the induction and development of an immune response, and further there have only been a limited number of studies which have directly examined the functional capacity of freshly isolated cells to present antigen to T cells in *in vitro* assays. Understanding the specialized features of different APC and the roles they play in immune regulation will hopefully facilitate the design of immunotherapeutic reagents that might be able to alter the size or nature of an established immune response.

Elucidating the events occurring in standard *in vitro* assays is also of importance because many conclusions on lymphokine production and epitope usage have been based on responses of lymph node cultures. In the studies reported here it was found that lymph node T cells from mice primed with immunogenic peptides responded very poorly or not at all to the protein antigen, but showed strong responses when stimulated with the immunizing peptide *in vitro*. In one case no responses could be obtained to either the protein or the peptide after peptide immunization even though this peptide was known to contain a T-cell epitope. The difference between presentation in cultures from protein- and peptide-primed mice was therefore investigated. The results show that antigen presentation in lymph node cells from mice primed with protein is mediated by antigen-specific B cells. Peptides were presented by both B cells and non-B-adherent cells. Since priming with peptide did not necessarily induce antigen-specific B cells the T cells from these mice could not be stimulated with protein except after the addition of antigen-specific B cells or fresh spleen dendritic cells.

Abbreviations: APC, antigen-presenting cell; CFA, complete Freund's adjuvant; DC, dendritic cells; *Der p* I and *Der p* II, the group I and II allergens of *Dermatophagoides pteronyssinus*; mAb, monoclonal antibody; MLR, mixed lymphocyte reaction; NH_4Cl, ammonium chloride.

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MATERIALS AND METHODS

Mice

B10 MHC congenic and C57BL/6J mice were obtained from Animal Resource centre (Murdoch, Western Australia) at 6–8 weeks of age and were kept under specific pathogen-free conditions.

Antigens

Der p I and *Der p* II (the group I and II allergens of *Dermatophagoides pteronyssinus*) were purified by affinity chromatography from 20% spent mite medium. The anti-*Der p* I and anti-*Der p* II monoclonal antibodies (mAb) were provided by Dr M. Chapman (Charlottesville, VA). Synthetic peptides based on the *Der p* I and *Der p* II sequence were produced using a standard t-BOC chemistry program. The peptides were passed over a G10-Sephadex column (Pharmacia, Uppsala, Sweden) and the peptide fraction was further purified as a single peak by high-performance liquid chromatography (HPLC) on a preparative C8 column with a solvent system of 0·1% trifluoroacetic acid and an increasing gradient of acetonitrile. The purity of the cleaved peptides was checked using reverse-phase HPLC and the N-terminal amino acid sequence was determined.

Immunizations

Mice were immunized with 100 μg of protein emulsified in complete Freund's adjuvant (CFA). To study responses to peptide antigens mice normally received a single injection of 50 μg peptide in CFA. For secondary responses mice received two injections of 50 μg of peptide 7 days apart.

Culture media

All cells were cultured in Dulbecco's Modified Eagles medium (DMEM) supplemented with 50 mM glutamine, 50 μM 2-mercaptoethanol, and 100 μg/ml gentamycin. Lymphokine-dependent or sensitive cell lines were maintained in DMEM + 5% foetal calf serum (FCS). All T-cell assays utilizing lymph node cells were cultured in DMEM + 2% FCS.

T-cell assays

Periaortic and inguinal lymph nodes were collected at 8 days following immunization and cell suspensions made by mincing the cells through a stainless steel wire seive. Single-cell suspensions were washed and set up at 4×10^5 cell/ml in a volume of 200 μl of culture media in 96-well flat-bottom tissue culture plates 24 hr. The supernatants were collected and stored frozen at 20° until required.

Antigen-presenting cells

Spleen-adherent cells were isolated from naive mice using the method of Bland and Warren.[6] Briefly red blood cells were depleted from spleen cell suspensions using hypotonic lysis in sterile distilled water. The debris was removed by passing the cells through a sterile cotton wool column and the cells were pelleted and resuspended in DMEM + 10% FCS and cultured on a sterile plastic culture dish for a minimum of 2 hr at 37°. Non-adherent cells were removed and the surface of the dish was carefully washed twice with warm DMEM. An ice-cold solution of phosphate-buffered saline (PBS) + 5 mM EDTA was added and the cells were incubated on ice for 15 min. Adherent cells were then collected by vigorous pipetting using a wide bore pasteur pipette. These cells were > 90% class II major histocompatibility complex (MHC) positive, but were < 10% Mac-1 positive and < 5% positive for surface immunoglobulin (Ig). Lymph node B cells from naive or protein-immunized mice were obtained by collecting the periaortic and inguinal lymph nodes and preparing cell suspensions. Cells were cultured initially for 3 hr at 37° and the non-adherent cell population was removed. The cells were washed and cultured overnight in the presence of 20 U/ml recombinant IL-4 so as to enhance class II MHC expression on such cells. The next day cells were layered onto a discontinuous Percoll gradient and the B-cell fraction collected from the 40–50% interface. These cells were then incubated sequentially with anti-Thy-1 and anti-33D1 mAb and treated with baby rabbit complement to remove contaminating T cells and dendritic cells. The cells remaining displayed more than 90% staining with surface Ig and class II MHC.

Complement depletion of cells

The 33D1 mAb recognizes a cell surface marker expressed predominantly on spleen dendritic cells.[7] To selectively remove 33D1$^+$ cells from spleen-adherent cell preparations, the cells were incubated with a 1/10 dilution of 33D1 mAb on ice for 1 hr. Afterwards they were washed twice and resuspended in DMEM containing a 1/10 dilution of baby rabbit serum as a source of complement and incubated at 37° for 45 min. The remaining cells were washed three times and used in antigen presentation assays. Comparisons were made with APC incubated in complement alone or in the presence of an irrelevant mAb. The J11D mAb is specific for a marker expressed predominantly on immature thymocytes, as well as B cells and interdigitating cells of the lymph node.[8,9] Therefore to deplete J11D expressing cells from the lymph node, cells were incubated with the mAb on ice for 1 hr and washed twice. Lysis was performed at 37° using baby rabbit complement. The remaining cells were washed three times before use in culture. The 33D1 mAb was supplied by Dr P. G. Holt (W.A. Research Institute for Child Health, Perth, Australia) and the J11D hybridoma was supplied by Dr A. Scalzo (Dept. of Microbiology, University of Western Australia, Australia).

Purification of antigen-specific or peptide-specific T cells

Mice were killed 8 days after immunization and cell suspensions were made from a pool of periaortic and inguinal lymph nodes. T cells were enriched by incubating the lymph node cells on a nylon wool column for 2 hr at 37° and then culturing the T cells on a plastic culture dish for a further 1 hr at 37° to remove any contaminating accessory cells not retained on the column. The cells isolated by this method demonstrated > 95% staining with anti-Thy-1 mAb and < 5% staining for surface Ig or with the MAC-1 mAb.

Antigen presentation assays

Unless otherwise stated, cultures containing adherent cells 4×10^5 T cells were cultured with 8×10^4 APC. In comparison, when B cells were assessed for their APC activity, 2×10^5 B cells were cultured with 2×10^5 T cells.

Lymphokine assays

Supernatants were normally assessed for the release of IL-3/granulocyte–macrophage colony-stimulating factor (GM-CSF) using the lymphokine-dependent cell line FDC-P1.[10] These cells

Table 1. Lymph node cells from peptide-immunized mice respond poorly to proteins *in vitro*

In vitro antigen	Antigen used for immunizing				
	Der p I	p110-131	*Der p* II	p78-104	p105-129
Protein (µg/ml)					
20	101,598 ± 12,113*	8014 ± 2345	116,676 ± 18,316	2458 ± 519	2888 ± 845
2	81,997 ± 7301	464 ± 434	63,211 ± 5736	1837 ± 782	2698 ± 55
0·2	17,380 ± 3298	301 ± 102	21,639 ± 3134	1374 ± 1003	1485 ± 711
Peptide (µM)	42,104 ± 3704	37,712 ± 5252	83,712 ± 7518	54,931 ± 6780	3498 ± 501
0·1	8837 ± 2176	11,317 ± 1330	27,815 ± 3177	28,952 ± 3185	2911 ± 829
0·01	874 ± 219	11,317 ± 1330	3205 ± 1001	151 ± 723	3852 ± 889
0	799 ± 225	768 ± 218	1014 ± 219	959 ± 320	1777 ± 354

C57BL/6J mice were immunized subcutaneously with either 100 µg of native *Der p* I or *Der p* II allergen or 50 µg of peptide in CFA. Lymph node cells were collected 8 days later and cultured *in vitro* with either native allergen or peptide. The 24-hr supernatants were collected and assayed for IL-3/GM-CSF using the FDC-P1 cell line.

*The data shown represent the mean response of triplicate cultures ± SD.

grow maximally in IL-3 and GM-CSF but only poorly in interferon-γ (IFN-γ) or IL-4. Briefly 2×10^3 FDC-P1 cells were incubated in (1/2 dilution) 50 µl of culture supernatant in DMEM + 5% FCS for 48 hr at 37°. Cells were pulsed with 1 µCi of [^{3}H]thymidine ([^{3}H]Tdr) for a further 6 hr at 37° and the amount of label incorporated was assessed by liquid scintillation spectrometry. A culture supernatant of the WEHI 3D$^-$ cell line was used as a source of IL-3 for standards in these assays. The FDC-P1 and WEHI 3BD$^-$ cell lines was supplied by Dr A. Kelso (Walter and Eliza Hall Institute for Medical Research, Melbourne, Australia).

RESULTS

Response of lymph node cells from peptide-primed mice

It has previously been reported that lymph node cells from mice immunized with antigenic peptides from either the *Der p* II allergen or ovalbumin, ($pOVA_{323-339}$) either failed to or responded very poorly to protein antigen *in vitro* but in contrast showed strong responses when stimulated with peptide.[11] This was initially found for all three peptide epitopes of *Der p* II in H-2^b mice and also with two epitopes for H-2^k mice.[11] This is shown here for the response of C57BL/6J mice immunized with either native *Der p* I or *Der p* II proteins or immunogenic peptides derived from these two allergens. Using our standard assay for measuring T-cell activation, the release of IL-3/GM-CSF, it was found that lymph node cells from protein-primed mice mounted strong responses when challenged with protein or peptide *in vitro*. In comparison the lymph node cells from C57BL/6J mice primed with the immunogenic synthetic peptides p110–131 of *Der p* I, or the *Der p* II-derived peptide p78–104 showed virtually no response when stimulated with the relevant protein *in vitro*, but these same cells showed large responses when presented with the immunizing peptide (Table 1). The same phenomenon was found when IL-2 and IFN-γ were measured (not shown). Also, shown in Table 1 is the lymph node response normally obtained from mice primed with the C-terminal peptide of *Der p* II, p105–129. This peptide has previously been determined to contain a T-

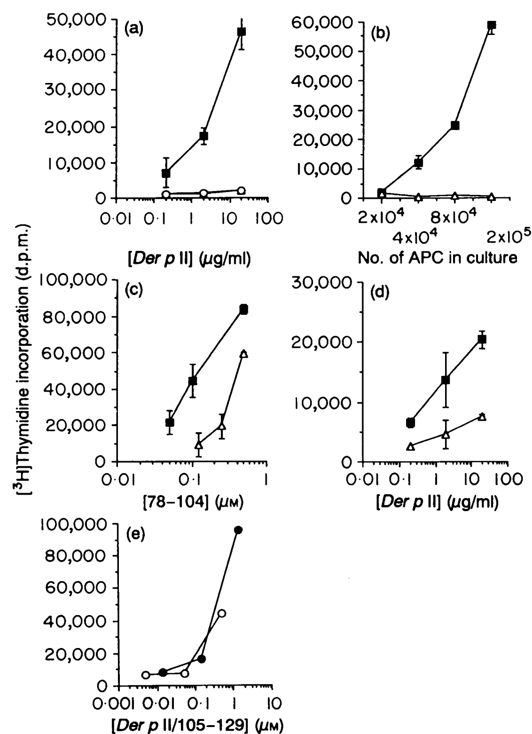

Figure 1. Splenic adherent cells present *Der p* II and peptide 105-129 to peptide-primed T cells. (a–d) B10.BR mice were immunized with the *Der p* II peptide 78-104 and IL-3/GM-CSF responses were measured (a) after presentation of *Der p* II with spleen-adherent cells (■) compared to lymph node adherent cells (○); (b) after presentation of *Der p* II (20 µg/ml/well) with graded doses of splenic (■) or lymph node (△) adherent cells; (c) after presentation of peptide 78-104 by spleen (■) or lymph node (△), and (d) stimulation by *Der p* II with spleen-adherent cells treated with 33D1 (△) or complement (■) alone. (e) B10 mice were immunized with either p105-129 (○) or *Der p* II (●) and the IL-3/GM-CSF response was measured after stimulation of peptide-primed T cells with splenic adherent cells. T cells for these experiments were purified according to the methods and did not respond to antigen without the addition of APC.

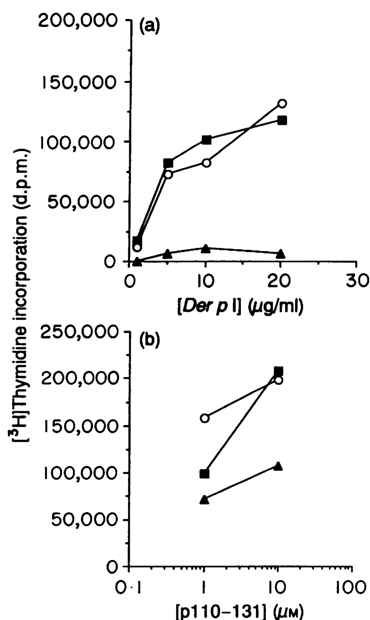

Figure 2. B cells present protein antigens to primed T cells in the murine lymph node. C57BL/6J mice were immunized with 100 μg of *Der p* I and 8 days later the draining lymph node cells were collected. Cell suspensions were treated with either medium (■), J11D mAb (▲) or 33D1 mAb (○) for 1 hr on ice and then washed and cultured with baby rabbit complement for 45 min at 37°. All cells were washed and cultured in the presence of (a) *Der p* I or (b) p110-131. The data represent the mean response of three individual wells and this experiment has been repeated twice with similar results. Background responses were <700 d.p.m.

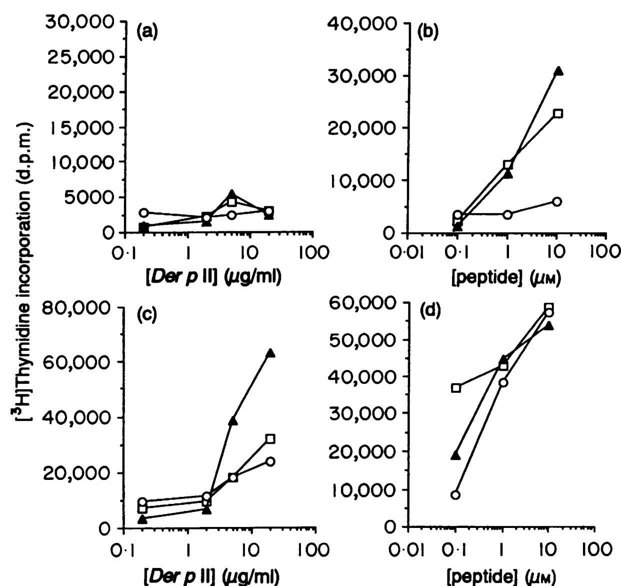

Figure 3. Immunizing mice twice with peptides enables lymph node T cells to respond to protein *in vitro*. C57BL/6J mice were immunized either once (a, b) or twice (c, d) with p11-35 (□), p78-104 (▲) or p105-129 (○). The draining lymph node cells were collected 8 days later and were cultured *in vitro* with either native *Der p* II (a, c) or peptide (b, d). The data shown are representative of one of three experiments performed. Data represent the mean response of three individual mice, background responses were <900 d.p.m.

Table 2. Immune B cells present protein antigens to lymph node T cells

	T cells primed with	
	Der p II	p78-104
+*Der p* II immune B cells		
Der p II	63,044 ± 5124*	30,356 ± 6529
p78-104	76,674 ± 8139	49,356 ± 7210
+CFA immune B cells		
Der p II	591 ± 278	1031 ± 322
p78-104	62,892 ± 5119	37,200 ± 4371

Lymph node T cells were collected from C57BL/6J mice immunized with either 100 μg of *Der p* II or 50 μg of p78-104. T cells were purified over a nylon wool column and were cultured with either *Der p* II immune B cells or CFA immune B cells with the addition of native allergen or peptide. Immune B cells were prepared as described in Materials and Methods.

* The data shown represent the mean response of triplicate wells (d.p.m. ± SD). *Der p* II was added into culture at 20 μg/ml and the p78-104 was added at 10 μM. Background responses were <1000 d.p.m. For simplicity only peak responses from the antigen titrations are presented.

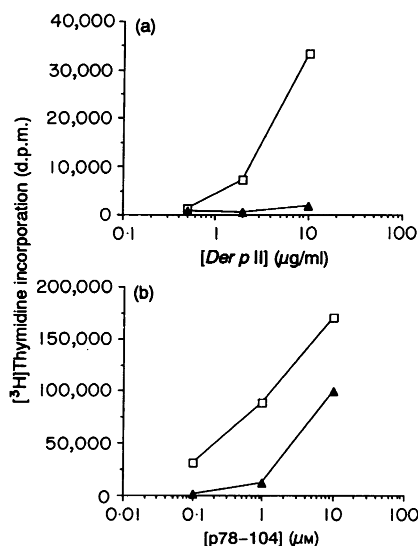

Figure 4. B cells are required for processing exogenous antigen in the lymph node of peptide-primed mice. C57BL/6J mice were immunized twice with p78-104. The lymph node cells were collected and incubated with a 1/10 dilution of J11D mAb (▲) or medium alone (□). The cells were then washed and treated with rabbit complement and set up in culture with either (a) *Der p* II or (b) p78-104. The supernatants were assayed for the presence of IL-3/GM-CSF and the data represent the mean response of triplicate cultures. These experiments have been repeated twice with equivalent results.

cell epitope[11] but following a single injection of this peptide the lymph node cells obtained could not be stimulated with either the protein or the peptide (Table 1).

Stimulation of lymph node T cells from peptide-primed mice with spleen DC

The initial findings in Table 1 suggested the possibility that immunization with peptide preferentially stimulated T cells which could not react to epitopes on the native antigen that were naturally presented by APC. This view was dispelled, however, when it was found that spleen-adherent cells could efficiently process and present the protein antigen to purified peptide-primed T cells. This is shown here for B10.BR mice immunized with the *Der p* II peptide 78-104 in Fig. 1a where lymph node cultures alone gave no response to the *Der p* II protein but had large responses on the addition of spleen-adherent cells. Increasing the dose of spleen-adherent cells in culture from 2×10^4 to 2×10^5 APC stimulated strong T-cell responses to the *Der p* II allergen *in vitro*, whereas in the same experiment lymph node-adherent cells were unable to stimulate T-cell respones to the protein at any dose (Fig. 1b). These lymph node-adherent cells could, however, present peptide efficiently although the spleen adherent cells required slightly ($< \times 5$) less peptide to achieve the same level of responses (Fig. 1c). The cells responsible for the presentation or stimulation in the spleen cells appeared to be a dendritic cells because treatment with 33D1 mAb and complement markedly reduced the activity below that of controls (Fig. 1d). As well, spleen-adherent cells could present both the peptide and protein to purified lymph node T cells collected from mice primed with p105–129 (Fig. 1e) which demonstrates that a single injection of this peptide does lead to T-cell sensitization in the draining lymph node but it cannot be demonstrated by lymph node cultures alone.

Evidence for B-cell presentation in protein-primed mice

When mice were immunized with protein the lymph node cells were able to recall responses *in vitro* to both protein and peptide but the preceding experiments clearly showed that the adherent cell population of the lymph node were unable to present protein antigens to peptide primed T cells. This therefore posed the question of which cells present protein in the lymph node cultures of protein-primed mice. This was examined firstly using the mAb J11D which primarily has specificity for B cells. Treating lymph node suspensions from *Der p* I-immunized mice with the J11D mAb and complement could totally abrogate the responses of the lymph node cells to the protein whereas treating the same cell suspensions with either the mAb 33D1 and complement or complement alone as a control had no effect (Fig. 2). The response to peptide was, however, only partially depleted. The ability of B cells to present protein and peptide to primed T cells was examined directly. B cells from the lymph node of mice immunized with *Der p* II in CFA or CFA alone were enriched by removing adherent cells on plastic and culturing the remaining cells overnight with IL-4. The next day cells were treated with anti-Thy-1 mAb and complement and the B cells were isolated on a discontinuous Percoll gradient. When these cells were added into culture with purified T cells the peptide 78-104 could be presented by both immune and non-immune B cells but the protein could only be presented by *Der p* II immune cells. Both peptide and protein-primed T cells responded to immune B cells in this way (Table 2).

Secondary response of mice to peptides

To examine further the role of B cells in the lymph nodes of mice, C57BL/6J mice were given either a single injection of peptide or two injections 1 week apart and the lymph node responses were measured after a further 10 days. As seen in Fig. 3 (a and b) cells from mice primed once did not respond to the protein but responded well to peptide. Lymph node cells taken after the second injection did in contrast respond to both protein and peptide (Fig. 3) for all the *Der p* II peptides tested including p105-129. To determine if immune B cells were indeed playing an active role in the lymph node that enabled the presentation of protein in mice that had received two injections, the responses to protein as illustrated for p78-104 were totally abrogated by treatment with the anti-B-cell monoclonal J11D and complement (Fig. 4). Again the response to peptide was only partially affected.

DISCUSSION

The studies described here began after it was discovered that lymph node cells from mice primed with immunogenic peptides, failed to respond to proteins using a standard *in vitro* culture assay. Since they could, however, respond very well to the peptide that been used for immunizing it seemed probable that this phenomenon could be of some importance especially since peptide-based strategies are being evaluated for their potential to modulate or divert ongoing responses to allergens. The basis of the non-responsiveness after peptide priming appeared to be at the level of the APC since it was found that T cells within the lymph nodes of peptide-primed mice could readily respond to proteins following the addition of splenic adherent cells to the cultures. Judging by the high sensitivity to 33D1 mAb the major APC activity of these spleen cell preparations could be attributed to the presence of dendritic cells. Of interest is the C-terminal *Der p* II peptide 105-129, which when used for immunization, induced sensitized T cells but they could not respond even to the peptide *in vitro* without the addition of splenic adherent cells. There is now evidence that this peptide requires processing because its presentation to peptide-primed T cells by spleen APC is sensitive to NH_4Cl treatment which is known for its ability to neutralize lysosome function. Thus in this sense the peptide, which is not necessarily a minimal epitope, is treated in the same way as if it were a protein.

Since cells from mice immunized with peptide are sensitized to respond to proteins, these studies highlight an important distinction which must exist between the presentation of antigen by lymph node APC during *in vitro* culture and that which occurs during *in situ* sensitization. The *in vitro* lymph node assay used here is a standard procedure for monitoring immune responses and the normal readout for T-cell activation can be either proliferation or lymphokine release. The results of such studies can be too easily extrapolated to *in vivo* events and lead to misunderstandings. It has, for example, been reported using lymph node cultures that HEL contains cryptic epitopes which respond to the peptide but do not respond to protein.[12] Results of the authors[11] show that the house dust mite allergens *Der p* I

and *Der p* II also have cryptic epitopes which are not usually recognized in immune responses to the protein allergen. Lymph node cells from mice primed with peptides containing these epitopes did however respond to the whole protein when spleen-adherent cells were present[11] demonstrating the ability of these peptides to prime for anti-protein T-cell responses *in situ*.

In this study it is shown that although the lymph node-adherent cell population and B cells can present peptide, the only cells which were capable of presenting protein to peptide-primed T cells were antigen-specific B cells. This was determined with depletion analysis using the J11D antibody which has a primary specificity for B cells and by positive purification and testing of B cells from mice immunized with *Der p* II or CFA alone. Cells from mice primed twice with peptide showed large responses to protein *in vitro* and experiments were performed to show that the APC activity resided in the $J11D^+$ population, i.e. B cells. The experiments performed here do not actually distinguish between the requirement for the second injection and the need to allow time for the B-cell response to develop, but do make the point that even for peptide immunization presentation of protein when it occurs is via the B cells. Although the antigen-presenting activity of B cells for soluble antigen is universally recognized the inactivity of the other cells in the lymph node has not been appreciated. It has, however, been previously reported by Janeway *et al.*[13] and by Malynn and Wortis[14] who demonstrated that the antigen-presenting activity in the murine lymph node resided only with antigen-specific B cells. The authors' results show that although the non-B-adherent cells do not present whole protein antigen they can present peptides bearing T-cell epitopes, as long as they do not require processing.

At this stage it appears that for sensitization to occur in the lymph nodes antigen will be endocytosed and processed by the epithelial Langerhans' cells at a peripheral site and these cells then receive a signal to migrate to the regional lymph node where they enter the afferent lymph as dendritic or veiled cells and thus eventually localize themselves to the paracortical region of the lymph node where they are able to present antigen to T cells.[15] Furthermore studies using contact sensitizers have found antigen-bearing dendritic ells (DC) in the draining lymph nodes.[15-18] These lymph node DC usually have up-regulated class II MHC expression[18] and are thought to be equivalent to a mature form of DC. It is shown here that the non-B-adherent cells within the node cannot process or present protein antigen but can present peptide. This sequence of events resembles that found for epithelial Langerhans' cells which are initially endocytic when freshly isolated but are poor at presentation as measured by their ability to stimulate a mixed lymphocyte reaction (MLR).[19-22] After short-term culture though these cells become potent stimulators of the MLR which is thought to reflect an up-regulation of their capacity to present foreign antigen, but this functional change coincides with a loss of endocytic vesicles from the cytoplasm of these cells and they are no longer able to process exogenous antigen.[20-24] Several studies have shown that DC are likely to be involved in stimulating primary T-cell responses.[25,26] Earlier studies suggested B cells might also play an important role in priming T cells in the lymph node[13,27,28] but this seems unlikely in light of the recent findings by Sunshine *et al.*[29] who found that lymph node cells from *scid* mice repopulated with T cells could mount a cell-mediated immune response following antigenic challenge with protein. Therefore this latter result strongly suggests that B cells are not essential for T-cell priming, but rather as previously proposed by Ron and Sprent[28] immune B cells might play a more important role in regulating the clonal expansion of T cells within the node and when required they may also be able to function as APC. The participation of antigen-specific B cells, which can present protein, in secondary responses as shown here could have important influences on the maturation of responses. In conclusion these studies have provided some insight into the nature and measurement of peptide-induced immune responses and hence will be important for the design of experiments that will aim to modulate specifically the activity of antigen-reactive T cells.

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