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Inhibition of T-cell responses by feeding peptides containing major and cryptic epitopes: studies with the *Der p* I allergen

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SUMMARY

H-2^b mice respond to the 222 residue allergen *Der p* I by producing T cells sensitized to the dominant epitopes encompassed in peptides 21–49, 78–100, 110–131 and 197–212. Immunization with the synthetic peptides 120–143 and 144–169, however, revealed cryptic epitopes which could sensitize T cells for responses to the respective peptides and, providing splenic adherent cells were added to lymph node cultures, to the whole allergen. It is shown that feeding recombinant fusion peptides can markedly inhibit the ability of the whole antigen to immunize mice, as measured by the *in vitro* interleukin-2 (IL-2) and granulocyte–macrophage colony-stimulating factor (GM-CSF)/IL-3 release on stimulation with protein or peptides, although inhibition measured by IL-2 release was more marked. The inhibition extended to epitopes other than those in the fusion peptides used for feeding. Thus feeding peptide 101–154 inhibited responses to 110–131 and 78–100. Fusion peptides 1–14 and 188–222 did not inhibit responses, although 188–222 did contain an epitope. Inhibition was also obtained when mice were fed a fusion containing the cryptic epitope 144–169. The ability of peptides containing the cryptic epitopes to inhibit responses has significant implications for peptide-based immunotherapy.

INTRODUCTION

Feeding antigens has been a classical method for inducing immunological unresponsiveness.[1–6] It has the hallmarks of being an active procedure, since unresponsiveness can be transferred to naive recipients by T cells.[1,7–9] Furthermore we have shown that although BALB/c mice fed ovalbumin (OVA) become profoundly tolerant to a subcutaneous injection of OVA in complete Freund's adjuvant (CFA), they nevertheless remain highly sensitized in that T cells from their mesenteric lymph nodes (MLN), spleen and Peyer's patches (PP) release large amounts of interferon-γ (IFN-γ) and granulocyte–macrophage colony-stimulating factor (GM-CSF) on *in vitro* challenge.[10] Although an important physiological response to dietary antigens,[11] oral tolerance could be used to control aberrant immunological responses such as those found in autoimmune disease[12] and allergy. The most extensively studied model system for autoimmune disease has been that of experimental allergic encephalomyelitis (EAE). It has been shown that rats fed a tolerizing dose of myelin basic protein (MBP) prior to sensitization can be protected from an encephalitogenic challenge with MBP[13–15] but there have been conflicting views as to the mechanisms involved in inducing tolerance. Whitacre *et al.*[14] were unable to transfer suppression with T cells from tolerized animals, but showed that clonal anergy may be an important mechanism for down-regulating the effector function of $CD4^+$ T cells, whereas Miller *et al.*[13] could transfer suppression using $CD8^+$ T cells. These suppressor (Ts) cells were able to release a cytokine,[13] later identified as transforming growth factor-β (TGF-β),[15] which inhibited an *in vitro* response of a MBP-specific $CD4^+$ T-cell line and also mediated bystander suppression of unrelated T cells.[13] The authors did not investigate a possible contribution by clonal anergy. The active component of oral tolerance, however, suggests it may be possible to use peptides containing epitopes to induce unresponsiveness. Because of our interests with house dust mite allergy we have examined this using peptides made from cDNA encoding the major allergen *Der p* I expressed as fusions with glutathione S-transferase in pGEX vectors. The results show that not only can peptides containing major epitopes induce oral tolerance to the whole allergen, but it is also possible to induce tolerance with other peptides possibly via cryptic epitopes.

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MATERIALS AND METHODS

Animals

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

Antigens

The house dust mite allergen *Der p* I was affinity purified from spent mite medium (SMM)[16] using monoclonal antibody from Dr M. D. Chapman. OVA crystalline Grade V was purchased from the Sigma Chemical Company (St Louis, MO). Overlapping synthetic peptides derived from the *Der p* I sequence were synthesized using standard t-BOC chemistry, and peptides were purified by reverse-phase high pressure liquid chromatography (HPLC) and the sequence of individual peptides checked to verify identity. The peptides used in this study included: 1–20, 13–39, 21–49, 40–60, 50–71, 61–84, 78–100, 85–109, 101–119, 110–131, 120–143, 132–157, 144–169, 158–180, 170–191, 181–204, 197–212 and 197–222.

Preparation of recombinant proteins and peptides

Complementary DNA inserts encoding either the whole *Der p* I or *Der p* II protein or fragments of *Der p* I were ligated to the pGEX vector, which expresses the fusion proteins with glutathione S-transferase in *Escherichia coli*.[17] The procedures for the cloning and expression of the fragments have been described elsewhere.[18,19] Log phase *E. coli* cells transformed with pGEX-based constructs were induced by adding 0·1 mM isopropylthiogalactosidase (IPTG; Promega, Sydney, Australia). Because large quantities of fusion peptides were required they were prepared from solubilized inclusion bodies. Bacterial pellets were resuspended in Tris-buffered saline with 1 mM EDTA and homogenized by a Braun MSK Homogenizer with 0.1 mm glass beads (Braun, Melsungen, Germany) for 5 min. The lysate was removed after centrifugation (10 000 *g*, 10 min) and the pellet was washed twice with 1·75 M guanidine HCl containing 1 M NaCl and 1% Triton X-100. It was then dissolved by incubating in 8 M urea with 50 mM NaCl and 1 mM EDTA and 2% 2-mercaptoethanol (2-ME). The sample was dialysed in 3-(cyclohexylamino)-propanesulphonic acid (CAPS) buffer, pH 10·7, and the pH was slowly adjusted to pH 9·6. The recombinant material was clarified by centrifugation at 10 000 *g* and the concentration of the soluble material was estimated against standard quantities of bovine serum albumin (BSA) using SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and staining with Coomassie blue. Peptides used in this study included GEX *Der p* I (1–222), GEX *Der p* II (1–129), GEX p1–14, GEX p60–111, GEX p98–140, GEX p101–154, GEX p57–130 and GEX p188–222.

Induction of oral tolerance

Mice were lightly anaesthetized under ether and fed intragastrically by a tube with 3 mg of protein or peptide on 3 consecutive days. Antigens were dissolved in CAPS buffer, pH 9·6, and administered in a volume of 0·2 ml. Mice were immunized subcutaneously at the base of tail 7 days after the last feed with 100 μg of native protein emulsified in CFA (Difco, Detroit, MI) in a volume of 0·2 ml.

Culture medium

Lymph node cells were cultured in Dulbecco's minimal essential medium (DME) (Gibco Laboratories, Grand Island, NY) supplemented with 2% fetal calf serum [FCS; Commonwealth Serum Laboratories (CSL), Parkville, Victoria, Australia], 50 μM, 2-ME (Sigma Chemical Company), 2 mM L-glutamine (CSL) and 20 μg/ml gentamycin (David Bull Laboratories, Mulgrave, Victoria, Australia). FDC-P1 cells were maintained in DME + 5% FCS, while CTLL-2 cells were maintained in RPMI (Gibco Laboratories) + 10% FCS.

T-cell assays

The periaortic and inguinal lymph nodes were expressed through a stainless steel wire mesh, washed and cultured at 4×10^5 cells in a volume of 0·2 ml in culture medium in a 96-well flat-bottomed tissue culture plate (Becton Dickinson Labware, Lincoln Park, NJ). Protein or peptide antigens were added at various concentrations and the cells were incubated at 37° for 24 hr. Supernatants were collected and stored at −20° until required for assay. To measure responses after immunization with peptides, 4×10^5 nylon wool-purified T cells were cultured with 8×10^4 spleen adherent cells in the presence of protein or peptide antigens, and 24 hr supernatants were assayed for lymphokines. Spleen-adherent cells were collected from normal mice.[10] Although purified T cells were used in these studies this step is not necessary.

Lymphokine assays

Assays were performed as described elsewhere.[10,20] FDC-P1 cell proliferate maximally in response to interleukin-3 (IL-3) and GM-CSF and submaximally to IFN-γ or IL-4. 2×10^3 cells were added in 50 μl DME + 5% FCS to 50 μl of culture supernatant in 96-well flat-bottomed tissue culture plates. The cells were incubated for 40 hr at 37° and then pulsed with 1 μCi[^{3}H]thymidine ([^{3}H]TdR) (Amersham Australia, North Ryde, Sydney, Australia) for another 4–6 hr at 37°. The cells were then harvested onto glass fibre filter mats and samples counted for [^{3}H]thymidine incorporation, as assessed using liquid scintillation spectrometry or, for later experiments due to its acquisition, on a Packard Matrix 9600 direct β counter (Packard Instruments, Meriden, CT). This latter counter gives five times less counts than liquid scintillation counting but has correspondingly lower backgrounds. This machine was used because it offers considerable advantages in sample processing.

The CTLL-2 cell line proliferates maximally with IL-2 but only poorly in the presence of IL-4. Supernatants were cultured with 5×10^3 CTLL-2 cells/well for 24 hr at 37° and pulsed with 1 μCi of [^{3}H]TdR. Cells were harvested onto glass fibre filter mats and the radioactivity incorporated determined.

No IL-4 could be detected in supernatants of cultures from mice immunized with *Der p* I in CFA, as described by the CT4S cell assay which gives 50 000 c.p.m. with recombinant IL-4 in our assays.

Statistical analysis

Differences between the lymphokine responses of control and experimental groups were examined for significance using the Mann–Whitney *U*-test or Student's *t*-test.

RESULTS

Epitopes of *Der p* I

Preliminary experiments showed that H-2^b mice are high responders to *Der p* I, while H-2^k, H-2^d and H-2^q mice are low responders. To determine the location of T-cell epitopes, B10 mice were immunized subcutaneously with *Der p* I in CFA and after 8 days the periaortic and inguinal lymph nodes were examined for antigen-specific lymphokine release (IL-3/GM-CSF) using a panel of overlapping peptides. In three

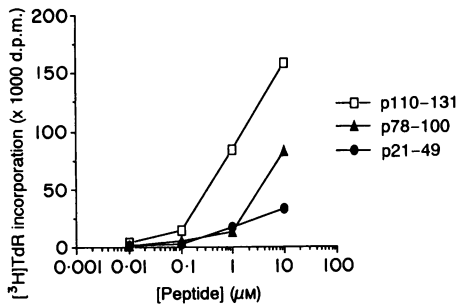

Figure 1. T-cell epitopes of *Der p* I. B10 mice were immunized with 100 μg of *Der p* I and 8 days later lymph node cells were cultured *in vitro* with the panel of 17 overlapping synthetic peptides. Twenty-four hour supernatants were assayed for IL-3/GM-CSF. The data are from a single representative experiment and only show the response to three of the four peptides which gave significant stimulation. This experiment has been performed on three occasions with equivalent results.

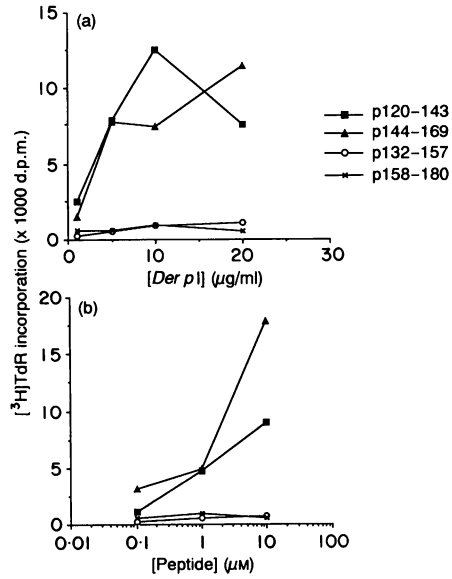

Figure 2. Identification of cryptic epitopes on *Der p* I. Groups of three C57BL/6J mice were immunized with 50 μg of peptides in CFA and 8 days later nylon wool-purified lymph node T cells were cultured with spleen adherent cells and either (a) protein or (b) peptide for 24 hr. The peptides 120–143 and 144–169 were able to induce responses, shown here in comparison to two representative negative peptides. The results show the mean IL-3/GM-CSF response to triplicate samples.

experiments the largest response was found to peptide 110–131, while lower responses were seen to peptides 197–212, 78–100 and 21–49. No other peptides stimulated a response significantly above the no antigen control, usually of 1000 c.p.m., similar to that described in more detail for *Der p* II.[21] An example of the stimulations produced in one experiment is shown in Fig. 1. The response of the cells to the peptide 110–131 was of about the same magnitude as the response to the whole protein (see Fig. 3 or ref. 16). We have previously described a cryptic epitope on *Der p* II[21] and so to test for cryptic epitopes on *Der p* I, mice were immunized with all the peptides individually and responses to *Der p* I and the immunizing peptide were measured in the presence of spleen adherent cells. The splenic adherent cells were added because it has been shown that lymph nodes from mice immunized with peptides can be poor at presenting protein in *in vitro* cultures. Peptides 120–143 and 144–169 were found to be able to sensitize mice so that they could recall responses to both the intact protein and the peptides, respectively (Fig. 2), even though they did not stimulate T cells from mice immunized with the whole *Der p* I allergen. The data obtained here with the mapping studies do not define minimal epitopes and hence finer mapping could be useful.

Oral tolerance induced by fusion peptides

A number of the recombinant fusion peptides chosen on the basis of the T-cell epitope data above were used for experiments on oral tolerance. Recombinant peptides GEX p1–14 did not contain any T-cell epitopes. GEX p57–130 contained two epitopes, while GEX p101–154, GEX p188–222 and GEX p98–140 contained the single epitope (110–131). GEX p131–187 contained cryptic epitopes.

Following a previously characterized regime for inducing oral tolerance,[10] mice were fed 3 mg of fusion peptide on 3 consecutive days and after a further 7 days were immunized subcutaneously with natural *Der p* I protein in CFA. Seven days later the periaortic and inguinal lymph nodes were harvested and stimulated with either protein or synthetic peptides and tested for lymphokine production. Experiments were performed which showed that feeding CAPS buffer or the recombinant GEX *Der p* I (1–222) fusion protein did not affect the IL-2 or IL-3/GM-CSF responses of mice to subcutaneous injection of OVA in CFA (data not shown). To test whether orally administered peptides could induce specific tolerance, mice were fed either CAPS buffer, GEX *Der p* I (1–222) or the fusion peptides GEX p57–130 or GEX p101–154 and the response to immunization with natural *Der p* I was determined. Mice from the group fed CAPS buffer showed strong responses to the *Der p* I protein *in vitro*, with their cells secreting both IL-3/GM-CSF and IL-2. On the other hand, mice fed GEX *Der p* I (1–222) or either GEX p57–130 or GEX p101–154

Table 1. Tolerance induced by one epitope reduces responses to others

Exp.	Fusion peptide fed	IL-2 response to p110–131	IL-2 response to p78–100
1	GEX *Der p* II	10·0 (3·2)	2·2 (0·8)
	GEX p101–154	0·9 (0·5)	0·6 (0·6)
	GEX p98–140	2·6 (2·6)	0·2 (0·3)
2	GEX *Der p* II	24·0 (2·9)	7·0 (3·5)
	GEX p131–187	8·5 (2·8)	2·2 (1·0)
	GEX p188–222	22·0 (7·2)	6·3 (3·0)

The results show mean (SD) of maximum IL-2 responses $\times 10^{-3}$ of cells from groups of three to five mice (usually occurring at 10 μM). Responses of the cells from mice fed GEX p101–154, p98–140 and p131–187 were significantly different from those of mice fed GEX *Der p* II and GEX p188–222 ($P < 0.05$ or less).

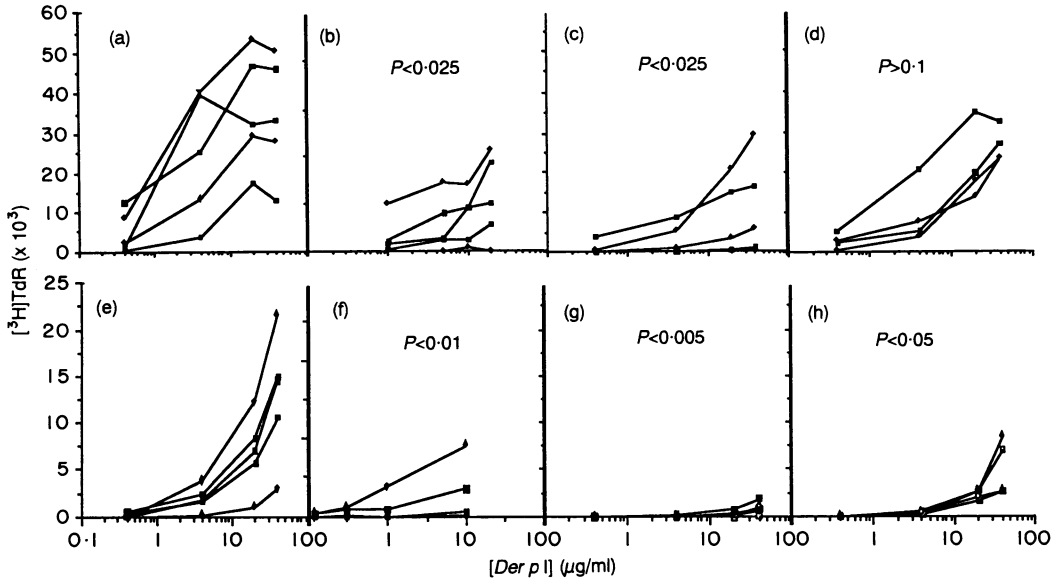

Figure 3. Feeding peptides induces oral tolerance. C57BL/6J mice (five per group) were either (a,e) fed CAPS buffer or 3 mg of either (b,f) GEX p57–130, (c,g) GEX p101–154, or (d,h) GEX *Der p* I (1–222) on 3 consecutive days and 1 week later immunized with *Der p* I in CFA. Seven days later draining lymph node cells were cultured *in vitro* with *Der p* I for 24 hr and the supernatants assayed for IL-3/GM-CSF (a–d) or IL-2 (e–h). The data show the response of individual mice in each group with the statistical significance shown in the panel.

displayed depressed responses, especially IL-2 responses (Fig. 3), although inhibition was found for IL-3/GM-CSF release. The more pronounced inhibition of IL-2 responses was a consistent feature of all subsequent experiments. To test for the number of feeds required to produce inhibition, mice were fed on 1, 2 or 3 consecutive days and immunized after 7 days. Inhibition was not found with one feed but was clearly evident after two (Fig. 4).

We were interested to see which fusion peptides could influence the immune response. Feeding GEX p131–187, which contains the cryptic epitopes found on peptides 144–169 and 120–143, could produce inhibition compared to control mice fed CAPS buffer (Fig 5). Feeding the fusion peptide GEX p188–222, which does have an epitope (197–222), however, did not produce suppression in the standard regime, and nor did feeding the fusion GEX p1–14, which does not have an epitope (Fig. 6).

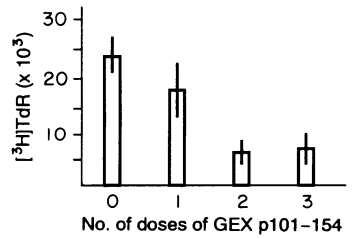

Figure 4. Number of feeds to induce oral tolerance. Groups of four to five C57BL/6J mice were fed 1, 2 or 3 doses of GEX p101–154 and 7 days after the last feed immunized with *Der p* I in CFA. The results show the mean (standard deviation) of the IL-2 release of *in vitro* stimulation with *Der p* I performed 7 days after immunization.

To examine how the development of oral tolerance affected responses to T-cell epitopes, mice were fed fusion peptides and immunized with the whole *Der p* I and responses to peptides measured. As shown for feeding GEX p101–154 (Fig. 7), marked inhibition of the response measured by *in vitro* stimulation with synthetic peptide p110–131 could be obtained at all doses. To measure the response to other epitopes, experiments were conducted in which mice were fed either GEX p101–154, GEX p98–140, GEX p131–187, GEX p188–222 or GEX *Der p* II. After immunization with *Der p* I responses were measured to the peptides *in vitro*. As seen in Table 1, feeding mice either GEX p188–222 or GEX *Der p* II did not affect the ability of

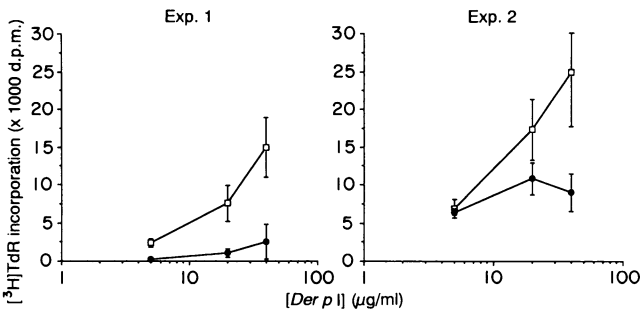

Figure 5. Tolerance induced by feeding peptide containing a cryptic epitope. C57BL/6J mice were fed either CAPS buffer (□) or 3 mg of GEX p131–187 (●) on 3 consecutive days and immunized 1 week later with *Der p* I in CFA. Lymph node cells were cultured *in vitro* with *Der p* I for 24 hr and the supernatants were assayed for IL-2. Shown are the results from two independent experiments. Each data point represents the mean response of five animals per group ± SD and the responses of cryptic-epitope fed mice were significantly different ($P < 0{\cdot}05$ Student's *t*-test).

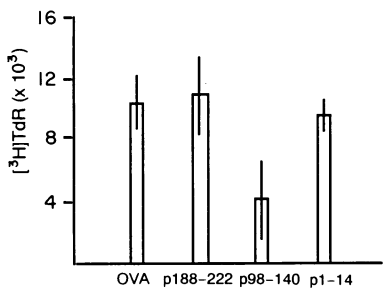

Figure 6. Ability of different fusion peptides to induce oral tolerance. Groups of four to five C57/BL6J mice were fed the fusion peptides shown (3 × 3 mg) or OVA and immunized with *Der p* I in CFA after 1 week. The *in vitro* IL-2 response of lymph node cells to *Der p* I was measured after 7 days. Results show mean (SD) of peak responses. The fusion GEX p98–140 gave significant inhibition, but not GEX p188–222 nor GEX p1–14.

their lymph node cells to secrete IL-2 upon *in vitro* challenge with the immunogenic peptides 110–131 or 78–100. However, the responses of mice fed GEX p131–187, GEX p101–154 and GEX p98–140 were reduced to both the peptides. Note here that tolerance induced by feeding a fusion peptide inhibited responses to epitopes not found on that peptide.

DISCUSSION

The results show that feeding fusion peptides can inhibit T-cell responses to subcutaneous immunization with the whole antigen. The inhibition was best measured by depressed IL-2 release from draining lymph node cells challenged *in vitro* with whole allergen or the immunodominant peptides. Some inhibition of GM-CSF/IL-3 could also be seen but interestingly this was less than the IL-2. Importantly, the inhibition was to the whole protein, including responses to peptides containing residues which were not present on the fusion protein used for feeding. For example, feeding the fusion GEX p101–154 inhibited the ability of *Der p* I immunization to induce T cells which react with the whole allergen and with synthetic peptides 110–131 and 78–100. At the present time we do not know the mechanism involved in causing down-regulation of immune responses by feeding antigen peptides, but Miller *et al.*[13,15] have found that oral tolerance to MBP was mediated by the release of TGF-β1 by Ts cells and, moreover, secretion of this cytokine by Ts cells was able to mediate bystander suppression in an *in vitro* model.

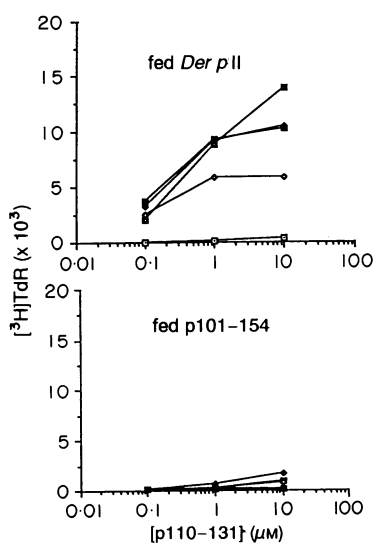

Figure 7. Inhibition of *in vitro* responses to peptide induced by feeding. C57BL/6J mice were fed the fusion peptide GEX p101–154 or GEX *Der p* II on 3 consecutive days immunized after 1 week with *Der p* I in CFA. After 7 days the *in vitro* response to the synthetic peptide 110–131 was measured by IL-2 release. The results show individual mice.

The fusion peptides were used for feeding because it was anticipated that the high doses required would make the use of synthetic peptides too expensive. The experiment shown here found that one 3 mg dose was not sufficient and in an experiment not shown it was found that feeding three doses of 1 mg gave incomplete inhibition.

Feeding fusion peptide GEX p1–14, which did not contain T-cell epitopes, did not inhibit the immune responses, but feeding the fusion GEX p131–187, which contained cryptic epitopes found in p144–169 and p120–143, did significantly inhibit. The cryptic epitopes were identified as peptides which could immunize mice but which themselves were not recognized by lymph node T cells after immunization with the natural protein. The degree of inhibition was not always as marked as that for the fusions containing dominant epitopes, but presumably could be increased by extending the feeding regime or increasing the dose.

Although the degree of inhibition induced by the peptides with dominant epitopes and cryptic epitopes is supportive of the effect being mediated via the epitopes, further evidence is required. This is made obvious by the inability to demonstrate inhibition with GEX p188–222 even though it does contain an epitope. This may, however, be due to instability of this fusion peptide in the gut or inappropriate processing to destroy the epitope. It is possible further doses could produce inhibition. In any case, the fact that the suppression can be obtained by feeding a peptide which does not contain an epitope recognized during immunization with the natural protein is significant. Peptides effective in immunotherapy may therefore not simply be limited to those identified by T-cell clones or polyclonal responses of sensitized individuals. Besides broadening the range of peptides which can be used, this could avoid the potential limitations inherent in administering epitopes to people containing sensitized cells. It also offers the potential for modifying immune responses without having to redirect the development of T-cell clones which have already progressed along helper type 1 (Th1) and Th2 or equivalent pathways because of antigenic stimulation. Cryptic epitopes were originally defined by T-cell responses to immunization with peptides which did not contain epitopes recognized during immunization with whole protein.[22,23] It appeared that T cells sensitized to these peptides did not respond to whole protein perhaps because the cryptic epitopes could not compete for presentation with the dominant epitopes or because they were destroyed during processing. Recently, Lehmann *et al.*[24] have reported that the late response to immunization with MBP is directed to epitopes previously considered to be cryptic and we have shown that T cells from mice immunized to peptides

containing cryptic epitopes of *Der p* II can respond to the whole allergen *in vitro* providing it is presented by splenic adherent cells.[21] It is proposed that the suppression here is similarly mediated by the recognition of a cryptic epitope from the processed protein.

In conclusion, we have shown here that feeding recombinant peptides which contain immunogenic determinants can induce antigen-specific tolerance in mice. These studies show that peptides may be useful in the treatment of immunological disorders such as allergy or autoimmunity. Furthermore, by understanding more about the presentation of protein antigens to the mucosal immune system, we may come to elucidate the molecular mechanisms involved in the development of oral tolerance.

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