

T Cell Epitope Mapping of Ragweed Pollen Allergen *Ambrosia artemisiifolia* (Amb a 5) and *Ambrosia trifida* (Amb t 5) and the Role of Free Sulfhydryl Groups in T Cell Recognition

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Ambrosia artemisiifolia (Amb a 5; Ra5S) and *Ambrosia trifida* (Amb t 5; Ra5G) are homologous allergens purified from short and giant ragweed pollen, respectively. Allergic human sera and hyperimmunized animal antisera directed against Amb a 5 or Amb t 5 show a high degree of species specificity, with little or no cross-reactivity between these two allergens, suggesting that the major Ab binding epitopes of Amb a 5 and Amb t 5 are distinct. Overlapping synthetic peptides derived from the allergen sequences were used to investigate the specificity of T cell responses in four strains of mice, BALB/c (H-2^d), CBA (H-2^k), C57BL/6 (H-2^b), and A/J (H-2^a). All four strains of mice responded to purified Amb a 5 and Amb t 5. Cross-reactivity was found at the T cell level between Amb a 5 and Amb t 5 in T cells from BALB/c, A/J, and CBA mice, but not in T cells from C57BL/6 mice. A T cell epitope from Amb a 5, residues 27–36 (PWQVVCYESS), was mapped using T cell hybridomas from BALB/c mice. A T cell epitope in Amb t 5 was mapped in the same strain to residues 24–34 (KYCVCYDSKAI). Disulfide bonds in Amb a 5 and Amb t 5 were found to be involved in T cell reactivity. Conversion of disulfide bridges into free sulfhydryl (SH) forms was required for the response of T cell hybridomas to peptide t5 (residues 27–40) from Amb t 5. Reduction of peptide a4 (residues 21–37) from Amb a 5 was essential for inducing the cross-reactivity observed with Amb t 5-specific T cell hybridomas. It is concluded that free sulfhydryl groups play a major role in the T cell recognition of cross-reactivity T cell epitopes within these related allergens. *The Journal of Immunology*, 1995, 155: 5064–5073.

Allergens, unlike most Ags that induce a protective IgG immune response, induce the production of IgE that results in allergic disease in a subset of individuals (1). Several ragweed allergens have been purified and sequenced (2–6), and have been shown to bind to IgE in atopic patient's sera. However, what makes an Ag an allergen is still a mystery, as no obvious allergic motif has emerged from identified allergens (2–10). The differentiation of B lymphocytes to IgE-producing plasma cells requires interaction with activated Th cells. Activation of helper activity is dependent on the recognition of T cell epitopes presented by MHC molecules on APCs (11–17). Therefore, the examination of the epitopes recognized by both T cells and B cells specific for allergens should aid our understanding of allergy.

Small allergens with well defined chemical structures and biochemical properties have provided excellent models for specificity studies. Amb a 5^{4,5} (Ra5S) and Amb t 5 (Ra5G) are low m.w. protein allergens purified from short and giant ragweed, respec-

tively (4, 5). The primary structure and immunochemical properties of Amb a 5 and Amb t 5 have been studied extensively (4–6, 18, 19). Amb a 5 and Amb t 5 show considerable sequence homology, with 50% identity in their primary structure. One striking feature of Amb a 5 and Amb t 5 is their high cysteine content. There are eight cysteines in each molecule, accounting for 20% of their m.w. The positions of these eight cysteines in Amb a 5 and Amb t 5 are conserved, and the high degree of intrachain interactions is suggested by the heat stability of these molecules (20). Amb a 5 has been reported to retain its skin test reactivity in allergic patients after boiling, while other allergens, such as Amb a I, Amb a II, and Amb a III, precipitate out of the solution and lose their skin test reactivity (20). The role of disulfide bonds in T cell recognition has been documented in the case of insulin, apamin, and snake toxin (21, 22, and 23). Due to the high cysteine content and homologous structure, we consider Amb a 5 and Amb t 5 to be good models for studying the role of disulfide bonds in immunorecognition by T and B cells.

Ab responses to Amb a 5 and Amb t 5 have been studied in man and animals (6, 19). The specific IgE Ab response to Amb a 5 and Amb t 5 was reported to be associated with distinct HLA-DR genes, with DR2 and DRw52, respectively (24–26). Among ragweed-allergic patients, most are sensitive to both Amb a 5 and Amb t 5, suggesting either cross-reactivity or exposure to both allergens. However, a significant number of patients only have IgE specific for binding to Amb a 5 but not Amb t 5, indicating that IgE generated to Amb a 5 is not cross-reactive to Amb t 5 (19). In murine studies, antisera specific for Amb a 5 does not crossreact with Amb t 5 and vice versa, indicating that there is no cross-reactivity in mice at the B cell level for Amb a 5 and Amb t 5.

Amb t 5 and Amb p 5 have been reported to be able to inhibit the stimulation by Amb a 5 to three human Amb a 5-specific T cell clones. Amb t 5 and Amb p 5, however, were unable to stimulate these human clones (27, 28). The authors suggested that the T cell

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⁴ Abbreviations used in this paper: Amb a 5, *Ambrosia artemisiifolia* (short ragweed); Amb t 5, *Ambrosia trifida* (giant ragweed); Amb p 5, *Ambrosia psilostachya* (Western ragweed); SH, sulfhydryl; DTNP, 5,5'-dithionitrobenzoic acid; MTT, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide.

⁵ Allergens are named according to the IUIS-approved nomenclature (T. P. King et al. 1994. *Int. Arch. Allergy Appl. Immunol.* 105:224).

epitopes in Amb t 5 and Amb p 5 are different from Amb a 5, but their agretopes, the region of an Ag in contact with MHC molecule, are able to bind to similar or identical regions of the class II molecule, HLA-DR2.

We have studied T cell cross-reactivity between Amb a 5 and Amb t 5 in four mouse strains. Cross-reactivity at the level of T cell recognition is demonstrated in BALB/c, CBA, and A/J mice, but not in T cells from C57BL/6 mice. We also generated a panel of Amb a 5- and Amb t 5-specific T cell hybridomas from BALB/c mice and studied the fine specificity of the T cell response. T cell epitopes within both allergens were mapped by examining reactivity in a panel of T cell hybridomas with a set of overlapping synthetic peptides from Amb a 5 and Amb t 5. The results demonstrate the presence of cross-reactivity between Amb a 5 and Amb t 5 at the T cell level. The cross-reactivity of Amb t 5-specific T cell hybridomas to peptide a4 (amino acid residues 21–37) from Amb a 5 was dependent on the conversion of Cys 32 in Amb a 5 into a free SH form.

Materials and Methods

Purification of Amb a 5 and Amb t 5

Defatted short and giant ragweed pollen was purchased from Greer Laboratories (Lenoir, NC). Pollen extracts were prepared as described elsewhere (18), with modifications. Briefly, 50 g of each pollen was stirred in 500 ml of 0.125 M $(\text{NH}_4)_2\text{CO}_3$ buffer containing 0.5 mg/ml leupeptin, 1 mM EDTA-Na, 0.7 mg/ml pepstatin, and 0.2 mg/ml PMSF at room temperature for 16 min, followed by centrifugation at $1800 \times g$ for 30 min at 10°C. The supernatants were clarified twice before filtration through a 0.45- μm filter, and concentrated 20-fold in an Amicon concentrator using a YM2 membrane. Amb a 5 and Amb t 5 were each further purified from these extracts by separation on a Sephadex G-50 column. The positions of Amb a 5 and Amb t 5 molecules in the elution profiles of each column were monitored by size on an 8 to 20% gradient SDS-PAGE gel. Fractions containing the 5000-m.w. molecular species were pooled, concentrated, and lyophilized. Amb a 5 and Amb t 5 were further purified on a C8 LKB Quantum HPLC column, a Vydac C18 semiprep column with an isocratic gradient, followed by a C18 mBondapak analytical HPLC. A bioassay testing the HPLC fractions using Amb a 5- or Amb t 5-specific T cell hybridomas (see below) was applied in the final step of the purification to determine the locations of Amb a 5 and Amb t 5 in the elution profiles.

Peptide synthesis and the synthesis of Amb a 5 and Amb t 5

A set of overlapping peptides derived from the sequence of Amb a 5 and Amb t 5, and the Amb a 5 and Amb t 5 proteins (4, 5) were synthesized with Applied Biosystems (Foster City, CA) 430 and Milligen 9095 peptide synthesizers using conventional Merrifield solid-phase chemistry with *t*-Boc or *f*-Moc amino acids. The derived peptides and proteins were cleaved from the synthesis substrate with hydrofluoric acid or trifluoroacetic acid, respectively. The peptides and proteins were desalted by gel filtration, lyophilized, and purified by reverse-phase HPLC. The purified peptides and proteins were subjected to amino acid analysis for sequence verification.

Reduced proteins and peptides

Amb a 5 and Amb t 5 were dissolved in 20 mM DTT and 8 M urea at a protein concentration of 10 mg/ml. Samples of Amb a 5 and Amb t 5 were heated for 5 min in boiling water and irradiated with 10,000 rad before they were used in T cell proliferation assays. Synthetic peptides of Amb a 5 and Amb t 5 were reduced with 20 mM DTT and filtrated through a 0.2- μm filter 1 h before use.

Peptides modified by 5,5'-dithionitrobenzoic acid

Five overlapping peptides of Amb t 5 were reduced with 20 mM DTT. Powdered DTNP (Sigma Chemical Co., St. Louis, MO) was added to each peptide solution to a final concentration of 40 μM . After 1 h, each peptide was passed through a Sephadex G-25 M column (PD-10 columns; Pharmacia). One-milliliter fractions were collected, and the OD at 200 to 450 nm was measured on a Beckman DU-44 spectrophotometer. Fractions of the first peak at OD₃₂₀ were pooled, and each peptide had its concentration determined by amino acid analysis.

Animals

Female BALB/c, C57BL/6J, CBA, and A/J mice 8 to 16 wk of age were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were used between 8 and 15 wk of age for the generation of T cell hybridomas, and up to 6 mo of age for the T cell proliferation assays.

Culture media and reagents

T cells were cultured in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) medium supplemented with 10% heat-inactivated FCS, 10 mM HEPES (Sigma Chemical Co.), 2 mM L-glutamine, 5×10^{-5} M 2-ME, and 1% penicillin/streptomycin (Life Technologies, Inc.). Purified Amb a 5 and Amb t 5 for the initial studies were kindly provided by Dr. L. Goodfriend (Thorp Division of Immunology, Royal Victoria Hospital, Montreal, Quebec, Canada). The cell line, BW5147, was provided by Dr. W. Born (National Jewish Center, Denver, CO), and A20 and CTLL20 cells were provided by Dr. D. Raulet (University of California, Berkeley, CA). MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) conversion was used as a readout for the IL-2 bioassay (29).

T cell proliferation assays

Each mouse was primed with 50 μg of Amb a 5 or Amb t 5 in CFA, s.c. injected at the base of the tail. Draining lymph nodes were removed 7 to 10 days later, and cultured at 4×10^5 cells/well in a 96-well flat-bottom plate (Costar, Cambridge, MA), with the indicated concentrations of various Ags in a final volume of 0.2 ml/well. After 48 h, cultures were pulsed with 1 μCi /well of [³H]methylthymidine (ICN Radiochemicals, Irvine, CA) and harvested onto glass fiber filters 16 h later with a semi-automated cell harvester (PHD, Cambridge, MA). T cell proliferation was assessed by the incorporation of [³H]methylthymidine.

Generation of T cell hybridomas

BALB/c mice were each primed with 50 μg of Amb a 5 or Amb t 5 emulsified in CFA, and s.c. injected at the base of the tail. Seven days later, draining lymph nodes were removed, and lymph node T cells were harvested and restimulated in vitro with Amb a 5 or Amb t 5 at 10 $\mu\text{g}/\text{ml}$. Fusions were performed 3 days later with a murine thymoma cell line, BW5147, in the presence of 40% PEG using standard techniques (30). Isolated hybridomas were selected with azaserine-hypoxanthine medium (31), then screened for the ability to produce IL-2 when cultured in the presence of Amb a 5 or Amb t 5 with A20 cells as APCs.

IL-2 assay

Cell supernatants were assessed for levels of IL-2 in a bioassay using the IL-2-dependent cell line, CTLL-20 (29). T cell culture supernatants were serially diluted twofold in duplicate wells in a 96-well tissue culture plate. Washed 5×10^3 CTLL-20 cells were then added and incubated in a total volume of 100 μl for 24 h at 37°C. IL-2-driven proliferation of CTLL-20 cells was then determined by an MTT assay. To each well, 50 μl of a 1-mg/ml MTT (Sigma Chemical Co.; M-2128) solution was added and incubated at 37°C for 4 h. Crystals were dissolved by the addition of 135 μl /well acid alcohol (4 ml 1 N HCL in 96 ml isopropanol). After thorough mixing, the OD of the wells was measured at 550 and 230 nm with an ELISA reader (Bio-Tek Instruments, Winooski, VT). Units of IL-2/ml were determined by comparison with a IL-2 standard curve.

Results

Effect of the MHC on the T cell response to Amb a 5 and Amb t 5

The T cell response to Amb a 5 and Amb t 5 was studied in BALB/c (H-2^d), CBA (H-2^k), C57BL/6 (H-2^b), and A/J (H-2^a) mice. Mice from each strain were immunized s.c. with Amb a 5 or Amb t 5 emulsified in CFA, and the T cell-proliferative response to Amb a 5 and Amb t 5 was assayed 9 days after immunization. All four strains of mice responded to Amb a 5 and Amb t 5. T cells from BALB/c mice primed with Amb a 5 (Fig. 1A) responded strongly to Amb a 5 with some cross-reactivity to Amb t 5, although the level of the response to Amb t 5 was much less than to the priming Ag, Amb a 5. Similar data were obtained when T cells from BALB/c mice were primed with Amb t 5 (Fig. 1B). The cross-reactive response to Amb a 5 in Amb t 5-primed T cells was

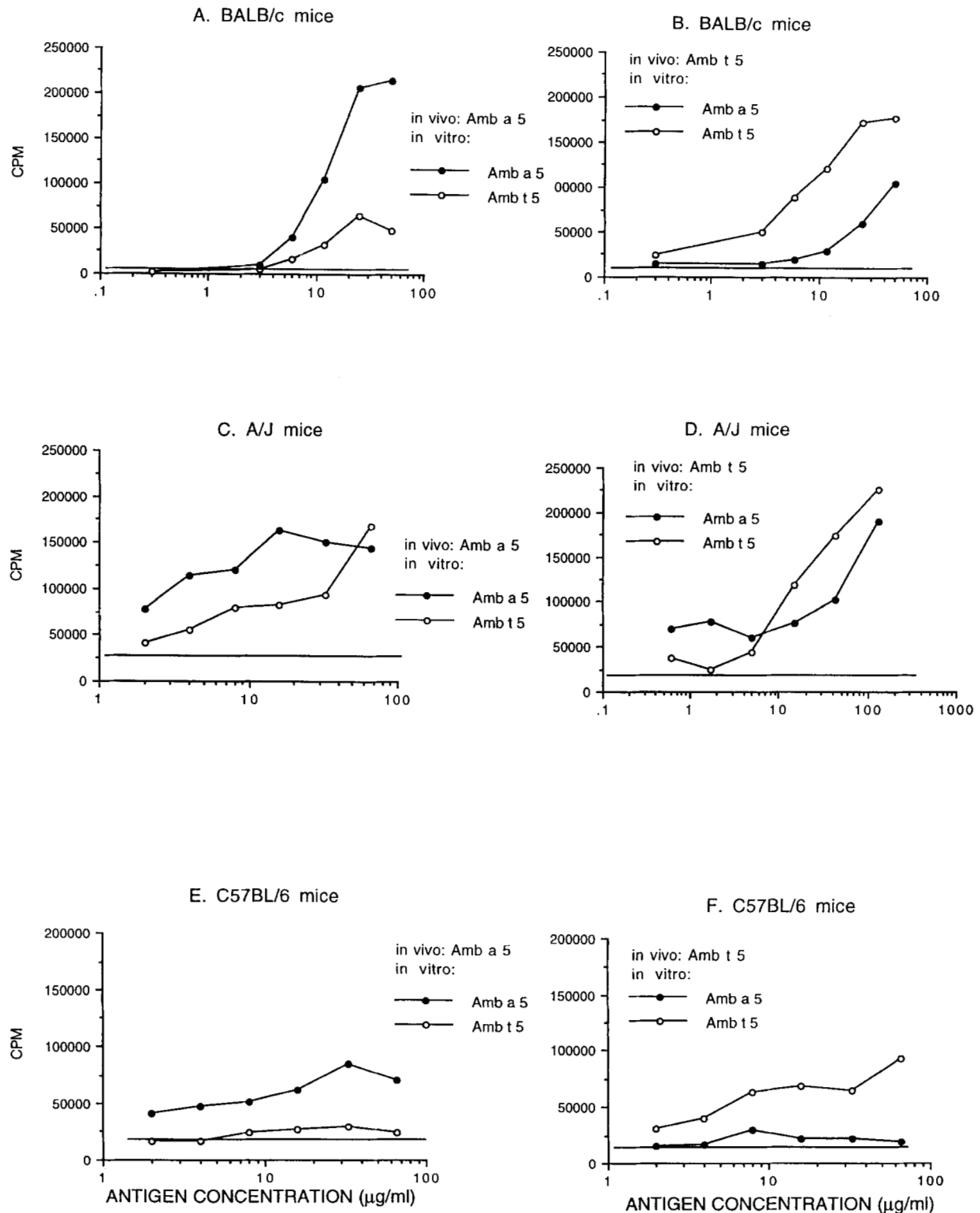


FIGURE 1. Proliferation of lymph node cells in response to Amb a 5 and Amb t 5. *A* and *B*, BALB/c mice were primed with Amb a 5 or Amb t 5 emulsified in CFA. *C* and *D*, A/J mice were primed with Amb a 5 or Amb t 5 emulsified in CFA. *E* and *F*, C57BL/6 mice were primed with Amb a 5 or Amb t 5 emulsified in CFA. Nine days after CFA challenge, the regional draining lymph node cells were collected and tested for their responses to medium alone (no symbol), Amb a 5 (closed circles), and Amb t 5 (open circles), as indicated in the figures. Proliferation was measured by [^{3} H]thymidine incorporation, as described in *Materials and Methods*. Results are expressed as the mean cpm values of triplicate cultures. Similar results were obtained in two other separate experiments.

greater than the cross-reactive response to Amb t 5 in Amb a 5-primed T cells. The level of maximum response to Amb a 5 and Amb t 5 was similar in BALB/c mice. Therefore, it is con-

cluded that at a higher Ag concentration (40 μ g/ml) the Amb t 5-induced response is more cross-reactive than the Amb a 5-induced response.

Amino Acid Sequence of Amb a 5

Amino Acid # 1 10 20 30 40
 Sequence : L V P C A W A G N V C G E K R A Y C C S D P G R Y C P W Q V V C Y E S S E I C S K K C G K

a1 (1-15) L V P C A W A G N V C G E K R
 a2 (8-22) G N V C G E K R A Y C C S D P
 a3 (16-30) A Y C C S D P G R Y C P W Q V
 a4 (21-37) D P G R Y C P W Q V V C Y E S S E
 a5 (31-45) V C Y E S S E I C S K K C G K

Amino Acid Sequence of Amb t 5

Amino Acid # 1 10 20 30 40
 Sequence : D D G L C Y E G T N C G K V G K Y C C S P I G K Y C V C Y D S K A I C N K N C T

t1 (1-15) D D G L C Y E G T N C G K V G
 t2 (8-22) G T N C G K V G K Y C C S P I
 t3 (16-30) K Y C C S P I G K Y C V C Y D
 t4 (21-34) P I G K Y C V C Y D S K A I
 t5 (27-40) V C Y D S K A I C N K N C T

FIGURE 2. Amino acid sequences of Amb a 5, Amb t 5 and 10 overlapping peptides derived from Amb a 5, Amb t 5 used to induce T cells and T cell hybridomas.

In A/J mice, the T cells appeared to be more cross-reactive than T cells from BALB/c mice (Fig. 1, *C* and *D*). T cells from A/J mice primed with Amb a 5 responded well to both Amb a 5 and Amb t 5 (Fig. 1*C*). The response to Amb t 5 was less than the response to Amb a 5 at lower Ag concentrations, but approached the same maximum level of response at higher Amb t 5 concentrations. This suggests that in A/J mice, the entire population of T cells that recognize Amb a 5 can also recognize Amb t 5. A similar pattern was obtained from T cells of A/J mice primed with Amb t 5 (Fig. 1*D*). The response pattern of T cells to Amb a 5 and Amb t 5 from CBA (H-2^k) mice was similar to the pattern of the response in A/J mice (data not shown). In contrast, T cells from C57BL/6 mice showed no cross-reactivity (Fig. 1, *E* and *F*), and the level of maximum proliferation was approximately one-half of the maximum response from the other mouse strains tested. Therefore, both Amb a 5 and Amb t 5 induced T cell responses in four strains of mice studied. BALB/c (H-2^d), A/J (H-2^a), and CBA (H-2^k) mice are high responders, and C57BL/6 (H-2^b) mice are low responders. Cross-reactivity at the T cell level was detected between Amb a 5 and Amb t 5 in BALB/c, A/J, and CBA mice, but not in C57BL/6 mice.

Polyclonal T cell responses to the overlapping peptides of Amb a 5 and Amb t 5

BALB/c, C57BL/6, and A/J mice were primed with Amb a 5 or Amb t 5, and the lymph node T cells were stimulated later with overlapping peptides derived from either Amb a 5 or Amb t 5 (Fig. 2). The proliferative responses to Amb a 5, Amb t 5, and the overlapping peptides were measured (Fig. 3). One peptide, a4 (amino acid residues 21–37) from Amb a 5, induced a strong T cell

response from BALB/c mice primed with Amb a 5 (Fig. 3*A*). The remaining four peptides from Amb a 5 induced similar but much lower level of response than the one by the peptide a4. T cells from BALB/c mice primed with Amb t 5 responded to peptides t4 (amino acid residues 21–34) and t5 (amino acid residues 27–40) (Fig. 3*B*). Peptide t4 induced a dominant T cell response at a peptide concentration of 1 μ M. The response induced by peptide t5 was much weaker even at a peptide concentration 30-fold higher than that of peptide t4. No response was detected to the three other Amb t 5 peptides (Fig. 3*B*). T cells from A/J mice primed with Amb a 5 responded strongly to Amb a 5, but not to any Amb a 5 peptide (Fig. 3*C*). T cells from A/J mice primed with Amb t 5 gave a strong response to Amb t 5 and to the two peptides, t4 and t5 (Fig. 3*D*). None of the peptides from Amb a 5 induced a response from C57BL/6 mice (Fig. 3*E*), and none of the peptides from Amb t 5 induced a response from C57BL/6 mice (Fig. 3*F*). The response to the Amb t 5 was slightly higher than the response to the Amb a 5. Therefore, peptide a4 from Amb a 5 contained a major T cell epitope or epitopes presented by this set of overlapping peptides. The epitope(s) could be presented by H-2^d, but not H-2^a or H-2^b mice. Peptides t4 and t5 from Amb t 5 contained the major T cell epitopes and could be recognized by H-2^d and H-2^a mice, but not H-2^b mice (Fig. 3).

T cell epitope mapping by T cell hybridomas

A panel of T cell hybridomas was generated from BALB/c mice. They were first selected by their response pattern to the extracts from short and giant ragweed pollen. Selected hybridomas all responded to purified Amb a 5 and Amb t 5. Therefore, the T cell hybridomas generated recognized native forms of Amb a 5 and Amb t 5 in pollen extract.

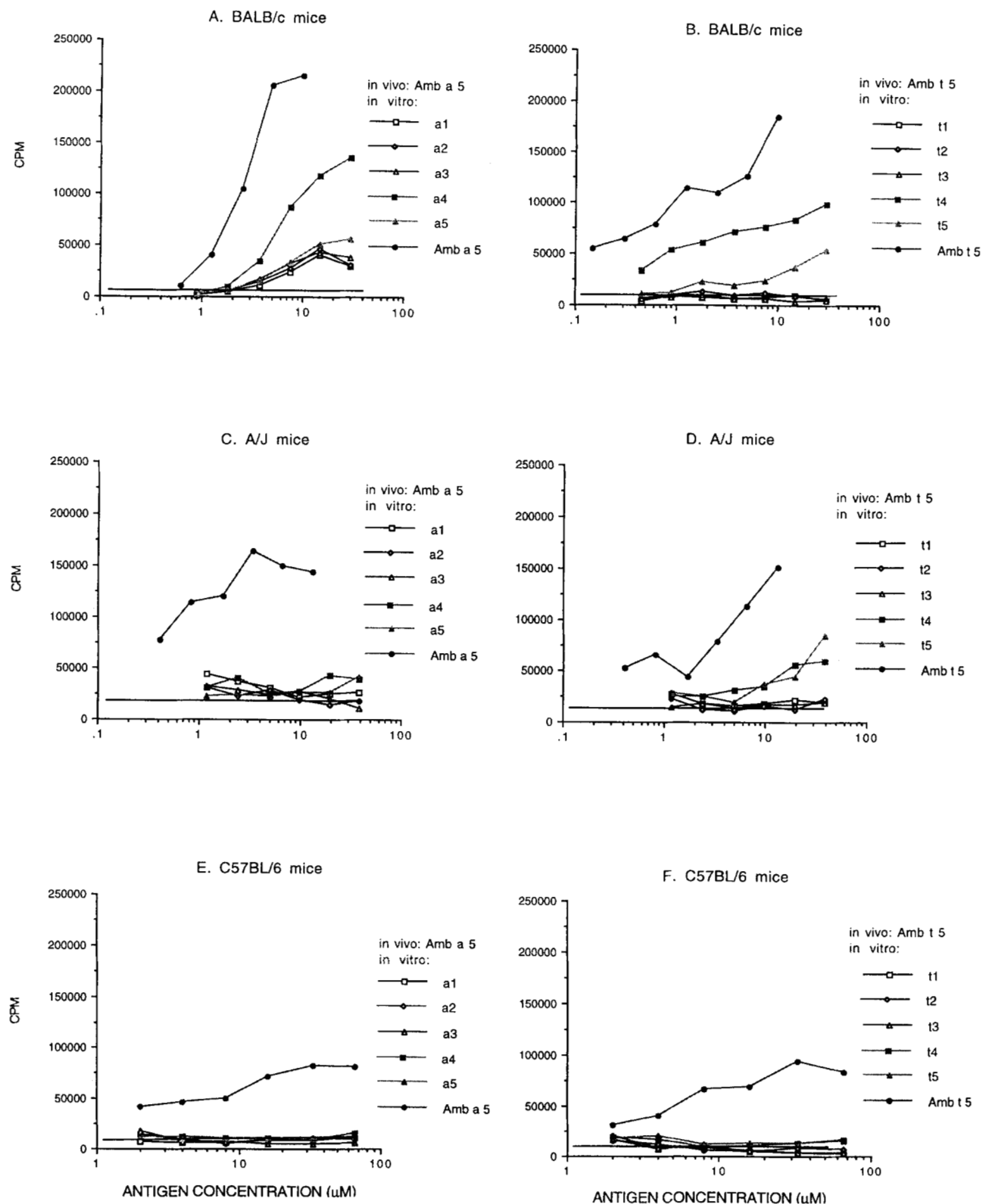
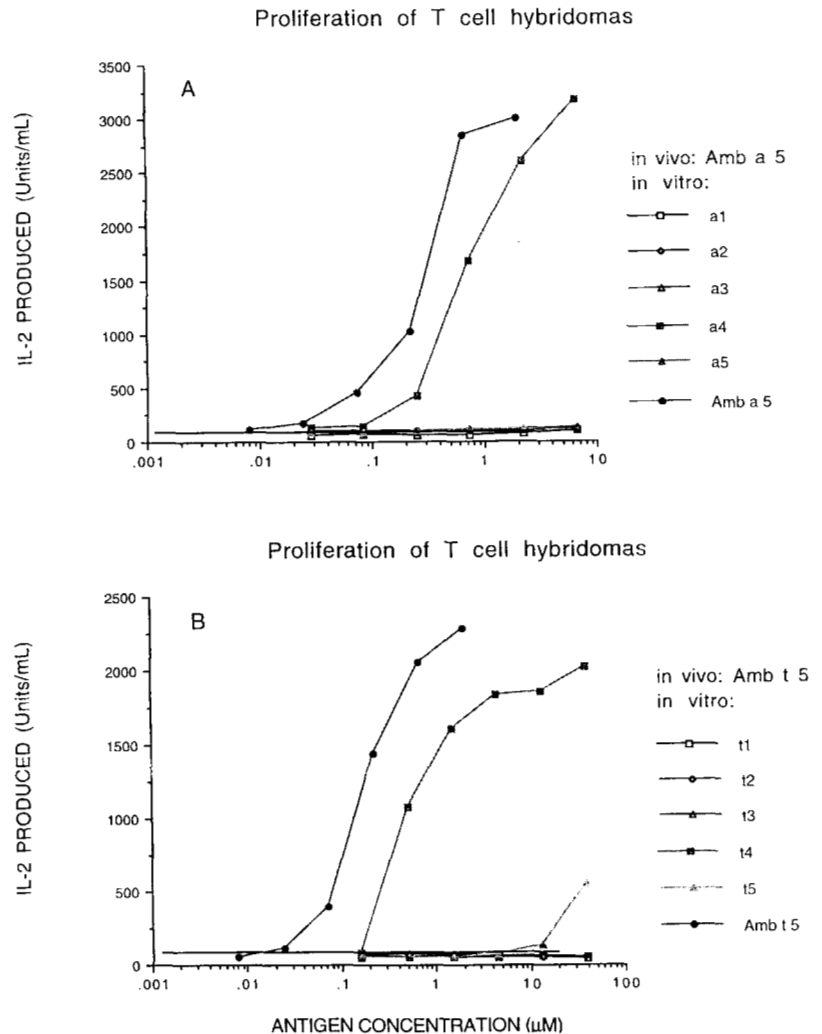


FIGURE 3. Proliferation of T cells to overlapping peptides of Amb a 5 and Amb t 5. A, BALB/c mice were immunized with Amb a 5. Lymph node cells were examined for their proliferation in response to the five overlapping peptides from Amb a 5 and Amb a 5 protein, as indicated in the figure. B, BALB/c mice were immunized with Amb t 5. Lymph node cells were examined for their proliferation in response to the five peptides from Amb t 5 and Amb t 5 protein, as indicated in the figure. Medium alone has no symbol. C, A/J mice were immunized with Amb a 5. The proliferation to the overlapping peptides from Amb a 5 was studied, as in A. D, A/J mice were immunized with Amb t 5. The response to five peptides of Amb t 5 was studied, as in B. E, C57BL/6 mice were immunized with Amb a 5. The response to five peptides of Amb a 5 was studied, as in A. F, C57BL/6 mice were immunized with Amb t 5. The response to five peptides of Amb t 5 was studied, as in B. Results are expressed as the mean cpm values of triplicate cultures. Similar results were obtained in two other experiments.

FIGURE 4. Proliferation of T cell hybridomas to peptides from Amb a 5 and Amb t 5. *A*, T cell hybridoma BaV3.2 cultured with APCs and the following Ags at the concentrations indicated: Amb a 5 (closed circles); medium alone (no symbol); and peptides from Amb a 5, a1 (open squares), a2 (open diamonds), a3 (open triangles), a4 (closed squares), and a5 (closed triangles). *B*, T cell hybridoma BtV12.1 was stimulated as above with five peptides from Amb t 5 and Amb t 5 protein, as indicated in the figures. Symbols used for the five overlapping peptides from Amb t 5 are the same as the ones used for the five peptides from Amb a 5. Results are expressed as the mean IL-2 units of duplicate cultures. Similar results were obtained in three other separate experiments.



T cell hybridomas were tested for their response to each of the two sets of five overlapping peptides from Amb a 5 and Amb t 5. Of the 30 T cell hybridomas tested, only one peptide from Amb a 5, a4, induced Ag-dependent IL-2 production. All 30 T cell hybridomas responded to peptide a4. Representative results obtained with hybridoma BaV3.2 are shown in Figure 4A. Peptides a1, a2, a3, and a5 of Amb a 5 did not induce any response. Not surprisingly, peptide a4 was the only peptide that induced a polyclonal T cell response in BALB/c mice. The response patterns of four Amb a 5-specific T cell hybridomas are shown in Table I.

To define the boundary of the epitopes in Amb a 5, three overlapping peptides from a4 were synthesized and tested against the Amb a 5-specific hybridomas. Peptide a4.3 (amino acid residues 27–36) induced a T cell response from the Amb a 5-specific T cell hybridomas. Results from two hybridomas are shown in Figure 5. A dose-dependent response to peptide a4.3 was demonstrated by hybridoma BaV3.2, and peptides a4.1 and a4.2 had no stimulatory activity (data not shown). The level of the response induced by peptide a4.3 at 100 μ g/ml was fivefold lower (512 U/ml IL-2) than the response induced by Amb a 5 (2720 U/ml).

All 25 BALB/c T cell hybridomas generated against Amb t 5 responded to peptide t4 from Amb t 5, but one of three hybridomas responded to peptide t5, in addition to peptide t4. The same result was obtained when T cell hybridomas were subcloned (data not shown). Representative data obtained with T cell hybridomas BtV12.1 are shown in Figure 4B. The proliferative response in-

Table I. T cell hybridomas responding to Amb a 5, Amb t 5, and their peptides

Hybridomas ^c	IL-2 Produced (U/ml) ^a		
	Media	Amb a 5	a4 ^b
Amb a 5-Specific Hybridomas			
BaV3.2	50	2920	2760
BaV7	29	2027	3291
BaV11.7	80	4533	4213
BaV14.1	80	4107	5120
Amb t 5-Specific Hybridomas			
BtV10.8	42	2743	2571
BtV12.1	43	3200	2743
BtV14	72	3252	2985
BaV39.2	<10	2773	2693

^a Supernatants of each hybridoma from the assay, as described in Figure 4, were collected after 24 h and assayed for levels of IL-2 in a bioassay. The concentration used for each antigen was 10 μ g/ml.

^b Peptide of Amb a 5 with amino acid residues 21–37.

^c T cell hybridomas were generated as described in Materials and Methods.

^d Peptide of Amb t 5 with amino acid residues 21–34.

duced by peptide t4 reached a similar level to that induced by the Amb t 5 protein. Peptide t5 also induced a response. This response was weak and only detected at the peptide concentration of 40 μ M

Fine mapping of the a4 peptide region of Amb a 5						Amb a 5 SPECIFIC T CELL HYBRIDOMAS	
						IL-2 PRODUCED UNITS/ML	
Amino Acid #	1	10	20	30	40	BaV3.2	BaV14.1
Sequence : L V P C A W A G N V C G E K R A Y C C S D P G R Y C P W Q V V C Y E S S E I C S K K C G K						2720	622
a4.1 (23-32)				G R Y C P W Q V V C		18	44
a4.2 (25-34)				Y C P W Q V V C Y E		7	48
a4.3 (27-36)				P W Q V V C Y E S S		512	215

Fine mapping of the t4 and t5 peptide region of Amb t 5						Amb t 5 SPECIFIC T CELL HYBRIDOMAS	
						IL-2 PRODUCED UNITS/ML	
Amino Acid #	1	10	20	30	40	BtV10.8	BtV12.1
Sequence : D D G L C Y E G T N C G K V G K Y C C S P I G K Y C V C Y D S K A I C N K N C T						1837	1719
t4.1 (23-34)				G K Y C V C Y D S K A I		2548	1541
t4.2 (24-34)				K Y C V C Y D S K A I		1748	696
t4.3 (26-34)				C V C Y D S K A I		19	54
t4.4 (27-35)				V C Y D S K A I C		37	37
t4.5 (27-36)				V C Y D S K A I C N		33	26
t4.6 (27-37)				V C Y D S K A I C N K		26	22

FIGURE 5. Fine specificity of T cell epitopes in Amb a 5 and Amb t 5. Responses of two Amb a 5-specific T cell hybridomas to the three overlapping peptides from a4, and of two Amb t 5-specific T cell hybridomas to the six overlapping peptides from t4 and t5 are shown. T cell hybridomas were stimulated with each peptide at a concentration of 100 μ g/ml in the presence of APCs. Amb a 5 and Amb t 5 were used as controls at 5 μ g/ml. The response was measured as in Figure 4. Results are expressed as mean IL-2 U/ml of triplicate cultures. Similar results were obtained in another experiment.

(60 μ g/ml), more than 100-fold higher than the concentration needed to induce a half-maximal response by peptide t4. No response was induced by the other peptides from Amb t 5. Typical results from four Amb t 5-specific T cell hybridomas are listed in Table I.

Six overlapping peptides covering the length of t4 and t5 from Amb t 5 were examined (Fig. 5). The response to peptide t4.1 reached a similar level to that induced by the Amb t 5 protein. Peptide t4.2, which has one amino acid deleted from the amino terminus of t4.1, also induced a strong response, though weaker than t4.1. The other four peptides (peptide t4.3, t4.4, t4.5, and t4.6) did not induce a response. Therefore, the dominant T cell epitope of Amb a 5, defined by this set of BALB/c T cell hybridomas, resided in the boundaries of peptide a4 (residues 21–37) and peptide a4.3 (residues 27–36) and contains essential residues of the T cell epitope to induce a T cell response. The T cell epitope of Amb t 5 was defined by amino acid residues 24–34 of Amb t 5.

Role of sulfhydryl group in T cell recognition

There are two to four cysteines in each of the five overlapping peptides from Amb a 5 and Amb t 5 (Fig. 2). When the peptides are oxidized, the disulfide bonds in these peptides are likely to be linked randomly. To ensure that the random disulfide bond linkage did not interfere with T cell response, the five overlapping peptides from Amb t 5 were reduced and modified with DTNB. The proliferative response from two T cell hybridomas is shown in Table II. Reduced peptide t5 induced a similar level of response as unreduced peptide t4. This is in contrast to the response to the unreduced form of peptide t5, which in several experiments induced a fraction of IL-2 production compared with peptide t4 by the same

Table II. The responsiveness of T cell hybridomas to reduced Amb t 5 peptides

Hybridomas	IL-2 Produced (U/ml) ^a						GPE ^c
	Media	rt1 ^b	rt2	rt3	rt4	rt5	
BtV10.8	33	54	58	43	2850	3258	2618
BtV12.1	80	40	43	46	2793	2618	3124

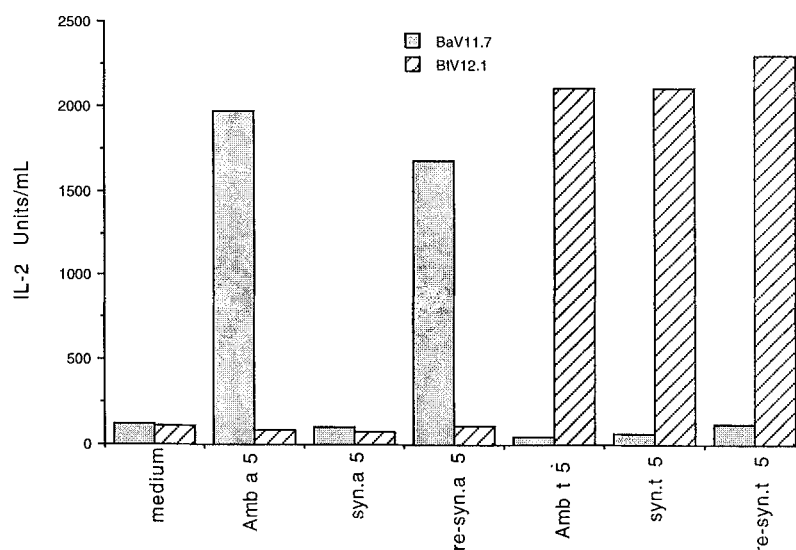
^a T cell hybridomas were stimulated with reduced peptides of Amb t 5 or giant ragweed pollen extract for an IL-2 assay. T cell responses were measured by IL-2 production.

^b Reduced peptides from Amb t 5 at a concentration of 36 μ M. Peptides were reduced by 20 mM DTT and then chemically modified by DTNB (5',5'-dithio-nitrobenzoic acid). Reduced peptides were purified on Sephadex G25 columns and irradiated before use.

^c Giant ragweed pollen extract was used as a control at 1:250 in the T cell assays. Similar results were obtained when reduced Amb t 5 was used in a separate experiment.

hybridoma cells (a representative result is shown in Fig. 4B). The finding that the same T cell hybridoma responded to both peptides indicated that the epitope it recognized was near the overlapping region of peptides t4 and t5. However, when a peptide extending over the overlapping region of peptides t4 and t5 was tested, no response to the octapeptide VCYSKAI was obtained from the T cell hybridomas that responded to both peptides (data not shown). Similar response patterns were obtained from the eight Amb t 5-specific T cell hybridomas tested (data not shown). The octapeptide was not tested for its ability to inhibit the response of T cell hybridoma to peptide t4 or t5.

FIGURE 6. The effect of disulfide bond in the proliferative response of T cell hybridomas to synthetic Amb a 5 and Amb t 5. Synthetic Amb a 5 and Amb t 5 were reduced (re-syn.aV and re-syn.tV) in the same way as Amb a 5 and Amb t 5 proteins, as described in *Materials and Methods*. T cell hybridomas BaV11.7 and BtV12.1 were studied for their Ag-specific IL-2 production to reduced and unreduced synthetic Amb a 5 and Amb t 5 at 100 μ g/ml. Amb a 5 (aV) and Amb t 5 (tV) were used as controls at 10 μ g/ml. The proliferation was measured as in Figure 4. Data represent the mean of triplicate cultures. Similar results were obtained with four other T cell hybridomas.



Five reduced overlapping peptides from Amb a 5 were tested for their ability to induce a response from Amb a 5-specific hybridomas. No peptide other than reduced peptide a4 induced a T cell response in these T cell hybridomas, even at 100 μ g/ml (data not shown). This is consistent with the data obtained using unreduced peptides (Fig. 4A). Therefore, reducing the disulfide bond in peptide t5 (which contains three cysteines) enhanced its T cell-stimulatory activity, indicating cysteine residues are involved in T cell recognition or in the binding of the peptide to MHC molecule.

To further explore the role of disulfide bonds in the T cell responses to Amb a 5 and Amb t 5, synthetic Amb a 5 and Amb t 5 were reduced and tested for their abilities to induce a response from six hybridomas, three each specific for Amb a 5 or Amb t 5. Representative results from one of each are presented in Figure 6. Amb a 5-specific T cell hybridoma BaV11.7 responded to Amb a 5 and reduced synthetic Amb a 5, but not to unreduced synthetic Amb a 5. Amb t 5-specific T cell hybridoma BtV12.1 responded to Amb t 5, reduced synthetic Amb t 5, as well as to unreduced synthetic Amb t 5. Assuming that the disulfide bonds are formed randomly in both proteins, this result showed that the reduction in Amb a 5, but not in Amb t 5, has made a dramatic change in the stimulation of T cells. The response to synthetic Amb t 5 without reduction could be explained by the T cell epitope in peptide t4 that stimulates T cells with or without reduction (Tables I and II). The response to purified Amb a 5 could be explained by the presence of reduced form of Amb a 5 in the sample.

Crossreactivity of T cell hybridomas for Amb a 5 and Amb t 5

In an effort to investigate the cross-reactivity of the T cell hybridomas, 10 Amb a 5-responsive T cell hybridomas were tested for their response to Amb t 5 and the five Amb t 5 peptides. None of the Amb t 5 peptides nor Amb t 5 induced a response from the Amb a 5-specific T cell hybridomas (data not shown). Ten Amb t 5-responsive hybridomas were tested for their response to Amb a 5 and the five peptides from Amb a 5. Again, neither Amb a 5 nor peptides of Amb a 5 induced a response (data not shown), indicating these T cell hybridomas exhibited a species-specific response to unreduced peptides.

To explore the cross-reactivity of the species-specific T cell hybridomas to reduced peptides, Amb a 5-specific T cell hybridomas were tested against the reduced peptide t4 from Amb t 5, and

Table III. T cell hybridomas responding to reduced synthetic peptides from Amb a 5 and Amb t 5

Hybridomas	IL-2 Production (U/ml) ^a						
	Media	a4	ra4 ^b	t4	rt4 ^c	Amb a 5	Amb t 5
Amb a 5-specific							
BaV3.2	55	2760	2783	55	113	2094	55
BaV11.7	<10	2693	2635	63	107	2338	30
Amb t 5-specific							
BtV12.1	75	88	624	2731	2693	88	2107
BtV14	73	83	780	2371	2459	102	3840

^a T cell hybridomas were stimulated with peptides and reduced peptides from Amb a 5 and Amb t 5 at 100 μ g/ml and 10 μ g/ml for Amb a 5 and Amb t 5 proteins.

^b Peptide a4 from Amb a 5 was reduced with 20 mM DTT.

^c Peptide t4 from Amb t 5 was reduced with 20 mM DTT.

Amb t 5-specific T cell hybridomas were tested against the reduced peptide a4 from Amb a 5 (Table III). Amb a 5-specific T cell hybridomas BaV3.2 and BaV11.7 responded to peptide a4 and reduced a4 from Amb a 5 with the same intensity as Amb a 5. They did not respond to the peptide t4 and weakly (approximately two-fold of the background) to reduced peptide t4 from Amb t 5 (Table III). Reduction of peptide t4 did not significantly change its T cell-stimulating ability to Amb a 5-specific T cell hybridomas. Amb t 5-specific hybridomas BtV12.1 and BtV14 responded to reduced and unreduced peptide t4 at a similar level to Amb t 5 whole protein. Neither of the T cell hybridomas responded to peptide a4 from Amb a 5. Unexpectedly, they both responded significantly to the reduced peptide a4. Therefore, reduction of peptide a4 made it capable of inducing a cross-reactive response from Amb t 5-specific T cell hybridomas (approximately eightfold above background).

Discussion

This study has examined the murine immune response to the closely related ragweed pollen allergens Amb a 5 and Amb t 5, and defined a fine map of the major T cell epitopes used by T cells from BALB/c mice. Both Amb a 5 and Amb t 5 are immunogenic in the four strains of mice studied. The fact that peptide a4 (amino

acid residues 21–37) of Amb a 5 and peptide t4 (amino acid residues 21–34) of Amb t 5 induced similar levels of maximum response as the intact proteins indicates that peptide a4 contains the intact major T cell epitope in Amb a 5 and peptide t4 contains the intact major T cell epitope in Amb t 5 (Fig. 4). Furthermore, all 30 T cell hybridomas generated against Amb a 5 responded to a4 peptide, and all 25 T cell hybridomas generated against Amb t 5 responded to t4. Therefore, peptides a4 and t4 contain dominant BALB/c T cell epitopes in Amb a 5 and Amb t 5.

The results from studying the polyclonal T cell response to Amb a 5 and Amb t 5 showed cross-reactivity of Amb a 5 and Amb t 5 in BALB/c, A/J, and CBA (Fig. 1; data not shown for CBA). The structural basis of cross-reactivity in H-2^d, H-2^a, and H-2^k mice may be explained by the sequence identity in peptide a4.3 and peptide t4.2, which share the sequences VCYES and VCYDS. The cross-reactivity to Amb a 5 and Amb t 5 observed from lymph node T cells, however, was not observed from T cell hybridomas when A20 cells were used as APCs. The response generated from lymph node cells against Amb a 5 in Figure 1A was much higher than the response to its homologous Amb t 5. It is possible that only minor population of T cells responded to both Ags, and the T cell hybridomas generated did not include those cross-reactive T cells. The lack of cross-reactivity seen with the T cell hybridoma may also be attributed to the difference in the APCs, as lymph node cells may function differently from the A20 cell line. A 10-fold increase in response was obtained from Amb t 5-specific T cell hybridomas when peptide a4 was reduced (Table III). Of the two cysteine residues (Cys 26 and Cys 32) in peptide a4, Cys 32 is in the identical position as Cys 28 from peptide t4. Thus, reduction of cysteine at position 32 of Amb a 5 appears to be essential for Amb t 5-specific T cell hybridomas to crossreact with the major T cell epitope in Amb a 5. Peptides presented by A20 cells are 14 to 17 amino acids in length and can presumably bind to MHC class II in the absence of processing.

The effect of sulfhydryl groups on T cell epitopes has been studied in insulin, apamin, and snake toxin (21–23). In the case of insulin, an A chain fragment (A1–14) contains the T cell epitope, but needs processing and presentation by APCs for full T cell activity (32, 33). Subsequent studies showed that conversion of the Cys A7, which forms a disulfide bridge with the B chain (Cys B7) in insulin, into a free SH form was sufficient to induce a T cell response without the need for processing by APCs (34). The authors proposed that disulfide reduction was critical for peptide presentation and stimulation of insulin-reactive T cells. In the case of Amb a 5 and Amb t 5, there are three cysteine residues in peptide t5 and two in a4 and t4, which presumably can form random disulfide linkages (Fig. 2). The two cysteine residues in peptide t4 are separated by one valine. The formation of the intrapeptide disulfide linkages in peptide t4 may be unfavorable because of the proximity of the cysteine residues. Interpeptide disulfide linkages may be less likely to alter the availability and peptide configuration of the T cell epitope. This assumption is supported by our data presented in Figure 6 and Table III; synthetic Amb t 5 and peptide t4 were recognized in both reduced and unreduced states by Amb t 5-specific T cell hybridomas (Fig. 6).

The three cysteine residues in peptide t5 are separated by three and six amino acid residues, and are interspersed throughout the peptide. The formation of an intrachain disulfide linkage between the first and the third cysteine residues would bring two ends of the peptide together, which could alter the peptide configuration and presumably affect the T cell reactivity. If T cell recognition of Amb t 5 requires a free SH group, as in the case of insulin and apamin, only a small portion of peptide t5 would be in the reduced state and recognized by T cell hybridomas generated against Amb

t 5 protein. We found that T cell hybridomas did not always respond to peptide t5, even at a peptide concentration of 100 μ g/ml (Fig. 4B and unpublished observation). The two cysteine residues in peptide a4 are separated by five residues, and the reduction of peptide a4 enhanced T cell activation by Amb t 5-specific T cells (Table III). This data suggested a requirement for free SH groups in Ag processing for T cell recognition and the binding to MHC molecules.

The results from the responses of T cell hybridomas to synthetic Amb a 5 and Amb t 5 showed that the response of Amb a 5-specific T cell hybridomas to synthetic Amb a 5 required the reduction of synthetic Amb a 5 (Fig. 6). It is likely that this synthetic protein needs to be reduced to allow correct processing for T cell recognition, as Amb a 5-specific hybridomas recognize the a4 epitope in both reduced and unreduced forms. The response of the T cell hybridomas to purified Amb a 5 may be explained by the presence of reduced Amb a 5 in the purified proteins. Interestingly, although reduced synthetic Amb a 5 was expected to contain a reduced form of the a4 epitope, it was not recognized by Amb t 5-specific T cells. A20 cells as APCs may not have processed a reduced a4 epitope to present on their MHC molecule. No cross-reactivity was obtained from Amb t 5-specific hybridomas to reduced purified Amb a 5 (data not shown). Although the recognition by Amb a 5-specific T cells can still occur, the a4 epitope produced by intracellular processing may not remain reduced or in a form that can be recognized by Amb t 5-specific T cells.

Sequence analysis of MHC class II binding molecules has revealed that the length of peptide bound to MHC class II is 12 to 18 amino acids and has ragged termini (35–37). The electron density in the binding groove of the HLA-DR1 crystallographic structure suggested a length of 15 amino acid residues in contact with HLA-DR (38). The minimal epitope of Amb a 5 (Fig. 5), peptide a4.3 (residues 27–36), is 10 amino acids. The response of T cell hybridomas to this peptide was much weaker than the response to Amb a 5, indicating that peptide a4.3 contains the essential residues for binding of TCR and MHC molecule, but needs additional supporting residues to induce a strong response. The T cell epitope mapped in Amb t 5 (Fig. 5), peptide t4.2 (residues 24–34), is 11 amino acid residues in length. Peptide t4.3 (Fig. 5), with residues K and Y at the N terminus deleted from t4.2, had no T cell reactivity. Peptides t4 and t5 both induced responses from polyclonal T cells (Fig. 3) and T cell hybridomas (reduced t5, Table II). The octapeptide that covers the overlapping region of peptides t4 and t5 failed to induce a response. It may contain a partial agetope, resulting in the lack of T cell-stimulatory activity.

It is interesting to note that the T cell epitopes recognized by our murine T cell hybridomas may be the same or similar to the epitope recognized by three human DR2-restricted T cell clones from a ragweed-allergic patient (27, 28). Three Amb a 5-specific human T cell clones were inhibited by peptide analogues of Amb a 5, residues 31–44 but not 12–26, with the cysteines substituted by alanine residues. The authors suggested that Amb a 5 and Amb t 5 may share an identical agetope, but have distinct T cell epitopes. The T cell epitope of Amb a 5 defined in BALB/c mice overlaps with the reported human T cell epitope (amino acid sequence 31–36). This region of Amb a 5, VCYESS, shares a high sequence identity with Amb t 5, VCYDSK.

We have demonstrated the cross-reactivity of Amb a 5 and Amb t 5 at the T cell level. This finding is in contrast with previous results in which no cross-reactivity was found at the B cell level for these Ags. We have mapped the T cell epitopes used by BALB/c mice in both proteins. The two T cell epitopes identified, a 10-mer in Amb a 5 and an 11-mer in Amb t 5, are among the shortest peptides reported for MHC class II T cell epitopes, and

also bear no resemblance to the reported I-A^d binding core region (37). This study demonstrates that free sulfhydryl groups are necessary for crossrecognition of an Amb a 5 epitope (a4) by Amb t 5-specific T cell hybridomas (Table III).

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