T-cell activation by peptide antigen: Effect of peptide sequence and method of antigen presentation

(Ia antigens/supported planar membranes)

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ABSTRACT A series of synthetic peptide analogues of a determinant recognized by the ovalbumin-specific, I-Ak-restricted, T-cell hybridoma 3DO-54.8 were synthesized. The resulting peptides were tested for activation of 3DO-54.8 cells by using glutaraldehyde-fixed cells as well as reconstituted membranes as antigen-presenting surfaces. The results show that the minimum epitope for activation of this T cell is between 7 and 11 amino acids in length. This region includes two important histidine residues. The order of preference of the various peptide analogues was the same regardless of the method of antigen presentation. However, the amount of peptide required for T-cell activation was considerably higher when reconstituted membranes, rather than fixed cells, were used as antigen-presenting surfaces.

The activation of helper T cells by antigen requires that the antigen be presented by an accessory cell that also expresses self-molecules encoded by the I region of the murine major histocompatibility complex, the Ia antigens (1–3). Current evidence suggests that a single T-cell receptor molecule is responsible for the recognition of both Ia and foreign antigen (4). This implies that either the T-cell receptor sees a complex of Ia and antigen or that a single receptor molecule has two sites for binding the two components. Some evidence for the former has been obtained in experiments that show that different repeating copolymers can compete for sites on antigen-presenting cells (5, 6).

For several protein antigens, an obligate step in the presentation process is the fragmentation and/or denaturation of antigen (7–9). Studies with purified Ia have shown that peptide fragments (added free in solution) together with I-Ak, reconstituted into planar membranes, are sufficient for activation of an ovalbumin-specific T-cell hybridoma, 3DO-54.8 (10). It is not known whether peptide antigen actually associates with Ia during T-cell activation. Studies with 125I-labeled peptide antigen have shown that the peptide interaction with either T cells or antigen-presenting cells alone is below the limits of detection (11). This suggests that if Ia and peptide interact directly, they do so with high affinity only when present in a ternary complex with T-cell receptor.

Current work in this laboratory is directed toward investigating the interactions between T cells, Ia, and peptide antigens using model membranes (10). Our model system involves the T-cell hybridoma 3DO-54.8, purified I-Ak (reconstituted in supported planar membranes), and the antigen ovalbumin. Shimonkevitz et al. (11) have described a 17-residue tryptic fragment of ovalbumin responsible for the activation of this hybridoma. Delineation of the precise residues involved in T-cell activation is useful both in placing spatial limits on the putative ternary complex and in identifying amino acids that can be modified with crosslinking and fluorescent reagents. To this end, we have synthesized a series of peptide analogues of ovalbumin 323–339 and tested them for T-cell activation by using glutaraldehyde-fixed cells as well as reconstituted membranes as the antigen-presenting surface.

MATERIALS AND METHODS

Materials. Protected amino acids and resins were purchased from Peninsula Laboratories (Belmont, CA). All solvents and chemicals were reagent grade and are listed with their respective suppliers: methylene chloride, 2-propanol, and triethylamine, from Baker; anisole, dicyclohexylcarbodiimide, dimethylformamide, dioxane, indole, methyl sulfide, and trifluoroacetic acid, from Aldrich (Milwaukee, WI).

Cells. The T-cell hybridoma 3DO-54.8 (9) was obtained from P. Marrack and J. Kappler, National Jewish Hospital and Research Center, Denver, CO. Fresh cells were thawed periodically to ensure that the antigen specificity did not drift. Growth of 3DO-54.8, A20-1.11 (12), and CTLL (13) cells was as described (10).

Antigen-Presenting Material. A20-1.11 cells were fixed with glutaraldehyde as described (9). Membrane vesicles consisting of the total membrane proteins of A20-1.11 cells were prepared as described in ref. 10. I-Ak was purified from A20-1.11 cells and reconstituted as described except that I-A was eluted from the MKD6 antibody-affinity column with 30 mM octyl glucose at pH 2 in phosphate buffer. I-Ak was reconstituted at 50 µg/ml of protein and 400 µg/ml of lipid (egg phosphatidylcholine/cholesterol at a 7:2 molar ratio). When the lipid-to-protein ratio was lowered beyond this point (in an attempt to increase the I-A density in the planar membranes), no increase in T-cell stimulation was obtained. This is probably due to a lower efficiency of reconstitution and/or incorporation into planar membranes at low phospholipid-to-protein ratios.

Synthetic Peptides. Peptides were synthesized by the solid-phase method (as reviewed by Erickson and Merrifield, ref. 14) on a Beckman 990B peptide synthesizer. A detailed description of the methodology used in synthesizing these peptides was published recently (15). Briefly, the syntheses were performed on a copoly(styrene/1% divinylbenzene) resin support. The protected amino acids were coupled starting at the COOH-terminal end of the sequence. The NH2-terminal tert-butoxycarbonyl (Boc)-protecting group of the growing peptide chain on the resin was removed by treating the peptide resin with 33% (vol/vol) trifluoroacetic acid in methylene chloride. The deprotected peptide resin was then neutralized with 10% (vol/vol) triethylamine in methylene chloride. The α-COOH group of the next Boc-amino acid to be coupled was then activated in the presence of dicyclohexylcarbodiimide to form a peptide bond with the free NH2 terminus of the peptide resin. The performance of

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Abbreviation: IL-2, interleukin 2.
each coupling step was monitored by treating a few resin beads with ninhydrin (16) and the coupling was repeated if necessary. After completing the desired sequence, the peptide was detached from the resin and all side chain blocking groups were removed by treating the peptide resin with anhydrous hydrofluoric acid in the presence of anisole. The peptides were then extracted from the resin with 5% acetic acid and lyophilized.

Peptides were used directly after lyophilization. Peptide concentration was determined by weight. To check on the validity of using crude peptides, peptides 323–339 and 326–339 were purified on HPLC by using a Vydac C18 reversed-phase column equilibrated in 0.1% trifluoroacetic acid/water. Peptides were eluted with a 50-minute gradient ending at 40% acetonitrile/0.1% trifluoroacetic acid in water. Fractions were collected and assayed for T-cell activation by using glutaraldehyde-fixed antigen-presenting cells as described (11). The active fractions represented 90% of the peptide material. The dose-response curves were indistinguishable (using 1:10 serial dilutions of antigen) between the crude and HPLC-purified synthetic antigens (data not shown).

Acetylation of peptide 329–339 was carried out by using acetic anhydride as described in ref. 17. Acetylation was >90% complete as measured by reactivity with trinitrobenzenesulfonic acid (18).

A tyrosine was added at the COOH terminus of the longer peptides to facilitate labeling by radioliodination. Shimonekivitz et al. (11) have shown previously that this modification does not affect antigenicity of the peptide.

**T-Cell Activation.** Stimulation of T cells by fixed A20-1.11 cells was carried out as described (9). Experiments with planar membranes were carried out as described in ref. 10. Interleukin 2 (IL-2) assays were carried out with the IL-2-dependent cell line CTLL. CTLL cell proliferation was measured by adding 1 μCi (1 Ci = 37 GBq) of [3H]thymidine (NEN; 6.7 μCi/mmol) for the final 12 hr of a 24-hr incubation with IL-2-containing supernatant. The results reported are for the full-strength supernatant. Dilutions of the supernatants showed that the amount of IL-2 was not saturating.

### RESULTS

**Mapping of the Epitope Recognized by 3DO-54.8 Cells.** The abilities of a number of synthetic peptide analogues of ovalbumin 323–339 to stimulate the T-cell hybridoma 3DO-54.8 are compared in Fig. 1. The results shown are for peptides presented by glutaraldehyde-fixed cells. Fig. 1a shows that the peptide can be shortened from its NH2 terminus by three residues with only a small loss of activity. This suggests that the presence of residues 323–326 may enhance binding somewhat but that these residues are not essential for activation. Removal of the next three residues (326–328) results in a loss in sensitivity to antigen by a factor of ~100. To test whether the introduction of a positive charge (by creating a new NH2 terminus) was responsible for the decrease in activity of peptide 329–339 as compared to peptide 326–339, we tested the effect of acetylating the NH2 terminus of the truncated peptide (data not shown). The stimulation activity of the resulting peptide (acetyl-329–339) was indistinguishable from the unacylated analogue. This suggests that at least one of residues 326–328 is involved in activation.

Fig. 1b shows that the peptide can also be shortened from the COOH terminus with only a small decrease in activity. To test whether the small decrease in activity upon removal of residue 337–339 was due to loss of specific side chains or due to an end effect (the introduction of a charged COOH group near the epitope), we synthesized an analogue with three alanines in place of these residues. No increase in stimulation was obtained, suggesting that the loss in activity was due to loss of either glycine-338 or arginine-339. The results of these experiments suggest that the 11-residue peptide (326–336) is sufficient for T-cell stimulation but that a small increase in activity is obtained upon lengthening the peptide in both directions. It was shown previously (11) that removal of residues 334–336 causes a complete loss of activity. This places a limit on the minimum size required for T-cell activation of between 7 and 11 residues—that is, the end points of the epitope are between residues 326 and 328 at the NH2 terminus and between residues 334 and 336 at the COOH terminus.

Fig. 1c shows the effect of amino acid substitution within the 11-residue peptide. Alanine was chosen for these substitutions because it is a residue that is frequently found in a helices (19) and because it is the smallest amino acid with hydrophobic character. Replacement of histidine-331 with alanine completely abolished activity, suggesting that this residue is essential for activation of 3DO-54.8 cells. Valine-327 can be replaced by alanine with only a small decrease in activity (this slight decrease was reproducible in several experiments). Substitution of histidine-328 with alanine caused a slightly bigger decrease, but the effect was not as large as for histidine-331. However, when both valine-327 and histidine-328 were substituted simultaneously, the effect was synergistic. This suggests that residues 327 and 328 contribute to the stimulation activity of the peptides but that neither residue is absolutely essential for T-cell activation.

Although the substitution experiments suggest that two histidines are important in the T-cell activation, peptide 332–339 in Fig. 1a shows that there is some residual activity at high peptide concentrations even in the absence of these residues. This might be due to a small contribution to binding provided by arginine-339, a residue that is absent in peptide 326–336.

**Effect of Method of Antigen Presentation on T-Cell Responsiveness to Antigen.** We have previously described alternate methods of antigen presentation to T-cell hybridomas involving model membranes in place of glutaraldehyde-fixed cells. When the antigen-presenting material consisted of the total membrane proteins of A20-1.11 cells it was found that antigen presentation was successful when the membranes were provided in vesicle form or when provided in planar form on glass supports. For purified I-A, on the other hand, supported planar membranes containing Ia were effective, whereas vesicles were not (10). It was of interest to compare the response of T cells to synthetic peptides by using these different methods of antigen presentation. All 10 of the synthetic peptide analogues described above were tested with total membrane preparations from A20-1.11 cells. In addition, we tested 5 of the truncated peptides with purified I-A* in planar membranes. In all cases, the order of preference of the T cells for the various peptide analogues was the same regardless of the method of antigen presentation (data not shown). However, the amount of peptide required to stimulate IL-2 release from 3DO-54.8 cells was considerably higher in the reconstituted systems than for fixed cells.

Fig. 2 shows the response of 3DO-54.8 cells to peptide 323–339 presented by various model membranes as compared to fixed cells. It can be seen that the midpoint of the titration of peptide 323–339 is 0.02 μM for fixed cells. For purified I-A in planar membranes the midpoint is 2 μM. The amount of I-A present in the two experiments would be comparable if the efficiency of I-A interactions was the same and if all of the I-A in the membrane were correctly oriented and retained a native conformation. This seems unlikely. However, we have not succeeded in incorporating higher amounts of I-A (see Materials and Methods).

Fig. 2, groups B and D, shows the response to peptide obtained when crude membrane preparations of A20-1.11
cells were in the planar (group D) or vesicular form (group B). The amount of material added in the planar form is limited by the size of the solid support. Therefore, the I-A to T-cell ratio for planar A20-1.11 membranes is only \(1.5 \times 10^3\) per 10\(^6\) T cells, or 1/10th of that in the experiment using fixed cells. The midpoint of the peptide titration in this experiment was 63 \(\mu\)M, or less active by a factor of 300 than with fixed antigen-presenting cells. In Fig. 2, group B, 5 \(\times\) 10\(^6\) equivalents of membrane were added. We previously reported that this was the point at which vesicles gave the same response as fixed cells at saturating antigen concentrations (10). In this case one obtains a midpoint of 2 \(\mu\)M. Since the surface for binding antigen is 50 times larger in this experiment due to the addition of more vesicles, it is not surprising that one requires additional antigen to get comparable stimulation.

**DISCUSSION**

Recently, the specific peptide fragments responsible for activation of a number of T-cell clones and hybridomas have been identified (for example, see refs. 11, 21–23). Studies of the determinant recognized by cytochrome c-specific T cells have led to the suggestion by Hansburg et al. (22) that the peptides recognized by T cells can be divided into two domains: an epitope responsible for T-cell interaction and an agretope (24), which interacts with I-A. Similarly, Allen et al. (23) have suggested that a 16-residue antigenic fragment of lysozyme can be divided into two domains: a hydrophobic segment for interaction with I-A or antigen-presenting membrane and a hydrophilic epitope responsible for interaction with T-cell receptor.

Our results show that for the T-cell hybridoma 3DO-54.8, an 11-residue fragment is sufficient for activation. Within this region, histidine-331 appears to be essential and histidine-328 makes a significant contribution. An alanine can be substituted for valine-327, suggesting that the requirement in this position may be for a hydrophobic side chain. However, upon examination of such a short linear sequence, it is difficult to delineate two spatially distinct domains for the binding of T-cell receptor and Ia separately. Therefore, for this antigen we propose the following possibilities. It is possible that the peptide does not interact directly with I-A but that an interaction that occurs between I-A and T-cell receptor leads to a transient allosteric change that allows T-cell receptor to bind peptide. Such a model is suggested by the model of T-cell receptor proposed by Patten et al. (25). On the other hand, it is also possible that peptide binds to a site formed by residues contributed both by T-cell receptor and by the I-A molecule. This would require that peptides presented by a given responder strain have a common feature that would allow such binding. One possibility is a hydrophobic surface, as has been suggested previously (23, 26). Although the linear sequence of ovalbumin peptide 323–339 does not show a particularly obvious hydrophobic stretch, one can generate a hydrophobic surface by modeling the peptide as an α helix.

Fig. 3 shows an α-helical representation of the ovalbumin peptide 323–339. The projection shows the view one obtains by cutting the helix along its long axis and opening it flat. A possible hydrophobic stretch is outlined. Removal of the nonessential residues 323–325 and 337–339 shortens this stretch to four residues. Alternatively, alanine-326, -329, and -330 together with valine-327 and isoleucine-323 could be viewed as a hydrophobic cluster. The allowed substitution of alanine for valine-327 does not disrupt the hydrophobic stretch. This view emphasizes the spatial proximity of the two important histidines. These residues are not contained within the hydrophobic cluster. One could speculate that histidine-328 and -331 plus additional neighboring residues form the T-cell binding site and that a cluster of four to six hydrophobic residues forms the I-A binding domain. Such a small site would not be expected to form a stable binding site. However, it could function to stabilize the interaction of...
T-cell receptor and peptide. Further stabilization of the complex might be obtained through additional I-A/T-cell receptor contacts. Therefore, the fact that the epitope recognized by 3DO-54.8 is contained within such a small fragment does not completely rule out the possibility that there are two functional sites on the peptide. It will be of interest to compare a variety of epitopes in detail to see if such a model holds up.

It is also worth noting that the likelihood of the formation of a ternary complex through a three-way collision is very small. We suggest that the third component of the complex (whether it be T-cell receptor, peptide, or I-A) interacts with a preformed binary complex (of lower stability) to form the more stable ternary complex.

The interaction of T cells with I-A and peptide antigens has been inferred from experiments that show a direct relationship between T-cell activation and the presence of these molecules. However, there are no direct binding data (at least for peptide antigens) demonstrating a physical interaction between these components. In attempting to investigate these interactions by using biophysical approaches, it will be important to bear in mind that considerably more peptide is required when reconstituted membranes are used for antigen presentation as compared to fixed cells. It is not clear why the reconstituted membranes are less effective than the fixed cells in antigen presentation. In the case of membrane vesicles, in which no actual fractionation of membrane proteins is done, one could attribute the effect to a poorer surface for contact. For the purified I-A in planar membranes, we do not know how much of the I-A is accessible and in its native conformation in the final preparation. It is also possible that other accessory molecules that are absent in the purified preparations contribute to T-cell activation. However, it is unlikely that such molecules play a direct role in the binding of peptide, since the order of peptide preference for stimulation was the same in the reconstituted system as with glutaraldehyde-fixed antigen-presenting cells.

Note Added in Proof. It has recently come to our attention that Delisi and Berzofsky (27) have proposed a similar role for amphipathic structures in antigen presentation.

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