RECONSTRUCTION OF THE IMMUNOGENIC PEPTIDE RNase(43–56) BY IDENTIFICATION AND TRANSFER OF THE CRITICAL RESIDUES INTO AN UNRELATED PEPTIDE BACKBONE

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The recognition of foreign antigen is one of the key events in the induction of an immune response. It is now accepted that T lymphocytes recognize a fragment of antigen bound to an MHC molecule (1–7). The discovery that the antigen fragments that bind to MHC molecules and are recognized by T cells can be represented by linear synthetic peptides has greatly facilitated the elucidation of the exact nature of antigen recognition (8–11). In this model, the antigen must be able to simultaneously contact the MHC molecule as well as the TCR (12), features that have been termed agretopes and epitopes, respectively, by Heber-Katz et al. (13). There have now been many different peptide determinants that have been analyzed in detail, including hen egg-white lysozyme (14), ovalbumin (15), and moth/pigeon cytochrome c (16). Several different approaches have been used in these studies; however, they all share a common feature in that they determined the effect of single amino acid substitutions on the function of a peptide antigen. The main conclusions reached by these authors are that individual residues in an immunogenic peptide can be identified as TCR contact residues or MHC molecule binding residues. These amino acid residues are not grouped together, but are instead interspersed among each other and among nonessential residues. Despite these studies, it is still unclear as to how and where a peptide precisely binds to a MHC molecule and what is the conformation of a peptide as it is bound and recognized by the TCR.

In the studies described below, we have approached this problem of MHC-peptide-TCR interaction by determining which residues in an immunogenic peptide are crucial for antigen-specific TCR recognition. Our working hypothesis was that if certain amino acid residues in a peptide are critical for T cell recognition, then these crucial residues should be sufficient to completely reconstitute antigen-specific T cell recognition when the residues are put in the context of an unrelated peptide backbone. We have taken the experimental approach of initially substituting an alanine residue at each position of a peptide antigen, and then testing these peptides for activity. From these results, a second generation of peptides was designed, synthesized, and tested, with the overall goal of this work being to identify the residues

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that are critical for the formation of a peptide determinant. In this manuscript, we report on the analysis of the I-Ak-restricted determinant RNase(43-56), from which we were able to identify the critical residues. These five identified residues were then substituted onto an unrelated peptide backbone and the resulting peptide was tested for its ability to stimulate an RNase-specific T cell hybridoma. The 15 amino acid hybrid peptide, which contains only five residues from the RNase determinant, was able to stimulate the RNase(43-56)-specific T cell hybridoma in a manner similar to that of the RNase(43-56) peptide. In addition, when tested in vivo this chimeric peptide was able to prime T cells to respond to the bovine RNase molecule.

Materials and Methods

**RNase-specific T Cell Hybridoma.** The generation and characterization of the I-Ak-restricted, RNase-specific T cell hybridoma TS12 has been previously described in detail (17).

**T Cell Stimulation Assay.** The stimulation of T cell hybridomas was performed exactly as previously described (17, 18). The source of APCs were peptone elicited peritoneal macrophages from CBA/J mice that had been previously infected with *Listeria monocytogenes*. The macrophage monolayers were fixed in 0.1% gluteraldehyde before exposure to antigen. To these fixed monolayers of macrophages, various concentrations of the peptide antigens were added along with $10^5$ TS12 T cell hybridomas. The level of stimulation was then determined by quantitating the level of IL-2 production by[^H]thymidine incorporation into the IL-2-dependent cell line CTLL-2. For competition assays, the competing and stimulating antigens were added sequentially and were present throughout the entire culture period.

**T Cell Proliferation Assay.** Two CBA/J mice were primed in each of the rear foot pads with 20 nmol or 50 nmol of antigen emulsified in CFA (H37Rv, Adjuvant Complete Difco Laboratories, Inc., Detroit, MI). 7-10 d later the popliteal lymph nodes were removed, pooled, and a single cell suspension was prepared. These cells were cultured in 96 well microtiter plates (No. 3596; Costar, Cambridge, MA) in a total volume of 0.2 ml. The culture medium consisted of RPMI 1640 supplemented with 2 mM l-Glutamine, 1 mM Hepes, 1 mM sodium pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin, 5 x 10^{-5} M 2-ME, and 0.5% (vol/vol) of freshly obtained autologous normal mouse serum. Each antigen was tested at concentrations from 100 to 0.01 μM. The background proliferation was determined from cultures that contained medium alone. The cultures were incubated at 37°C for 96 h, with each well being pulsed with 0.4 μCi of[^H]thymidine for the last 18 h of the culture. Each group contained a positive control that consisted of wells containing purified protein derivative (PPD, 100 μg/ml).

**Peptide Synthesis, Purification, and Analysis.** All of the peptides were synthesized manually using the FMOC strategy on a RmPS™ multiple peptide synthesis system (DuPont Co., Wilmington, DE) (19). Each peptide was purified using reverse-phase, high-pressure liquid chromatography (14, 20). The composition of each peptide was verified by fast atom bombardment mass spectrometry (21).

**Results**

**Localization of the RNase(43-56) Determinant.** We had previously shown that TS12 cells recognize a determinant of bovine RNase contained in residues 40–61 (17). To further localize this determinant we generated a series of overlapping 15-mers covering the entire 40–61 sequence. These peptides were then tested for their ability to stimulate TS12 (Fig. 1). Four of these peptides (41-55, 42-56, 43-57, and 44-58) stimulated the TS12 cells, whereas the 45-59 and 46-60 (data not shown) did not

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1 Abbreviations used in this paper: cIR, bacteriophage XcI; HA, hemagglutinin; Matrix, influenza matrix protein; Nase, staphylococcal nuclease; PPD, purified protein derivative; RNase, bovine ribonuclease.
stimulate even at 100 μM. Of the peptides tested, 42–56 and 43–57 were the most stimulatory, although both were 10-fold less potent than 41–61. From these results we chose the 14-mer 43–56 to use as the core sequence for our further investigations (Table I).

Identification of the Critical Residues of RNase(43–56). To identify the amino acid residues in the RNase(43–56) molecule that were critical in the formation of the determinant recognized by the TS12 cells, we used the strategy of testing peptides containing single amino acid substitutions. Initially a series of single alanine substituted peptides were tested to identify potential critical residues. At positions that contained an alanine residue in the native sequence, a serine residue was substituted. If a substitution with an alanine residue significantly altered the activity of the peptide, then that position was then further examined by a series of peptides that contained conservative amino acid substitutions. Shown in Fig. 2 are the results when each of the alanine substituted peptides were tested for their ability to stimulate TS12 cells. Single alanine substitutions at four positions dramatically decreased the potency of the peptides, those being at positions 46, 47, 48, and 51. All of the other peptides

**Table I**

| Designation | Sequence |
|-------------|----------|
| RNase(43-56) | V N T F V H E S L A D V Q A |
| Y′46         | Y        |
| R′48         | R        |
| I′51         | I        |

The amino acid sequence of the bovine ribonuclease determinant RNase(43–56) and that of peptides containing substitutions are shown. The standard single-letter codes for the amino acids are used. The numbers refer to the position numbers in the native bovine ribonuclease molecule (30). The designation of the peptide and the position of the substituted residues in the peptides are shown. The solid line indicates homology with the residues in the native RNase(43–56) molecule.
An alanine scan of the RNase(43-56) determinant. A series of peptides were synthesized that contained single alanine substitutions at each of the indicated positions. A serine residue was substituted for the alanine residues found in the native sequence. Each peptide was tested for its ability to stimulate TS12 cells over a concentration range of 0.01 to 100 μM using the assay conditions described in the Materials and Methods. The relative stimulatory capacity of each peptide was determined from the ratio of the concentration of the substituted peptide required for a 50% maximal response to the same value determined for the native RNase(43-56) molecule. The 100% value was the maximal value obtained from stimulation with the native RNase(43-56). Thus, a value of 0.1 indicated that 10 times more of the substituted peptide than of RNase(43-56) was required to achieve the same level of stimulation. The solid bar represents the response of the native peptide RNase(43-56). The values shown are from single experiment and this experiment has been performed twice with identical results.

stimulated TS12 in an unaltered or slightly decreased manner. Eight positions, including the four identified from the alanine scan, were then further examined by conservative substitutions. A summary of these results is shown in Fig. 3. Of the four potentially critical residues, three of them showed remarkable specificity. The Phe-46 when replaced with a tyrosine residue was totally inactive, and likewise, the substitution of the His-48 with either an arginine or a glutamine residue also resulted in completely inactive peptides. The Leu-51 position showed remarkable specificity in that isoleucine and valine substitutions were not active, while a norleucine substitution was only very weakly active. At position Val-47 there appeared to be less specificity in that an isoleucine residue was fully active, while a leucine or alanine substitution was much less active. Other positions which were similarly examined were the Glu-49, Ser-50, Ala-52, and Val-54. The Glu-49 residue could be replaced with nonconservative substitutions including an alanine or a glycine residue. In a similar manner, the Ala-52 could be replaced with a serine or a glycine and the Val-54 could be replaced with an alanine or isoleucine. We had previously examined position 50 in detail since this is the only position that differs from the self murine RNase molecule. We had found that a wide variety of residues could be substituted, and only the extreme substitution of a proline residue (which is the corresponding residue in the murine RNase sequence) resulted in the loss of activity (17). Substitutions at two positions, an isoleucine for the valine at position 47 and a glycine for
the alanine at position 52 resulted in peptides with enhanced stimulatory characteristics. From this analysis we concluded that three of the four potentially critical positions; residues Phe-46, His-48, and Leu-51 had very stringent requirements and could not be replaced even with very conservative substitutions. In contrast, the Val-47 showed less specificity in that it could be replaced with an isoleucine residue.

Reconstruction of the RNase(43-56) Determinant by Transferring the Critical Residues into an Unrelated Peptide. To directly demonstrate the crucial nature of these four potentially critical residues, we generated chimeric peptides in which these residues were substituted onto an unrelated peptide backbone. If these residues were the crucial residues for the formation of the RNase(43-56) determinant then we should be able to insert only these four residues into another peptide backbone, and consequently transfer the RNase(43-56) specificity. The peptide backbone that we chose was the determinant of influenza hemagglutinin (HA) contained in the residues HA(130-142) (22). This peptide had been previously shown to bind to both I-A^d and I-A^d molecules and to contain an amino acid motif found in I-A^d binding peptides (VTAACS) (23). The I-A^d motif is a sequence of amino acid residues initially observed in the ovalalbumin(232-339) sequence. From an examination of several different I-A^d binding peptides, Sette et al. (23) found that they all shared a form of this common sequence. The RNase(43-56) peptide also contained an I-A^d motif (VHESLA) and this motif was used initially to align these two peptide sequences (Fig. 4A). In this alignment the two peptides only had a single identical residue, a valine. We then generated a series of peptides that contained different assortments of these four critical residues. Each of these peptides was then tested for its ability to stimulate the TS12 hybridoma (Fig. 4B). Substitution of residues Phe-46, His-48, and Leu-51, which were the positions with absolute specificity, did not result in an active peptide (RN/HAI). Likewise substitution of the residues FVHESL (positions 46-51 in bovine RNase) did not generate a stimulatory peptide (data not shown). We concluded from these initial results that these four residues were not sufficient to transfer RNase (43-56) recognition to the HA molecule. To elucidate what other positions in
The amino acid sequence of RN/HA chimeric peptides. The sequence of the influenza HA peptide determinant HA(130-144) and RNase(43-56) are shown along with the two chimeric peptides RN/HAI and RN/HA2. The amino acid residues are indicated by the standard single-letter code. The highlighted residues are those from the RNase(43-56) sequence.

(B) The response of TS12 cells to the RN/HA chimeric peptides RN/HAI and RN/HA2. The T cell stimulation assay was performed as described in the Materials and Methods. The values represent the mean ± SD of triplicate values.

RNase(43–56) were critical for formation of the T cell determinant, we generated a peptide that included 9 amino acids identical to RNase out of 15 positions. This hybrid molecule did stimulate the TS12 hybridoma (data not shown). To further investigate this initial positive result, a series of chimeric peptides with fewer amino acids derived from the RNase peptide were generated and functionally tested. When the Phe-46, His-48, and Leu-51 were transferred along with the Val-54 an active peptide resulted (RN/HA2). It should be noted that although only four positions in the HA backbone were replaced with amino acids from RNase, there are actually five residues that correspond to the RNase peptide. This is due to the fact that the Val-47 is shared by both peptide backbones. Significantly, the RN/HA2 peptide was as stimulatory as the RNase(43–56) peptide. Thus a chimeric peptide in which only 5 of the 15 residues were from the RNase(43–56) sequence was fully able to stimulate the RNase(43–56)-specific T cells. This result confirms that these five residues confer all of the specificity of the RNase(43–56) determinant. It should be pointed out that the Val-47 cannot be identified as a crucial residue using this chimeric model, as it is shared by both the original RNase peptide and the HA backbone. One other interesting observation from this chimeric approach is that the Val-54 was identified as an important residue for TS12 recognition, even though the other experimental methods did not highlight this as a crucial position. From the results reported here, we cannot determine whether the two valine residues Val-47 and Val-54 are crucial because of their specific side chains or whether they are important because of factors such as hydrophobicity.

The Reconstructed Peptide RN/HA2 Can Prime In Vivo for RNase. Since we had shown that the peptide RN/HA2 could stimulate the TS12 cells as strongly as the
RNase(43-56) molecule, we wanted to determine if this chimeric peptide could be used to prime in vivo for reactivity to the native RNase molecule in vitro. CBA/J mice were primed with 20 nmol of RN/HA2 and then stimulated in vitro with either RN/HA2 or native RNase (Fig. 5). The RN/HA2 peptide was able to prime RNase-specific T cells, thus confirming the functional crossreactivity of these two peptides. Therefore, even though the RNase(43-56) and RN/HA2 peptides only shared 5 residues, they were functionally equivalent in that they could stimulate RNase(43-56)-specific T cells in vivo and in vitro.

Role of the Critical Residues. The individual amino acid residues of a peptide determinant could play one of several different roles. The amino acid residues could be involved in binding to the Ia molecule, contacting the TCR, determining the conformation of the peptide, or simply acting as spacer residues. We next examined the critical residues of RNase(43-56) and tried to determine which of these roles they played. We initially determined if the substituted peptides still could bind to I-A\(^b\) using a functional competition assay (4). In the autologous system we could only test the nonstimulatory peptides Y46, R48, and I51 (see Table I for a complete description of these peptide designations). These results are shown in Table II. The Y46 peptide was strongly able to compete for the presentation of RNase(43-56), whereas the R48 and I51 peptides did not compete. These results indicated that the R48 and I51 peptides were nonstimulatory because they were unable to bind to Ia, while the Y46 peptide could bind and must be involved in contacting the TCR. By definition, the substituted peptides that can stimulate the TSI2 hybridoma are binding the I-A\(^b\) molecule, and since the isoleucine substitutions at positions 47 and 54 were stimulatory they could not be tested in this assay. Thus, of the three positions directly tested, only the Phe-46 could be unequivocally assigned a role of contacting the TCR, and the His-48 and Leu-51 were either involved in contacting Ia or assisting in the conformation of the peptide.

Examination of Phe-46, His-48, Leu-51 Using In Vivo Primed T Cells. The identification of the RNase(43-56) determinant in general used a single T cell hybridoma. To further examine these findings in bulk T cell populations, we tested the ability of these substituted peptides to stimulate in vivo primed populations of T cells. This general approach has been used previously by Schwartz and his colleagues to examine the role of each of the residues in several cytochrome \(c\) peptides (16, 24). We examined the three critical residues Phe-46, His-48, Leu-51 using the nonstimulatory analogues Y46, R48, and I51. Primed lymph node cells were tested for their ability to be stimulated by either the native RNase(43-56) or the three substituted peptides.

**Figure 5.** The ability of the RN/HA2 peptide to prime bovine RNase-reactive T cells. CBA/J mice were primed with 20 nmol of RN/HA2 and a lymph node proliferation assay was performed as described in Materials and Methods. The lymph node cells were stimulated in vitro with the indicated concentrations of either RN/HA2 or native RNase. The values shown are the mean ± SD of triplicate values from which the background values from culture wells containing no antigen were subtracted (1,623 ± 189). This is a representative experiment from two separate determinations.
Table II

| Competitor | Molar excess | T cell response | Percent inhibition |
|------------|--------------|-----------------|--------------------|
|            | cpm x 10^{-3} |                  |                    |
| Medium     |              | 43.7 ± 0.8      |                    |
| Y46        | 32           | 31.7 ± 1.5      | 27.4               |
|            | 100          | 8.6 ± 4.2       | 80.4               |
| R48        | 32           | 45.5 ± 2.0      | -6.4               |
|            | 100          | 44.8 ± 1.7      | -2.6               |
| I51        | 32           | 44.7 ± 0.8      | -2.3               |
|            | 100          | 41.1 ± 2.1      | 5.9                |

Three nonstimulatory analogues of RNase(43-56) were tested for their ability to compete for the presentation of RNase(43-56) to TS12 cells as described in the Materials and Methods. To monolayers of fixed macrophages, 1 µM of RNase(43-56) was added along with either culture medium, or 32 µM or 100 µM of the indicated peptide. The values represent the mean ± SD of triplicate determinations. The percent inhibition was calculated from the formula 100 x [(cpm of stimulator alone - cpm of stimulator plus competitor/cpm of stimulator alone)]. The sequences of the substituted peptides are given in Table I.

Figure 6. The ability of RNase(43-56) and single amino acid substituted peptides to prime lymph node cells in vivo and to stimulate in vitro. CBA/J mice were primed with either 20 nmol of RNase(43-56) or Y46 or with 100 nmol of R48, or I51. The T cell proliferation assay was performed as described in the Materials and Methods. The lymph node cells were stimulated with the indicated peptides in vitro at concentrations from 0.01 to 100 µM. For simplicity, only the maximal values which were obtained from the 10 µM concentration are shown. The response of the lymph node cells to 100 µg/ml of PPD was included as the positive control and was 116,013 ± 11,486, 31,727 ± 4,367, 57,733 ± 7,185, and 62,970 ± 1,219 for RNase(43-56), Y46, R48, and I51, respectively. The values shown are the mean ± SD of triplicate values from which the background responses to medium alone have been subtracted (31).
When the cells were in vivo primed with RNase(43-56) and then stimulated in vitro with either RNase(43-56) or the three single amino acid substituted peptides, it was observed that the RNase(43-56) showed the best recall stimulation, but that all three other peptides were able to stimulate (Fig. 6). When the Y46 peptide was used for priming, again a good response was detected; however, the RNase(43-56) did not stimulate nearly as well as the priming antigen Y46. Thus the Y46 peptide was eliciting a new population of T cells, indicating that this residue was involved in contacting the TCR. In contrast, the R48 and L51 peptides did not appear to be able to prime in vivo, even when a fivefold higher concentration of peptide was used for priming.

The results obtained in these experiments are identical to those obtained using the single cell line and the competition assay, in that the Phe-46 residue appears to be contacting the TCR, while the His-48 and Leu-51 residues are involved in the binding/conformation of the peptide.

Discussion

We have previously demonstrated that TS12, the bovine RNase-specific T cell hybridoma used in this study, was specific for the 22-mer RNase(40-61). In this report, we have shown that the 14 amino acid peptide RNase(43-56) can also stimulate the TS12 hybridoma. Through the use of a panel of synthetic peptides containing single amino acid substitutions, we have determined that there are four residues crucial to the formation of the TS12 determinant, the Phe-46, Val-47, His-48, and Leu-51. The Phe-46 was determined to be a TCR contact residue, while the other three residues either function in Ia contact or peptide conformation. To test the critical nature of these four amino acids, they were substituted into the unrelated peptide backbone HA(130-144). This chimeric peptide (RN/HA1) was not able to stimulate the TS12 hybridoma. However, when only one additional amino acid residue from the RNase peptide, Val-54, was substituted into the HA backbone, stimulation of the TS12 hybrid was seen. The activity of this peptide was equivalent to the native RNase(43-56) in vitro and it was able to prime RNase-specific T cells in vivo. In brief, our studies have shown that these five amino acids from the RNase sequence are necessary and sufficient for RNase(43-56)-specific T cell stimulation.

There have been two previous reports of generating chimeric peptides. Guillet et al. were able to transfer three residues of the 12-26 determinant of the cIR to the very similar sequence of Nase(66-78) and create an active peptide (25). The residues that were substituted were chosen because they were the only three residues that were not either identical or conservative substitutions between the cIR and Nase determinants. They were also able to transfer the same three residues to the unrelated ovalbumin sequence (324-336) and again create an active neo-peptide. Both of these chimeric peptides were active, but ~1,000-fold weaker than the native 12-26 molecule. Rothbard et al. generated chimeric peptides between the HA(307-319) determinant and the matrix(17-29) (26). The overall strategy used involved the substitution of residues predicted to be on one face of an α-helix. Using this procedure, they were able to generate an active chimeric peptide by substituting 7 of 13 residues. The resulting peptide was ~300-fold weaker than the native HA(307-318) molecule. When 10 of the 13 residues were substituted, the resulting peptide was nearly as active as the native HA(307-318) peptide. Although our strategy for identifying the critical residues was different, our results are similar to those of Guillet et al. (25)
and Rothbard et al. (26). A major difference is that we were able to generate chimeric peptides that were nearly as active as the native sequence, and that we were able to show that in vivo the native and the chimeric peptide appeared to be functionally identical in their ability to prime for T cell responses.

During our examination of chimeric peptides, we have gained an appreciation of the sensitive nature of the antigenic determinants recognized by T cells. We have found that many of the nonstimulatory chimeric peptides are not able to bind to I-A<sup>k</sup>. Thus it appears that in order to successfully generate a chimeric peptide, one has to transfer the residues that contact the TCR or the I<sub>a</sub> molecule, as well as neighboring residues that will permit the correct conformation to be formed. An example of the fragile nature of these determinants is seen if one takes the RN/H2A peptide and replaces the Gly-55 with Gln-55 from the RNase(43-56) sequence, the resulting peptide is now completely inactive (data not shown). By replacing an additional residue of the chimeric peptide RN/H2A, one has completely destroyed the activity of the peptide. This phenomenon has been previously observed by Rothbard and his colleagues during their examination of the series of HA/matrix chimeric peptides. Replacing 7 of 13 residues generated an active peptide, whereas replacing 8 of 13 resulted in an inactive peptide (26). Therefore, our previous classification of the residues of a determinant into T cell contact, I<sub>a</sub> contact, and spacer residues should now be extended to include residues that are involved in allowing the peptide to adopt the correct conformation. To generate active chimeric peptides one has to include other residues, in addition to the T cell and I<sub>a</sub> contact residues. These additional amino acids permit or assist the peptide into adopting the correct conformation to bind to an I<sub>a</sub> molecule and be recognized by the T cell.

As we have discussed previously, the RNase(43-56) determinant is predicted by several of the current methods for predicting T cell epitopes. It is predicted by the DeLisi and Berzofsky amphipathic α-helix algorithm (27) and it contains a Rothbard motif (ESLAD) (28). The RNase(43-56) determinant also possesses the I-A<sup>d</sup> motif (23), although H-2<sup>d</sup> mice do not respond to the RNase(43-56) peptide (data not shown). The three predictive methods are not mutually exclusive and for the RNase(43-56) determinant are probably identifying the same common feature.

In this study, we observed that substitution of conservative amino acid residues for the His-48 or Leu-51, dramatically decreased the resulting peptide's ability to bind to the I-A<sup>k</sup> molecule. Similar results were obtained when the HEL(52-61) determinant was analyzed (14). Thus for these two determinants, the replacement of a single critical residue could almost completely abrogate a peptide from binding to I-A<sup>k</sup>. These results are contrasted by those obtained from detailed examinations of two other determinants. Sette et al. found that for the ovalbumin(323-339) determinant (15), no single conservative substitution dramatically affected a peptide's ability to bind. When the determinant HEL(34-45) was similarly analyzed, again the substitution of no single amino acid residue completely abolished binding (Lambert, L. E., and E. R. Unanue, manuscript submitted for publication). Thus, from this limited sample it appears that determinants can be divided into two groups; one in which an individual amino acid residue appears to play a major role in binding to an I<sub>a</sub> molecule, and another in which the binding to an I<sub>a</sub> molecule must be due to the collective binding of several different amino acid residues.

During the examination of the critical residues, we identified that the Phe-46 ap-
peared to contribute the majority of the T cell specificity. This residue could not be replaced with even the closely related tyrosine residue and in vivo priming with substitutions at this position elicited unique populations of T cells. When we have examined other peptide determinants we have also found that, in general, one or two positions seem to predominate in determining the T cell specificity (14; our unpublished observation). There are other residues in RNase(43–56) that most likely are contacting the TCR, but the Phe-46 residue appears to determine most of the specificity.

Another interesting aspect from this examination involved the length of the RNase(43–56) determinant. Four of the five critical residues are contained in the 6-mer 46–51, and when tested, this peptide did not stimulate the TS12 cells, even at a concentration of 10 mM. All five of the critical residues were contained in the 9-mer 46–54 (Table I). When this peptide was tested for its ability to stimulate TS12 cells, we detected only a very weak response at 100 μM, which made the stimulation by this peptide 10,000 times weaker than the native RNase(43–56) (data not shown). Even though this 46–54 peptide contained all of the critical residues, there appeared to be a requirement for residues on either side of this core. The specificity of these residues was not very stringent since they could individually be replaced with alanine residues, or collectively replaced with the residues from the HA(130–144) sequence. We believe that these other residues could be involved in secondary binding events to the Ia molecule or be required for the peptide to adopt the correct conformation. There has been a recent report of a class I-restricted epitope that could be represented by a pentapeptide which contained the Rothbard motif (29). Our findings with the RNase(43–56) determinant are different, in that either a 6-mer or a 9-mer, both of which contained the Rothbard motif, were essentially not active. The difference between these two results could be due to the sensitivity of the assay systems used or simply reflects a difference between the two determinants.

Since we were able to regenerate the RNase(43–56) determinant by transferring only five residues, the question arises as to how frequently this pattern of amino acid residues occurs. One could envision the potential for crossreactive T cell determinants if only five residues are needed. We searched the Protein Identification Resource database (Release 17.0; June 30, 1988; 5407 sequences; 1,448,175 residues) for the occurrence of the FVHXXLXXV sequence, where the X represents any amino acid residue. Excluding related ribonuclease molecules, only a single example of this sequence was found, that being residues 834–842 from the glycoprotein precursor of the Rift Valley fever virus (FVHTYLQSV). Thus, even though a determinant can be recreated from five residues, the rare occurrence of this sequence pattern is consistent with the observation that T cell responses are highly specific.

Summary

The involvement of each of the amino acid residues of the I-Ak-restricted T cell determinant RNase(43–56) was examined in detail using a series of peptides containing single amino acid substitutions. Four positions were identified as being essential for the formation of the determinant, Phe-46, Val-47, His-48, and Leu-51. When these four residues were substituted into the backbone of the unrelated peptide HA(130–144), a nonstimulatory peptide was obtained. The inclusion of an ad-
ditional residue, Val-54, resulted in a chimeric peptide, RN/HA2, which was nearly as active as the native molecule. The peptide RN/HA2 was able to prime in vivo for RNase reactivity, confirming that these five residues contained all of the specificity of the RNase(43–56) determinant. The role of three of these critical residues was examined using both a functional competition assay and an in vivo priming assay. It was ascertained that the Phe-46 was directly involved in contacting the TCR, while the His-48 and Leu-51 were either involved in binding to the I-A<sup>+</sup> molecule or in determining the conformation of the peptide. Thus, by critically evaluating the contribution of each of the amino acid residues in a T cell determinant, we were able to generate a chimeric peptide only containing 5 of 15 residues from the RNase(43–56) sequence that was functionally identical to the native RNase(43–56) molecule both in vitro and in vivo.

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