THE T CELL REPERTOIRE FOR RECOGNITION OF A PHYLOGENETICALLY DISTANT PROTEIN ANTIGEN

Peptide Specificity and MHC Restriction of Staphylococcal Nuclease–specific T Cell Clones

BY ALISON FINNEGAN,* MELANIE A. SMITH,* JOHN A. SMITH,‡ JAY BERZOFSKY,§ DAVID H. SACHS,* AND RICHARD J. HODES*

*From the *Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892; the ‡Departments of Molecular Biology and Pathology, Massachusetts General Hospital, and the Department of Pathology, Harvard Medical School, Boston, Massachusetts 02114; and the §Metabolism Branch, National Cancer Institute, National Institutes of Health, Bethesda Maryland 20892*

The diversity of the T cell repertoire specific for foreign antigens has been evaluated by a number of experimental approaches. To this end, monoclonal T cell populations have recently been used to probe the fine specificity of the antigen and Ia epitopes recognized by T cells, in particular where the overall antigen molecule encountered by the T cell is a potentially complex foreign protein. Previous analyses of the antigen fine specificity of cloned T cells have used variants of protein antigens, cleavage fragments derived from intact antigen molecules, or synthetic peptides made to represent native or variant amino acid sequences expressed on the antigen molecule (reviewed in references 1 and 2). These studies have identified the antigenic peptides recognized on protein antigens by T cells in several model systems, including the responses of cloned T cells to cytochrome *c* (3–5), myoglobin (6–9), lysozyme (10–11), OVA (12, 13), and influenza virus hemagglutinin (14). In each of the systems in which a panel of T cells was studied, it has appeared that only a small number of antigenic epitopes on the overall protein antigen are recognized by T cells (3–13). Moreover, when the association between antigen specificity and Ia specificity has been evaluated, an additional constraint on the T cell repertoire has been recognized since strong preferential associations appear to exist between the antigenic epitope recognized and the self Ia molecule recognized by the T cell in association with the antigen.

The reason(s) for this apparently limited antigen plus Ia fine specificity repertoire expressed by T cells is not yet clear. Structural constraints at the level of antigen processing, presentation, or antigen and Ia association have been proposed to account for at least some examples of immunodominance (15, 16). The other major parameter that might be expected to influence apparent immunodominance is the extent of the expressed T cell receptor repertoire. This

J. A. Smith was supported by a grant from Hoechst Aktiengesellschaft. Address correspondence to Alison Finnegan, Bldg. 10, Rm. 4B10, National Institutes of Health, Bethesda, MD 20892.

repertoire matures under influences that include strong effects of the T cell maturation environment, resulting in both self tolerance and in skewing toward self MHC restriction. It therefore follows that experimental systems used to study the diversity of the T cell repertoire for foreign antigens will be influenced by the effects of self tolerance on that repertoire. Thus, for antigens that are structurally and antigenically similar to the self proteins of a responding organism, it is likely that the number of epitopes on that antigen that will be recognized by T cells will be limited to those that differ from the self proteins to which physiologic tolerance has been induced. It is notable that the antigens that have to date been used most extensively to study the fine specificity of the T cell repertoire are cytochrome *c*, myoglobin, lysozyme, and OVA. Each of these antigens differs from the homologues expressed in responding experimental mice by only a few amino acids. Although this limited species variation has been exploited in defining antigenic epitopes, it has also left open the possibility that apparent limitations in the number of immunogenic epitopes recognized by T cells on complex antigenic molecules reflect the outcome of self tolerance to most of the determinants on these closely related molecules.

The present studies evaluate the diversity expressed in the T cell repertoire specific for an antigen that is more distant from mammalian proteins. A series of T cell clones was generated specific for the antigen staphylococcal nuclease (Nase),[1] a bacterially derived protein that is 149 amino acids in length. The use of a series of overlapping 20-amino-acid synthetic peptides spanning the entire Nase molecule has allowed the assignment of antigen fine specificities to these clones and has permitted an analysis of the relationship between the antigenic determinant recognized by a given T cell and the self MHC product used as a restricting element by that T cell.

Materials and Methods

Animals. C57BL/10 (B10), B10.A (5R), B10.A (4R), and (C57BL/6 × A/J)F_1 mice were purchased from The Jackson Laboratory, Bar Harbor, ME. [B10.A (5R) × B10.A (4R)]F_1 mice were bred in our own colony.

Antigens. Lysozyme was purchased from Cooper Biomedical, Inc. (Malvern, PA), and Nase was either purchased (Boehringer Mannheim, Penzberg, Federal Republic of Germany) or prepared in this laboratory. Nase fragments 1-126, 127-149, 6-48, 49-149, and 99-149 were prepared by trypsin digestion and CNBr cleavage of Nase, as previously described (17).

Nase peptides were synthesized by the solid-phase method (18). The *t*-butoxycarbonyl(Boc)-amino acid coupled to hydroxymethyl-phenyl-acetaminodomethyl(Pam)-polystyrene was purchased from Applied Biosystems, Inc. (Foster City, CA). The peptide assembly at a 0.5 mmol scale was carried out automatically with an Applied Biosystems, Inc. 430A Peptide Synthesizer, using Boc-amino acids (Peninsula Laboratories, Inc., Belmont, CA) to give Boc-Arg (Tos)-Thr (Bzl)Asp-(OBzl)-Lys(Cl-Z)-Tyr(Br-Z)-Gly-Arg(Tos)-Gly-Leu-Ala Ala-Asp(OBzl)-Gly-Lys (Cl-Z)-Met-Val-Asn-OCH_2-Pam-resin (Nase 81-100) and Boc-Tyr(Br-Z)-Ile-Tyr (Br-Z)-Ala-Asp(OBzl)-Gly-Lys(Cl-Z)-Met-Val-Asn-Glu(OBzl)-Ala-Leu-Val-Arg (Tos)-Gln-Gly-Leu-Ala-Lys (Cl-Z)-OCH_2-Pam-resin (Nase 91-110). After treatment of the peptidyl-resin with anhydrous hydrogen fluoride (10 ml/g resin) in the presence of *p*-cresol (1 ml/g resin) and *p*-thiocresol (1 g/g resin) for 1 h at 0°C (19), the resin was washed with diethyl ether (20 ml × 5) to remove scavengers, and with 30% glacial acetic acid (20 ml × 5) to solubilize the synthetic peptide. The lyophilized

[1] *Abbreviations used in this paper:* Nase, staphylococcal nuclease; TFA, trifluoroacetic acid.

FIGURE 1. Overlapping peptide sequences of the Nase molecule.

peptide recovered in the aqueous phase was desalted on a G-25 column (2.5 × 90 cm) in 1 M glacial acetic acid, and was lyophilized. Aliquots of each peptide were hydrolyzed in 6 M HCl containing 0.2% phenol at 110°C for 24 h, and hydrolysates were analyzed with a 6300 amino acid analyzer (Beckman Instruments, Inc., Fullerton, CA), and the following compositions were determined for the synthetic peptide corresponding to Nase (81-100): Asx 3.1 (3), Thr 0.8 (1), Gly 3.2 (3), Ala 2.1 (2), Val 1.2 (1), Ile 1.0 (1), Leu 1.1 (1), Tyr 3.1 (3), Lys 2.1 (2), Arg 1.9 (2); and for the synthetic peptide corresponding to Nase (91-110): Asx 1.8 (2), Glx 2.1 (2), Gly 2.0 (2), Ala 3.1 (3), Val 2.0 (2), Met 0.9 (1), Ile 0.9 (1), Leu 2.1 (2), Tyr 1.8 (2), Lys 2.1 (2), Arg 0.9 (1). The theoretical number of residues expected in each peptide is shown in parenthesis. Subsequently, an aliquot (0.2 mg) of each crude peptide mixture was separated on an analytical Vydac C_4 reverse-phase HPLC column (0.46 × 25 cm; 5 μm particle size; 300 A pore size) (The Separations Group, Hesperia, CA) using 0.1% trifluoroacetic acid (TFA) (Pierce Chemical Co., Rockford, IL) and a 60 min, linear gradient of 0–60% acetonitrile (J. T. Baker Chemical Co., Phillipsburg, NJ) containing 0.1% TFA. After optimization of isocratic elution conditions for each peptide mixture (0.2 mg) using an analytical Vydac C_4 and systems of 0.1% TFA and acetonitrile containing 0.1% TFA, semipreparative purification of each peptide was carried out on a Vydac C_4 (2.2 × 25 cm) (15–20 μm particle size; 300 A pore size) under isocratic elution conditions shown to give the maximum chromatographic resolution (20; J. C. Ford and J. A. Smith, manuscript in preparation). The other peptides were synthesized in a similar fashion. A schematic drawing depicting the overlapping peptides of the Nase molecule is shown (Fig. 1).

Antisera. We used the supernatant ascites form of the mAbs 34-5-3 (anti-I-A^b cross-reactive on I-A^d) and 14-4-4 (anti-I-E^k crossreactive on H-$2^{d,p,r}$) (21).

Culture Medium. The medium used for assays of proliferation and maintenance of T cell clones was RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM Hepes, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 5×10^{-5} M 2-ME and 10% FCS.

Proliferation. Cultures were performed in a total volume of 200 μl in flat-bottomed wells (3040; Falcon Labware, Oxnard, CA) incubated for 3.5 d at 37°C in 5% CO_2-humidified air. T cell proliferation was assayed by culturing 10^4 T cells with irradiated (3,300 rad) spleen cells in complete medium containing 10% FCS. We assessed amount

TABLE I
Antigen Specificity of Nuclease-specific T Cell Clones

T cell clones*	cpm × 10^{-3} + SEM		
	Medium	Nuclease	Lysozyme
G31	0.33 ± 0.05	5.0 ± 0.5	0.43 ± 0.04
G60	0.27 ± 0.03	5.0 ± 0.3	0.30 ± 0.02
G56	0.32 ± 0.05	36.4 ± 4.2	0.64 ± 0.08
G28	0.50 ± 0.04	16.4 ± 1.5	0.64 ± 0.07
G46	0.50 ± 0.06	11.8 ± 1.0	0.14 ± 0.02

* 10^4 cloned T cells were cocultured with 4 × 10^5 irradiated (B6A)F1 irradiated spleen cells in the presence of 12 μg/ml nuclease, 50 μg/ml lysozyme, or medium for 3 d.

of [^{3}H]TdR incorporated during a 12 h pulse (1 μCi/well, specific activity 2 Ci/μM; New England Nuclear, Boston, MA). Results are given as the arithmetic mean CPM of triplicate cultures ± SEM.

T Cell Clones. T cell clones were prepared as previously described (22). T cell clones were derived from lymph node cells from mice primed with 100 μg of Nase in the footpads. 4 × 10^5 nylon-nonadherent T cells were cultured in the presence of 3 × 10^6 irradiated (3,300 rad) syngeneic spleen cells and antigen. This feeding was repeated every 10–14 d. To remove dead cells from the T cell lines, the cells were layered and centrifuged over lymphocyte separating medium (Litton Bionetics, Charleston, SC) at the end of each cycle. After 2–4 cycles of stimulation the T cell lines were cloned by limiting dilution. Positive wells were expanded and clones were maintained in the presence of antigen, syngeneic stimulator cells, and a source of IL-2 if required for growth. All the T cell clones used in this study were obtained from T cells plated at 1 cell per well or 0.3 cell per well with 50 and 20% of wells positive, respectively.

Results

Antigen Specificity and MHC Restriction of Nase-reactive T Cell Clones. Two independent anti-Nase lines were generated from B6AF$_1$ T cells. From these two lines, we derived a total of nine anti-Nase clones by limiting dilution. The antigen specificity of these clones was assessed by determining the proliferative responses of each clone to Nase or to control antigen in the presence of irradiated syngeneic spleen cells. As shown in the representative experiment presented in Table I, each of the Nase-specific T cell clones proliferated significantly in the presence of the specific antigen Nase. In contrast, there was no significant proliferative response to lysozyme, a protein similar to Nase in overall size and charge.

The MHC restriction pattern of each Nase-specific T cell clone was next evaluated by comparing the responses to Nase generated in the presence of APC of different MHC types. APC were derived from B6AF$_1$ (H-2^b × H-2^a) or parental (H-2^b or H-2^a) strain mice, or from mice expressing recombinant H-2 types. Some Nase-specific clones (G28 and N36) appear to be H-2^b-restricted since they responded to Nase in the presence of irradiated B10 (H-2^b), but not B10.A (H-2^a) spleen cells (Table II). Other B6AF$_1$ clones (e.g., G60, N24.3, or N40) were H-2^a-restricted, responding to Nase plus B10.A, but not B10 APC. Intra-H-2 recombinant APC were used to further map these MHC restrictions. Each of the two H-2^b-restricted clones studied responded in the presence of

TABLE II

H-2 Subregion Specificity of Nuclease-specific T Cell Clones

Spleen cells*	H-2								(B6 × A/J)F_1 T cell clones (cpm × 10^{-3} + SEM)				
									Exp. 1		Exp. 2		
	K	A	B	J	E	C	S	D	G60	G28	N24.3	N40	N36
B10	b	b	b	b	b	b	b	b	0.26 ± 0.02	12.3 ± 0.7	0.20 ± 0.05	0.3 ± 0.01	3.7 ± 0.2
B10.A	k	k	k	k	k	d	d	d	2.1 ± 0.2	0.35 ± 0.3	7.0 ± 1.7	11.9 ± 0.6	0.4 ± 0.1
B10.A(5R)	b	b	b	k	k	d	d	d	0.53 ± 0.1	9.4 ± 1.0	0.7 ± 0.03	10.6 ± 0.7	1.6 ± 0.09
B10.A(4R)	k	k	b	b	b	b	b	b	0.17 ± 0.5	0.31 ± 0.6	0.4 ± 0.1	0.7 ± 0.09	0.18 ± 0.04
(5R × 4R)F_1	b	b	b	k	k	d	d	d	5.0 ± 0.4	16.4 ± 1.5	14.6 ± 1.8	16.9 ± 0.7	2.3 ± 0.1
	k	k	b	b	b	b	b	b					

* Irradiated spleen cells (4×10^5) from various intra-H-2 recombinant strains were incubated with nuclease-specific T cell clone cells (10^4) in the presence of 12 μg/ml of nuclease for 3 d.

TABLE III

Inhibition of Nuclease-specific T Cell Clones with Anti-Ia Antibodies

Antigen	mAbs	Concentration	T cell clone proliferation (cpm × 10^{-3} ± SEM)	
			G28.4*	N24.3
		%		
Nuclease:	—		16.2 ± 0.5	12.9 ± 1.1
	Anti-I-A^b	25	1.8 ± 0.3	9.8 ± 0.4
		8	5.5 ± 0.1	10.9 ± 1.5
		2	6.9 ± 0.6	17.3 ± 1.2
	Anti-I-E^k	1.2	26.1 ±4.8	2.3 ± 0.4
		0.4	29.8 ± 2.2	3.6 ± 0.7
		0.1	25.8 ± 3.1	3.6 ± 0.7

* 10^4 T cells from clone G28.4 and N24.3 were incubated from the onset of culture with 4×10^5 (B10 × B10.A)F_1 stimulator cells in the presence of nuclease (12 μg/ml) and anti-Ia antibodies.

B10.A(5R), but not B10.A(4R) spleen cells, suggesting that the relevant restricting element was K- or I-A-encoded. In contrast, each of the seven H-2^a-restricted B6AF_1-derived clones was unresponsive to antigen presentation by B10.A(4R) spleen cells. Six of these seven clones were also unresponsive to B10.A(5R) cells, but responded to Nase in the presence of [B10.A(5R) × B10.A(4R)F_1 APC (Table II and data not shown). This pattern of genetic complementation suggested that the restriction element recognized by these clones was on the E^k_α E^k_β product. One H-2^a-restricted clone, N40, responded to Nase in association with B10.A or B10.A(5R) APC, but not to Nase in association with B10 or B10.A(4R) APC, suggesting restriction to a determinant expressed by both E^k_α E^k_β and E^k_α E^b_β.

The MHC restriction specificity of Nase-specific T cell clones was further evaluated by examining the effects of anti-Ia mAbs on T cell proliferative responses (Table III). The response of the H-2^b-restricted clone G28.4 to (B10 × B10.A)F_1 APC was strongly inhibited by the anti-I-A^b antibody 34-5-3, but was not inhibited by the anti-I-E^k antibody 14-4-4, suggesting that the clone G28.4 is responsive to Nase in association with A^b_α A^b_β. The H-2^a-restricted clone

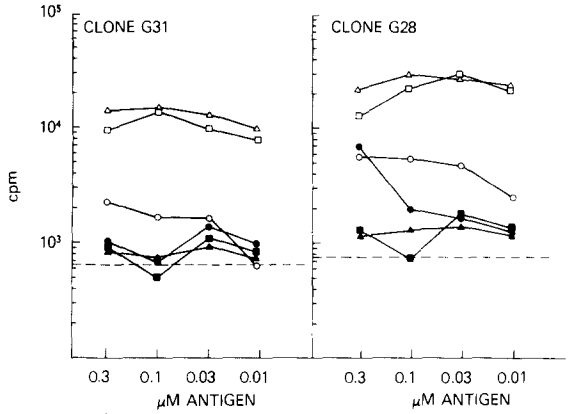

FIGURE 2. T cell clone responses to fragments of the Nase molecule. 10^4 cloned T cells were incubated with various concentrations of cyanogen bromide cleavage fragments of the nuclease molecule in the presence of 4×10^5 irradiated spleens. O, 1-149; ●, 6-48; □, 49-149; ■, 99-149; △, 1-126; ▲, 127-149. The response of T cells in the absence of antigen and in the presence or absence of irradiated stimulator cells was consistently $<10^3$ cpm.

N24.3 was inhibited reciprocally by anti-I-E^k, but not anti-I-A^b antibody, indicating its specificity for Nase in association with E^k_α E^k_β.

Responses of Nase-specific Clones to Cleavage Fragments of Nase. The initial attempts to better define the Nase molecule's antigenic structures that are recognized by T cells used cyanogen bromide and tryptic fragments of the Nase molecule, fragments that were previously used to study the responses of heterogeneous Nase-specific T cell populations (23, 24). Cloned T cells were cultured with syngeneic APC and titrated molar quantities of intact Nase (1-149) or Nase fragments. For all clones tested, including H-2^b-restricted and H-2^a-restricted clones, we observed responses to the large fragments 1-126 and 49-149, and these responses were uniformly higher in magnitude than those generated to intact Nase (Fig. 2). It is unclear whether the enhanced antigenicity of these fragments is due to purely immunologic factors, e.g., the availability of antigenic determinants for processing and presentation, or relates to the abrogation of Nase enzymatic activity in the fragments (17). Fragments 99-149 and 127-149 failed to stimulate any of the Nase-specific clones, and fragment 6-48 gave responses that were inconsistent and seen only at the highest antigen dose studied.

The results of these studies using Nase cleavage fragments thus failed to reveal any differences in the antigen specificities of the T cell clones tested. The fact that only fragments 1-126 or 49-149 were stimulatory suggested either that: (*a*) the sequences included in the area of amino acids 49–98 are critical for T cell recognition (since this sequence is present in each of the stimulatory peptides but absent from nonstimulatory peptides), or (*b*) the determinants recognized by T cells are conformational and require contributions by multiple sequences only present in the large 1-126 or 49-149 peptides.

Responses of Nase-specific Clones to Synthetic Peptides. To further pursue the analysis of cloned T cell fine specificity, a series of synthetic peptides were analyzed. We synthesized peptides 20 amino acids in length which corresponded to the entire sequence of Nase, and which overlapped one another by 10 amino

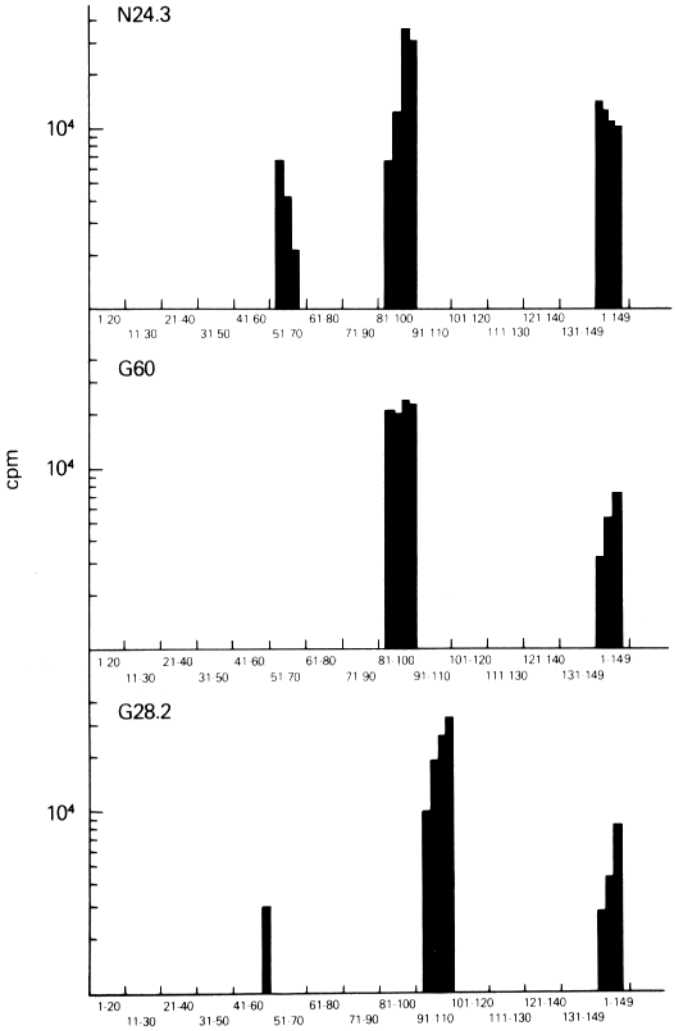

FIGURE 3. T cell clone responses to 20-amino-acid peptides of the Nase molecule. 10^4 cloned T cells were incubated with various concentrations (0.9–0.03 μM) of nuclease peptides of 20 amino acids in the presence of 4×10^5 irradiated spleen cells. The response of T cells in the absence of antigen and in the presence or absence of irradiated stimulator cells was consistently $<10^3$ cpm.

acids, thus corresponding to Nase amino acid residues 1-20, 11-30, 21-40, . . . 131-149. Nase-specific T cell clones were assayed for responsiveness to titrated molar quantities of each of the 14 peptides derived from the Nase amino acid sequence.

Two independently derived H-2^b-restricted clones responded strongly to the synthetic peptide 91-110 over the entire range of peptide concentrations studied, and the magnitudes of these responses were greater than those generated to the native Nase molecule (Fig. 3). These clones were completely unresponsive to the other peptides tested, with the sole exception of smaller and inconsistent re-

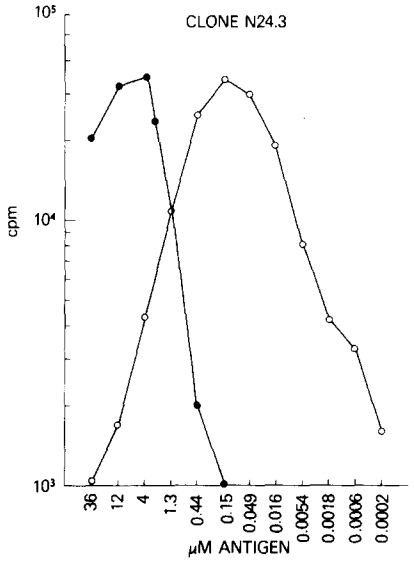

FIGURE 4. T cell clone responses to crossreactive Nase peptides. 10^4 cloned 24.3 T cells were incubated with peptide 51-70 and 81-100 at concentrations of 9 –0.01 μM in the presence of 4×10^5 irradiated spleen cells to determine the quantitative difference in stimulator capacity. ●, 51-70; ○, 81-100. The response of T cells in the absence of antigen and in the presence or absence of irradiated stimulator cells was consistently $<10^3$ cpm.

sponses to single concentrations of the peptide 41-60. Each of three subclones derived at 0.3 cells per well gave a response pattern identical to that expressed by the H-2^b-restricted clone G28 (data not shown).

The seven H-2^a-restricted Nase-specific clones gave response patterns that were distinct from those observed for H-2^b-restricted clones. Each of these clones was completely unresponsive to peptide 91-110, the peptide to which the H-2^b-restricted clones were consistently responsive (Fig. 3). Reciprocally, each of the H-2^a (E^k_α E^k_β)–restricted clones was strongly responsive to peptide 81-100, whereas the H-2^b (A^b_α A^b_β)–restricted clones were completely unresponsive to this overlapping peptide. It was striking that a number of H-2^a-restricted clones responded to peptide 51-70, in addition to responding to 81-100 and not responding significantly to other peptides. The monoclonal nature of the T cell lines responding to these two peptides was supported by the derivation of clones N24.1, N24.3, and G31.2, each of which was generated by initial limiting dilution cloning followed by subcloning at 0.3 cells per well. This pattern of apparent crossreactivity was observed in clones derived from two independent T cell lines (lines G and N). The dose-response curve to titrated molar amounts of peptides 51-70 and 81-100 indicated that peptide 51-70 was significantly less efficient in triggering T cell responses in the dual reactive clones, suggesting a range of 27-fold to 300-fold difference in different clones in repeated titrations (Fig. 4).

Recombinant strain APC were then used to determine whether the responses of T cell clones to synthetic peptides showed the same MHC restrictions as their responses to the native Nase molecule (Table IV). Responses of H-2^b-restricted clones G28.4 and N36 to 91-110 were observed in the presence of B10 or B10.A(5R), but not B10.A or B10.A(4R) spleen cells, paralleling the mapping to

TABLE IV

The Responses of T Cell Clones to Nuclease Peptides Are Under the Same I Region Genetic Control as Responses to the Whole Nuclease Molecule

Spleen cells	Peptide	T cell clone proliferation* (cpm × 10^3 ± SEM)		
		N40	N24.3	N28.4
B10	1–149	0.6 ± 0.09	0.2 ± 0.09	5.9 ± 0.3
	81–100	0.8 ± 0.1	0.2 ± 0.02	ND
	91–110	ND	ND	15.1 ± 0.8
B10.A	1–149	18.9 ± 2.8	7.6 ± 0.1	0.4 ± 0.01
	81–100	46.4 ± 4.2	9.5 ± 0.9	ND
	91–110	ND	ND	0.7 ± 0.08
B10.A(5R)	1–149	6.7 ± 1.5	0.9 ± 0.09	5.1 ± 0.4
	81–100	6.6 ± 1.9	0.4 ± 0.05	ND
	91–110	ND	ND	13.9 ± 0.6
B10.A(4R)	1–149	1.1 ± 0.5	0.1 ± 0.006	0.3 ± 0.1
	81–100	0.6 ± 0.1	0.7 ± 0.1	ND
	91–110	ND	ND	0.7 ± 0.05
(5R × 4R)F_1	1–149	15.0 ± 0.5	5.6 ± 0.2	9.0 ± 1.5
	81–100	49.4 ± 3.7	6.8 ± 1.2	ND
	91–110	ND	ND	9.9 ± 1.8

* T cell clones were incubated in the presence of whole nuclease 1–149 (0.7 μM), peptide 81–100 (0.1 μM), or 91–110 (0.1 μM) in the presence of irradiated spleen cells.

K^b or I-A^b of responses to intact Nase. Responses of the H-2^a-restricted clones to both 81-100 and 51-70 also showed the same genetic restrictions as the responses to native Nase, with responses by clones G31 and N24 in the presence of B10.A, but not B10, B10.A(4R), or B10.A(5R) APC, and response by clone N40 in the presence of B10.A or B10.A(5R) APC (data not shown).

A Nase-specific T cell line was also generated from BALB/c spleen cells, and clones were derived from that line by limiting dilution. One cloned BALB/c T cell line (C30) and its subcloned derivative (C30.1) were studied for their reactivity patterns to Nase peptides. In contrast to any of the H-2^a-restricted or H-2^b-restricted B6AF_1 clones, these BALB/c T cell lines responded to peptide 61-80, and not to other Nase peptides, including 81-100 and 91-110 (data not shown).

Discussion

In the present study we used cloned T cells and synthetic peptides to analyze MHC-restricted, antigen-specific T cell recognition of the protein antigen Nase. We evaluated a total of 10 Nase-specific clones, derived from two independent B6AF_1 lines and one BALB/c line. Each of these T cell clones was shown to be specific for one peptide component of the overall Nase protein (with the exception of the crossreactivity described below). Two of the B6AF_1 clones were A^b_α A^b_β-restricted. Seven were restricted by complementing *H-2*^{a} genes, which most probably are those encoding the E^k_α E^k_β product, and one of these seven also crossreactively recognized the B10.A(5R) (E^k_α E^b_β) product as a restriction element.

Moreover, in the panel of T cell clones studied here, the identity of the peptide recognized by a given Nase-specific T cell correlated with the MHC restriction pattern of that T cell. Both A^b_α A^b_β-restricted clones were highly responsive to peptide 91-110, and not to the other synthetic Nase peptides; whereas all of the E^k_α E^k_β clones were consistently responsive to peptide 81-100 but not to 91-110 or other Nase peptides. Certain of these E^k_α E^k_β T cells responded to peptide 51-70 as well as to 81-100, although the markedly shifted dose-response curve indicated a reduced efficiency of activation by peptide 51-70.

The finding that Nase-specific T cells are responsive to discrete Nase peptides is consistent with previous data, indicating that protein antigen–specific T cells recognize limited peptide determinants (1, 2, 5, 8–11). The present findings also suggest that for Nase, as for other antigens, a limited number of peptides may represent the bulk of immunodominant sites recognized by T cells on a protein antigen. The reason for this limited repertoire is not clear. The most extensive previous analyses of this area were carried out with species variants of cytochrome *c* (3–5), myoglobin (6–9), lysozyme (10, 11), and OVA (12, 13). Since each of these protein antigens differs by only a few amino acids from the homologue present in mice (the source of responding T cells), it was possible that self tolerance contributed significantly to the limitation in the number of immunogenic sites recognized on these antigens. That is, self tolerance to determinants shared by homologous foreign and self proteins may have left only a small number of unique foreign determinants to be recognized as immunogenic. In the present studies, the foreign antigen used was Nase, a bacterial product without extensive sequence homology to known mammalian proteins (C. C. Yue, NIH; personal communication). The fact that in clonal T cell responses to Nase a limited number of immunodominant peptides are recognized in association with a given Ia molecule suggests that self tolerance is not a universally important component in the limited repertoire of T cells for sites on foreign proteins.

These findings are consistent with the concept that structural properties intrinsic to peptide molecules may play a critical role in determining immunogenicity. Recently, DeLisi and Berzofsky (15) have reported evidence that the majority of immunodominant epitopes recognized by T lymphocytes are amphipathic structures, i.e., structures with separated hydrophobic and hydrophilic domains. Most of these had a periodic variation in hydrophobicity consistent with the ability to fold into an amphipathic α helix in which hydrophobic residues are primarily on one side of the helix and hydrophilic ones on the other. The data so far available for T cell recognition of Nase peptides do not as yet permit a statistically powerful test of the ability to predict immunodominant epitopes on the basis of amphipathicity. Nevertheless, all four of the peptides that stimulated T cell clones in the present study (51-70, 61-80, 81-100, and 91-110) were compatible with predictions made on the basis of amphipathicity. It is anticipated that a finer analysis of the epitopes that are recognized on these peptides by T cells will allow a more powerful test of this hypothesis.

The data presented here suggest that, for a complex protein antigen, different peptide determinants may be recognized by T cells predominantly in association with different Ia-restricting elements: e.g., peptide 81-100 in association with E^k_α E^k_β and 91-110 in association with A^b_α A^b_β. A number of previous studies have

similarly indicated that different antigenic determinants on a complex protein are recognized by T cells in association with different class II molecules. Shastri et al. (11) showed this finding in a clonal analysis of T cell responses to the synthetic peptides of hen egg lysozyme. Lysozyme peptide 81-96 was recognized in association with I-A^b, whereas peptide 74-86 was recognized in association with I-A^k, analogous to the associations described in the present study for Nase peptides. However, when a more extensive evaluation of antigen specificity was carried out using substituted lysozyme peptides, it was found that T cells that superficially expressed the same peptide specificity were in fact quite heterogeneous in their antigen fine specificity. We are currently making a similar analysis of clonal fine specificity for Nase peptides. Berkower et al. (8) have also used T cell clones in a panel of species variants of myoglobin to evaluate the effect of Ia restriction on "epitope dominance," and have similarly observed that T cell clones specific for one epitope were restricted to I-A^d, whereas clones specific for a second epitope were restricted to I-E^d. All of these findings are consistent with the earlier studies of Heber-Katz et al. (3) and Matis et al. (4), which showed a correlation between the Ia restriction element and the fine specificity of antigen recognition by T cells, and are in fact consistent with the earliest observations of MHC-linked immune response gene control.

Although the mechanisms underlying the preferential association of a given antigen specificity with a given Ia-restricting element remain unknown, two alternative models have been proposed. In the first of these models, a given antigen epitope may be selectively associated, at the level of the APC, with a given Ia molecule. Direct evidence supporting the existence of such selective associations in the binding of antigen to Ia molecules has recently been presented by Babbitt et al. (16). It remains to be determined whether this phenomenon will be widely generalized, and whether it is functionally important in instances of selective recognition of antigen plus Ia by T cells. The second model is based upon an hypothesized absence in the T cell repertoire of receptors that can recognize certain epitope plus Ia combinations, despite the existence of these epitope/Ia complexes in potentially antigenic form (25). A number of mechanisms, either germline- or differentiation-dependent, might underlie such deficiencies in the T cell repertoire. More direct and detailed analyses of specific interactions between antigenic peptides and Ia molecules, and ultimately with the T cell receptor, may help to distinguish among these alternatives.

The finding that several E^k_α E^k_β-restricted T cell clones derived from two independent lines responded most efficiently to peptide 81-100, and consistently (although less efficiently) to peptide 51-70 was unexpected. Since these were independent synthetic peptides, and not fragments prepared from a common Nase molecule, it is not likely that the reactivity we saw represents a contamination of one peptide with another. In addition, since several clones and subclones displayed this crossreactivity, it is unlikely that it is due to responses of distinct 51-70-reactive and 81-100-reactive cells contained within each T cell line. The basis for crossreactive recognition by a given T cell of both 81-100 and 51-70 in association with E^k_α E^k_β is unclear. The amino acid sequences of these peptides do not reveal extensive homologies. However, it is striking that there are two tripeptides in common between these sequences, Lys-Tyr-Gly and Lys-Met-Val,

and these are spaced almost the same distance apart (8 and 10 residues) in the two peptides. Moreover, immediately adjacent to the first Lys, 81-100 has an Asp whereas 51-70 has a Glu. As this substitution is very conservative, the peptides may share homologous tetrapeptides Glu or Asp-Lys-Tyr-Gly. Thus 7 out of 20 residues of each are in these homologous regions. Thus, we would hypothesize that the peptides 51-70 and 81-100 may fold in such a way as to form a homologous epitope containing either or both of these homologous regions. Additional studies using variants of the 51-70 and 81-100 peptides may better define the structural basis of this crossreactivity.

The findings presented here have shown that T cell clones specific for the bacterially derived antigen Nase appear to be specific for one or another synthetic peptide of the Nase sequence. Moreover, the identity of the peptide recognized by a T cell clone correlated strongly with the MHC restriction specificity of that T cell in the panel studied to date. These findings thus suggest a preferential association between antigen fine specificity and Ia restriction in T cell responses, and suggest that a limited T cell repertoire exists for immunogenic epitopes on even phylogenetically and structurally distant protein molecules. The mechanism underlying the selective association of antigenic epitopes with specific Ia molecules is a subject of ongoing studies.

Summary

Previous studies (1) have indicated that the repertoire of murine T cells specific for a potentially complex protein antigen is in fact specific for a limited number of antigenic epitopes on that antigen in association with a given Ia molecule. Since those studies generally analyzed responses to antigens that differ in only a few amino acids from homologous murine molecules, it was possible that tolerance to self proteins was responsible for the limited T cell repertoire seen in responses to closely related proteins. It was therefore of interest to determine whether T cell recognition of a structurally and phylogenetically more distant protein molecule would also show specificity for a limited number of immunodominant peptides on that molecule. A series of experiments was designed to study the antigen fine specificity and MHC restriction of T cell clones specific for the bacterially derived antigen staphylococcal nuclease (Nase). T cell clones generated in (H-2^b × H-2^a)F_1 (B6AF_1) T cells were shown to be specific for Nase and to be restricted by either A^b_α A^b_β or E^k_α E^k_β. The fine specificity of these clones was then analyzed using cyanogen bromide and tryptic fragments and a series of overlapping 20-amino-acid synthetic peptides corresponding to and spanning the entire sequence of the Nase molecule. Two A^b_α A^b_β-restricted clones were highly responsive to peptide 91-110, and not to other synthetic Nase peptides. In contrast, seven E^k_α E^k_β-restricted clones were consistently responsive to peptide 81-100 and not to 91-110 or to other Nase peptides. Certain of these E^k_α E^k_β-restricted T cells expressed an interesting crossreactivity, in that they responded to peptide 51-70 as well as to 81-100, although the response to 51-70 was characterized by a markedly shifted dose-response curve, indicating a reduced efficiency of activation by this peptide. Analysis of the amino acid sequences of these regions indicates that this unexpected crossreaction may have a structural basis. A single Nase-specific T cell line generated from BALB/c T cells was, in

contrast to any of the $B6AF_1$ clones studied, responsive only to peptide 61-80 and not to other peptides, including 81-100 or 91-110. Collectively, these findings show that Nase-specific T cells are responsive to discrete Nase peptides. Moreover, the present findings suggest that in T cell recognition of a complex and highly foreign protein antigen, a limited number of peptide epitopes are preferentially recognized by T cells in association with a given Ia molecule.

The authors thank Drs. Ira Berkower and Ronald Schwartz for their very constructive suggestions during the preparation of this manuscript; Ms. Judith Kress for her excellent editorial assistance; and Lerzan Kizilay and Margaret Flynn for characterization of the synthetic peptides.

Received for publication 6 May 1986.

References

1. Benjamin, D. C., J. A. Berzofsky, I. J. East, F. R. N. Gurd, C. Hannum, S. J. Leach, E. Margoliash, J. G. Michael, A. Miller, E. M. Prager, M. Reichlin, E. E. Sercarz, S. J. Smith-Gill, P. E. Todd, and A. C. Wilson. 1984. The antigenic structure of proteins: a reappraisal. *Annu. Rev. Immunol.* 2:67.
2. Atassi, M. Z., and C. R. Young. 1985. Discovery and implications of the immunogenicity of free small synthetic peptides: Powerful tools for manipulating the immune system and for production of antibodies and T cells of preselected submolecular specificities. *CRC Crit. Rev. Immunol.* 5:387.
3. Heber-Katz, E., R. H. Schwartz, L. A. Matis, C. Hannum, T. Fairwell, E. Appella, and D. Hansburg. 1982. Contribution of antigen-presenting cell major histocompatibility complex gene products to the specificity of antigen-induced T cell activation. *J. Exp. Med.*155:1086.
4. Matis, L. A., D. L. Longo, S. M. Hedrick, C. Hannum, E. Margoliash, and R. H. Schwartz. 1983. Clonal analysis of the major histocompatibility complex restriction and the fine specificity of antigen recognition in the T cell proliferative response to cytochrome *c*. *J. Immunol.* 130:1527.
5. Corradin, G., R. H. Zubler, and H. D. Engers. 1981. Clonal analysis of the BALB/c T cell proliferative response to APO beef cytochrome *c*. *J. Immunol.* 127:2442.
6. Infante, A. J., M. Z. Atassi, and C. G. Fathman. 1981. T cell clones reactive with sperm whale myoglobin. Isolation of clones with specificity for individual determinants on myoglobin. *J. Exp. Med.* 154:1342.
7. Yoshioka, M., G. S. Bixler, and M. Z. Atassi. 1983. Preparation of T-lymphocyte lines and clones with specificities to preselected protein sites in vitro passage with free synthetic peptides: demonstration with myoglobin sites. *Mol. Immunol.* 20:1133.
8. Berkower, I., H. Kawamura, L. A. Matis, and J. A. Berzofsky. 1985. T cell clones to two major T cell epitopes of myoglobin: effect of I-A/I-E restriction on epitope dominance. *J. Immunol.* 135:2628.
9. Berkower, I., G. K. Buckenmeyer, and J. A. Berzofsky. 1986. Molecular mapping of a histocompatibility-restricted immunodominant T cell epitope with synthetic and natural peptides: Implications for T cell antigenic structure. *J. Immunol.* 136:2498.
10. Allen, P. M., D. J. Strydom, and E. R. Unanue. 1984. Processing of lysozyme by macrophages: identification of the determinant recognized by two T-cell hybridomas. *Proc. Natl. Acad. Sci. USA* 81:2489.
11. Shastri, N., A. Oki, A. Miller, and E. E. Sercarz. 1985. Distinct recognition phenotypes exist for T cell clones specific for small peptide regions of proteins. Implications for the mechanisms underlying major histocompatibility complex–restricted antigen

recognition and clonal deletion models of immune response gene defects. *J. Exp. Med.* 162:332.

12. Shimonkevitz, R., S. Colon, J. W. Kappler, P. Marrack, and H. M. Grey. 1984. Antigen recognition by H-2-restricted T cells. II. A tryptic ovalbumin peptide that substitutes for processed antigen. *J. Immunol.* 133:2067.
13. Watts, T. H., J. Gariepy, G. K. Schoolnik, and H. M. McConnell. 1985. T-cell activation by peptide antigen: effect of peptide sequence and method of antigen presentation. *Proc. Natl. Acad. Sci. USA.* 82:5480.
14. Hackett, C. J., B. Dietzschold, W. Gerhard, B. Ghrist, R. Knorr, D. Gillessen, and F. Melchers. 1983. Influenza virus site recognized by a murine helper T cell specific for H1 strains. Localization to a nine amino acid sequence in the hemagglutinin molecule. *J. Exp. Med.* 158:294.
15. DeLisi, C., and J. A. Berzofsky. 1985. T-cell antigenic sites tend to be amphipathic structures. *Proc. Natl. Acad. Sci. USA.* 82:7048.
16. Babbitt, B. R., P. M. Allen, G. Matsueda, E. Haber, and E. R. Unanue. 1985. Binding of immunogenic peptides to Ia histocompatibility molecules. *Nature (Lond.).* 317:359.
17. Taniuchi, H., and C. B. Anfinsen. 1966. The amino acid sequence of an extracellular nuclease of Staphylococcus aureus. I. Linear order of the fragments produced by cleavage with cyanogen bromide. *J. Biol. Chem.* 241:4366.
18. Merrifield, R. B. 1963. Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. *J. Am. Chem. Soc.* 85:2149.
19. Sakakibara, S. 1971. The use of hydrofluoride in peptide chemistry. *In* Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins. Vol. I. B. Weinstein, editor. Marcel Dekker, Inc., New York. 51–85.
20. Rivier, J., R. McClintock, R. Galyean, and H. Anderson. 1984. Reversed-phase high-performance liquid chromatography: preparative purification of synthetic peptides. *J. Chromatogr.* 288:303.
21. Sachs, D. H., N. Mayer, and K. Ozato. 1981. Hybridoma antibodies directed toward murine H-2 and Ia antigens. *In* Monoclonal Antibodies and T Cell Hybridomas. G. J. Hammerling, U. Hammerling, and J. F. Kearney, editors. Elsevier Scientific Publishing Co., Amsterdam. 95–101.
22. Finnegan, A., B. Needleman, and R. J. Hodes. 1984. Activation of B cells by autoreactive T cells: cloned autoreactive T cells activate B cells by two distinct pathways. *J. Immunol.* 133:78.
23. Schwartz, R. H., J. A. Berzofsky, C. L. Horton, A. N. Schechter, and D. H. Sachs. 1978. Genetic control of the T lymphocyte proliferative response to staphylococcal nuclease: evidence for multiple MHC-linked Ir gene control. *J. Immunol.* 120:1741.
24. Sachs, D. H., J. A. Berzofsky, D. S. Pisetsky, and R. H. Schwartz. 1978. Genetic control of the immune response to Staphylococcal nuclease. *Springer Semin. Immunopathol.* 1:51.
25. Ishii, N., Z. A. Nagy, and J. Klein. 1982. Absence of Ir gene control of T cells recognizing foreign antigen in the context of allogeneic MHC molecules. *Nature (Lond.).* 295:531.