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Class II-Restricted T-Cell Clones to a Synthetic Peptide of Influenza Virus Hemagglutinin Differ in Their Fine Specificities and in the Ability To Respond to Virus

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Fifteen T-cell clones were derived from BALB/c or DBA/2 mice immunized with a synthetic peptide corresponding to the C-terminal 24 residues (residues 305 to 328) of the HA_1 chain of H3 subtype influenza virus hemagglutinin. All of the clones proliferated when the peptide was presented in association with I-E^d. By using shorter homologs, it was shown that the T-cell response was focused predominantly on the region at the N-terminal end of the peptide encompassed by residues 306 to 319. Individual clones recognizing this region differed in their absolute requirements for residues at the extremities of the site and also in their patterns of efficiency of recognition of shorter homologs. One particular clone defined another site of T-cell recognition within residues 314 to 328. The response of the clones to peptide analogs identified certain residues within the sites that were critical for recognition, with the substitution Gln-311→Ser having a differential effect on clones responding to the N-terminal site. Only one of the clones responded well to influenza virus itself. This clone also required relatively low concentrations of the parent peptide for optimum stimulation and was suppressed by higher concentrations. The data demonstrate striking heterogeneity in the T-cell response even to a short synthetic peptide, with different T-cell clones recognizing slightly different but overlapping areas of the molecule.

In a previous study (4), we determined that the murine class II-restricted T-cell response to influenza virus, H3 subtype, was predominantly directed towards a particular region of the viral hemagglutinin (HA) molecule (site E) but that individual T cells recognizing this site differed markedly in their fine specificities. A total of 14 T-cell clones specific for site E were divided into at least four groups according to their patterns of reactivity with different naturally occurring strains of influenza virus and with monoclonal antibody (MAb)-selected escape mutants. All of these clones recognized antigen presented in association with I-A^d. Heterogeneity of the T-cell response to a particular site has also been reported by others (1, 2, 17, 21, 24, 33) and has been interpreted by Cease et al. (7) as representing recognition by the receptors on different T-cells of distinct "views" of a single conformation of the antigen presented by the class II molecule, rather than reflecting multiple conformations or binding sites.

The site E-specific T-cell clones were thought to recognize overlapping epitopes, although the exact nature of these epitopes has not yet been precisely determined within this complex region of HA. Site E consists of two antiparallel beta chains with a carbohydrate attachment site on one strand and, in particular strains and escape-mutants, a potential attachment site on the other strand, possibly affecting the processing and presentation of the site (4). In order to investigate the heterogeneity of the T-cell response to a particular region in greater detail, we used for the present study a less complex antigen in the form of a short synthetic peptide. This synthetic peptide, H3 HA_1(305-328), representing the C-terminal 24 amino acid residues of the heavy chain (HA_1) of A/Memphis/1/71 (Mem 71) influenza virus HA (H3 subtype), has been used by us as a model antigen in an ongoing study of the physical and functional relationships between epitopes recognized by B cells and T cells (6, 18, 31, 35). The amino acid sequence of the region of HA represented by this peptide is generally conserved within each subtype. Peptide H3 HA_1(305-328) is not recognized by antiviral antibody. It does, however, elicit antibodies in rabbits and mice without the need for coupling to a carrier protein, and these antibodies bind to influenza viruses of the H3 subtype but not to viruses of other subtypes (26).

Mice of the *H-2*^{d} haplotype are high responders to peptide H3 HA_1(305-328) (6), producing antibodies to three distinct sites on the peptide (31). The primary response is directed principally to the site 314 to 318, whereas the secondary response after a boost is predominantly directed to site 320 to 328. The third site, 305 to 314, elicits only a small fraction of the total antibody (6). The ability of mice to mount an antibody response and a T-cell proliferative response to the peptide requires the presence of I-E^d. Polyclonal T cells from mice primed to H3 HA_1(305-328) do not recognize a peptide corresponding to the equivalent region of the H2 virus A/Japan/305/57 (Jap 57) [H2 HA_1(305-328)] or a peptide analog in which residues 311 to 315 have been replaced with the corresponding residues of the H2 sequence (6).

Although bulk cultures of BALB/c (*H-2*^{d}) and CBA (*H-2*^{k}) T cells primed to H3 viruses do not respond to peptide H3 HA_1(305-328) (25; unpublished data) and BALB/c T cells primed to H3 HA_1(305-328) show only weak reactivity with the intact virus (6), this same peptide contains the immunodominant site of recognition for human T cells primed to H3 influenza virus (19). The corresponding region of the H1 subtype also contains one of the three sites on the HA recognized by H1 virus-primed BALB/c T cells (17).

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In this study we investigated the recognition of peptide H3 HA_1(305-328) by *H-2*^{d} T cells at the clonal level; here we describe the heterogeneity of the response. The ability of individual T-cell clones to recognize virus was also examined.

MATERIALS AND METHODS

Viruses. Influenza viruses used in this study were reassortants bearing the N1 neuraminidase of A/Bellamy/42 and either the H3 HA of Mem 71 or A/Duck/Ukraine/1/63 or the H2 HA of Jap 57. The H3N2 virus A/Philippines/2/82 was also used. The viruses were grown for 2 days in the allantoic cavity of 10-day embryonated hen eggs, harvested, and then stored at −70°C. Purified Mem 71 virus was obtained from Alan Hampson (Commonwealth Serum Laboratories, Parkville, Victoria, Australia).

Mice. All of the mice used were females between 6 and 8 weeks old. BALB/c and D2.GD strains were obtained from the animal facility of this department, and DBA/2 mice were obtained from the Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia.

Immunizations. DBA/2 mice were immunized intraperitoneally with 25 μg of peptide H3 HA_1(305-328) in complete Freund adjuvant. After 3 months the mice were given a boost with the same dose of peptide in complete Freund adjuvant, and 4 days later, the spleens were removed for culture. BALB/c mice were inoculated in the hind footpad with 25 μg of H3 HA_1(305-328) in complete Freund adjuvant, and 7 days later, popliteal and inguinal lymph nodes were removed for culture.

Production of T-cell clones. The immune spleen or lymph node cells were cultured (5×10^6/ml) with 4 μM peptide H3 HA_1(305-328) for 5 days. Subsequent passages followed a regimen of 5 days of culture in the presence of 2 μM peptide and gamma-irradiated (2,200 R) syngeneic normal spleen cells (2×10^6/ml) as a source of antigen-presenting cells (APC) alternated with 7 days in medium containing interleukin 2, which was prepared from concanavalin A-stimulated BALB/c spleen cells as previously described (4). At each passage the cells were reseeded at 2×10^5/ml. These T-cell lines were cloned by limiting dilution (5); clones were expanded only from wells in which the T-cell number used yielded less than 30% of replicate wells positive for growth.

T-cell proliferation assay. T-cell proliferation assays were performed as described previously (4) with microcultures containing 10^4 T cells, 3×10^5 APC, and various concentrations of peptide or virus. Proliferation was measured by [^{3}H]thymidine incorporation.

Culture medium. RPMI 1640 (Commonwealth Serum Laboratories, Parkville, Victoria, Australia) was supplemented with 10% heat-inactivated (56°C, 30 min) fetal calf serum, 2 mM L-glutamine, 2 mM sodium pyruvate, 0.1 mM 2-mercaptoethanol, 100 IU of penicillin per ml, 100 μg of streptomycin per ml, and 30 μg of gentamicin per ml.

Synthetic peptides. The synthetic peptides H3 HA_1(305-328) and H2 HA_1(305-328), which represent the C-terminal 24 amino acid residues of the HA_1 polypeptides of the HA of Mem 71 and Jap 57 viruses, respectively, together with analog A and analog B (Fig. 1), were synthesized by using an Applied Biosystems Inc. Synthesizer 430A as previously described (18). Briefly, assembly was carried out in the solid phase with a 1% cross-linked phenylacetamidomethyl polystyrene resin and *t*-butyloxycarbonyl derivatives of the amino acids. Peptides were cleaved from the resin with hydrogen fluoride containing 10% anisole. After cleavage, peptides were extracted into 50% acetic acid. Shorter peptides were synthesized manually with 9-fluorenylmethoxycarbonyl derivatives of amino acids in place of *t*-butyloxycarbonyl derivatives by using either a Cambridge Research Biochemicals flow synthesizer or a DuPont Rapid Multiple Manual Peptide Synthesizer system. Composite polydimethylacrylamide-Kieselguhr support (Pepsyn K) was used as the resin for flow syntheses (10), and 2,4-dimethoxybenzhydrylamine-polystyrene (Rapidamide) was used as the support for Rapid Multiple Manual Peptide Synthesizer-based syntheses. Synthetic peptides were purified by reverse-phase chromatography with a PepRPC column (1 by 10 cm) in a fast protein liquid chromatography (Pharmacia South Seas Pty. Ltd.) system. The purity of the final product was monitored by amino acid analysis.

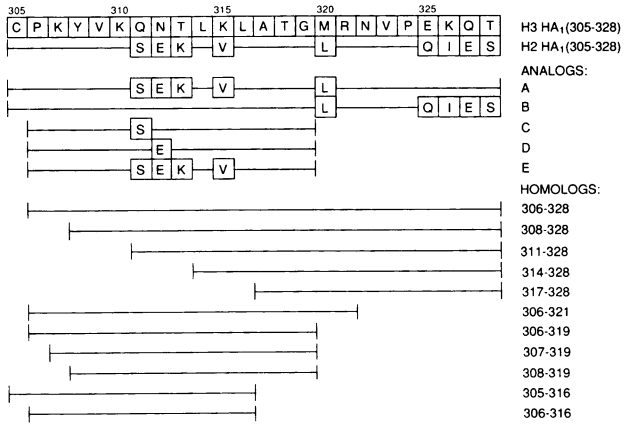

FIG. 1. Amino acid sequences, in single-letter code, of the synthetic peptide H3 HA_1(305-328), representing the C-terminal 24 residues of the HA_1 chain of Mem 71 virus (subtype H3) and of analogs and homologs of this peptide. The amino acid differences in the peptide H2 HA_1(305-328), which represents the equivalent region of the HA_1 of Jap 57 virus (subtype H2), are shown in boxes. The substituted amino acids in synthetic analogs A through E are also boxed. Amino acids in common with the H3 HA_1(305-328) sequence in analogs and homologs are shown as a line.

MAbs. Ascitic fluids containing MAbs to *H-2*^{d} gene products were used in blocking assays. Hybridoma cell line 14-4-4 (anti-I-$E^{k,d}$; specificity, la.7) (27) was obtained from I.F.C. McKenzie (University of Melbourne), and lines 34-2-12S (anti-D^d) and 34-1-2S (anti-K^dD^d) (28) were from N. Koch (Walter and Eliza Hall Institute, Melbourne). MAb 34-5-3S (anti-I-A^d) (28) was purchased as ascitic fluid from Litton Bionetics (Charleston, S.C.).

RESULTS

Reactivity of T-cell clones with synthetic homologs of influenza peptide H3 HA_1(305-328). T-cell lines were prepared from lymph node or spleen cells of BALB/c (lines 7 and 8) and DBA/2 (lines 3 and 4) mice immunized with peptide H3 HA_1(305-328). After 2 weeks in culture, each line proliferated in response to H3 HA_1(305-328), and in addition, one DBA/2 line showed reactivity to Mem 71 virus (H3 subtype). T cells from these lines were subsequently cloned in the presence of H3 HA_1(305-328). DBA/2 line 4 was also cloned with Mem 71 virus as the stimulating antigen, and the resulting clone, 4.51, was maintained in culture with virus.

Proliferation of each of the clones in response to shorter homologs of the parent peptide H3 HA_1(305-328) is shown in

TABLE 1. Proliferative response of T-cell clones to synthetic homologs of H3 HA_1(305-328)

Peptide	% Proliferative response of T-cell clone[a]														
	8.1[b]	3.23[b]	4.27[b]	4.28[b]	8.6	3.21	4.5	4.4	7.2	7.6	3.33[b]	7.5	7.11[b]	8.2	4.51
305-328	100.0	100.0	100.0	100.0[c]	100.0[c]	100.0	100.0	100.0[c]	100.0[c]	100.0[c]	100.0[c]	100.0[c]	100.0[c]	100.0[c]	100.0[d]
306-328	0.3	423.3	206.0	44.6	16.0	66.6[e]	57.5	51.6	48.1	46.2	70.7[e]	99.7	89.2	113.8[e]	84.2[e]
308-328	0.1	0.3	0.6	0.0	0.0	0.3	0.4	0.0	0.2	13.1	69.5	111.5	107.4[e]	123.5[e]	136.9[e]
311-328	0.0	0.0	0.0	0.1	0.3	0.1	0.0	0.0	0.0	0.0	0.1	0.0	0.4	0.2	0.0
314-328	40.5	0.0	1.0	0.1	0.2	0.1	0.0	0.0	0.1	0.0	0.3	0.0	0.1	0.3	0.0
317-328	0.0	0.0	0.3	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.2	0.0
306-321	0.0	174.6	109.5	29.4	16.0	103.6	67.3	76.3	32.8	39.6	73.0	77.6	140.4	126.6[e]	125.8[e]
306-319	0.0	87.0	60.4	28.9	70.0	83.1	282.5	163.5[e]	84.4[e]	104.0	78.9	130.4	122.8	85.5	178.6[e]
307-319	0.0	0.0	7.1	2.7	36.2	15.2	192.3	52.8	44.1	60.0	1.5	134.0	156.2[e]	106.0	180.2[e]
308-319	0.0	0.0	0.0	0.0	0.4	0.2	3.2	0.0	0.1	17.2	0.5	83.9	104.0	100.5[e]	145.3[e]
305-316	0.0	0.0	3.2	0.0	0.5	0.2	0.4	2.6	5.4	64.0	0.4	0.0	0.2	0.6	2.8
306-316	0.0	0.0	0.3	0.3	0.8	0.1	0.2	28.4	21.7	65.5	0.3	0.0	0.2	0.8	45.2

[a] Values represent the maximum proliferative responses over the dose range, which, unless indicated, occurred at 10 μM. Data are expressed as mean counts per minute of triplicate cultures containing antigen minus counts per minute in the absence of antigen and are given as a percentage of the value obtained for T cells stimulated by H3 HA_1(305-328).
[b] Tested at 10 and 2 μM only. All other clones were tested at 10, 2, 0.2, and 0.02 μM.
[c] Maximum proliferation occurred at 2 μM.
[d] Maximum proliferation occurred at 0.02 μM.
[e] Maximum proliferation occurred at 0.2 μM.

Table 1. These results indicate that the T-cell response was directed predominantly to the N-terminal region of the peptide; with the exception of clone 8.1, which proliferated in response to peptide 314-328, the clones did not react to peptides 311-328, 314-328, or 317-328. Within the N-terminal region, peptide 306-319 stimulated all of the clones, again with the exception of 8.1. The dose response data indicated that this 14-residue peptide was, in the majority of cases, able to stimulate the clones as efficiently as did H3 HA_1(305-328).

Even though the region 306 to 319 contains the information necessary for recognition by 14 of the 15 clones, different residues are critical for different clones. Compared with the response to peptide 306-319, the proliferation of some clones (3.23, 4.27, and 4.28) was dramatically reduced in response to peptide 307-319, whereas the absence of residue 306 in this peptide caused only a partial loss of activity of clones 8.6, 3.21, 4.5, 4.4, 7.2, and 7.6. Clones 7.5, 7.11, 8.2, and 4.51 were efficiently stimulated by peptide 308-319, as well as by peptide 307-319. Clone 3.33 had an unusual reactivity pattern; it responded well to peptides 308-328 and 306-319 but not to 307-319 or 308-319 within the dose range tested. The shortest peptide capable of stimulating a response was 306-316, which induced proliferation of clones 4.4, 7.2, 7.6, and 4.51 at the highest concentration of peptide only.

These results indicate that there is a focusing of the T cells toward a particular site, with heterogeneity of this response displayed by the absolute requirement for different residues at the extremities of the site and by differences in the relative efficiency of recognition of the various peptide homologs.

Reactivity of T-cell clones with synthetic analogs of H3 HA_1(305-328). We have previously shown that bulk cultures of *H-2*[d] T cells raised to H3 HA_1(305-328) do not respond to a peptide representing the C-terminal 24 residues of HA_1 of the H2 subtype (6). The results in Table 2 show that this subtype specificity was also manifested by individual T-cell clones. Only clone 4.51 responded to H2 HA_1(305-328) but required >500-fold more of this peptide than H3 HA_1(305-328) for maximum proliferation (discussed below).

The H2 HA_1(305-328) peptide differs in sequence from the H3 peptide by only nine residues (Fig. 1). One or more of these residues must therefore be critical to the reactivity of the T cells. This was examined further by testing the ability of each clone to respond to synthetic analogs in which particular residues unique to the H3 subtype were replaced by those of the H2 subtype (Fig. 1). The clones recognizing the N-terminal region of the H3 HA_1(305-328) responded equally well to the parent peptide and to analog B, in which residues 320, 325, 326, 327, and 328 were replaced by those characteristic of the H2 subtype (Table 2). Analog A, which has substitutions at residues 311, 312, 313, 315, and 320, failed to stimulate the majority of these clones. Clone 8.2 recognized this analog but did so very inefficiently. Clone 4.51 showed a good proliferative response to analog A, but as with its response to H2 HA_1(305-328), this was apparent

TABLE 2. Proliferative response of T-cell clones to analogs of peptide H3 HA_1(305-328)

Peptide	% Proliferative response of T-cell clone[a]														
	8.1	3.23	4.27	4.28	8.6	3.21	4.5	4.4	7.2	7.6	3.33	7.5	7.11	8.2	4.51
H3 HA_1(305-328)	100.0	100.0	100.0	100.0[b]	100.0[b]	100.0	100.0	100.0[b]	100.0[b]	100.0[b]	100.0[b]	100.0[b]	100.0[b]	100.0[b]	100.0[c]
H2 HA_1(305-328)	0.1	0.0	0.4	0.3	0.0	0.2	0.0	0.0	0.1	0.3	0.3	0.0	0.4	0.2	40.6
Analog A	0.2	0.0	1.6	0.0	0.3	0.7	0.0	0.0	0.1	0.5	2.7	0.8	0.0	12.0	146.7
Analog B	0.0	157.3	170.0	102.3	124.0	84.0	76.3	120.1	87.9	147.0	78.9	104.0	100.5[b]	77.4[b]	87.4[c]

[a] Values represent the maximum proliferative responses over the dose range (0.02 to 10 μM), which, unless indicated, occurred at 10 μM. Data are expressed as mean counts per minute of triplicate cultures containing antigen minus counts per minute in the absence of antigen and are given as a percentage of the value obtained for T cells stimulated by H3 HA_1(305-328).
[b] Maximum proliferation occurred at 2 μM.
[c] Maximum proliferation occurred at 0.02 μM.

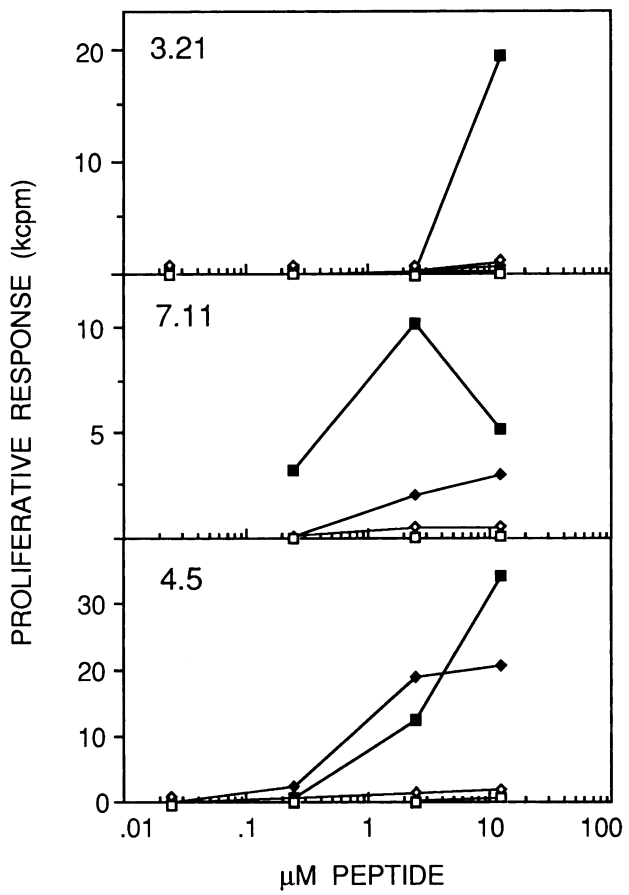

FIG. 2. Proliferative response of T-cell clones 3.21, 7.11, and 4.5 to analogs of peptide 306-319. Data are expressed as the mean counts per minute incorporated in triplicate cultures containing peptide 306-319 (■), analog C (◆), analog D (◇), or analog E (□) minus mean counts per minute in the absence of antigen.

only at relatively high concentrations of peptide. Clone 8.1, which was unique in its ability to recognize peptide 314-328, failed to respond to analog A or B.

To investigate the contribution of individual residues within the N-terminal half of the peptide, analogs of peptide 306-319 were constructed. Analog C contained the amino acid substitution Gln-311→Ser, analog D had Asn-312→Glu, and analog E had all four of the substitutions that occur in this region of the H2 subtype (Fig. 1). Figure 2 shows the response of selected clones to these analogs. None of the clones responded to analog E or analog D, which was substituted at residue 312. The clones differed in ability to tolerate the substitution of residue 311 in analog C. Clones 3.23, 4.28, 3.21, and 4.4 were not stimulated by this analog (for example, see Fig. 2, clone 3.21), clone 7.11 did respond but only weakly, whereas clone 4.5 efficiently recognized this analog. From these data and the data in Table 1, it can be seen that the clones not responding to analog C were also relatively inefficient in their recognition of peptide 307-319, whereas the clones that could recognize this analog also gave a good proliferative response to the 307-319 homolog.

Virus-reactive T-cell clones. As noted above, not all of the T-cell lines were able to proliferate when presented with whole virus. Only 2 of the 15 clones in this study, 4.51 and 8.2, were able to recognize the parent virus Mem 71 when tested in the range of 1 to 100 hemagglutinating units per

TABLE 3. Proliferative response of antipeptide T-cell clones to virus

Antigen	Proliferative response of T-cell clone[a]		
	8.2	4.51	4.28
H3 HA_1(305-328)	6,340	9,309	58,072
Mem 71 virus (H3)	541	40,741	0
A/Duck/Ukraine/1/63 virus (H3)	560	31,077	0
A/Philippines/2/82 virus (H3)	638[b]	34,088	0
Jap 57 virus (H2)	0	0	0

[a] Data represent the mean counts per minute of triplicate cultures containing antigen minus counts per minute in the absence of antigen, and each value is the maximum obtained over the following ranges: peptide, 0.2 to 10 μM; virus, 1 to 100 hemagglutinating units per culture. Background incorporation in the absence of antigen was 38 cpm for 8.2 (23 cpm in separate experiment), 265 cpm for 4.51, and 93 cpm for 4.28.

[b] Separate experiment.

culture (Table 3). Two other viruses of the H3 subtype, A/Duck/Ukraine/1/63 and A/Philippines/2/82, were also able to stimulate these two clones, even though these viruses each have a single amino acid change in the region between residues 306 and 319; A/Duck/Ukraine/1/63 has Val-309→Ile (36), and A/Philippines/2/82 has Lys-307→Arg (R. S. Daniels, personal communication). The H2 subtype virus, Jap 57, was unable to stimulate any of the clones.

Clone 4.51, which gave a very strong response to virus, was examined in more detail. While the other clones in this study, represented in Fig. 3 by 4.4, proliferated best in response to the higher concentrations of peptide tested (2 to 20 μM), clone 4.51 showed maximum response to H3 HA_1(305-328) at 0.02 μM. At higher concentrations of peptide, inhibition of the response was observed, a phenomenon previously described by Lamb et al. (20) for human clones responding to this peptide.

The dose response of 4.51 to analog B was indistinguishable from its response to H3 HA_1(305-328), while analog A induced proliferation only at 1,000 times the concentration (Fig. 3). Peptides 306-319 and 307-319 stimulated the clone to maximum proliferation at 0.2 μM, 10-fold more than required for the parent peptide. A further 10-fold increase in peptide concentration was required in the absence of residue 307 (peptide 308-319).

The results of testing 4.51 and 4.4 with purified Mem 71 virus over a wide range of concentrations are shown in Fig. 4. Clone 4.4 failed to respond to virus even at concentrations at least 10-fold greater than that producing maximum proliferation by the equivalent molar amount of peptide. Clone 4.51, however, was stimulated by virus at concentrations corresponding to 0.6 nM to 0.6 μM HA_1 C terminus, with the peak response occurring at a concentration similar to that obtained with peptide.

***H-2* restriction of the proliferative response of the clones.** All of the clones used in this study responded only when peptide was presented in association with $I\text{-}E^d$. This was deduced from their inability to proliferate when peptide was presented on APC from D2.GD mice, which bear $I\text{-}A^d$ and no I-E molecule (data not shown). Confirmation of $I\text{-}E^d$ restriction was obtained by blocking the proliferative response to H3 HA_1(305-328) with anti-$I\text{-}E^d$ MAbs. The proliferation of clone 8.2 was inhibited 99% by the addition of anti-$I\text{-}E^d$, with little or no blocking by MAbs against other *H-2* gene products (Fig. 5A).

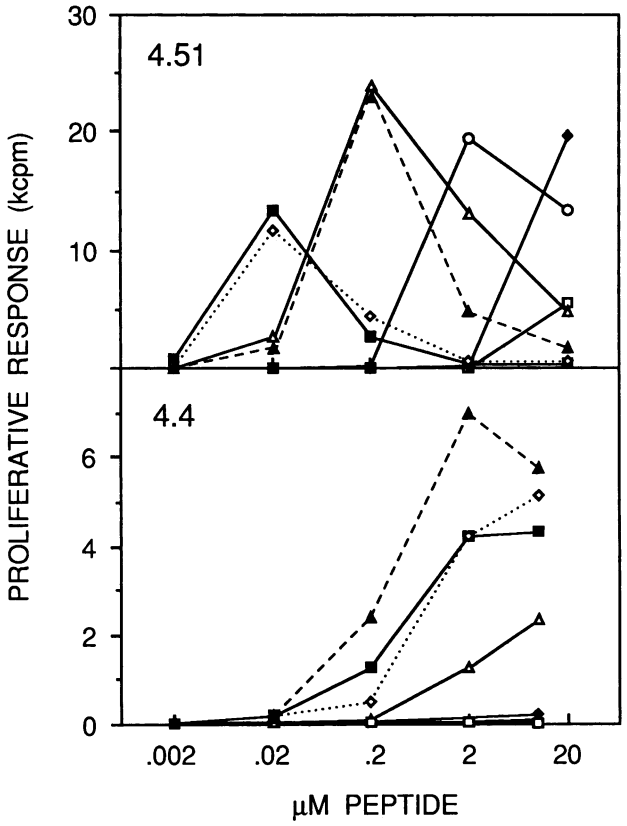

FIG. 3. Proliferative response of T-cell clones 4.51 and 4.4 to selected short homologs and analogs of peptide H3 HA_1(305-328). Data are expressed as the mean counts per minute in triplicate cultures containing H3 HA_1(305-328) (■), H2 HA_1(305-328) (□), analog A (◆), analog B (◇), 306-319 (▲), 307-319 (△), or 308-319 (○) minus mean counts per minute in the absence of antigen.

Clone 4.51 was also tested in this way, and as expected, at a peptide concentration of 0.2 μM, proliferation was inhibited by the anti-I-E^d MAb (Fig. 5B). However, at a 100-fold-greater concentration of peptide, the very low level of proliferation was actually enhanced (by approximately 550%) by addition of the anti-I-E^d MAb (Fig. 5C). None of the other MAbs had this ability to increase the response.

DISCUSSION

The T-cell response of mice primed to the peptide H3 HA_1(305-328) is focused predominantly on an antigenic region encompassed by residues 306 to 319. Within this region, a number of overlapping epitopes were revealed by the distinct reactivity patterns of individual clones with shorter homologs and analogs of the parent peptide. Some clones had an absolute requirement for residue 306, while others could proliferate in the absence of 306 and even 307. Substitution of residue Gln-311→Ser also had a differential effect on the response of the clones. All of the clones recognized their respective epitopes presented by the class II molecule I-E^d.

Heterogeneity of the T-cell response to a particular antigenic site may be considered in two ways. First, the processed antigen, in the form of a peptide, may bind the class II molecule in different places or in different orientations, each recognized by a different population of T cells. Second, processed antigen may be presented in a single orientation, but different clones of T cells may recognize slightly different regions of the antigen-class II molecule complex (7). If the structure of the class II molecule resembles that of the class I molecule (3), this would involve binding of the peptide in a particular conformation within the groove of the binding site, thus creating a single surface of residues from the antigen and from the class II molecule; T cells of different fine specificities may recognize overlapping areas of this surface. The first of these hypotheses would imply that within the antigenic site there are several different agretopes, that is, particular sets of residues capable of interacting with the class II molecule (32), while the second hypothesis predicts that only one agretope is necessary.

The data in the present study can be interpreted to mean that different T-cell receptors recognize different views of the 306- to -319 region of peptide H3 HA_1(305-328) presented in combination with I-E^d. For example, the clones whose reactivity was dependent on residues 306 and 311 were probably interacting with a part of the antigen-class II molecule surface different from, yet overlapping, the part that interacts with clones not dramatically influenced by these residues. In fact, the many different fine specificities of the receptors on the T cells described here may indicate several different views of this surface; the 14 clones respond-

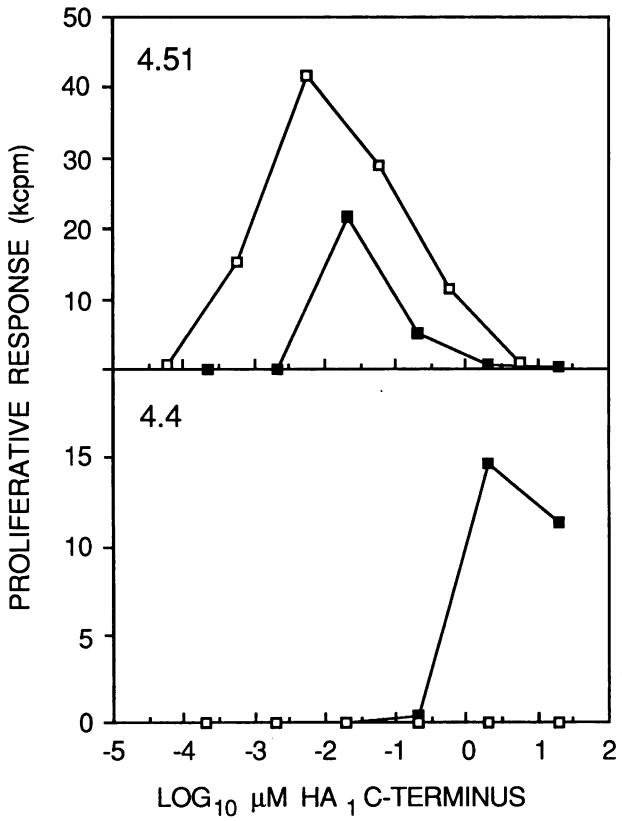

FIG. 4. Proliferative response of T-cell clones 4.51 and 4.4 to Mem 71 virus (□) and peptide H3 HA_1(305-328) (■). Approximate molar equivalents of HA_1 C termini in virus and in peptide were calculated assuming 10^7 virions per hemagglutinating unit of virus (9) and 10^3 HA trimers per virion (37). Data are expressed as mean counts per minute of triplicate cultures containing antigen minus mean counts per minute in the absence of antigen.

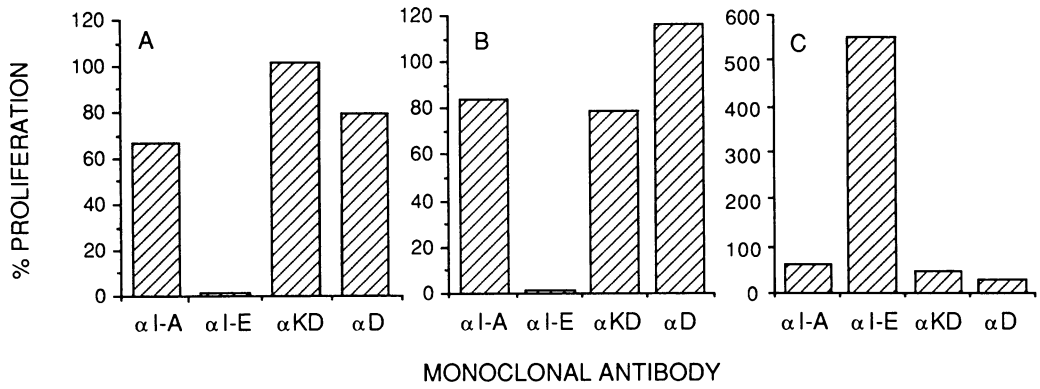

FIG. 5. Proliferative response of T-cell clones 8.2 and 4.51 in the presence of anti-major histocompatibility complex MAbs. Clones were tested with a stimulating dose of peptide H3 HA_1(305-328): 2 μM for 8.2 (A) and 0.2 μM for 4.51 (B). Clone 4.51 was also tested with a supraoptimal dose (20 μM) (C). MAbs 34-5-3S (anti-I-A), 14-4-4 (anti-I-E), 34-1-2S (anti-KD), and 34-2-12S (anti-D) were present at a final dilution of 10^{-2} for the entire culture period. Results were calculated as the mean counts per minute of triplicate cultures and expressed as a percentage of the response in the absence of antibody.

ing to peptide 306-319 (Table 1) display 12 different reactivity patterns their abilities to recognize different homologs and the relative efficiency of this recognition. The number of subtle differences in the fine specificities of T cells is analogous to the differences in specificities described for MAbs binding to a particular antigenic site (12). It is not improbable that the repertoires of T-cell receptors and antibodies have a similar degree of diversity, even though the mechanisms used to generate this diversity are not identical (22).

The 306- to -319 region contains a sequence pattern found by Rothbard and Taylor (30) to be common to many T-cell epitopes. In fact, a slightly shorter version of this same peptide (306-318 in the numbering of Mem 71 used here) was recently used by Rothbard et al. (29) in a study with two human DR1-restricted T-cell clones, one specific for this region of HA and the other specific for a peptide of influenza virus matrix protein. When the HA and matrix peptides were modeled on a helical wheel, in both cases the residues that comprise the allele-specific sequence pattern were found to be aligned on one face of the wheel. These residues, which are postulated to form the agretope of the peptides that interacts with DR1, can be exchanged from one peptide to the other, with the resulting hybrid peptides being presented by DR1 and recognized by the appropriate T-cell clone. In the HA peptide, the residues postulated by Rothbard et al. to be involved in binding to DR1 are 310, 313, 314, and 317. In the present study, analogs in which residues 311 and 312 were replaced by those in the corresponding position of the Jap 57 HA sequence were tested for their ability to stimulate the clones. The substitution at residue 311 was tolerated by some clones that recognize the 306- to -319 region but not by others. If each clone responding to this region recognizes antigen bound to $I\text{-}E^d$ via the same agretope, as we predict, then a change in an agretope residue might be expected to influence the response of all of the clones. Residue 311 is therefore likely to be within the epitope recognized by the T cells that do not tolerate this substitution, rather than part of the agretope. In contrast, the change at residue 312 was not tolerated by any of the T cells, and therefore this is a critical residue within either the agretope or the epitope recognized by each of the clones or is necessary for correct folding of the processed antigen. The fact that analog D, which is substituted at residue 312, fails to block presentation of the parent peptide 306-319 to the T-cell clone 4.51 even when it is present in the cultures at 200-fold molar excess (data not shown) suggests that residue 312 may be important for interaction of the peptide with the $I\text{-}E^d$ molecule.

One T-cell clone, 8.1, does not recognize peptide 306-319. Of all the shorter homologs of H3 HA_1(305-328) tested, this clone responded only to peptide 314-328, indicating the presence of a second site of T-cell recognition on the parent peptide. The lack of recognition by this clone of longer homologs that include the stimulatory region is similar to the pattern of response of clones observed in other systems (16, 24) and may possibly be explained by conformational constraints imposed by the additional residues. The fact that this clone failed to respond to analogs A or B suggests that the Met→Leu substitution at residue 320, which is common to both these analogs, may be responsible for the lack of reactivity, although it is equally possible that the Lys-315→Val substitution in analog A and the substitution of residues 325 to 328 in analog B are independently capable of abrogating activity. Despite the unique specificity of clone 8.1, this clone, like all the others in the panel, is $I\text{-}E^d$ restricted. Peptide H3 HA_1(305-328) may therefore have another agretope capable of interacting with this class II molecule in addition to the one used in the presentation of antigen to T cells specific for residues 306 to 319. It is also possible, however, that all clones recognize a peptide bound via an agretope within residues 314 to 319.

T cells from Mem 71 virus-primed BALB/c mice show no detectable response to peptide H3 HA_1(305-328) (unpublished data), even though, as shown in the present study, T cells capable of responding to this peptide are present in the repertoire of BALB/c mice. This phenomenon is unlikely to be due to competition for class II molecules to present the region of HA corresponding to the peptide, because the immunodominant response to Mem 71 virus is composed of T cells restricted by $I\text{-}A^d$ rather than $I\text{-}E^d$ (4). Furthermore, the majority of peptide-specific clones in this study did not proliferate in culture with virus at the doses tested. This lack of cross-reactivity may point to a difference in processing of the C-terminal 24 residues of HA_1 in the two forms of antigen. When virus is processed by murine splenic APC, this region, and residues 306 to 319 in particular, may not be fragmented in a manner appropriate for efficient presentation to T cells. This would be unlike the processing by human APC that can present the 306- to -318 region of HA_1 from virus via DR1 (29).

Two peptide-specific clones did, however, respond to virus; clone 8.2 gave a very poor response to virus, while 4.51 appeared to recognize virus as efficiently as the isolated peptide. One possibility is that any differences in the processing of the C-terminal region of HA_1 of the whole virus and that of peptide H3 HA_1(305-328) are irrelevant to recognition by the T-cell receptor of clone 4.51. An alternative possibility takes into account the observation made by Hackett et al. (13, 14) that the stimulation efficiency of peptide 111-119 of A/PR/8 (H1) HA for virus-specific or peptide-specific T-cell clones recognizing this region was 10^6- to 10^7-fold less than that of viral HA. This was later shown (11) to be due to the much more efficient presentation of the virus due to its specific focusing onto APC via interaction with sialic acid residues on the surface of the cell. If this is a general truth for presentation of influenza virus, then clone 4.51 is recognizing virus approximately 10^6-fold less efficiently than expected. This would suggest that the 306- to -319 region as it is seen in H3 HA_1(305-328) is not a normal product of processing or presentation of H3 virus by BALB/c APC. Only the high affinity of clone 4.51, as evidenced by its ability to recognize lower concentrations of peptide H3 HA_1(305-328) than the other clones, would allow this clone to detect a small amount of antigen that may be presented in the required form.

The fact that clone 4.51 could be stimulated at low antigen concentrations allowed examination of the response under supraoptimal conditions. As found by Matis et al. (23) with T-cell clones to cytochrome *c*, high antigen concentrations were inhibitory and this high-dose suppression could be overcome by addition of antibody against the relevant class II molecule. Suppression was thought to result from blockage of the mitogenic signal delivered by interleukin 2 due to restimulation of the activated T cells 20 h after initial stimulation (34). In those studies, suppression was observed with a 65- and a 21-residue fragment but not with a 14-residue synthetic peptide having full antigenic potency, suggesting that size was a critical factor. Synthetic peptides of 12, 13, and 14 residues can inhibit proliferation of clone 4.51 (Fig. 3).

In a previous study (31), the epitopes recognized by BALB/c antibodies on peptide H3 HA_1(305-328) were identified. Antibodies directed to the B-cell epitope 314-LKLAT-318 dominated the primary response, whereas antibodies to 320-MRNVPEKQT-328 were predominant during the secondary response (6). The immunodominant site of recognition by T-cell clones, residues 306 to 319, encompasses LKLAT, while the 314- to -328 region recognized by clone 8.1 encompasses both of the major epitopes recognized by B cells. Celada and Sercarz (8) have proposed that T-cell–B-cell cooperation for antibody production is not random but that the B cell selects the T cell with which to cooperate. This selection is postulated to take place at the level of antigen processing and presentation by the B cell. The B cell binds antigen via its immunoglobulin receptors, and then the antigen-receptor complex is internalized and the antigen is processed for presentation to T cells. The region of the antigen bound by the paratope of the immunoglobulin may be protected from enzymatic degradation during processing and thus will be presented intact. Therefore the residues recognized by the receptor on a B cell may overlap those recognized by the T cell with which the B cell interacts, as found in the present study. In this study the T-cell clones were derived after a primary immunization or very soon after a booster. It will be of interest to determine whether T cells obtained late in the secondary response to H3 HA_1(305-328) have a specificity different to those of the T cells described here, possibly with an increased frequency of clones such as 8.1 that recognize the C-terminal end of the peptide, which is associated with the B-cell epitope 320 to 328.

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LITERATURE CITED

1. **Allen, P. M., G. R. Matsueda, E. Haber, and E. R. Unanue.** 1985. Specificity of the T-cell receptor: two different determinants are generated by the same peptide and the I-A^k molecule. J. Immunol. **135**:368–373.
2. **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**: 2498–2503.
3. **Brown, J. H., T. Jardetzky, M. A. Saper, B. Samraoul, P. J. Bjorkman, and D. C. Wiley.** 1988. A hypothetical model of the foreign antigen binding site of class II histocompatibility molecules. Nature (London) **332**:845–850.
4. **Brown, L. E., R. A. Ffrench, J. M. Gawler, D. C. Jackson, M. L. Dyall-Smith, E. M. Anders, G. W. Tregear, L. Duncan, P. A. Underwood, and D. O. White.** 1988. Distinct epitopes recognized by *I-A*^{d}-restricted T-cell clones within antigenic site E on influenza virus hemagglutinin. J. Virol. **62**:305–312.
5. **Brown, L. E., J. M. Katz, R. A. Ffrench, E. M. Anders, and D. O. White.** 1987. Characterization of subtype-specific and cross-reactive helper-T-cell clones recognizing influenza virus hemagglutinin. Cell. Immunol. **109**:12–24.
6. **Brown, L. E., J. M. Murray, E. M. Anders, X.-L. Tang, D. O. White, G. W. Tregear, and D. C. Jackson.** 1988. Genetic control and fine specificity of the immune response to a synthetic peptide of influenza virus hemagglutinin. J. Virol. **62**:1746–1752.
7. **Cease, K. B., I. Berkower, J. York-Jolley, and J. A. Berzofsky.** 1986. T cell clones specific for an amphipathic α-helical region of sperm whale myoglobin show differing fine specificities for synthetic peptides. A multiview/single structure interpretation of immunodominance. J. Exp. Med. **164**:1779–1784.
8. **Celada, F., and E. E. Sercarz.** 1988. Preferential pairing of T-B specificities in the same antigen: the concept of directional help. Vaccine **6**:94–98.
9. **Donald, H. B., and A. Isaacs.** 1954. Counts of influenza virus particles. J. Gen. Microbiol. **10**:457–464.
10. **Dryland, A., and R. C. Sheppard.** 1988. Peptide synthesis. II. A system for continuous flow solid-phase peptide synthesis using fluorenylmethoxycarbonyl-amino acid pentafluorophenyl esters. Tetrahedron **44**:859–876.
11. **Eisenlohr, L. C., W. Gerhard, and C. J. Hackett.** 1987. Role of receptor-binding activity of the viral hemagglutinin molecule in the presentation of influenza virus antigens to helper T cells. J. Virol. **61**:1375–1383.
12. **Gerhard, W., J. Yewdell, M. E. Frankel, and R. Webster.** 1981. Antigenic structure of influenza virus haemagglutinin defined by hybridoma antibodies. Nature (London) **290**:713–717.
13. **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**:294–302.
14. **Hackett, C. J., J. L. Hurwitz, B. Dietzschold, and W. Gerhard.** 1985. A synthetic decapeptide of influenza virus hemagglutinin elicits helper T cells with the same fine recognition specificities as occur in response to whole virus. J. Immunol. **135**:1391–1394.
15. **Hackett, C. J., J. L. Hurwitz, C. Moller, and W. Gerhard.** 1985. Fine specificity of antigen recognition by influenza hemagglutinin-specific helper T cells: heterogeneity of clones and the role of a single amino acid position in cross-reactive responses, p.

48-55. *In* W. G. Laver and G. M. Air (ed.), Immune recognition of protein antigens. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

16. **Heber-Katz, E., M. Hollosi, B. Dietzschold, F. Hudecz, and G. D. Fasman.** 1985. The T cell response to the glycoprotein D of the herpes simplex virus: the significance of antigen conformation. J. Immunol. **135**:1385–1390.
17. **Hurwitz, J. L., E. Heber-Katz, C. J. Hackett, and W. Gerhard.** 1984. Characterization of the murine T_H response to influenza virus hemagglutinin: evidence for three major specificities. J. Immunol. **133**:3371–3377.
18. **Jackson, D. C., X.-L. Tang, L. E. Brown, J. M. Murray, D. O. White, and G. W. Tregear.** 1986. Antigenic determinants of influenza virus hemagglutinin. XII. The epitopes of a synthetic peptide representing the C-terminus of HA_1. Virology **155**: 625–632.
19. **Lamb, J. R., and N. Green.** 1983. Analysis of the antigen specificity of influenza hemagglutinin-immune human T lymphocyte clones: identification of an immunodominant region for T cells. Immunology **50**:659–666.
20. **Lamb, J. R., B. J. Skidmore, N. Green, J. M. Chiller, and M. Feldmann.** 1983. Induction of tolerance in influenza virus-immune T lymphocyte clones with synthetic peptides of influenza hemagglutinin. J. Exp. Med. **157**:1434–1447.
21. **Manca, F., J. A. Clarke, A. Miller, E. E. Sercarz, and N. A. Shastri.** 1984. A limited region within hen egg-white lysozyme serves as the focus for a diversity of T-cell clones. J. Immunol. **133**:2075–2078.
22. **Marrack, P., and J. Kappler.** 1988. The T-cell repertoire for antigen and MHC. Immunol. Today **9**:308–315.
23. **Matis, L. A., L. H. Glimcher, W. E. Paul, and R. H. Schwartz.** 1983. Magnitude of response of histocompatibility-restricted T-cell clones is a function of the product of the concentrations of antigen and Ia molecules. Proc. Natl. Acad. Sci. USA **80**: 6019–6023.
24. **Mills, K. H. G., D. S. Burt, J. J. Skehel, and D. B. Thomas.** 1988. Fine specificity of murine class II-restricted T-cell clones for synthetic peptides of influenza virus hemagglutinin. Heterogeneity of antigen interaction with the T cell and the Ia molecule. J. Immunol. **140**:4083–4090.
25. **Mills, K. H. G., J. J. Skehel, and D. B. Thomas.** 1986. Extensive diversity in the recognition of influenza virus hemagglutinin by murine T helper clones. J. Exp. Med. **163**:1477–1490.
26. **Nestorowicz, A., G. W. Tregear, C. N. Southwell, J. Martyn, J. M. Murray, D. O. White, and D. C. Jackson.** 1985. Antibodies elicited by influenza virus hemagglutinin fail to bind to synthetic peptides representing putative antigenic sites. Mol. Immunol. **22**:145–154.
27. **Ozato, K., N. M. Mayer, and D. H. Sachs.** 1980. Hybridoma cell lines secreting monoclonal antibodies to mouse H-2 and Ia antigens. J. Immunol. **124**:533–540.
28. **Ozato, K., N. M. Mayer, and D. H. Sachs.** 1982. Monoclonal antibodies to mouse major histocompatibility complex antigens. IV. A series of hybridoma clones producing anti-H-2^d antibodies and an examination of expression of H-2^d antigens on the surface of these cells. Transplantation **34**:113–120.
29. **Rothbard, J. B., R. I. Lechler, K. Howland, V. Bal, D. D. Eckels, R. Sekaly, E. O. Long, W. R. Taylor, and J. R. Lamb.** 1988. Structural model of HLA-DR1 restricted T cell antigen recognition. Cell **52**:515–523.
30. **Rothbard, J. B., and W. R. Taylor.** 1988. A sequence pattern common to T cell epitopes. EMBO J. **7**:93–100.
31. **Schoofs, P. G., H. M. Geysen, D. C. Jackson, L. E. Brown, X.-L. Tang, and D. O. White.** 1988. Epitopes of an influenza viral peptide recognized by antibody at single amino acid resolution. J. Immunol. **140**:611–616.
32. **Schwartz, R. H.** 1985. T-lymphocyte recognition of antigen in association with gene products of the major histocompatibility complex. Annu. Rev. Immunol. **3**:237–261.
33. **Schwartz, R. H., B. S. Fox, E. Fraga, C. Chen, and B. Singh.** 1985. The T lymphocyte response to cytochrome *c*. V. Determination of the minimal peptide size required for stimulation of T cell clones and assessment of the contribution of each residue beyond this size to antigenic potency. J. Immunol. **135**:2598–2608.
34. **Suzuki, G., Y. Kawase, S. Koyasu, I. Yahara, Y. Kobayashi, and R. H. Schwartz.** 1988. Antigen-induced suppression of the proliferative response of T cell clones. J. Immunol. **140**:1359–1365.
35. **Tang, X.-L., G. W. Tregear, D. O. White, and D. C. Jackson.** 1988. Minimum requirements for the immunogenic and antigenic activities of homologs of a synthetic peptide of influenza virus hemagglutinin. J. Virol. **62**:4745–4751.
36. **Ward, C. W.** 1981. Structure of the influenza virus hemagglutinin. Curr. Top. Microbiol. Immunol. **94**:1–74.
37. **White, D. O.** 1974. Influenza viral proteins: identification and synthesis. Curr. Top. Microbiol. Immunol. **63**:1–48.