

# NATURE OF T LYMPHOCYTE RECOGNITION OF MACROPHAGE-ASSOCIATED ANTIGENS

## V. Contribution of Individual Peptide Residues of Human Fibrinopeptide B to T Lymphocyte Responses\*

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Many of the mechanisms proposed to explain proliferating or helper T lymphocyte recognition of exogenous antigens are predicted on the four following basic observations: (a) soluble antigen alone fails to stimulate T cells (1); (b) antigen-specific T cell stimulation depends on accessory cells, which in the guinea pig are macrophage-like (1-3); (c) T cell stimulation is genetically restricted for the Ia antigens expressed by the accessory cell (4-9); and (d) Ir gene function is correlated with Ia expression by accessory cells (10-14). The various hypotheses accommodating these observations seem to fall into two general categories. The first category encompasses determinant selection-type proposals (15-17). For antigens not under Ir gene control, determinant selection suggests that accessory cell Ia antigens bind exogenous antigen, where different Ia species bind different antigenic regions, and that T cells express a single antigen-combining receptor that interacts with distinct antigenic determinants of the exogenous antigen. For antigens under Ir gene control, the nonresponder accessory cell Ia fails to bind the antigen and/or there are no T cell antigen-combining receptors for those particular antigenic determinants. The second category of proposals for T cell recognition are of the dual recognition type (18-20). These mechanisms suggest that exogenous antigens need not bind to accessory cell Ia antigens, and that T cells express two distinct receptors for recognition; one directed against Ia antigens, and a second directed against exogenous antigenic determinants. For antigens under Ir gene control, T cells exhibit allelic exclusion such that the antigen-specific receptor is expressed concordantly only with structures directed against Ia of the responder haplotype.

One approach to examine the nature of T cell recognition in light of these theories is to use small well defined peptide antigens to determine which regions of the antigen interact with T cells and which regions, if any, specifically interact with accessory cell Ia. Initial studies using this approach have employed the 14 residue peptide antigen,

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human fibrinopeptide B (hFPB),<sup>1</sup> in guinea pig T cell-proliferative responses. We have previously found that strain 2 guinea pigs responded to hFPB (B $\beta$ 1-14), that most of the antigenic regions were in the B $\beta$ 5-14 fragment, and that the minimum antigenic and immunogenic fragment was B $\beta$ 7-14 (21). However, strain 2 animals failed to produce immune responses with the des-Arg hFPB homologue B $\beta$ 1-13. By contrast, strain 13 guinea pigs produced immune responses with B $\beta$ 1-13, but were unresponsive with B $\beta$ 1-14. Thus, genetic control of T cell responsiveness to hFPB is determined by a single residue, Arg<sup>14</sup>, and is regulated by Ia antigens of the stimulator macrophages (17). In this communication we examined the contributions of individual residues in the hFPB homologue B $\beta$ 5-14 (the peptide fragment containing most of the antigenic regions involved in T cell responses) to T cell responses using various synthetic analogues with single residue substitutions. Several residues were identified that determined the specificity of the T cell responses, and most likely serve as contact points for a clonally distributed T cell antigen-combining receptor. Several other residues were identified that appear to serve as contact points for structures with more conservative combining properties.

### Materials and Methods

**Animals.** Inbred strain 2 and strain 13 guinea pigs were obtained from Biological Systems, Toms River, N. J.

**Antigens.** Human fibrinopeptide B (hFPB, B $\beta$ 1-14) was purchased from Bachem, Inc., Torrance, Calif., and purified protein derivative of tuberculin (PPD) from Connaught Medical Research Laboratory, Willowdale, Ontario, Canada.

**Reagents.** All chemicals were of reagent grade. *N*<sup>α</sup>-tert-butoxycarbonyl (Boc) amino acids were purchased from Bachem, Inc. Boc amino acids with protected side chains were  $\beta$ -benzylaspartic acid,  $\gamma$ -benzylglutamic acid, *O*-benzylserine, *N*<sup>ε</sup>-tosylarginine, and *N*<sup>ε</sup>-trifluoroacetyl lysine. All Boc amino acids were of the L-configuration with the exception of glycine. Purity of Boc amino acids was assessed by melting points (uncorrected) and thin-layer chromatography. DEAE-Sephacel and SP-Sephadex C-25 were obtained from Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J. Bio-Gel P2, 100–200 mesh, was purchased from Bio-Rad Laboratories, Richmond, Calif.

**Peptide Synthesis.** Analogues of human fibrinogen sequence B $\beta$ 5-14 were synthesized manually using Merrifield's solid-phase method as previously described (22). Boc-Asp and Boc-Gln were introduced using a *N,N*-dicyclohexylcarbodiimide coupling in the presence of equimolar 1-hydroxybenzotriazolemonohydrate. The analogue Arg<sup>14</sup> → Lys was prepared by treating the analogue Arg<sup>14</sup> → Lys (Tfa) with 1 M piperidine to remove the TFA-blocking group (23). All analogues with the exception of Arg<sup>14</sup> → Lys (Tfa) were purified definitively by ion-exchange chromatography on a 1.2- × 34-cm DEAE-Sephacel column equilibrated with a 0.005 M borate buffer (24) followed by desalting on a 2.0- × 70-cm Bio-Gel P-2 column equilibrated with 0.05 M NH<sub>4</sub>HCO<sub>3</sub>. Analogue Arg<sup>14</sup> → Lys (Tfa) was purified on a 1.2- × 68-cm SP-Sephadex C-25 column using a pH gradient as described before (22). Purity and identity of synthetic peptides were assessed by thin-layer chromatography, thin-layer electrophoresis, and amino acid analysis (22, 24).

**Preparation of Cells.** 2–6 wk after immunization with 400  $\mu$ g of hFPB or of the various analogues in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) guinea pigs were injected intraperitoneally with 25 ml of sterile mineral oil (Marcol 52; Exxon Company, U. S. A., Houston, Tex.), and the resulting peritoneal exudate was harvested 3–4 d later. A T lymphocyte-enriched peritoneal exudate lymphocyte (PEL) population was prepared by passing cells over a rayon wool adherence column (3).

<sup>1</sup> *Abbreviations used in this paper:* BOC, *N*<sup>α</sup>-tert-butoxycarbonyl; DNP, dinitrophenyl; hFPB, human fibrinopeptide B; <sup>3</sup>H-TdR-[<sup>3</sup>H]methyl thymidine; PEL, peritoneal exudate lymphocyte; PPD, purified protein derivative of tuberculin; Tfa, trifluoroacetyl.

*In Vitro Assay of DNA Synthesis.* Immune PEL ( $3-4 \times 10^5$  cells/well) were cultured in round-bottom microtiter plates (Cooke Laboratory Products Div., Dynatech Laboratories, Inc., Dynatech Corp., Arlington, Va.) with soluble hFPB or the B $\beta$ 5-14 analogues (5-40  $\mu$ g/well) or PPD (10  $\mu$ g/well), in a total volume of 0.2 ml or RPMI-1640 medium (Grand Island Biological Co., Grand Island, N. Y.) containing L-glutamine (300  $\mu$ g/ml) penicillin (100 U/ml), 2-mercaptoethanol ( $5 \times 10^{-5}$  M), and 5% heat-inactivated normal guinea pig serum. After incubation for 2 d at 37°C in 5% CO<sub>2</sub> in air, 1  $\mu$ Ci of tritiated thymidine (<sup>3</sup>H-TdR; sp act 6.7 Ci/mmol; Research Products International Corp., Elk Grove Village, Ill.) was added to each well. The amount of radioactivity incorporated into cellular DNA was determined after an additional 18-h incubation with the aid of a semiautomated microharvesting device (Titer-Tek; Flow Laboratories, Inc., Rockville, Md.). Each <sup>3</sup>H-TdR counts per minute value represents the mean of triplicate cultures, and the standard error was always within 10% of the mean. The representative experiments shown were each performed two to six times with similar results, and 40  $\mu$ g of the peptide antigens was the dose producing the highest proliferative responses.

### Results

To examine the contributions of individual residues to the antigenicity and immunogenicity of hFPB, various analogues of B $\beta$ 5-14 were synthesized as listed under Fig. 1. The three following different regions of the B $\beta$ 5-14 peptide were investigated: the basic residue arginine<sup>14</sup>, the hydrophobic residues phenylalanine<sup>10</sup> and phenylalanine<sup>11</sup>, and the acidic residues aspartic acid<sup>5</sup>, glutamic acid<sup>7</sup>, and glutamic acid<sup>8</sup>. Each analogue was tested for antigenicity by culture with hFPB-immune strain 2 guinea pig T cells. In addition, strain 2 and strain 13 animals were immunized with each analogue and T cell responsiveness determined by culture with the homologous analogue used for immunization, as well as B $\beta$ 1-14.

*Substitutions for Arginine<sup>14</sup>.* The basic residue Arg<sup>14</sup> was replaced with another basic residue, lysine, or the  $\epsilon$ -blocked neutral Lys (Tfa), and these analogues were examined for antigenicity and immunogenicity (Table I). T cells from hFPB strain 2 animals showed proliferative responses to B $\beta$ 1-14 and 5-14, but were unresponsive to either the B $\beta$ 5-14 Lys<sup>14</sup> or Lys(Tfa)<sup>14</sup> analogues (Table I, lines 1 and 2). By contrast, T cells from strain 2 guinea pigs immunized with the Lys<sup>14</sup> analogue responded to the Lys<sup>14</sup> analogue, but showed no responses to either B $\beta$ 1-14 or the Lys(Tfa)<sup>14</sup> analogue (Table I, line 3). In similar fashion, T cells from strain 2 animals immunized with the Lys(Tfa)<sup>14</sup> analogue responded to the Lys(Tfa)<sup>14</sup> analogue, but were unresponsive to B $\beta$ 1-14 and  $\beta$ 5-14, and showed a low response to the Lys<sup>14</sup> analogue (Table I, line 4).

	5	6	7	8	9	10	11	12	13	14	Antigenicity		Immunogenicity		
											for hFPB-immune		Guinea Pig		
												Strain 2	T cells	Strain 2	Strain 13
B $\beta$ 5-14	H <sub>2</sub> N-Asp-Asn-Glu-Glu-Gly-Phe-Phe-Ser-Ala-Arg-OH											+++		+++	-
	Lys											-		+++	-
	Lys(Tfa)											-		+++	-
	Tyr											-		-	+
	Phe(4-NO <sub>2</sub> )											-		-	-
	Tyr											-		+++	-
	Phe(4-NO <sub>2</sub> )											-		+++	-
	Gln-Gln											+		+	-
	Asn											+++		+++	-

FIG. 1. Primary structure of B $\beta$ 5-14 and B $\beta$ 5-14 analogues, and summary of antigenicity and immunogenicity of these peptides for strain 2 and strain 13 guinea pig T lymphocyte responses.

TABLE I  
Antigenicity and Immunogenicity of Analogues Containing Substitutions for Arginine<sup>14</sup>

Guinea pig	Immunization	Antigen in culture					
		None	PPD	B $\beta$ 1-14	B $\beta$ 5-14	B $\beta$ 5-14 Arg <sup>14</sup> → Lys	B $\beta$ 5-14 Arg <sup>14</sup> → Lys(Tfa)
<sup>3</sup> H-TdR cpm							
Strain 2	B $\beta$ 1-14	1,452	<u>103,490</u>	<u>37,107</u>	<u>21,918</u>	1,656	
Strain 2	B $\beta$ 1-14	1,708	<u>71,919</u>	<u>28,583</u>		1,786	
Strain 2	B $\beta$ 5-14 Arg <sup>14</sup> → Lys	2,290	<u>76,425</u>	3,269		<u>21,875</u>	4,007
Strain 2	B $\beta$ 5-14 Arg <sup>14</sup> → Lys(Tfa)	1,301	<u>88,553</u>	1,394	823	<u>7,793</u>	<u>26,341</u>
Strain 13	B $\beta$ 5-14 Arg <sup>14</sup> → Lys	1,461	<u>65,646</u>	1,682		1,556	1,384
Strain 13	B $\beta$ 5-14 Arg <sup>14</sup> → Lys(Tfa)	2,612	<u>47,566</u>	1,005			2,409

Strain 2 and strain 13 guinea pigs were immunized with hFPB (B $\beta$ 1-14) or the B $\beta$ 5-14 analogues as indicated in the Table. 2-6 wk after immunization T cell-enriched PEL were cultured in vitro with PPD, as a positive control, and B $\beta$ 1-14, B $\beta$ 5-14, and the B $\beta$ 5-14 analogues (40  $\mu$ g/well) as described under Materials and Methods. The <sup>3</sup>H-TdR counts per minute were determined on the 3rd d of culture, and each value represents the mean counts per minute from triplicate cultures. Underlined values indicate those cultures in which positive stimulation occurred, whereas a dashed line indicates those cultures showing relatively less stimulation.

It is not known if the small response of Lys(Tfa)<sup>14</sup>-immune T cells to the Lys<sup>14</sup> analogue represents antigenic cross-reactivity or, rather, results from contamination of the blocked lysine peptide with small amounts of free lysine. In any case, it is clear that most of the strain 2 T cell responsiveness is uniquely directed against the Lys(Tfa)<sup>14</sup> analogue. Neither of these analogues, however, was immunogenic for strain 13 guinea pigs (Table I, lines 5 and 6).

*Substitutions for Phenylalanine.* In the first series of experiments, residues Phe<sup>10</sup> and Phe<sup>11</sup> in B $\beta$ 5-14 were replaced by tyrosine, and the antigenicity and immunogenicity of the Tyr<sup>10</sup> and Tyr<sup>11</sup> B $\beta$ 5-14 analogues were determined as described above (Table II). T cells from hFPB-immune strain 2 animals showed no responsiveness to either the Tyr<sup>10</sup> or Tyr<sup>11</sup> analogues (Table II, line 1). On the other hand, T cells from strain 2 guinea pigs immunized with the Tyr<sup>10</sup> analogue were responsive to the Tyr<sup>10</sup> analogue, but were unresponsive to the Tyr<sup>11</sup> analogue and showed low responses to B $\beta$ 1-14 and 5-14 (Table II, line 2). By contrast, T cells from strain 2 animals immunized with the Tyr<sup>11</sup> analogue were unresponsive to all of the analogues tested (Table II, line 3). This lack of responsiveness did not seem to be a result of dominant suppression because mixtures of the Tyr<sup>11</sup> analogue with either hFPB or the Tyr<sup>10</sup> analogue for immunization and in vitro challenge did not diminish responsiveness to hFPB or the Tyr<sup>10</sup> analogue, respectively (data not shown). The Tyr<sup>10</sup> analogue was

TABLE II  
*Antigenicity and Immunogenicity of Analogues in which Phenylalanine<sup>10</sup> and Phenylalanine<sup>11</sup> Are Replaced by Tyrosine*

Guinea pig	Immunization	Antigen in Culture					
		None	PPD	B $\beta$ 1-14	B $\beta$ 5-14	B $\beta$ 5-14 Phe <sup>10</sup> → Tyr	B $\beta$ 5-14 Phe <sup>11</sup> → Tyr
				<sup>3</sup> H-TdR cpm			
Strain 2	B $\beta$ 1-14	1,416	<u>151,730</u>	<u>17,334</u>	<u>11,508</u>	1,876	1,726
Strain 2	B $\beta$ 5-14 Phe <sup>10</sup> → Tyr	2,030	<u>153,637</u>	<u>15,730</u>	<u>10,589</u>	<u>111,690</u>	3,410
Strain 2	B $\beta$ 5-14 Phe <sup>11</sup> → Tyr	2,898	<u>178,955</u>	3,929	3,892	3,563	3,724
Strain 13	B $\beta$ 5-14 Phe <sup>10</sup> → Tyr	4,993	<u>181,983</u>	4,850	4,366	6,748	4,114
Strain 13	B $\beta$ 5-14 Phe <sup>11</sup> → Tyr	3,893	<u>248,827</u>	4,043	4,412	4,885	<u>8,652</u>

See legend for Table I.

not immunogenic for strain 13 guinea pigs, as found for the residue 14 analogues, but T cells from strain 13 animals immunized with the Tyr<sup>11</sup> analogue showed a low response to the Tyr<sup>11</sup> analogue (Table II, lines 4 and 5).

In a second series of experiments, residues Phe<sup>10</sup> and Phe<sup>11</sup> were replaced by Phe(4-NO<sub>2</sub>), and the Phe(4-NO<sub>2</sub>)<sup>10</sup> and Phe(4-NO<sub>2</sub>)<sup>11</sup> B $\beta$ 5-14 analogues were examined for antigenicity and immunogenicity as before. The patterns of T cell responsiveness were essentially identical with those observed with the Tyr<sup>10</sup> and Tyr<sup>11</sup> analogues. Briefly, neither the Phe(4-NO<sub>2</sub>)<sup>10</sup> nor Phe(4-NO<sub>2</sub>)<sup>11</sup> analogues stimulated hFPB-immune strain 2 T cells (Table III, line 1). Strain 2 animals immunized with the Phe(4-NO<sub>2</sub>)<sup>10</sup> analogue responded to the Phe(4-NO<sub>2</sub>)<sup>10</sup> analogue but not to B $\beta$ 1-14 or to the Phe(4-NO<sub>2</sub>)<sup>11</sup> analogue (Table III, line 2), and the Phe(4-NO<sub>2</sub>)<sup>11</sup> analogue was nonimmunogenic for strain 2 guinea pigs (Table III, line 3). Neither analogue was immunogenic for strain 13 animals (Table III, lines 4 and 5). Again, mixtures of the Phe(4-NO<sub>2</sub>)<sup>11</sup> analogue with hFPB for immunization and in vitro challenge did not diminish the hFPB-specific strain 2 T cell responses (data not shown).

*Substitutions for Glutamic Acid.* To examine the contribution of residues Glu<sup>7</sup> and Glu<sup>8</sup> to T cell responses to hFPB both of these residues were substituted with glutamine, and the antigenicity and immunogenicity of the Gln<sup>7,8</sup> B $\beta$ 5-14 were analogue determined. T cells from hFPB-immune strain 2 animals respond to the Gln<sup>7,8</sup> analogue at levels from 20 to 30% of the response observed to B $\beta$ 1-14 (Table IV, line 1). By contrast, T cells from strain 2 guinea pigs immunized with the Gln<sup>7,8</sup> analogue responded poorly to the Gln<sup>7,8</sup> analogue, but showed good responses to B $\beta$ 1-14, B $\beta$ 5-14, and 7-14 (Table IV, line 2). This pattern of responsiveness after Gln<sup>7,8</sup> analogue immunization has been consistent in five different experiments. In addition, mixtures of the Gln<sup>7,8</sup> analogue with hFPB for immunization and in vitro challenge

TABLE III  
*Antigenicity and Immunogenicity of Analogues in which Residues Phenylalanine<sup>10</sup> and Phenylalanine<sup>11</sup> Are Replaced by Phenylalanine (4-NO<sub>2</sub>)*

Guinea pig	Immunization	Antigen in culture				
		None	PPD	B $\beta$ 1-14	B $\beta$ 5-14 Phe <sup>10</sup> → Phe (4-NO <sub>2</sub> )	B $\beta$ 5-14 Phe <sup>11</sup> → Phe (4-NO <sub>2</sub> )
				<sup>3</sup> H-TdR cpm		
Strain 2	B $\beta$ 1-14	2,452	<u>57,927</u>	<u>52,228</u>	3,772	2,852
Strain 2	B $\beta$ 5-14 Phe <sup>10</sup> → Phe(4-NO <sub>2</sub> )	2,418	<u>50,262</u>	1,862	<u>29,644</u>	1,909
Strain 2	B $\beta$ 5-14 Phe <sup>11</sup> → Phe(4-NO <sub>2</sub> )	4,859	<u>154,400</u>	4,399	3,529	3,662
Strain 13	B $\beta$ 5-14 Phe <sup>10</sup> → Phe(4-NO <sub>2</sub> )	1,164	<u>148,983</u>	2,036	1,570	1,874
Strain 13	B $\beta$ 5-14 Phe <sup>11</sup> → Phe(4-NO <sub>2</sub> )	702	<u>72,066</u>	519	676	979

See legend for Table I.

TABLE IV  
*Antigenicity and Immunogenicity of an Analogue in which Glutamic Acid<sup>7</sup> and Glutamic Acid<sup>8</sup> Are Replaced by Glutamine*

Guinea pig	Immunization	Antigen in culture					
		None	PPD	B $\beta$ 1-14	B $\beta$ 5-14	B $\beta$ 7-14	B $\beta$ 5-14 Glu <sup>7,8</sup> → Gln
				<sup>3</sup> H-TdR cpm			
Strain 2	B $\beta$ 1-14	5,020	<u>98,322</u>	<u>36,677</u>			<u>11,645</u>
Strain 2	B $\beta$ 5-14 Glu <sup>7,8</sup> → Gln	3,063	<u>133,291</u>	<u>43,576</u>	<u>48,935</u>	<u>37,323</u>	6,967
Strain 13	B $\beta$ 5-14 Glu <sup>7,8</sup> → Gln	1,077	<u>144,946</u>	879			1,038

See legend for Table I.

did not alter the magnitude or pattern of T cell responsiveness to hFPB or to the Gln<sup>7,8</sup> analogue (data not shown). As was found for the other analogues tested, strain 13 guinea pigs failed to respond to the Gln<sup>7,8</sup> analogue (Table IV, line 3).

*Substitution for Aspartic Acid.* The contribution of residue Asp<sup>5</sup> to T cell responses was examined by replacing Asp<sup>5</sup> with asparagine (Table V). The Asn<sup>5</sup> B $\beta$ 5-14 analogue stimulated hFPB-immune strain 2 T cells to around half of the response observed with B $\beta$ 1-14 and 80% of that observed with B $\beta$ 5-14 (Table V, line 1). T cells from strain 2 animals immunized with the Asn<sup>5</sup> analogue produced proliferative

TABLE V  
*Antigenicity and Immunogenicity of an Analogue in which Aspartic Acid<sup>5</sup> is Replaced with Asparagine*

Guinea pig	Immunization	Antigen in culture				
		None	PPD	B $\beta$ 1-14	B $\beta$ 5-14	B $\beta$ 5-14 Asp <sup>5</sup> → Asn
				<i><sup>3</sup>H-TdR cpm</i>		
Strain 2	B $\beta$ 1-14	2,952	<u>121,801</u>	<u>47,254</u>	<u>33,117</u>	<u>26,865</u>
Strain 2	B $\beta$ 5-14					
	Asp <sup>5</sup> → Asn	2,366	<u>85,399</u>	<u>46,437</u>	<u>47,302</u>	<u>56,661</u>
Strain 13	B $\beta$ 5-14					
	Asp <sup>5</sup> → Asn	2,780	<u>103,960</u>	1,280		1,162

See legend for Table I.

responses to the Asn<sup>5</sup> analogue that were slightly greater than the responses observed with B $\beta$ 1-14 and 5-14 (Table V, line 2). Again strain 13 guinea pigs failed to respond to the Asn<sup>5</sup> analogue (Table V, line 3).

### Discussion

In the present study we examined the involvement of individual residues of hFPB in guinea pig T cell-proliferative responses by using synthetic analogues containing residue substitutions. A summary of T cell responsiveness to the hFPB homologue B $\beta$ 5-14 and various B $\beta$ 5-14 analogues is shown in Fig. 1. Substitutions for residues 14, 11, and 10 totally eliminate antigenicity for hFPB-immune strain 2 T cells, whereas substitutions for residues 7, 8, and 5 retain antigenicity. These results suggest that the COOH-terminal region of the B $\beta$ 5-14 molecule is more critical for T cell responsiveness than the NH<sub>2</sub>-terminal region. However, as based on immunogenicity, the residues seem to play different roles in T cell responses. Analogues containing substitutions for residues 14 and 10 are immunogenic for strain 2 animals and elicit specific T cell responses that are noncross-reactive with the B $\beta$ 5-14 peptide. By contrast, analogues with substitutions for residues 11 and 7 and 8 are non- or poorly immunogenic, respectively, as based on in vitro T cell responses to the homologous antigen. Thus, it would appear that the residue substitutions fall into two broad categories: (a) substitutions that create new immunogenic and antigenic determinants; and (b) substitutions that destroy or alter the immunogenicity of the peptide. It is likely that these substitutions define at least two different roles for residues in T cell responses to the B $\beta$ 5-14 antigen, as will be discussed below.

*Substitutions for Arginine<sup>14</sup> and Phenylalanine<sup>10</sup>.* Some of the most striking observations of T cell responses to the various B $\beta$ 5-14 analogues is that substitutions for residues 14 and 10 create distinct antigenic specificities. Replacement of Arg<sup>14</sup> in B $\beta$ 5-14 with either intact or blocked lysine results in analogues with unique noncross-reactive antigenic determinants. In similar fashion, replacing Phe<sup>10</sup> with Tyr or Phe(4-NO<sub>2</sub>) produced unique T cell responses specific for the substituted residues. It is clear, therefore, that residues 10 and 14 are responsible for the antigenic specificity of the T cell responses to B $\beta$ 5-14. In addition, the T cell responses elicited by each of these

residue 10 and 14 analogues seem to be highly specific for the side chain of that residue, as shown schematically in Fig. 2. Thus, the T cell responses can discriminate subtle parasubstitutions on the phenol ring of Phe<sup>10</sup>, and in alterations of the amino group of residue 14. These substitutions clearly indicate that the antigenic specificity of the T cell responses to the B $\beta$ 5-14 analogues is determined primarily by the configuration of the side chains of residues 10 and 14. For this reason, the preferred conformation of these peptide antigens in solution does not seem to be critical for the specificity of the T cell responses as suggested by others for protein antigens (25), although it cannot be ruled out that conformation has other roles in antigenicity of these small peptides.

Because each residue 10 and 14 substitution elicits distinct T cell clones in strain 2 guinea pigs, the simplest explanation is that these residues serve as contact residues for clonally distributed T cell receptors. If the observed clonal selection resided with macrophages, then macrophages must exhibit highly specific antigen-combining structures, and each of these must specifically interact with different T cell subpopulations, all of which express similar antigen-specific receptors. The most likely macrophage structures with these properties may be Ia antigens. However, the number of

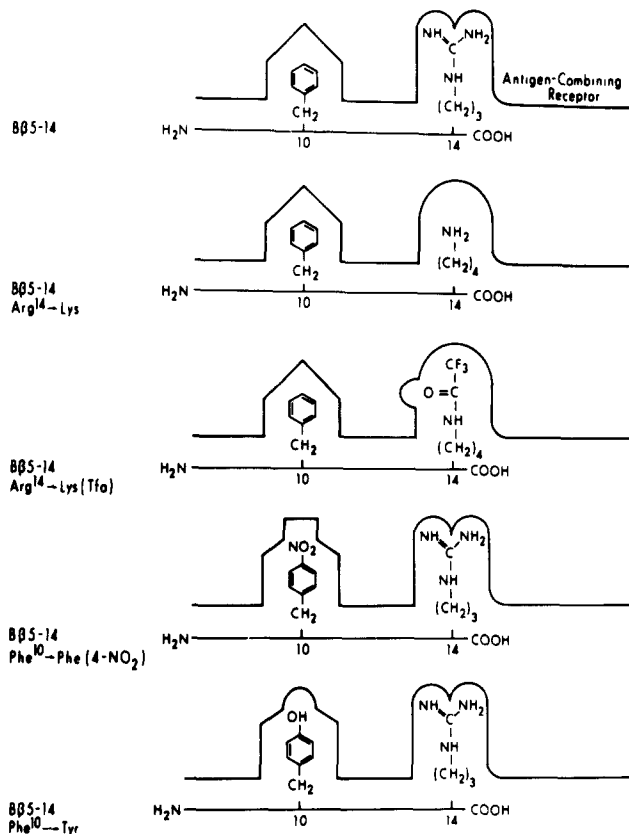


FIG. 2. Representation of the specificity of T lymphocyte responses for the side chains of residues 10 and 14. Each structure is a unique antigenic species specific for parasubstitutions of phenylalanine<sup>10</sup> (4-OH, or 4-NO<sub>2</sub>), or alterations of the  $\epsilon$ -amine of arginine<sup>14</sup> (lysine, or lysine [Tfa] with a blocked amine).



residue 10 and 14 analogues tested thus far (five) already exceeds the number of strain 2 guinea pig Ia specificities (two) showing limited heterogeneity (26). Therefore, we favor the interpretation that Arg<sup>14</sup> and Phe<sup>10</sup> serve as contact residues for strain 2 T cell antigen-combining receptors. As observed with the analogues tested thus far, the clonally distributed T cell receptor may contain a minimum of two hypervariable-like regions allowing specific interactions with residues 14 and 10. Thus, either one of these residues can be kept constant while replacing the other residue to select for distinct T cell clones.

*Substitution for Phenylalanine*<sup>11</sup>. By contrast with the roles of residues 14 and 10 as discussed above, replacement of Phe<sup>11</sup> with Tyr or Phe(4-NO<sub>2</sub>) completely destroys the immunogenicity of the Bβ5-14 analogues. This is particularly intriguing because the same substitutions for Phe<sup>10</sup> created unique immunogenic analogues. It is also of interest to note that a rabbit anti-hFPB antibody directed against the Bβ5-14 region of hFPB bound the Bβ5-14, Tyr<sup>11</sup> analogue to the same extent as Bβ5-14, indicating that the Tyr<sup>11</sup> substitution does not radically alter the antigen, and that there are differences in recognition of the same antigenic region by rabbit antibody and guinea pig T cells (D. W. Thomas and G. D. Wilner. Unpublished observations.). Two explanations for the observations that alterations of residue 11 destroy immunogenicity for T cell responses are: (a) Tyr or Phe(4-NO<sub>2</sub>) substitutions add bulk to the Phe<sup>11</sup> side chain that make these analogues too big to fit within the spatial constraints of the antigen-combining pocket; or (b) residue 11 serves as a contact residue for more conservative antigen-combining regions of cellular receptors. The cellular expression of this receptor could reside with either T cells or macrophages. These possibilities will be examined by constructing a series of residue 11 analogues with side chains of varying sizes.

*Substitution for Glutamic Acid*. Replacing residues Glu<sup>7</sup> and Glu<sup>8</sup> with glutamine creates an analogue that behaves like the Phe<sup>11</sup>-substituted analogues in that the Gln<sup>7,8</sup> analogue is poorly immunogenic. However, one important difference is that the Gln<sup>7,8</sup> analogue primes strain 2 T cells for subsequent responses to Bβ1-14, 5-14 and 7-14. Clearly, the Gln<sup>7,8</sup> analogue retains the antigenic determinants responsible for T cell priming, but itself only poorly stimulates those T cells in vitro. Thus, residues Glu<sup>7</sup> and Glu<sup>8</sup> are not involved in the specificity of T cell responses, but nonetheless provide a role in T cell stimulation. These findings suggest that antigen presentation may be more restricted for the Gln analogue, rather than interference with T cell recognition of the antigenic determinants per se. For this reason, we favor the explanation that Glu<sup>7</sup> and/or Glu<sup>8</sup> may be important for antigen presentation by accessory cells. Again, analogues with a variety of substitutions for glutamic acid will allow determination of the specificity and chemical requirements for the function of these residues.

*Substitution for Aspartic Acid*. All of the residues discussed above appear to serve important functions in strain 2 guinea pig T cell responses to hFPB. Residue Asp<sup>5</sup>, however, seems to be less important in its contribution to T cell responses because replacement with asparagine does not markedly alter the Bβ5-14 antigen. Thus, the Asn<sup>5</sup> analogue retains antigenicity for in vitro hFPB-immune T cell responses, and primes strain 2 animals for T cell responses to hFPB. This result is not surprising because we previously found that the major antigenic region of hFPB was contained in the Bβ7-14 homologue with only minor contributions by the rest of the peptide.<sup>2</sup>

<sup>2</sup> Thomas, D. W., J. Schauster, and G. D. Wilner. Manuscript submitted for publication.

*Genetic Control and T Lymphocyte Recognition.* We previously found that genetic control of T cell responsiveness to hFPB was determined solely by the presence or absence of Arg<sup>14</sup>; strain 2 guinea pigs required Arg<sup>14</sup>, and strain 13 animals responded to only the des-Arg homologue (21). In addition, we found that this Ir gene function corresponded with Ia antigen expression by antigen-presenting macrophages (17). Initially, we thought that this Ir gene function was a property of Arg<sup>14</sup> itself because strain 2 guinea pigs are known to respond to basic antigens such as poly-L-lysine and poly-L-arginine (27). Therefore it was anticipated that neutralization of the basic charge of residue 14, as with the blocked lysine group, may abrogate Ir gene function. This was not observed because strain 2 animals responded to the Lys(Tfa) analogue, whereas strain 13 guinea pigs remained nonresponders. In fact, strain 13 animals failed to respond well to any of the B $\beta$ 5-14 analogues examined thus far. It would therefore seem that the presence of residues in position 14 irrespective of charge somehow prohibits strain 13 T cell responsiveness to these peptides. Thus, the peptide analogues tested thus far indicate that it is possible to alter completely the specificity of the strain 2 clonal T cell response while preserving genetic control. Schlossman et al. reached a similar conclusion based on their elegant studies of cellular immune responses in guinea pigs to dinitrophenyl (DNP)-oligolysine antigens (28-31). In these studies, strain 2 animals responded to antigens as small as DNP-heptalysine and could distinguish between the length of the DNP-oligolysines antigens as well as the position of the DNP hapten. However, strain 13 animals remained unresponsive, demonstrating that it is possible to alter the specificity of the strain 2 T cell response while retaining Ir gene control. An examination of the contribution of residues involved in strain 13 T cell responses to des-Arg hFPB and further substitutions for residue 14 are critical for defining the mechanisms of Ir gene function in T cell responsiveness to the hFPB peptide antigens.

To approach an understanding of strain 2 guinea pig T cell recognition of the major antigenic determinant contained in residues 7-14 several observations must be considered: (a) there is an absolute requirement for Arg<sup>14</sup>; (b) Arg<sup>14</sup> determines the specificity of the T cell response; and (c) there is an absolute correlation for responsiveness between Arg<sup>14</sup> and Ia antigen expression by strain 2 stimulator macrophages. These latter two observations initially seem discrepant because on the one hand, Arg<sup>14</sup> appears to be a contact residue for a T cell receptor, and on the other hand is closely associated with macrophage Ia antigens. Taken together, these observations suggest that macrophage Ia antigens dictate or permit T cell interaction with residue 14. The implication of this suggestion is that macrophage Ia antigens are intimately involved in T cell recognition of these peptide antigens. One model to accommodate these observations is schematically shown in Fig. 3. Basically, a single antigen-combining

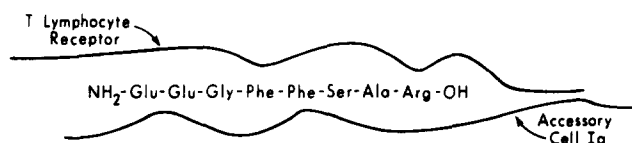


FIG. 3. Schematic representation of T lymphocyte recognition of the octapeptide antigen B $\beta$ 7-14. A single antigen-combining site is formed by the interaction of a T cell receptor with accessory cell Ia antigens. Optimal binding of the peptide within this site requires contributions by both structures; the T cell receptor contacts residues Arg<sup>14</sup> and Phe<sup>10</sup>, and Ia may contact other peptide residues.

pocket is formed by an interaction between stimulator or accessory cell Ia antigen and the T cell receptor, perhaps analogous to heavy-light chain combining in antibodies. Constraints on antigen recognition are thereby imposed in part by both structures, and the residue and spatial accommodation of the antigen-combining site determine responsiveness. Based on T cell responses to DNP-oligolysine antigens, Stashenko and Schlossman (31) similarly reasoned that the Ir gene may cooperate with or modify the T cell antigen-combining receptor. As shown for the B $\beta$ 7-14 antigen, the T cell receptor probably contacts both Arg<sup>14</sup> and Phe<sup>10</sup>, whereas accessory cell Ia may interact with other regions of the peptide, e.g., with Phe<sup>11</sup> and Glu<sup>7,8</sup>. The fine specificity of antigen responsiveness is therefore contributed by the hypervariable-like regions of the T cell antigen-combining receptor. The Ia antigens may be much more restricted in their interaction with the peptide antigen and exhibit limited antigen-combining specificities. According to this model, the minimum number of interactions required for antigen recognition by T cells would be two; the first being the interaction between the T cell receptor and Ia antigen to create the combining pocket, and the second is the binding of antigen within that pocket. Exogenous antigen may not bind well to either of these structures individually, or may be bound to Ia before T cell interactions, as suggested before (16). For alloreactivity, this model would predict a single interaction, the residues contacted by hypervariable-like regions of the T cell receptor in this case being inherent in the allogeneic Ia as allelic residue substitutions. Obviously, resolution of this or other models of T cell recognition will require isolation and biochemical characterization of the recognition complex. Nevertheless, the approach presented here of using small peptide antigens to examine more carefully the specificity and antigenic requirements for T cell responses allows a better understanding of the recognition processes involved, and provides insight into immunocompetent cellular interactions and mechanisms for genetic regulation of immune responsiveness.

### Summary

Guinea pig T lymphocyte responses to a decapeptide antigen (NH<sub>2</sub>-Asp<sup>5</sup>-Asn<sup>6</sup>-Glu<sup>7</sup>-Glu<sup>8</sup>-Gly<sup>9</sup>-Phe<sup>10</sup>-Phe<sup>11</sup>-Ser<sup>12</sup>-Ala<sup>13</sup>-Arg<sup>14</sup>-OH) of human fibrinopeptide B (hFPB) were examined using various synthetic peptide analogues containing single residue substitutions. Each analogue was examined for antigenicity as determined by *in vitro* proliferative responses of hFPB-immune strain 2 guinea pig T cells. In addition, both strain 2 and strain 13 animals were immunized with each analogue and immunogenicity assessed by *in vitro* T cell-proliferative responses with the homologous immunizing analogue and the parent peptide. Replacement of arginine<sup>14</sup> with lysine formed an immunogenic analogue which showed no antigenic cross-reactivity with the native peptide in strain 2 T cell responses. In addition, substitution of arginine<sup>14</sup> with blocked lysine again produced a unique immunogenic analogue that showed little or no antigenic identity with the intact lysine analogue or the native peptide. In similar fashion, substitution of residue phenylalanine<sup>10</sup> with tyrosine or Phe(4-NO<sub>2</sub>) created unique immunogenic analogues with little or no antigenic identity to the native peptide with strain 2 T cells. By contrast, replacement of phenylalanine<sup>11</sup> with either tyrosine or Phe(4-NO<sub>2</sub>) resulted in analogues with a total loss of immunogenicity and antigenicity in strain 2 T cell responses. An analogue in which glutamic acid<sup>7,8</sup> were replaced with glutamine retained a small degree of antigenicity with hFPB-immune

T cells, but T cells from strain 2 animals immunized with the Gln analogue responded only marginally to the Gln analogue while producing good proliferative responses with the native peptide. On the other hand, an analogue in which asparatic acid<sup>5</sup> was replaced with asparagine retained most of the antigenic identity with hFPB for strain 2 T cell responses. None of these analogues were immunogenic for strain 13 guinea pigs. These observations are discussed with respect to the contribution of each substituted residue to T cell responses, mechanism of Ir gene function, and a model for T cell recognition of small peptide antigens.

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