FINE SPECIFICITY OF REGULATORY T CELLS

II. Suppressor and Helper T Cells Are Induced by Different Regions of Hen Egg-White Lysozyme in a Genetically Nonresponder Mouse Strain*

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Immune response genes (Ir genes)[1] associated with the major histocompatibility complex (MHC), H-2 in the mouse, control the immune response to a variety of thymus-dependent antigens (1). This control is expressed at multiple levels: presentation of selected determinants by macrophages to T cells (2), induction of specific suppressor and helper T cells (3), T-B collaboration (4), and fine specificity of antibodies (5). The dissection of the immune response in terms of Ir-gene control and determinant selection may well be best achieved by studying small protein antigens whose amino acid sequences and three-dimensional structures are known. Obvious advantages are the availability, in some of these systems, of closely related proteins, and the possibility of preparing defined peptides.

Previous studies in our laboratory have demonstrated H-2-linked genetic control, in mice, of the immune response to a set of closely related gallinaceous egg-white lysozymes (6, 7). Lysozymes are tight globular proteins of mol wt close to 14,300. All gallinaceous lysozymes so-far examined are composed of 129 amino acid residues, with the exception of ring-necked pheasant egg-white lysozyme (REL), which has an extra glycine residue at the amino terminus. The structural conformation of at least two gallinaceous and one mammalian lysozyme is known, and for most lysozymes we have studied, the amino acid sequence has been determined (8). C57BL/10Sn (B10) mice ($H\text{-}2^b$) are nonresponders to hen (chicken) egg-white lysozyme (HEL), although the congenic B10.A/SgSn ($H\text{-}2^a$) mice (B10.A) are responders; nevertheless, both strains respond to the closely related REL, and several other lysozymes (6, 7). Evidence has been presented that HEL, but not REL, is able to induce suppressor T cells in B10 mice (9). HEL and REL differ only at 10 amino acid residues, thus suggesting that a limited region on the nonimmunogenic HEL, absent on REL, can account for

* Supported in part by U.S. Public Health Service grants AI 11183 and CA 24442.

‡ Recipient of a Cancer Research Institute postdoctoral fellowship.

§ Recipient of Postdoctoral Fellowship PF-1309 of the American Cancer Society.

[1] *Abbreviations used in this paper:* BRBC, burro (donkey) erythrocytes; B10, C576BL/10Sn mice; B10.A, B10.A/SgSn mice; CFA, complete Freund's adjuvant; CNBr, cyanogen bromide; HEG, Hepes-buffered Eagle's minimum essential medium containing 0.25% gelatin; HEL, hen (chicken) egg-white lysozyme; HuRBC, erythrocytes; IR genes, immune response genes; JEL, Japanese quail egg-white lysozyme; L_{II}, mixed disulfide form of L_{II} peptide of HEL (a.a. 13–105); MHC, major histocompatibility complex; N-C, N-terminal, C-terminal peptide of HEL (a.a. 1–17:cys 6–cys 127:120–129); PFC, plaque-forming cell; PT-LN, parathymic lymph node(s); REL, ring-necked pheasant egg-white lysozyme; RBC, erythrocytes; SRBC, sheep erythrocytes. TEL, turkey egg-white lysozyme.

the induction of suppressor T cells in H-2^b mice.

In this study, we demonstrate that the N-terminal, C-terminal peptide (N-C), obtained from HEL by mild acid hydrolysis (a.a. 1–17:cys 6–cys 127:120–129) mimics the intact HEL molecule in the induction of suppressor cells in B10 and can induce helper cells in B10.A mice. The mixed disulfide peptide (L_{II}) derived by cyanogen bromide (CNBr) treatment of HEL followed by disulfide exchange (a.a. 13–105) induces helper cells in both strains. Therefore, the genetic nonresponsiveness of the B10 strain to HEL seems to be a consequence of the activation of suppressor T cells by a restricted portion of this antigen. One manifestation of MHC gene activity appears to be the intramolecular selection of different antigenic determinants leading to activation of functionally different T-cell subpopulations.

Materials and Methods

Mice. Female B10 and B10.A were obtained from The Jackson Laboratory, Bar Harbor, Maine, and maintained in our animal facilities. All mice were between 10 and 15 wk of age when immunized.

Lysozymes. HEL was purchased from Societá Prodotti Antibiotici, Milan, Italy. REL was isolated from egg-white by adsorption on carboxymethyl-Sephadex (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) and elution with a linear-concentration gradient of 0.05–0.8 M ammonium carbonate at pH 9.0. It was further purified by chromatography on the weak-cation-exchange resin, Bio-Rex 70 (Bio-Rad Laboratories, Richmond, Calif.) with 0.2 M sodium phosphate buffer at pH 7.18 (10). Purity for both lysozymes was established by column chromatography and slab gel electrophoretic analysis.

Preparation and Characterization of the N-C Peptide from HEL (a.a. 1–17:cys 6–cys 127:120–129). The N-C peptide was prepared by mild-acid cleavage, a procedure that results in the destruction of peptide bonds at aspartic acid and asparagine residues (11). 1 g of lysozyme was dissolved in 0.03 N HCl and extensively dialyzed using a Spectrapor No. 1 membrane (Spectrum Medical Industries, Inc., Los Angeles, Calif.) against the same solution. The solution was then transferred to a two-necked round-bottom flask, placed in a heating mantle and refluxed for 36 h while passing a stream of nitrogen through the solution. The digest was then concentrated by flash evaporation and loaded on a G-25 Sephadex column (Pharmacia Fine Chemicals, Inc.) (3.5 × 90 cm) in 5% acetic acid. Because HEL has only one histidine, at amino acid residue 15, the purification of the peptide was followed by using the Pauli reagent to detect the presence of histidine. The first major polypeptide peak eluted from the G-25 Sephadex column contained the only histidine detected. This material was concentrated and passed over a Bio-Gel P6 column (Bio-Rad Laboratories) (3.5 × 90 cm) in 5% acetic acid. The largest molecular-weight material from this column was lyophilized and further purified by binding to a CM-Sephadex C-25 column (Sigma Chemical Co., St. Louis, Mo.) (1 × 20 cm) and elution with a linear (salt) gradient from 0.01 to 0.2 M ammonium acetate in 8 M urea, pH 9.0. After desalting, the material was concentrated by lyophilization. This peptide fraction had no detectable enzymatic activity at a concentration of 6 mg/ml. The threshold for this assay is <10 μg/ml HEL.

The peptide exhibited only one spot on high-voltage electrophoresis at pH 1.9. This spot contained histidine and tryptophan and no detectable tyrosine. Reduction and carboxymethylation of this peptide revealed two ninhydrin-positive spots on high-voltage electrophoresis. One of these spots contained histidine and no detectable tryptophan, corresponding to the N-terminal portion, the other tryptophan and no detectable histidine, corresponding to the C-terminal portion. Amino acid analyses were performed with a Beckman 120B amino acid analyzer (Beckman Instruments, Inc., Fullerton, Calif.). Samples were hydrolyzed in sealed and evacuated Beckman tubes (Beckman Instruments, Inc.) in 6 N HCl at 110°C for 22 h. Table I contains the amino acid composition of the peptide. These results indicate that this peptide fraction consists of a.a. residues 1–17:cys 6–cys 127:120–129 (Fig. 1).

Preparation and Characterization of the L_{II} Peptide from HEL (a.a. 13–105). The L_{II} peptide was prepared by CNBr cleavage of native HEL as previously described (12), followed by disulfide

TABLE I

Amino Acid Analysis of N-C and L_{II} Peptides

Amino acid residues	N-C		L_{II}	
	Expected	Found	Expected	Found
Lys	2	2.4	4	3.7
His	1	1.1	1	0.9
Arg	4	4.3	6	6.4
Asx	0	0.2	18	19.7
Thr	0	0.1	6	4.4
Ser	0	0.3	10	9.3
Pro	0	0.1	2	2.0
Glx	2	1.6	3	2.9
Gly	3	3.5	9	9.4
Ala	4	3.5	6	6.5
Val	2	1.9	3	2.9
Met	1	0.7	0	0
Ile	1	0.9	5	4.5
Leu	3	2.7	6	6.7
Tyr	0	0	3	2.7
Phe	1	0.8	2	1.9
H Ser	0	ND	1	ND
½ Cys	2	ND	5	ND

ND, not determined.

exchange according to the method of Smithies (13). Briefly, 1 g of HEL was dissolved in 100 ml of 70% formic acid and 720 mg of CNBr was added. After 24 h, the digestion was stopped by adding 10 volumes of distilled water and the preparation was lyophilized. 200 mg of CNBr-digested HEL was dissolved in 10 ml of borate buffer (0.1 M borate, 0.04 M EDTA in 8 M urea, pH 8.4). This solution was then added to 10 ml of the same buffer containing 0.2 M 2-hydroxyethyl disulfide (Aldrich Chemical Co., Inc., Milwaukee, Wis.) and 0.008 M 2-mercaptoethanol. The mixture was rocked at 37°C for 4 h and then loaded onto a G-25 Sephadex column (3.5 × 90 cm) equilibrated with 5% acetic acid. The first protein peak eluted was concentrated and passed over a G-75 Sephadex column (1 × 100 cm) equilibrated with 5% acetic acid. The major peak from this column was lyophilized and used without further purification. This peptide demonstrated a single band of apparent mol wt close to 10,000 on 10–20% polyacrylamide gel containing 0.1% SDS. No enzymatic activity was detected at 6 mg/ml, indicating that the fraction was at least 99.8% free of intact lysozyme. The amino acid analysis of the L_{II} peptide is shown in Table I and its sequence in Fig. 1.

Immunizations. Mice were immunized i.p. with 100 μg HEL/mouse in 0.1 ml of saline emulsified with an equal part of complete Freund's adjuvant (CFA, Grand Island Biological Co., Grand Island, N. Y.). Immunizations with peptides were carried out with doses equivalent in moles to 100 μg HEL: 20 μg N-C or 70 μg L_{II} peptide/mouse in CFA. Mice primed with a saline-CFA emulsion served as control. 4 wk after immunization, mouse spleens were used for in vitro cultures, or the mice were boosted with 100 μg HEL in saline i.p. and their parathymic lymph nodes (PT-LN) assayed for PFC response after 6 d.

Assay of In Vivo Response. Suspensions were gently teased from PT-LN and sequentially passed through coarse and fine mesh screens into cold Hepes-buffered Eagle's Minimum Essential Medium (Grand Island Biological Co.) containing 0.25% gelatin (HEG). The cell suspensions were washed two times in HEG, resuspended in cold medium and assayed for hemolytic plaque formation using the Cunningham and Szenberg technique (14). As indicator cells, HEL coupled to guinea pig erythrocytes (Colorado Serum Co., Denver, Colo.) were used at a final concentration of 1%. HEL-erythrocyte (RBC) coupling procedures have been previously described (9). Goat anti-mouse Ig-developing serum was used at a final dilution of 1:400 to detect IgG plaque-forming cells (PFC). Fresh guinea pig serum, adsorbed on RBC,

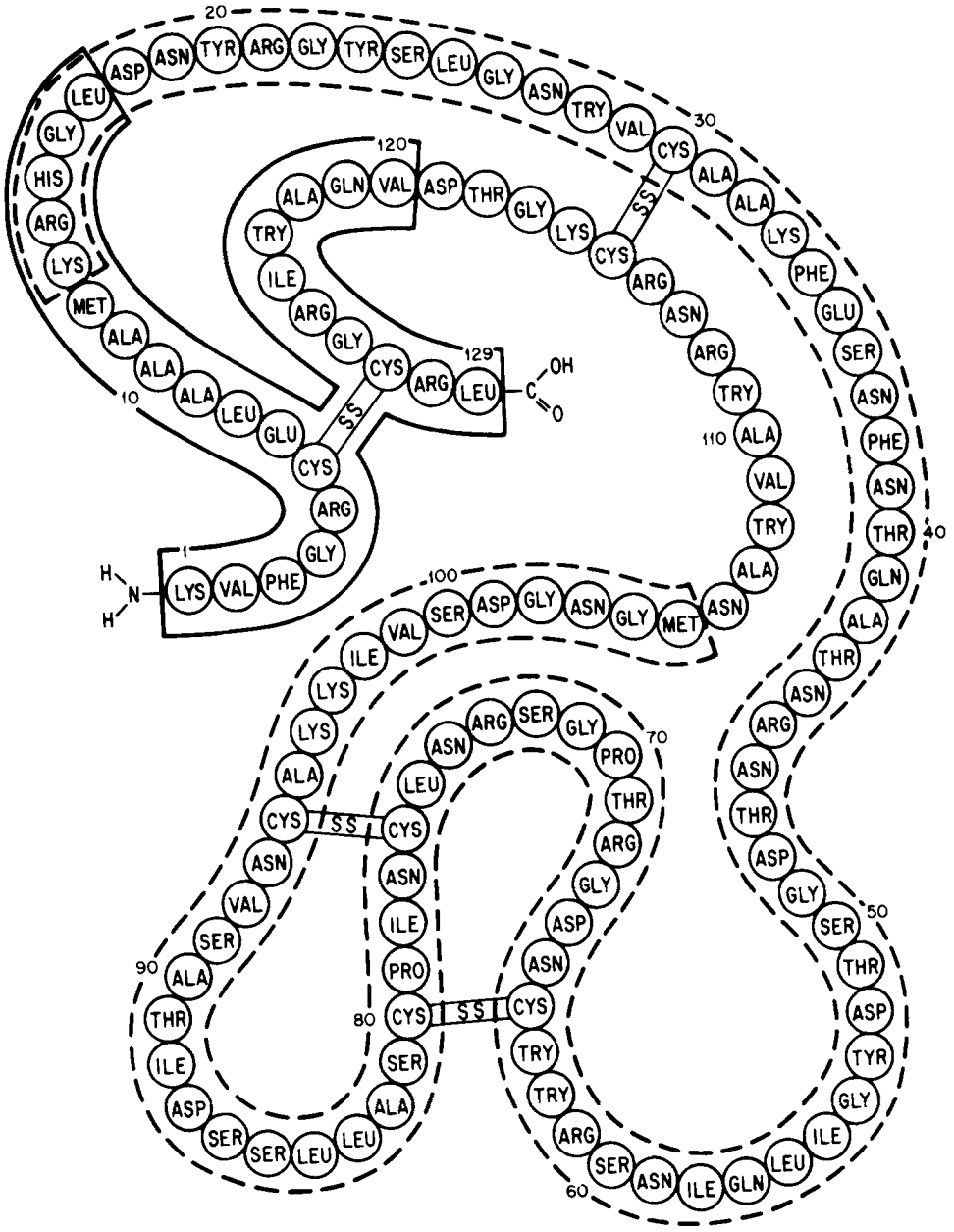

FIG. 1. Primary structure of the HEL molecule. Amino acid residues included in solid box represent the N-C peptide (a.a. 1–17:Cys 6-Cys 127:120–129). Amino acid residues included in dotted box represent the L_{II} peptide (a.a. 13–105).

was used as a source of complement at a final dilution of 1:64. The results are presented as the geometric mean of PFC/10^6 nucleated PT-LN cells. An SE coefficient was calculated from the logarithmically transformed PFC, and denotes a factor which multiplies and divides the mean to give the upper and lower limit of the SE.

In Vitro Culture. In vitro experiments were performed using a miniaturized two-chamber diffusion culture system recently developed in our laboratory (15). Culture medium was RPMI

1640 (Grand Island Biological Co.) supplemented with 10% fetal calf serum (Flow Laboratories, Inc., Rockville, Md.), 2 mM L-glutamine (Grand Island Biological Co.), 5×10^{-5} M 2-mercaptoethanol and 10 μg/ml gentamicin (Shering Corp., Bloomfield, N. J.). Briefly, 2×10^6 spleen cells were cultured together with 2×10^6 HEL-sheep RBC (SRBC) (Grand Island Biological Co.) or HEL-human RBC (HuRBC) (from a single donor) in 0.1 ml in the inner chamber, separated by a dialysis membrane from the reservoir in which 1 ml of medium was placed. On day 4 of culture, triplicate cultures were harvested and tested for direct PFC against HEL-burro (donkey) RBC (BRBC), (BRBC, Colorado Serum Co.), or SRBC using the Cunningham and Szenberg technique (14). Results are expressed as PFC/culture. SE represents the SE of the arithmetic mean PFC from triplicate cultures.

Treatment of T Cells with Antisera. Anti-T serum was rabbit anti-mouse thymocyte serum (Microbiological Associates, Walkersville, Md.) adsorbed on XS63 (a Balb/c, non-Ig-producing plasmacytoma) according to Kappler and Marrack (16). Anti-T serum additionally adsorbed twice on B10 thymocytes (10^8 thymocytes/ml serum for 30 min at 4°C) was used as control. Anti-I-J^b serum was prepared in our laboratory by multiple intraperitoneal injections of B10.A (3R) spleen cells into B10.A (5R) recipients. As a specificity control, anti I-J^b serum was adsorbed four times on B10 (I-J^b) or B10.A (I-J^k) spleen cells (10% vol:vol) for 30 min at 4°C. All antisera were incubated at a 1:10 final dilution with spleen cells (2×10^7/ml) for 30 min at 4°C. The cells were then washed once, resuspended in rabbit complement (Cedarlane Laboratories, London, Ontario, Canada) at a final dilution of 1:8, incubated for 30 min at 37°C, and then washed three times.

Results

N-C Priming followed by HEL Challenge In Vivo Induces Antibody Formation in B10.A but Not in B10 Mice. Previous experiments from our laboratory demonstrated that after HEL-CFA priming, B10.A mice respond to a soluble-HEL challenge, whereas B10 mice do not (6). To test the effect of N-C priming, six B10 or B10.A mice/group were primed i.p. with a saline-CFA emulsion or with 100 μg of HEL in CFA or 20 μg of N-C (molar equivalent to 100 μg HEL) in CFA. After 28 d, all mice were challenged with 100 μg of soluble HEL i.p. and the anti-HEL-IgG PFC response assayed 6 d later in the PT-LN. This lymphatic organ was chosen on the basis of previous work that demonstrated that, after this immunization protocol, the highest concentration of PFC is found in the PT-LN (17). Results shown in Table II demonstrate that in both experimental groups, the response of B10 mice was <1 PFC/10^6 PT-LN cells. In B10.A mice, no response was observed after saline-CFA priming and soluble-HEL challenge, but comparable anti-HEL PFC responses were obtained after priming with either HEL-CFA or N-C-CFA.

The N-C Portion of HEL Mimics the Intact Molecule in the Induction of Suppressor Cells in B10 and Helper Cells in B10.A Mice. Although B10 mice do not respond to HEL when immunized with soluble antigen or antigen in CFA, a primary anti-HEL response can be obtained, both in vivo and in vitro, if HEL is coupled to an immunogenic carrier, such as RBC (6, 9). HEL-CFA i.p. priming in B10 mice was found to induce suppression of the anti-HEL response to HEL-RBC (9). Results in Fig. 2 demonstrate that in vitro cultures of spleen cells from CFA-primed mice, with HEL-RBC as antigen, give a primary anti-HEL PFC response both in B10 and B10.A mice, although in the latter strain, the response is of lower magnitude. Spleen cells from B10 mice primed 4 wk before culture with 100 μg HEL-CFA give a small anti-HEL PFC response: only 15% of the primary response. Spleen cells from B10 mice primed with 20 μg of N-C-CFA give <20% of the anti-HEL PFC response attained by spleen cells from CFA-primed mice. However, when 10% of spleen cells from HEL- or N-C-

TABLE II

HEL and N-C Priming followed by HEL Challenge Induce Anti-HEL Response In Vivo in B10.A but Not in B10 Mice

Priming	Challenge	Anti-HEL IgG PFC/10^6 PT-LN cells	
		B10	B10.A
CFA	HEL	<1	<1
HEL-CFA	HEL	<1	132 (1.21)
N-C-CFA	HEL	<1	218 (2.31)

Six B10 or B10.A mice/group were primed i.p. with saline-CFA or HEL-CFA (100 μg/mouse) or N-C-CFA (20 μg/mouse) and challenged i.p. 4 wk later with 100 μg/mouse soluble HEL. PT-LN from individual mice were assayed for anti-HEL IgG PFC 6 d after challenge. Data are expressed as geometric mean PFC and (in parentheses) SE coefficients.

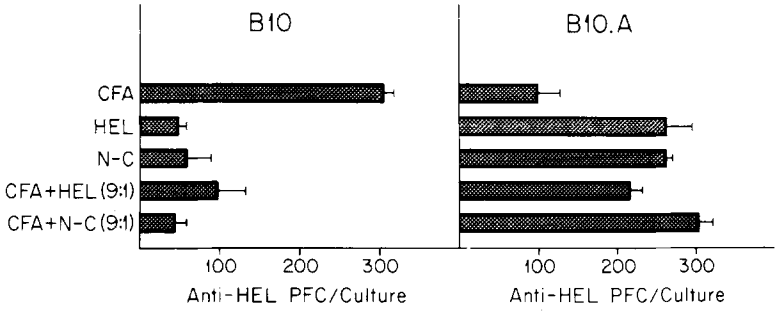

FIG. 2. The N-C peptide derived from HEL mimics the intact molecule in the induction of suppressor cells in B10 and helper cells in B10.A mice. Mice were primed with saline-CFA, or HEL-CFA (100 μg/mouse) or N-C-CFA (20 μg/mouse) 4 wk before culture. 2×10^6 spleen cells and 2×10^6 HEL-HuRBC were cultured in triplicate miniaturized diffusion culture chambers and separately assayed, on day 4 of culture, for direct anti-HEL PFC. Cell mixtures were 90% spleen cells from saline-CFA-primed mice and 10% spleen cells from HEL-CFA- or N-C-CFA-primed mice. Data are presented as arithmetic means PFC/culture and bars represent SE from triplicate cultures.

primed B10 mice are mixed with 90% of spleen cells from CFA-primed mice, the same degree of suppression is observed.

The right panel of Fig. 2 shows the response obtained in the congenic B10.A strain under the same experimental conditions used for B10 cultures. Spleen cells from B10.A mice, primed 4 wk before culture with 100 μg HEL-CFA, when challenged in vitro with HEL-RBC, exhibit almost a threefold increase over the primary anti-HEL PFC response. The same increase is evident if spleen cells are from N-C-CFA-primed mice. Coculture of 10% spleen cells from HEL-primed or N-C-primed mice with 90% of spleen cells from CFA-primed mice leads to a comparable increase in anti-HEL PFC response, suggesting a helper effect of the same magnitude.

L_{II} Priming Induces HEL-Specific Helper Cells in B10 and B10.A Mice. B10 and B10.A mice were primed with CFA or HEL-CFA (100 μg/mouse), or L_{II}-CFA equivalent, in moles, to 100 μg of HEL (70 μg/mouse). 4 wk after priming, spleen cells were cultured with HEL-SRBC as antigen and 4 d later, triplicate cultures were assayed for direct anti-HEL and anti-SRBC PFC. As shown in Table III, primary anti-HEL and anti-SRBC response are present in both B10 and B10.A spleen-cell cultures. Spleen-cell

TABLE III

L_{II} Priming Induces HEL-Specific Helper Cells in B10 and B10.A Mice

Spleen cells in culture from mice primed with			Direct PFC/culture			
			B10		B10.A	
CFA	HEL-CFA	L_{II}-CFA	Anti-HEL	Anti-SRBC	Anti-HEL	Anti-SRBC
	$\times 10^6$ *cells*					
2	—	—	370 ± 62	1,583 ± 344	166 ± 46	900 ± 57
—	2	—	106 ± 56	1,466 ± 176	287 ± 47	983 ± 116
—	—	2	820 ± 128	1,716 ± 130	373 ± 18	933 ± 60
1.8	.2	—	73 ± 33	1,150 ± 225	391 ± 96	800 ± 57
1.8	—	.2	603 ± 85	1,700 ± 327	533 ± 264	800 ± 264

Mice were primed with saline-CFA, or HEL-CFA (100 μg/mouse) or L_{II}-CFA (70 μg/mouse), 4 wk before culture. 2×10^6 spleen cells and 2×10^6 HEL-SRBC were cultured in triplicate miniaturized diffusion culture chambers and separately assayed, on day 4 of culture, for direct anti-HEL and anti-SRBC PFC. PFC data are presented as arithmetic means and SE from triplicate cultures.

cultures from HEL-CFA-primed B10 mice give a lower response, while cultures from B10.A mice produce an augmented PFC response, as compared to the respective primary responses. After L_{II}-CFA priming, however, both strains exhibit a significant increase in the PFC response ($P < 0.05$). HEL-specific suppression in B10 is demonstrated by mixing 10% of spleen cells from HEL-CFA-primed mice with 90% of cells from CFA-primed mice. In the B10.A strain, HEL-specific helper activity is demonstrated by mixing 10% of spleen cells from HEL-CFA-primed mice with 90% of spleen cells from CFA-primed mice. A substantial amount of helper activity is obtained in both strains by coculturing 10% of spleen cells from L_{II}-primed mice with 90% of spleen cells from CFA-primed mice. Specificity of the HEL-induced suppression or help is illustrated by the anti-SRBC response, which is in any case very similar in the different experimental groups in each strain. Consistently, the primary anti-HEL and anti-SRBC PFC responses are lower in B10.A, as compared to the congenic B10 strain.

Results in Fig. 3 demonstrate that helper activity induced by L_{II} in B10 mice is suppressed by HEL-induced suppressor T cells. B10 mice were primed with CFA, HEL-CFA (100 μg/mouse) or L_{II}-CFA (70 μg/mouse), and 4 wk later, their spleen cells cultured with HEL-HuRBC as antigen in vitro. Direct anti-HEL PFC assayed on day 4 demonstrate that, as expected, a lower response is obtained in spleen-cell cultures from HEL-CFA-primed mice and an augmented response in cultures from L_{II}-CFA-primed mice. Coculture of 5% spleen cells from HEL-CFA-primed mice with 95% spleen cells from CFA-primed mice results as expected, in strong suppression of the primary anti-HEL in vitro response. On the other hand, 5% of spleen cells from L_{II}-primed mice, when added to 95% spleen cells from CFA-primed mice, are able to generate the same amount of helper activity present in cultures containing 100% spleen cells from L_{II}-primed mice. However, the activity of the L_{II}-induced helper cells is almost completely suppressed by the addition of 5% spleen cells from HEL-CFA-primed mice.

N-C-Induced T-Suppressor Cells in B10 Mice Are HEL-Specific and I-J Positive, and Their Suppressive Activity Is Indistinguishable from HEL-Induced Suppressor Cells. We have previously demonstrated that suppressor cells induced in B10 mice by HEL-CFA

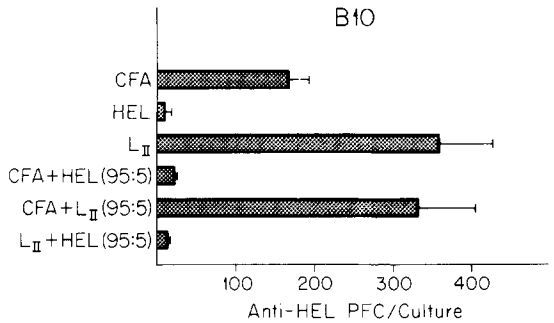

FIG. 3. Helper cells induced by L_{II} in B10 mice are suppressed by HEL-induced suppressor cells. Mice were primed with saline-CFA, or HEL-CFA, (100 μg/mouse) or L_{II}-CFA (70 μg/mouse) 4 wk before culture. 2×10^6 spleen cells and 2×10^6 HEL-HuRBC were cultured in triplicate miniaturized diffusion culture chambers and separately assayed, on day 4 of culture, for direct anti-HEL PFC. Cell mixtures were 95% spleen cells from saline-CFA or L_{II}-CFA primed mice and 5% spleen cells from HEL-CFA or L_{II}-CFA primed mice. Data are presented as arithmetic means PFC/culture and bars represent SE from triplicate cultures.

priming bear T and I-J surface markers and do not suppress the response to the closely related REL (9). It was therefore of interest to examine the specificity and the surface phenotype of N-C-induced suppressor cells. Characterization and fine specificity of N-C-induced suppressor cells are depicted in Fig. 4. Spleen cells from mice primed with HEL-CFA (100 μg/mouse) or N-C-CFA (20 μg/mouse) 4 wk before culture, when cultured with HEL-HuRBC exhibit a very low anti-HEL PFC response as compared to the primary response. Mixing 5% of spleen cells from HEL-CFA- or N-C-CFA-primed mice with 95% of spleen cells from CFA-primed mice results in a similar degree of suppression. Treatment of N-C-CFA-primed cells with anti-T serum and complement is able to abolish suppression, although anti-T serum adsorbed on thymocytes and complement treatment is ineffective. Furthermore, suppression exerted by spleen cells from N-C-primed mice is removed by treatment with anti-I-J^b (B10.A [5R] anti-B10.A[3R]) serum and complement. The specificity of anti-I-J^b serum was assessed by adsorbing it on B10 (I-J^b) or B10.A (I-J^k) spleen cells. The effect of anti-I-J^b serum treatment on N-C-induced suppressor T cells is specifically removed by adsorption with B10 spleen cells, although its activity is retained after adsorption with B10.A spleen cells.

The fine specificity of HEL- or N-C-induced suppressor cells is demonstrated by lack of suppression of the anti-HEL response when cells are cultured with REL-HuRBC. This confirms our previous findings of high cross-reactivity between HEL and REL at the B-cell level and extends the lack of recognition of REL to N-C-specific suppressor T cells (9).

A titration of HEL- and N-C-induced suppressor cells is presented in Fig. 5. In the miniaturized in vitro culture system, 2×10^6 spleen cells and 2×10^6 HEL-SRBC were cultured alone or mixed with graded numbers of spleen cells from HEL-primed (100 μg/mouse) or N-C-primed (20 μg/mouse) mice. Spleen-cell density in all cultures was kept to 2×10^6 and triplicate cultures were assayed on day 4 of culture for direct anti-HEL PFC response. Maximal suppression of the anti-HEL response is still present when 2×10^4 spleen cells from HEL-CFA- or N-C-CFA-primed mice are added, although 10^4 cells suppress only one-third of the response in both cases. The titration curves obtained with suppressor cells from HEL-CFA- or N-C-CFA-primed mice are

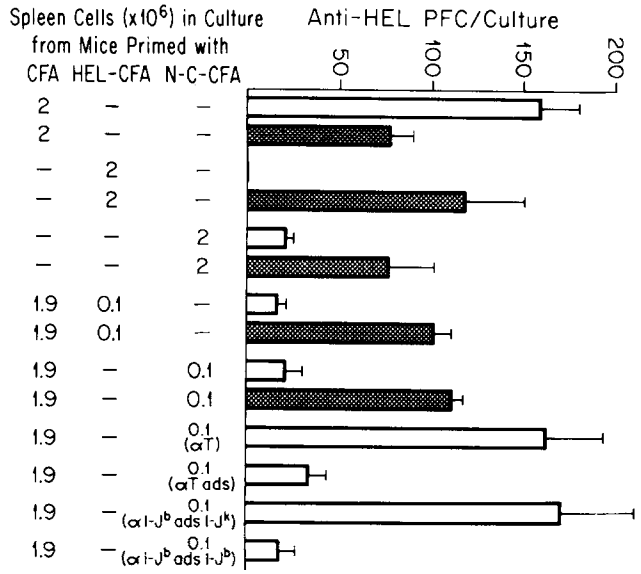

FIG. 4. Suppressor cells induced by the N-C peptide in B10 mice are T and I-J positive and specific for HEL. Mice were primed with saline-CFA, or HEL-CFA (100 μg/mouse) or N-C-CFA (20 μg/mouse) 4 wk before culture. 2 × 10^6 spleen cells and 2 × 10^6 HEL-HuRBC □, or REL-HuRBC □, were cultured in triplicate miniaturized diffusion culture chambers and separately assayed, on day 4 of culture, for direct anti-HEL PFC. Cell mixtures were 95% spleen cells from saline-CFA-primed mice and 5% spleen cells from HEL-CFA or N-C-CFA-primed mice. Spleen cells from N-C-CFA-primed mice were sequentially incubated with antisera (final dilution 1:10) for 30 min at 4°C and rabbit complement (final dilution 1:8) for 30 min at 37°C. αT refers to rabbit anti-mouse thymocyte serum and αTads to αT serum adsorbed twice on B10 thymocytes. αI-J^b ads I-J^k refers to B10.A (5R)-anti-B10.A (3R) serum adsorbed 4 times on B10.A (I-J^k) spleen cells. αI-J^b ads I-J^b refers to B10.A (5R)-anti-B10.A (3R) serum adsorbed 4 times on B10 (I-J^b) spleen cells. Data are presented as arithmetic means PFC/culture and bars represent SE from triplicate cultures.

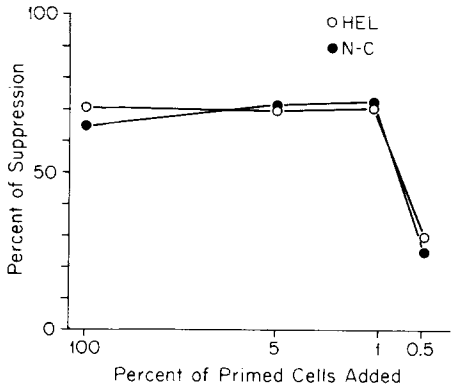

FIG. 5. Titration of suppressor cells induced by HEL-CFA or N-C-CFA in B10 mice. Mice were primed with saline-CFA, or HEL-CFA (100 μg/mouse) or N-C-CFA (20 μg/mouse) 4 wk before culture. 2 × 10^6 spleen cells from saline-CFA-primed mice and 2 × 10^6 HEL-HuRBC were cultured alone or mixed with graded numbers of spleen cells from HEL-CFA- (○——○) or N-C-CFA- (●——●) primed mice. Triplicate cultures were assayed for direct anti-HEL PFC on day 4 of culture. Results are expressed as the percentage of suppression of spleen cell anti-HEL compared to saline-CFA-primed mice. Control value (saline-CFA) was 178 ± 42 anti-HEL PFC/culture.

virtually superimposable, indicating that an equally effective suppressive activity is generated.

Discussion

We have analyzed the ability of HEL and two of its peptides to induce suppressor T cells and helper T cells in a pair of congenic nonresponder-responder mouse strains. HEL, when injected i.p. in CFA, induces predominant suppression in B10, H-2^b nonresponder mice, and help in the congenic responsive strain B10.A, H-2^a. The N-C peptide (a.a. 1–17:cys 6–cys 127: 120–129) derived from HEL (a.a. 1–129) induces suppressor T cells in B10 and helper cells in B10.A, whereas the L_{II} peptide (a.a. 13–105) induces helper cells in both strains. These results indicate that, in the response to thymus-dependent antigens such as HEL, helper T cells may be directed against certain antigenic determinants and suppressor T cells against others. The evidence from these experiments also points to the identity, in the B10 strain, of HEL-induced and N-C-induced suppressor T cells. Therefore, it appears that the potential L_{II}-specific helper activity in antagonized by suppressor T cells induced by a suppressive epitope present on the same HEL molecule, in the N-C region.

In B10 mice, HEL-specific suppressor T cells do not recognize the closely related, immunogenic REL, indicating that a limited region on the nonimmunogenic HEL, absent on REL, could account for the induction of suppression (9). The presence of tyrosine at amino acid residue 3, as in REL, Japanese quail egg-white lysozyme (JEL), or turkey egg-white lysozyme (TEL), seems to correlate with immunogenicity in B10 mice. The nonimmunogenic lysozymes (e.g., HEL) have phenylalanine at this position (7, 8). The extra glycine residue, recently detected at the amino terminus of the REL molecule (18) is probably irrelevant to the composition of the critical determinant because REL vis-a-vis JEL and TEL, which lack the extra glycine, show a similar responsiveness pattern, as judged by in vivo antibody response (6), and in vitro T-cell proliferation (7). Thus, the induction by the N-C fragment of HEL-specific suppressor T cells in B10 mice, and of helper cells in B10.A mice, strongly suggests that this region is presented, under H-2-linked Ir gene control, primarily to either suppressor T cells or helper cells. Although it seems likely that no suppressor T cells can be raised in the B10.A strain to N-C, it is possible that this peptide of mol wt 3,000 contains both helper and suppressor determinants and that hidden suppressors (19, 20) could be revealed even in the responder strain. One approach to solving this question would be to dissociate a helper- from a suppressor-inducing portion of the N-C peptide, although it is possible that suppression and help are induced by the same epitope present in the N-C region. In any event, the use of N-C and L_{II} peptides has allowed analysis of almost the entire HEL molecule. The five amino acid overlap between the N-C and the L_{II} peptide does not seem to be relevant because different cell populations are induced, in B10 mice, by these two fragments. Fine analysis of the crucial determinant site included within the N-C fragment is currently in progress by using the isolated N-terminal and C-terminal peptides obtained from reduction of the N-C fragment, as well as other small, nonoverlapping CNBr-derived HEL peptides.

The existence of limited regions on antigenic molecules able to activate suppressor T cells to nullify the positive effect induced by helper cells reactive with other epitopes has been demonstrated in the response to β-galactosidase where a single CNBr peptide of β-galactosidase-induced T-cell-mediated suppression specific for haptens coupled

to the native enzyme (21). Furthermore, a peptic fragment of bovine serum albumin has been demonstrated to induce suppressor T cells able to suppress the primary anti-BSA IgE response (22). The regulatory role of limited portions on a protein antigen in affecting the overall response to the entire molecule has also been reported in the myelin basic protein system (23). Myelin basic protein, which can induce experimental allergic encephalomyelitis, can be cleaved into distinct regions, one of which can cause the encephalitis; whereas a different one can specifically induce suppressor T cells to prevent the disease upon subsequent challenge with myelin basic protein. A similar interpretation has been given to the finding that a polypeptide of glutamic acid and alanine, which is immunogenic in H-2^s mice, can be converted into a nonimmunogenic antigen by the addition of 4–10% tyrosyl residues (24).

Although we have attributed the lack of response to HEL in B10 mice to suppression induced by an epitope included in the N-C region, the B10 lesion may be viewed from a different perspective. We know that the potential to produce L_{II}-specific help is not expressed in the nonresponder strain, presumably owing to N-C-induced suppressors. It is conceivable that N-C induction of help, demonstrable in the B10.A responder, may be the key deficit in the B10 mouse repertoire. If N-C-specific help is resistant, even to some extent, to T suppressor cells directed against the same N-C peptide, B10.A could be a responder despite the existence of an adequate suppressive machinery (19, 20).

H-2-linked Ir gene control of the immune response is exerted at multiple levels of cell interaction events (1) and the ability of the N-C peptide to mimic the intact HEL molecule in the induction of suppressor T cells in B10 and helper cells in the congenic B10.A strain implies that Ir gene control is exerted at the level of individual antigenic determinants rather than the entire molecule. The work of Berzofsky et al. has demonstrated the existence of H-2-linked Ir gene control operating at the level of individual regions of the staphylococcal nuclease molecule in terms of restriction of antibody specificity and T-cell proliferation (25). The well-established role of macrophages in the intramolecular selection of critical antigenic determinants for presentation to T cells (26) and the results presented here are compatible with the hypothesis of a directive influence of macrophages not only in determinant selection but also in the MHC-controlled activation of different T-cell subpopulations by the same antigen fragment (27).

Two major modes of cellular interaction, via antigen bridging (28) and idiotypic complementarity (29), are currently utilized to explain cellular interactions in the immune response. Most of the results presented in this paper could be easily explained by antigen bridging (i.e., in B10 mice, N-C-specific receptors on suppressor T cells bind native HEL and then interact with L_{II}-specific helper cells across the HEL molecule). Recently, we have found common idiotypic determinants present on HEL-specific suppressor T cells and on the large majority of anti-HEL antibodies produced in the secondary response, almost all with specificity for the N-C region (30). Thus, the induction of idiotype-positive B-cell clones could be controlled by suppressor and helper T cells bearing idiotypically complementary receptors. The precise interaction between these two different regulatory pathways, which evidently coexist and determine the course of the immune response (31–33), is currently under scrutiny in our laboratory.

In conclusion, the genetic nonresponsiveness of the B10 strain to HEL seems to be

a consequence of the predominant activation of suppressor T cells by a small fragment of this antigen. Furthermore, this same restricted region of the molecule is able to induce, in the congenic B10.A responder partner of this strain, prevalent activation of helper-T-cell activity. Therefore, one of the levels of Ir gene control appears to be the ability to select specific antigenic determinants for the predominant activation of functionally different T-cell subpopulations.

Summary

We have examined the ability of two purified peptide fragments derived from hen (chicken) egg-white lysozyme (HEL); *N*-terminal, Co-terminal peptide (a.a. 1–17:cys 6–cys 127:120–129) and mixed disulfide L_{II} peptide (L_{II}) (a.a. 13–105) to induce antigen-specific suppression or help in B10 (H-2^b) nonresponder and B10.A (H-2^a) responder mice. An anti-HEL primary in vitro antibody response can be obtained in either strain by stimulation with HEL coupled to erythrocytes (RBC). Preimmunization with HEL-complete Freund's adjuvant-(CFA) or N-C-CFA-induced suppression of the anti-HEL PFC response to HEL-RBC in spleen cell cultures from B10 mice, whereas helper activity was demonstrated in cultures from B10.A mice similarly immunized. L_{II}-CFA priming elicited helper cells in both C57BL/10 Sn (B10) and B10.A/SgSn (B10.A) mice. The genetic nonresponsiveness of B10 mice to HEL can therefore be attributed to the activation of suppressor T cells by a limited portion of the molecule (e.g., N-C) which prevent the potential response directed against other epitopes on the same molecule (e.g., L_{II}). One manifestation of major histocompatibility complex gene activity appears to be the intramolecular selection of different antigenic determinants leading to activation of functionally different T-cell subpopulations.

The excellent secretarial assistance of Ms. Julie Zackrison is gratefully acknowledged.

Received for publication 12 March 1979.

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