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SHORT COMMUNICATION

AN IMMUNOLOGICAL DETERMINANT FOR HELPER T CELLS AT THE N- AND C-REGION OF HEN EGG-WHITE LYSOZYME

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The carrier determinant in the N- and C-region of hen egg-white lysozyme (lysozyme), recognized by T cells, which helps antibody formation to a single DNP residue bound to the Lysine-33 of lysozyme, was analysed. P17 (sequence 1–27: Cys 6-Cys 127: 123–129) and P17t (sequence 1–Homoser 12: Cys 6-Cys 127: 123–129), which are known to be immunodominant antigenic determinants, could induce helper T cells. When P17t was reduced and alkylated, one of the resultant peptides, P17tN (sequence 1-Homoser 12) could still induce helper T cells, but P17tC (sequence 123–129) could not. A synthetic peptide SP1-14 (sequence 1-Ala 6–14) also induced helper T cells. Thus the carrier determinant helping in antibody formation to the DNP residue at Lysine-33 may be localized in the region corresponding to the sequence 1–12 of lysozyme.

The participation of T cells in the development of humoral immune responses to protein antigens has been established (Mitchison et al., 1970). So, the immunological determinants recognized by the T cells in the process of antibody formation are of great interest. However, little is known about the structures of these determinants. Differences in the mode of recognition by T cells from that by B cells have been suggested from the cross-reactions observed between serologically non-cross-reacting antigens with respect to several parameters of the cellular immune response, such as cross tolerance, delayed skin reactivity and stimulation of splenic lymphocytes (Thompson et al., 1972; Maron et al., 1972; Parish, 1972; Scibienski et al., 1972). As those phenomena might involve multiple determinants, it is hard to deduce events related to an individual determinant of T cells. This is also so for the determinants of helper T cells (carrier determinant) (Ichiki and Parish, 1972; Ishizaka et al., 1974).

To analyse a single carrier determinant, we

chose hen egg-white lysozyme (lysozyme) as a model antigen because much information is available about its primary, tertiary structure (Imoto et al., 1972) and antigenic determinants (Shinka et al., 1967; Wilson and Prager, 1974; Arnon, 1977; Atassi, 1978). We introduced a single hapten DNP residue onto a known site of the protein (Hirayama et al., 1981). This preparation, designated as DNP_1-33 lysozyme, was shown to bear a single DNP group at Lysine-33 of the molecule.

In this work, we tried to identify and localize the single carrier determinant in the N- and C-region of lysozyme recognized by T cells that helps in the antibody response to a chemically defined antigenic determinant on the molecule.

MATERIALS AND METHODS

1. *Animals*

Male and female A/J mice were obtained from Omura Experimental Animals, Kanagawa, and maintained in our animal facilities. All mice were immunized or used as recipients at 8–12 weeks of age.

2. *Preparation of DNP-Protein Conjugates*

Keyhole limpet hemocyanin (KLH) was obtained from Calbiochem, San Diego, Calif., and bovine γ-globulin (BγG) was purchased from Sigma Chemical Co., St. Louis, Mo. These proteins were dinitrophenylated by the method of Eisen et al. (1953). The resulting DNP-KLH and DNP-BγG contained 280 and 30 DNP groups per molecule, respectively. Six-times crystallized hen egg-white lysozyme was purchased from Seikagaku Kogyo Co., Tokyo. Substitution of lysozyme with a DNP at Lys-33 residue (DNP_1-33 lysozyme) was achieved as described previously (Hirayama et al., 1981).

3. *Preparations and Characterizations of P17 and Its Derivatives*

P17 (peptide with sequence 1–27 and sequence 123–129 linked by a single disulfide bond between Cys-6 and Cys-127) and its terminal portion, P17t (peptide with sequence 1-Homoserine 12 and sequence 123–129 linked by a single disulfide bond between Cys-6 and Cys-127) were prepared as described previously (Fujio et al., 1968; Ha et al., 1975). For preparation of P17tN (peptide with sequence 1-Homoserine 12) and P17tc (peptide with sequence 123–129), 10 mg of P17t was reduced and alkylated as described (Ha et al., 1975). The reaction mixture was applied to a Sephadex G-15 column (2×140 cm) equilibrated with 25% acetic acid, and fractions of 3 ml were collected. The optical density was measured at 280 nm and the relative fluorescence was recorded by the method of Nakai et al. (1974). The first fraction showed relative fluorescence but no absorbance at 280 nm. The second fraction showed absorbance at 280 nm. Amino acid analysis, performed by the method of Spackman et al. (1958), showed that the first and second fractions were P17tN and P17tC, respectively (Table 1). The enzymatic activity of each peptide at a concentration of 5 mg/ml was measured using *Micrococcus lysodeikticus* as substrate (Shinka et al., 1967). No peptide preparations used in this series of experiments had any enzymatic activity and

TABLE 1. *Amino Acid Compositions of P17tN and P17tC*

Amino Acid	P17tN		P17tC	
	found[a]	calc.[b]	found[a]	calc.[b]
Lys	0.95	1		
Arg	1.07	1	1.84	2
CM-Cys	1.08	1	0.92	1
Glu	1.09	1		
Gly	*1.0*	1	*1.0*	1
Ala	2.99	3	0.1	
Val	0.87	1		
Ile			0.98	1
Leu	1.0	1	0.94	1
Phe	0.92	1		
Homoser	0.89	1		
Trp			ND[c]	1

[a] One mg of each peptide was hydrolysed in 1 ml of constant boiling HCl (5.7 *N*) for 24 h. Values are expressed as molar ratios to glycine. Where no numbers are given, the values obtained were less than 0.05 moles per mole of peptide.

[b] Moles of amino acids in the given sequence of lysozyme were calculated from the data of Canfield and Liu (1965).

[c] Not determined.

therefore they were concluded to contain less than 0.01% of intact lysozyme.

The synthetic peptide of lysozyme residues 1–14 (SP1-14), in which Cys-6 was replaced by Ala, was synthetized by a method to be published (Shimonishi, Y. and Yamauchi, K., manuscript in preparation). The purity of the peptide was tested by thin layer chromatography on a silica gel G-60 plate and also by electrophoresis at pH 4.8. SP1-14 was found to be homogeneous by both methods. The amino acid composition of the peptide was as follows: Lys, 2.01 (2); Arg, 2.05 (2); Glu, 1.04 (1); Gly, 1.01 (1); Ara, 4.00 (4); Val, 0.93 (1); Met, 0.91 (1); Leu, 0.98 (1); Phe, 0.95 (1).

4. *Immunizations and adoptive cell transfers*

Donors of DNP-primed spleen cells and donors of peptide fragment-primed spleen cells were immunized 8 weeks before experiments by intraperitoneal (i.p.) injection of 100 μg of DNP-BγG or DNP-KLH in complete Freund's adjuvant (CFA) and i.p. injection of 50 μg of P17 or its derivatives in CFA, respectively. The primed or normal spleen cells were transferred intravenously to X-ray-irradiated (400 R) syngeneic recipients together with $1.5–2\times10^7$ DNP-primed B cells or T cell depleted DNP-primed spleen cells. Immediately after cell transfer, recipients were immunized by i.p. injection of 50 μg of DNP_1-33 lysozyme in incomplete Freund's adjuvant, and were bled 10 days later.

5. *Fractionation of spleen cells*

In some experiments, T cells were removed from the primed spleen cells by treatment with monoclonal anti-Thy-1.2 antibody (New England Nuclear, Boston, Mass.) plus guinea pig complement (complement). The monoclonal anti-Thy-1.2 plus complement used here lysed 95% of the thymocytes and 35–40% of the spleen cells, as measured by the trypan-blue dye exclusion test. T cells were purified from spleen by the method of Julius et al. (1973) with a nylon wool column. The yield of T-enriched spleen cells, more than 95% of which were Thy-1.2 antigen positive, was about 30% of the cells applied to the column.

6. *Measurement of antibodies*

Serum anti-DNP antibody levels were determined by a modification of the technique of Farr (Farr, 1958; Green et al., 1969) using ε-[^{3}H]DNP lysine. Using a standard curve constructed for the A/J strain by the method described by Katz et al. (1970) for inbred guinea pigs, the percentage binding was converted to the amount of anti-DNP antibody in micrograms per ml.

RESULTS AND DISCUSSION

1. *The P17 region is a carrier determinant helping in the anti-DNP response to challenge of DNP_1-33 lysozyme*

As P17, composed of the N- and C-region of lysozyme, is known to be an immunodominant peptide (Fujio et al., 1968), we first examined whether immunization with P17 would induce helper T cells, which cooperate with DNP-specific B cells on challenge with DNP_1-33 lysozyme. The protocol and results are shown in Figure 1. Untreated or treated spleen cells from donors primed with P17 eight weeks previously, or normal spleen cells (2×10^7) were transferred to X-irradiated recipients together with DNP-primed B cells (2×10^7) prepared by treatment of DNP-BγG primed

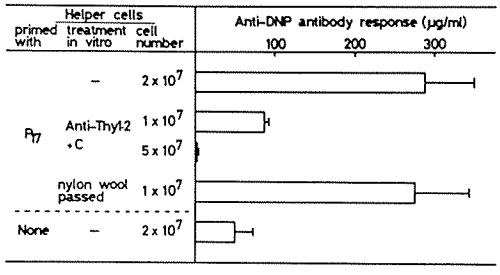

FIGURE 1. Helper function of spleen cells primed with P17.

Donors of helper cells were primed with P17 in CFA. A portion of primed spleen cells was treated with anti-Thy-1.2 plus complement or passed through a nylon wool column; $1–5\times10^7$ cells of each preparation or 2×10^7 normal spleen cells were transferred to each irradiated recipient together with 2×10^7 DNP-BγG primed B cells, and the recipients were challenged with DNP_1-33 lysozyme. Anti-DNP antibody responses 10 days after the challenge are illustrated as arithmetic means for groups of five mice and bars represent standard error (SE).

spleen cells with anti-Thy-1.2 plus complement. All recipients were challenged with DNP_1-33 lysozyme and the anti-DNP titers were measured 10 days after the challenge. Results showed that spleen cells of P17-primed animals had helper function for anti-DNP responses and that their helper function was abolished by treatment with anti-Thy-1.2 plus complement. Animals which had received 5×10^7 P17-primed spleen cells treated with anti-Thy-1.2 plus complement did not show any anti-DNP antibody response. Thus, the trivial response by 1×10^7 cells of this fraction was concluded not to be due to the specific help of T cells. Moreover, the T-enriched fraction of P17-primed spleen cells obtained by passage through nylon wool restored the helper function. These results clearly indicate that P17 was capable of priming helper T cells. In other words, the P17 portion of the antigen DNP_1-33 lysozyme is a carrier determinant helping in the anti-DNP response on challenge with DNP_1-33 lysozyme.

2. *The P17tN region of lysozyme as a minimal carrier determinant*

Next the minimum region for induction of helper T cells to augument the anti-DNP response was studied. Lysozyme, P17, P17t, P17tN, P17tC and the synthetic peptide SP1-14 were used as priming antigens and T cell enriched fractions of the spleen cells were tested. As shown in Figure 2, the magnitude of the antibody response to one antigenic determinant was dependent on the broadness of the carrier determinants of the antigen. P17t, P17 and lysozyme induced helper T cells efficiently. These data are consistent with our previous findings that P17t is a dominant antigenic determinant in guinea pigs with respect to both circulating antibodies against lysozyme (Ha et al., 1975) and delayed hypersensitivity (Miyagawa et al., 1975).

P17tN (sequence 1–12) and synthetic peptide SP1-14 could also induce helper T cells,

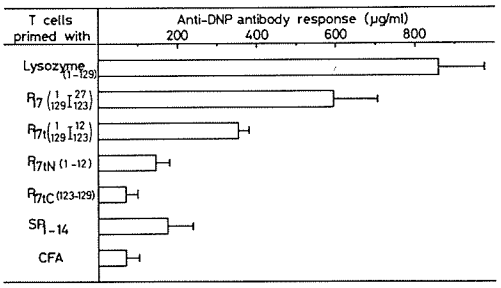

FIGURE 2. Comparison of helper functions of T cells primed with lysozyme, P17 and its derivatives.

Donors of helper cells were primed with lysozyme, P17 or its derivatives, whose sequences are shown in parentheses. 1×10^7 T cells purified from primed spleen cells by passage through a nylon wool column were transferred to irradiated recipients together with 1.5×10^7 DNP-KLH primed B cells, and the recipients were challenged with DNP_1-33 lysozyme. Anti-DNP antibody responses 10 days after the challenge are shown as arithmetic means for groups of five mice and bars represent SE. P values were calculated by Student's t test for differences from the group primed with CFA. P values of groups primed with P17tN, P17tC and SP1-14 were <0.01, >0.1 (not significant) and <0.0025, respectively.

whereas P17tC could not. Thus sequence 1–12 is a carrier determinant helping in the anti-DNP response to challenge with DNP_1-33 lysozyme. However, as reported previously by Young and Leung (1970) and also by Ha et al. (1975), the peptide with the sequence 1–12 did not react with antibodies to native lysozyme but it was active in delayed type hypersensitivity (Miyagawa et al., 1975). These results suggest that helper T cells, unlike B cells, could recognize determinants related to the primary structure of the antigen, that is, sequential determinants.

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