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ANTIGENIC DETERMINANTS OF HEN EGG-WHITE LYSOZYME IN DELAYED HYPERSENSITIVITY. II. ANTIGENICITY AND IMMUNOGENICITY OF THE N- AND C-TERMINAL PEPTIDE

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SUMMARY Various small fragments of P_{17} (sequence 1-[Cys·6]-27, 123-[Cys·127]-129), which is one of the immunodominant groups of hen egg-white lysozyme (HL), were tested for macrophage migration inhibition (MMI) of peritoneal exudate cells (PEC) from guinea pigs immunized with HL. P_{17} was split in the middle with cyanogen bromide. The terminal portion of P_{17}, P_{17t} (sequence 1-[Cys·6]-Homoser.·12, 123-[Cys·127]-129) showed positive MMI, whereas the non-terminal half of P_{17}, P_{17i} (sequence 13–27) only showed very weak MMI activity. A fragment derived from the middle portion of P_{17}, P_{17m} (sequence 11–22), was inactive. When P_{17} were reduced and alkylated, one of the resultant peptides, P_{17N} (sequence 1-[CM·Cys·6]-27) still has MMI activity with PEC taken from guinea pigs immunized with HL, although no antibody reacting with it was detected, but P_{17C} (sequence 123-[CM·Cys·127]-129) was inactive.

The peptides P_{17} and P_{17N} were both immunogenic in guinea pigs in respect to the delayed hypersensitivity response. Again P_{17t} and P_{17N} were immunodominant groups, but the reactivity of P_{17i} in MMI assay of this group of animals was greater than that in guinea pigs immunized with HL. The reactivities of HL with PEC taken from guinea pigs immunized with P_{17} or P_{17N} were generally weaker than those of the antigens used for immunization.

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Skin reactions in guinea pigs immunized with HL, P_{17} or P_{17N} were also tested. It was found that the results of the delayed skin reaction and that of MMI were approximately parallel.

INTRODUCTION

A high degree of specificity in delayed hypersensitivity is generally noted in the hapten-carrier system (Gell and Benacerraf, 1961; Gell and Silverstein, 1962; Bloom, 1971; Fleischman and Eisen, 1975). For manifestation of delayed hypersensitivity the haptenic group alone does not seem to be enough, and generally the contribution of a certain structure of carrier protein is essential. But an exceptionally high degree of contribution of the haptenic group in delayed hypersensitivity was reported by Leskowitz (1963 and 1971) using p-azobenzene arsonate as a hapten. It was reported that a molecule as small as p-azobenzene-arsonate-L-tyrosine can induce delayed hypersensitivity as immunogen and also as a test antigen in guinea pigs.

Although protein seems to be very important in delayed hypersensitivity as antigen itself and also as a carrier for hapten systems, relatively little information is available on the antigenic determinants in proteins responsible for delayed hypersensitivity. Parish (1971) and Ichiki and Parish (1971) reported that chemically modified flagellin or enzymatically degraded flagellin induced cell-mediated immunity as well as, or even better than unmodified antigens. Later Thompson et al. (1972) reported that there is no discrimination in delayed hypersensitivity between native lysozyme and reduced-alkylated lysozyme. These reports are somewhat contradictory to results obtained with the hapten-carrier system described above.

Therefore, further studies on the nature of antigenic determinant involved in delayed hypersensitivity seem necessary. Previously we reported (Miyagawa et al., 1975) that three or four kinds of peptides obtained from HL showed MMI activities when tested with PEC from guinea pigs immunized with HL. One of these, called P_{17}, which is composed of the N- and C-terminal portions of HL was studied further, because it was also shown to have antibody binding activity when tested with antibodies made to HL in rabbits (Fujio et al., 1968) and guinea pigs (Ha et al., 1975). This paper reports detailed studies on the localization of the specificity of delayed hypersensitivity in HL in comparison with that of circulating antibody.

MATERIALS AND METHODS

1. *Hen egg-white lysozyme (HL)*

Six times recrystallized HL preparation (Seikagaku Kogyo Co. Ltd.) was used without further purification for preparing the active peptide. For preparation of immunizing antigen, crystalline HL was further purified on SE-Sephadex C-25 in 0.2 M sodium phosphate buffer, pH 7.16 and then passed through QAE-Sephadex C-25 in 0.005 M sodium phosphate buffer, pH 8.0.

2. *Preparation of derivative peptides of P_{17}*

P_{17} (sequence 1-[Cys·6], 123-[Cys·127]-129) was prepared by limited digestion of HL by pepsin and purified by CM-cellulose chromatography, as described previously (Fujio et al., 1968 and Ha et al., 1975). The homogeneity of P_{17} used in the present work was estimated to be about 93% by amino acid analysis.

P_{17t} (sequence 1-[Cys·6]-Homoser·12, 123-[Cys·127]-129) and P_{17i} (sequence 13–27) were prepared by splitting the methionyl bond in P_{17} with cyanogen bromide. Two peptides were separated by chromatography on SE-Sephadex C-25 as described before (Ha et al., 1975). These peptides were further purified by rechromatography. Amino acid analyses showed that both peptides were at least 95% homogenous.

TABLE 1. *Amino acid sequences and minimal molecular weights of the peptide derivatives of HL tested for MMI and skin reactions*

Peptide	Amino acid sequence	Molecular weight[a]
P_8	Gln^{57}—Cys^{64}—Cys^{76}—Cys^{80}—Leu^{84} Ser^{85}—Cys^{94}—Ala^{107}	5,456
P_{17}	Lys^{1}—Cys^{6}—Asn^{27} Trp^{123}—Cys^{127}—Leu^{129}	3,927
P_{17t}	Lys^{1}—Cys^{6}—$Homoser^{12}$ Trp^{123}—Cys^{127}—Leu^{129}	2,203
P_{17i}	Lys^{13}—Asn^{27}	1,760
P_{17N}	Lys^{1}—CM·Cys^{6}—Asn^{27}	3,086
P_{17C}	Trp^{123}—CM·Cys^{127}—Leu^{129}	
P_{17m}	Ala^{11}—Gly^{22}	1,418

[a] The minimum molecular weight of each peptide was calculated from the results of amino acid analysis.

P_{17N} (sequence 1-[CM-Cys·6]-27) and P_{17C} (sequence 123-[CM-Cys-127]-129) were prepared by reduction and alkylation of the single disulphide bond in P_{17}. The resultant two peptides were separated by gel filtration on serial columns of a Sephadex G-15 (3×140 cm) and of a Bio-Gel P-10 (3×150 cm) in 25% (v/v) acetic acid as described previously (Ha et al., 1975). Amino acid analyses showed that one peptide was an N-terminal peptide (P_{17N}) of P_{17} and the other was a C-terminal peptide (P_{17C}) of P_{17}. On amino acid analysis of P_{17N} using 0.3 μm of peptide no isoleucine was detected and contamination of P_{17N} with intact P_{17} or P_{17C} was estimated as less than 1%.

P_{17m} (sequence 11–22) was prepared directly from the peptic digest of HL. The details of the purification procedure and the results of amino acid analysis were reported previously (Ha et al., 1975).

P_8 (sequence 57-[Cys·64]-[Cys·76]-[Cys·80]-83, 84-[Cys·94]-107) was also prepared by limited digestion of HL with pepsin as described before (Shinka et al., 1967; Fujio et al., 1974 and Miyagawa et al., 1975). The homogeneity of the peptide was also tested by amino acid analysis (Shinka et al., 1967) and disc electrophoresis in acid medium in the presence of 6 M urea (Fujio et al., 1974). Anino acid analysis indicate that the P_8 preparation was more than 95% homogeneous and only a single band was observed on disc electrophoresis of 20 μg of peptide on a 20% polyacrylamide gel column (5×80 mm).

Contamination of each peptide with intact HL was especially carefully checked by measuring the lytic activity of 5 mg/ml of each peptide with *M. lysodeikticus* as substrate (Shinka et al., 1967). None of the peptides showed lytic activity under the present conditions. Therefore, contamination of each of the peptides with HL was less than 0.01%. In the case of P_{17}, the peptide was subjected to gel filtration on Bio-Gel P-10 (2×150 cm) in 10% (v/v) acetic acid and the fraction corresponding to intact HL was concentrated and its lytic activity was measured. Again it was estimated that the degree of contamination of P_{17} by intact HL was less than 0.01%.

The amino acid sequences and minimal molecular weights of the various peptides of HL used in the present experiments are listed in Table 1.

3. *Immunization*

Non inbred guinea pigs, weighing 300 to 500 g, were used for immunization. One hundred μg of chromatographically puridied HL in complete Freund's adjuvant were injected into all four foot pads. Highly purified P_{17} or P_{17N} (100 μg each) in complete Freund's adjuvant was injected into guinea pigs by the same route. The animals were kept under similar conditions to those described in the preceeding paper (Miyagawa et al., 1975).

4. *Passive hemagglutination*

HL was coupled to sheep erythrocytes by glutaraldehyde. The preparation of HL coupled erythro-

cytes, HL-erythrocytes, was described in detail by Komatsu et al. (1975). The titer was given as the $\log_2$ of the end dilution of serum giving definitive positive agglutination. Using the present method of hemagglutination approximately 50 ng of precipitable antibody could be detected per ml of hyperimmune guinea pig anti-HL antiserum.

5. *Macrophage migration inhibition*

The method used was essentially the same as that reported by David et al. (1964). Guinea pigs immunized with each antigen were injected intraperitoneally with 30 ml of sterilized liquid paraffin 4 to 5 days before sacrifice. On the day of sacrifice, guinea pigs were exsanguinated by cardiac puncture and peritoneal exudate cells (PEC) were collected as described previously (Miyagawa et al., 1975). The sera were titrated by passive hemagglutination to detect circulating antibody to HL.

Triplicate capillaries containing known concentrations of particular antigen were prepared using PEC. The mean migration area under each condition was calculated from results in at least 3 capillaries under identical conditions. The migration index (%), that is

$$\frac{\text{the mean migration area in the presence of antigen}}{\text{the mean migration area in the absence of antigen}} \times 100,$$

was used in some cases and the weights of cut paper were used in some cases as described in the preceding paper (Miyagawa et al., 1975).

Dunnett's multiple comparison tests (1964) were performed using the weights of cut paper for statistical comparison of the values under each condition.

6. *Antigen-agarose plate*

To test the specificity of lymphocytes, a kind of immunoadsorbent was prepared using agarose and cyanogen bromide (Axen et al., 1967).

One hundred ml of 1% agarose solution in deionized water were poured into a glass petri dish of 21 cm diameter. One hundred ml of cyanogen bromide solution (50 mg per ml) were layered on the top of the agarose plate. The pH was maintained at 11 ± 0.5 for 8 min by addition of 1 N NaOH while the agarose plate was shaken by hand. The agarose plate was thoroughly washed 3 times with 300 ml

TABLE 2. *MMI of PEC from guinea pigs immunized*

Animal number	Mean migration indices (%) at the indicated HL ng/ml				P_{17} ng/ml				
	10	10^2	10^3	10^4	10	10^2	10^3	10^4	10^5
50	80.8 (I)[b]	50.0 (III)	36.5 (III)	38.5 (III)	82.7 (I)	60.8 (III)	55.8 (III)	50.0	
52	78.9 (III)	71.0 (III)	65.8 (III)	44.7 (III)			85.8 (I)	65.8 (III)	50.0 (III)
54		79.2 (II)	70.8 (III)	68.8 (III)		95.0 (I)	72.1 (III)	66.7 (III)	43.8 (III)
55	65.0 (III)	63.0 (III)	52.8 (III)	44.7 (III)		91.9 (I)	83.7 (I)	69.1 (III)	52.8 (III)
58	77.8 (I)	63.0 (III)	44.4 (III)	33.3 (III)	88.9 (I)	66.7 (III)	53.7 (III)	48.1 (III)	
60	70.4 (III)	61.1 (III)	57.4 (III)	63.0 (III)	85.2 (I)	77.8 (II)	66.7 (III)	55.6 (III)	
65	82.1 (I)	64.1 (II)	33.3 (III)	23.1 (III)		82.1 (I)	50.0 (III)	38.5 (III)	
66	90.0 (I)	67.5 (III)	45.0 (III)	35.0 (III)		85.0 (I)	51.5 (III)	41.3 (III)	37.5 (III)

[a] The concentration of antigen is given as ng of antigen per ml of culture medium for MMI.

[b] Dunnett's multiple comparison test (1955 and 1964) was performed using the migration areas in arbitrary different from that of the control. The symbols used are as follows; I: $P>0.05$, II: $0.05>P>0.01$, III:

volumes of chilled 0.1 M $NaHCO_3$ solution. One hundred ml of HL solution or pancreatic ribonuclease (RNase) solution containing 10 mg per ml of of 0.1 M $NaHCO_3$ were added in a cold room. The plates were shaken gently overnight in a cold room and then thoroughly washed with 0.1 M $NaHCO_3$ and 1 N acetic acid. The plates were then washed five times with 300 ml volumes of distilled water and finally with Hanks' solution until the pH of the washing fluid was neutral. On this way approximately 200 mg of HL or ribonuclease were coupled to one agarose plate.

The PEC taken from 2 guinea pigs immunized with HL were pooled and first incubated at 37 C in a glass petri dish in medium 199 containing 20% foetal calf serum to adsorb macrophages. The macrophages were roughly removed by adhesion to glass and half the lymphocyte rich fraction of PEC was poured into the HL-agarose plate at 4 C. The immunoplate was incubated at 4 C for 1 hr with occasional shaking by hand. The unbound fraction of lymphocytes were recovered by decantation and washing the plate several times with Hanks' solution and the cells were collected by centrifugation.

To elute bound cells, 100 ml of HL solution (1 mg/ml) in Hanks' solution were poured into the agarose plate at 4 C and the plate was shaken gently at 4 C for 1 hr. The eluted lymphocytes were collected by centrifugation and washed 3 times with Hanks' solution. As a control, the other half of the lymphocyte rich fraction of PEC was treated on an ribonuclease-agarose plate in exactly the same way as that of the HL-agarose plate.

Cell fractions obtained from immuno-plates were mixed with an equal packed volume of normal PEC and their MMI was measured.

7. *Skin reaction*

Albino guinea pigs were shaved and injected intradermally into the flank with 0.05 ml of antigen in saline solution. Skin reactions were read at 4 hr for immediate-type reactions and at 24 hr for delayed-type hypersensitivity reactions.

The diameters of the reaction areas were measured along the long and short axes and the mean of the two values was used as a measure of the skin reaction.

with HL by P_{17} and its derivative peptides

concentrations[a] of various antigens

P_{17t} ng/ml				P_{17i} ng/ml				P_{17m} ng/ml			
10^2	10^3	10^4	10^5	10^2	10^3	10^4	10^5	10^2	10^3	10^4	10^5
88.5 (I)	69.2 (III)	53.8 (III)		78.8 (I)	76.2 (II)	62.7 (III)	65.4 (III)	80.0 (I)	80.0 (I)	92.3 (I)	88.5 (I)
	84.2 (I)	76.3 (III)	50.0 (III)		92.1 (I)	78.9 (III)	73.7 (III)		100 (I)	86.8 (I)	92.1 (I)
91.7 (I)	78.3 (II)	66.7 (III)	47.9 (III)	106 (I)	108 (I)	93.8 (I)	83.3 (I)		87.5 (I)	87.5 (I)	95.8 (I)
89.4 (I)	85.4 (I)	69.1 (III)	69.1 (III)	93.5 (I)	93.5 (I)	93.5 (I)	85.4 (I)	89.4 (I)	83.7 (I)	89.4 (I)	93.5 (I)
85.2 (I)	63.0 (III)	44.4 (III)			85.2 (I)	77.8 (II)	77.8 (II)		85.2 (I)	81.5 (I)	92.6 (I)
81.5 (I)	53.7 (III)	59.3 (III)		85.2 (I)	70.4 (III)	77.8 (II)	77.8 (II)	88.9 (I)	81.5 (I)	92.6 (I)	81.5 (I)
82.1 (I)	66.7 (II)	55.4 (III)			92.3 (I)	94.9 (I)	84.6 (I)		92.3 (I)	76.9 (I)	94.9 (I)
100 (I)	87.5 (I)	67.5 (III)	60.0 (III)	105 (I)	103 (I)	97.5 (I)	103 (I)	110 (I)	100 (I)	100 (I)	90.0 (I)

units and the symbols in parentheses are the probabilities that the migration areas with antigens are not $P<0.01$

RESULTS

1. *MMI assays on P_{17} and its derivative peptides with PEC from guinea pigs immunized with HL*

Only a few PEC can be obtained from a single animal, so the MMI activities of various derivatives of P_{17} were tested in two groups of guinea pigs which had both been immunized with HL. The first group of animals were tested with HL, P_{17}, P_{17t}, P_{17i} and P_{17m} by measuring MMI at various concentrations of antigens. Representative results of these experiments are listed in Table 2.

The second group of animals were tested with HL, P_{17} and the N-terminal peptide of P_{17} (P_{17N}). The results are shown in Table 3.

The ranges of the molar ratios of HL and derivative peptides of P_{17} to that of P_{17} inducing 30% MMI were calculated from the data in Tables 2 and 3 and are listed in Tables 4 and 5, respectively.

Tables 2 and 3 show that the sensitivities of individual guinea pigs to various antigens differed. Nevertheless, P_{17N} and P_{17t} always showed significant of MMI activities and the molar ratios of the two peptides to that of P_{17} inducing 30% MMI were very similar. The MMI activities of P_{17i} and P_{17m} were far weaker than those of P_{17N} and P_{17t}, P_{17m} in particular being almost completely inactive, and showing no MMI activity, even at a concentration of 100 μg per ml. Only one animal (#50) of the 8 animals tested showed definite MMI activity against P_{17i}, although the reactivities to P_{17i} in the other animals shown in Table 2 tended to reach a statistically significant level. Although the MMI activity of P_{17i} was definitely weaker than those of P_{17N}, P_{17t} and P_{17} but P_{17i} still can be a second antigenic determinant of P_{17} in a certain individual guinea pigs.

TABLE 3. *MMI by P_{17} and P_{17N} of PEC from guinea pigs immunized with HL*

Animal number	Mean migration indices (%) at the indicated concentrations[a] of various antigens											
	HL ng/ml				P_{17} ng/ml				P_{17N} ng/ml			
	10	10^2	10^3	10^4	10	10^2	10^3	10^4	10	10^2	10^3	10^4
82	83.3 (I)[b]	75.0 (I)	79.2 (I)	66.7 (II)	104 (I)	83.3 (I)	83.3 (I)	66.7 (II)	87.5 (I)	75.0 (I)	70.8 (II)	62.5 (III)
86	83.9 (I)	64.5 (III)	59.0 (III)	51.6 (III)	96.8 (I)	90.3 (I)	87.0 (I)	67.7 (III)	96.8 (I)	90.3 (I)	80.6 (I)	59.0 (III)
87	76.1 (II)	52.2 (III)	43.5 (III)	37.0 (III)		84.8 (I)	80.4 (I)	63.0 (III)		109 (I)	73.9 (II)	65.2 (III)
88	76.5 (I)	55.9 (III)	36.8 (III)	38.2 (III)	91.2 (I)	94.1 (I)	67.6 (III)	50.0 (III)	100 (I)	94.1 (I)	64.7 (III)	58.8 (III)
89	72.2 (III)	64.2 (III)	56.1 (III)	42.8 (III)	74.9 (III)	56.1 (III)	56.1 (III)	48.1 (III)	72.2 (III)	58.8 (III)	56.1 (III)	46.8 (III)
93	100 (I)	93.5 (I)	73.9 (I)	63.0 (III)	115 (I)	100 (I)	76.1 (I)	58.7 (III)	104 (I)	84.8 (I)	71.7 (II)	67.4 (II)
95	78.6 (I)	60.7 (III)	60.7 (III)	57.1 (III)	89.3 (I)	100 (I)	64.3 (III)	60.7 (III)	85.7 (I)	71.4 (II)	68.3 (III)	60.3 (III)
96	75.9 (II)	69.3 (III)	59.4 (III)	52.8 (III)	89.0 (I)	82.5 (I)	49.5 (III)	52.8 (III)	89.1 (I)	79.2 (II)	59.4 (III)	56.1 (III)

[a] The concentration of antigen is given as ng of antigen per ml of culture medium for MMI.

[b] Dunnett's multiple comparison test (1955 and 1964) was performed using the migration areas in arbitrary units and the symbols in parentheses are the probabilities that the migration areas with antigens are not different from that of the control. The symbols used are as follows; I: $P>0.05$, II: $0.05<P<0.01$, III: $P<0.01$

TABLE 4. *Molar ratios of HL and derivative peptides of P_{17} to P_{17} inducing 30% MMI in PEC guinea pigs immunized with HL*

Animal number	Range of molar ratios inducing 30% MMI[a]			
	HL/P_{17}	P_{17t}/P_{17}	P_{17i}/P_{17}	P_{17m}/P_{17}
50	0.028 -2.8	1.9 -190	2.3-230	>2,800[b]
52	0.0028-0.28	1.9 -190	> 23	> 28
54	0.028 -2.8	0.19-19	> 23	> 28
55	<0.00028	0.19-19	> 23	> 28
58	0.028 -2.8	1.9 -190	>2,300	>2,800
60	0.028 -0.28	1.9 -190	>2,300	>2,800
65	0.0028-0.28	0.19-19	> 230	> 280
66	0.0028-0.28	1.9 -190	> 230	> 280

[a] Molar ratios were calculated from the results in Table 2.

[b] Where the symbol > or < is used more than 30% MMI could not be attained within the antigen concentration range used.

Since P_{17t} (1-[Cys·6]-Homoser·12, 123-Cys·127]-129) constitutes the terminal half of P_{17}, the results indicate that the terminal half of P_{17} may be a dominant antigenic determinant in P_{17} with respect to delayed hypersensitivity. On the basis of this result, the specificity of P_{17} in delayed hypersensitivity cannot be differentiated from that of circulating antibody (Ha et al, 1975).

TABLE 5. *Molar ratios of HL and P_{17N} to P_{17} inducing 30% MMI in PEC of guinea pigs immunized with HL*

Animal number	Range of molar ratios inducing 30% MMI[a]	
	HL/P_{17}	P_{17N}/P_{17}
82	0.028 -2.8	0.13-13
86	0.00028-0.028	0.13-13
87	0.00028-0.028	0.13-13
88	0.0028 -0.28	0.13-13
89	0.028 -2.8	0.13-13
93	0.028 -2.8	0.13-13
95	0.0028 -0.28	0.13-13
96	0.0028 -0.28	0.13-13

[a] Molar ratios were calculated from the results in Table 3.

On the other hand, P_{17N}, which originated from the N-terminal portion of P_{17}, had comparable MMI activity to that of P_{17}. This strongly suggests that the single disulphide bond in P_{17} was not important for its MMI activity. It was reported previously (Ha et al., 1975) that when the single disulphide bond in P_{17} was reduced and alkylated, the binding activity of P_{17} to guinea pig circulating antibody was completely abolished. Therefore, these results suggest that the determinant responsible for antibody specificity contained the disulphide bond or that splitting of the disulphide bond induced a conformational change necessary for the antibody binding activity. On the other hand the determinant responsible for delayed hypersensitivity must be located in another portion from that of circulating antibody or the structure of the determinant must be very insensitive to the conformational change induced by reduction of the disulphide bond in P_{17}.

The MMI of P_{17C}, derived from the C-terminal portion of P_{17}, was also tested using 10^3 to 10^5 ng per ml of P_{17C} with PEC from 4 guinea pigs immunized with HL 3 weeks previously. Results were negative in all the animals and hence the data are omitted from Tables 2 and 3.

As a control the MMI activities of the various antigen tested were also measured using PEC from normal guinea pigs and various concentrations of the antigens. The results are shown in Table 6. None of the antigens showed any significant MMI activity.

2. *Test of the specicity of P_{17}-reactive cell populations using HL-immunoadsorbent*

It has been postulated that the cell populations responsible for delayed hypersensitivity may be sensitized to degradation products of the antigen (Parish, 1971; Parish and Ichiki, 1971). Therefore, experiments were made to see whether any cell population responding to the MMI activity of P_{17} remained after treatment with the immunoadsorbent of undegrated antigen.

The PEC from 2 guinea pigs immunized with HL were pooled and treated on an HL-agarose plate. The experimental procedure used is summarized in Fig. 1.

Three populations of cells were mixed with PEC from normal guinea pigs and their suscep-

TABLE 6. *MMI of PEC from normal guinea pigs by HL and derivative peptide of P_{17}*

Antigen concentration[a]	Numbers of animals showing more than 30% MMI at the indicated concentrations of various antigens[b]					
ng/ml	HL	P_{17}	P_{17N}	P_{17t}	P_{17i}	P_{17m}
10	0/11	0/ 8	0/4	0/ 7	0/ 4	0/ 3
10^2	0/17	1/21	0/8	1/18	0/13	0/11
10^3	0/23	0/22	0/8	0/22	0/15	1/15
10^4	0/23	0/23	0/8	0/23	0/14	1/15
10^5	0/21	0/23	0/8	0/23	0/15	0/15

[a] Antigen concentration in culture medium for MMI.

[b] Triplicate capillaries were prepared for each concentration of each antigen and the migration index was calculated from the mean migration areas with and without antigen.

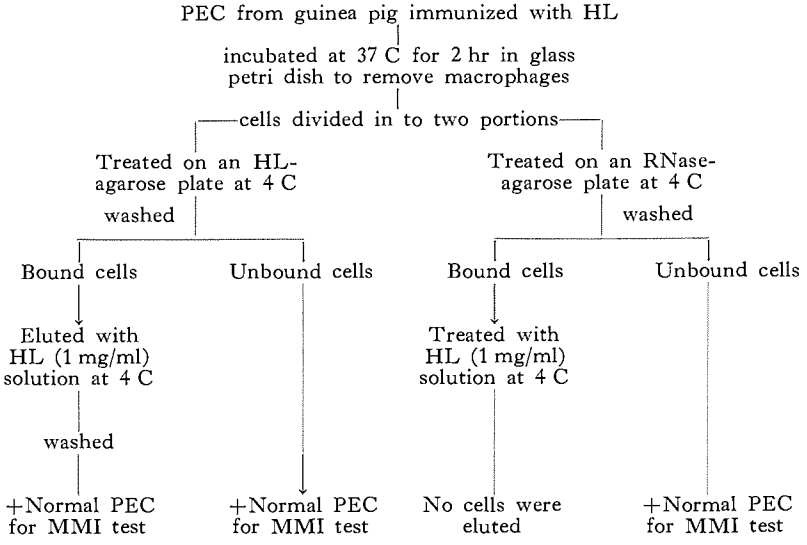

FIGURE 1. *Specific adsorption of PEC having macrophage migration inhibition activity.*

tibilities to MMI activity were measured. As shown in Table 7, after treatment of PEC from guinea pigs immunized with HL on the HL-agarose plate, no cell population remained which responded to HL or P_{17}. When the cells were eluted with HL from the HL-agarose plate, they were sensitive to the MMI activities of HL or P_{17} at various concentrations. On the other hand, when cells were treated on an RNase-agarose plate, no cells which were susceptible to HL or P_{17} were adsorbed on the plate but the unbound cells manifested strong susceptibility to the MMI activities of HL and P_{17}. These results indicate that all the cell population which is susceptible to P_{17} also seems to have the ability to bind to HL.

3. *MMI studies with PEC from guinea pigs immunized with P_{17}*

To test the immunogenicity of P_{17}, guinea pigs were immunized with 100 μg of P_{17} in complete Freund's adjuvant and then MMI was tested with HL and the peptide derivatives of P_{17}.

Preliminary experiments were performed to see the suitable times for testing MMI activities by various peptide derivative of P_{17}. The results indicated that at least 4 weeks were necessary to attain a certain level of sensitization to P_{17} for delayed hypersensitivity. Table 8 shows results on the MMI activities of HL, P_{17} and its derivative peptides with PEC from guinea pigs immunized with P_{17} 4 weeks previously.

The ranges of the molar ratios of HL and P_{17} derivative peptides to P_{17} inducing 30% MMI were calculated from the results in Table 8 and are listed in Table 9. Tables 8 and 9, show that the MMI responses of individual animals are more divergent than those of PEC taken from guinea pigs immunized with HL. The MMI of P_{17m}, at concentrations of 10^3 to 10^5 ng per ml of cultured medium, was tested on PEC from 9 guinea pigs, but none of the samples showed more than 30% migration inhibition. On the other hand, the susceptibilities of these cells in the MMI tests to P_{17t} and P_{17N} were much higher than that to P_{17i} and P_{17m}. In this respect, the MMI reactivities of PEC from guinea pigs immunized with P_{17} were similar to those of PEC from guinea pigs immunized with HL.

P_{17C}, which was derived from the C-terminal portion of P_{17}, was also tested at concentrations of 10^3 to 10^5 ng per ml of culture medium with PEC from 7 guinea pigs immunized in the same way as in the previous experiment. None of the samples showed more than 30% MMI

TABLE 7. *MMI activities of cell populations treated with various immunoadsorbents*

Species of cells[b]	Mean migration indices (%) at the indicted concentrations[a] of two antigens									
	HL (ng/ml)					P_{17} (ng/ml)				
	10	10^2	10^3	10^4	10^5	10	10^2	10^3	10^4	10^5
HL-bound	75.0 (III)[c]	70.3 (III)	57.8 (III)	53.1 (III)	54.7 (III)	87.5 (I)	76.6 (III)	54.7 (III)	45.3 (III)	35.9 (III)
HL-unbound	111 (I)	107 (I)	88.9 (I)	96.3 (I)	100 (I)	111 (I)	107 (I)	85.2 (I)	94.4 (I)	77.8 (I)
RNase unbound	95.2 (I)	95.2 (I)	57.1 (II)	38.1 (III)	38.1 (III)	85.7 (I)	71.4 (I)	38.1 (III)	38.1 (III)	28.6 (III)

[a] The concentration of antigen is given as ng of antigen per ml of culture medium for MMI.

[b] See text for details of the cell preparations.

[c] Dunnett's multiple comparison test (1955 and 1964) was performed using the migration areas in arbitrary units and the symbols in parentheses are the probabilities that the migration areas with antigens are not different from that of the control. The symbols used were as follows; I: $P>0.05$, II: $0.05>P>0.01$, III: $P<0.01$

TABLE 8. *MMI of PEC from guinea pigs*

Animal number	Mean migration indices (%) at the indicated HL ng/ml			P_{17} ng/ml					P_{17N}	
	10^3	10^4	10^5	10	10^2	10^3	10^4	10^5	10	10^2
201	90.7 (I)[b]	81.5 (I)	51.9 (III)		77.8 (II)	48.1 (III)	51.9 (III)			74.1 (III)
203	75.0 (I)	50.0 (III)	50.0 (III)	93.8 (I)	68.8 (II)	62.5 (III)	37.5 (III)		81.3 (I)	50.0 (III)
204	86.7 (I)	73.3 (II)	53.3 (III)	86.7 (I)	63.3 (III)	56.7 (III)				
205	107 (I)	85.2 (I)	66.7 (II)	104 (I)	100 (I)	96.3 (I)	66.7 (II)	48.1 (III)		98.5 (I)
207	77.8 (III)	77.8 (III)	63.0 (III)		96.3 (I)	77.8 (III)	50.0 (III)	40.7 (III)		88.9 (I)
208	88.1 (I)	85.7 (I)	83.3 (I)	110 (I)	98.1 (I)	81.0 (I)	73.8 (I)	61.9 (III)	114 (I)	110 (I)
209	70.6 (III)	55.9 (III)	50.0 (III)	79.4 (II)	73.5 (III)	61.8 (III)	47.1 (III)	61.8 (III)	85.3 (I)	73.5 (III)
210	91.2 (I)	100 (I)	82.4 (I)	94.1 (I)	82.4 (I)	70.6 (II)	52.9 (III)	64.7 (III)	106 (I)	82.4 (I)

[a] The concentration of antigen is given as ng of antigen per ml of culture medium for MMI.

[b] Dunnett's multiple comparison test (1955 and 1964) was performed using the migration areas in arbitrary different from that of the control. The symbols used are as follows; I: $P>0.05$, II: $0.05>P>0.01$, III:

TABLE 9. *Molar ratios of HL and P_{17} derivatives to P_{17} inducing 30% MMI in PEC of guinea pigs immunized with P_{17} 4 weeks previously*

Animal number	Range of molar ratios inducing 30% MMI[a]			
	HL/P_{17}	P_{17N}/P_{17}	P_{17t}/P_{17}	P_{17i}/P_{17}
201	2.8 -280	0.13- 13	19 -1,900	>230
203	2.8 -280	0.13- 13	0.19 - 19	2.3-2,300
204	2.8 -280	1.3 -130	1.9 - 190	>230
205	0.28- 28	1.3 -130	1.9 - 190	> 23
207	0.28- 28	0.13- 13	1.9 - 190	> 23
208	>0.28	>1.3	0.019- 1.9	> 2.3
209	0.28- 28	1.3 -130	0.19 - 19	2.3- 230
210	>2.8	0.13- 13	>19	> 23

[a] Molar ratios were calculated from the results of Table 8.

at any concentration of P_{17C} tested, and hence the data are omitted from Tables 8 and 9.

Although a high proportion of guinea pigs immunized with P_{17} responded to HL, the PEC of some guinea pigs completely lacked susceptibility to the MMI activity to HL even though they responded to P_{17} (e.g. #210 in Table 6). In addition, with the PEC of some guinea pigs the concentration of HL needed to induce 30% MMI was higher than the corresponding concentration of P_{17}.

Results using PEC from guinea pigs 6 weeks after immunization were essentially similar.

Next experiments were performed to con-

immunized with P_{17} 4 weeks previously

concentrations[a] of various antigens

ng/ml			P_{17t} ng/ml					P_{17i} ng/ml		
10^3	10^4	10^5	10	10^2	10^3	10^4	10^5	10^3	10^4	10^5
59.3 (III)	55.6 (III)	51.9 (III)			75.9 (II)	72.2 (III)	55.6 (III)	88.9 (I)	70.4 (III)	70.4 (III)
56.3 (III)	46.9 (III)	34.4 (III)	81.3 (I)	62.5 (III)	62.5 (III)	50.0 (III)	37.5 (III)	106 (I)	68.8 (II)	50.0 (III)
93.3 (I)	63.3 (III)	66.7 (III)			80.0 (I)	66.7 (III)	60.0 (III)	86.7 (I)	86.7 (I)	83.8 (I)
110 (I)	77.8 (I)	66.7 (II)		104 (I)	88.9 (I)	81.5 (I)	64.8 (II)	111 (I)	107 (I)	92.6 (I)
74.1 (III)	64.8 (III)	59.3 (III)		92.6 (I)	88.9 (I)	74.1 (III)	44.4 (III)		92.6 (I)	77.8 (III)
100 (I)	88.1 (I)	90.5 (I)		92.9 (I)	95.2 (I)	66.7 (II)	61.9 (III)	110 (I)	114 (I)	60.3 (III)
70.6 (III)	52.9 (III)	45.6 (III)		75.3 (III)	66.5 (III)	61.8 (III)	35.3 (III)	70.6 (III)	66.5 (III)	67.6 (III)
88.2 (I)	64.7 (III)	64.7 (III)		79.4 (I)	85.3 (I)	76.5 (I)	76.5 (I)	100 (I)	91.2 (I)	76.5 (I)

units and the symbols in parentheses are the probabilities that the migration areas with antigens are not $P<0.01$

TABLE 10. *Effect of contamination peptide preparations with intact HL in inducing MMI reactions and circulating antibodies against HL*

Antigens for immunization	MMI by HL		Circulating antibody to HL	
	Mean MI±SE[a] (%)	No. of responder[b]	Mean titer±SE[c]	No. of responder[d]
HL (10 ng)	91.7±17.1	0/6	<2	0/6
HL (10^2 ng)	86.8±12.2	0/6	<2	0/6
HL (10^3 ng)	69.6±15.9	3/6	7.2±2.9	6/6
HL (10^3 ng)+P_{17} (10_5 ng)	71.3±10.7	4/6	8.8±0.8	6/6
HL (10^3 ng)+P_{17N} (10^5 ng)	69.5±15.6	1/2	8.0±1.4	2/2

[a] Triplicate capillaries in three separate chambers containing 10^5 ng per ml of HL were prepared from each animal immunized 4 weeks previously with the various antigens. The mean values and standard errors of the mean migration indices for each animals under each condition were calculated.

[b] Number of animals of which PEC showed more than 30% MMI.

[c] $\log_2$ and standard error of the end dilution of serum which gave positive hemagglutination.

[d] Number of animals in which sera showed positive hemagglutination at more than two fold dilution of serum.

firm that the observed cross reactivity of the peptides was not due to contamination of the peptides preparations with undegraded antigen.

All the peptide preparations used were tested at high concentration (5 mg/ml) but no enzymic activity could be detected. Therefore, the degree of contamination of the peptide preparations with HL seemed to be less than

0.01%. Further tests were made to determine the lowest limits of HL required to sensitize guinea pigs with respect of MMI activity and to induce anti-HL antibody. Tests were also made on the effect of peptide on the immunizing ability of a sub-optimal doses of HL. The results are shown in Table 10.

When the amount of HL was less than 10^2 ng, no appreciable susceptibility of PEC to MMI nor any circulating antibody could be detected. On the other hand, when 10^3 ng of HL were used for immunization, some animals were sensitized against HL with respect of MMI activity and all animals showed a positive level of circulating antibody. The co-existence of peptides with intact HL did not seem to influence the appearance of susceptibility to the MMI of HL or the production of anti-HL antibody. Therefore, contamination of peptide preparations with less than 0.1% HL, was unlikely to induce susceptibility to MMI activity in PEC of guinea pigs immunized with 100 μg of peptide. If the level of contamination of peptide preparations with HL were 1%, circulating antibody should consistently be detected by passive hemagglutination. However in fact, when the sera from 8 guinea pigs 4 weeks after immunization with 100 μg of P_{17}, and the sera from 11 guinea pigs 6 weeks after immunization with 100 μg of P_{17}, were titrated by passive hemagglutination with HL-erythrocytes. Their hemagglutination titers ($\log_2$) were all less than 2. Therefore, it is very much unlikely that the reactivities of PEC taken from guinea pigs 4 or 6 weeks after immunization with P_{17} are due to contamination of the P_{17} preparation with undegraded HL.

4. *MMI studies using PEC from guinea pigs immunized with* P_{17N}

P_{17N} shows significant MMI activity on PEC from guinea pigs immunized with either HL or P_{17}. Therefore, we next tested the immunogenicity of P_{17N}, which is derived from the N-terminal portion of P_{17}. Guinea pigs were immunized with 100 μg of P_{17N} in com-

TABLE 11. *MMI of PEC from guinea pigs immunized with* P_{17N}

Animal number	Mean migration indices (%) at the indicated HL ng/ml				P_{17} ng/ml				
	10^2	10^3	10^4	10^5	10	10^2	10^3	10^4	10^5
213		92.3 (I)[b]	79.2 (II)	83.1 (I)			90.8 (I)	80.8 (II)	48.1 (III)
215	100 (I)	64.3 (III)	64.3 (III)	67.9 (III)	85.7 (I)	64.3 (III)	64.3 (III)	64.3 (III)	25.0 (III)
216		75.7 (I)	73.0 (II)	78.4 (I)	86.5 (I)	83.8 (I)	56.8 (III)	59.5 (III)	59.5 (III)
221		104 (I)	109 (I)	80.1 (I)	100 (I)	97.3 (I)	102 (I)	74.3 (II)	38.5 (III)
222		73.8 (III)	66.7 (III)	57.1 (III)		78.6 (II)	64.3 (III)	59.5 (III)	59.5 (III)
224	90.9 (I)	100 (I)	86.4 (I)	72.7 (I)		86.4 (I)	72.7 (I)	63.6 (III)	36.4 (III)
225	100 (I)	100 (I)	85.2 (I)	81.5 (I)		91.1 (I)	70.4 (III)	57.4 (III)	50.0 (III)
228		72.1 (III)	84.1 (I)	55.3 (III)	84.1 (I)	81.7 (I)	86.5 (I)	48.1 (III)	48.1 (III)

[a] The concentration of antigen is given as ng of antigen per ml of culture medium for MMI.

[b] Dunnett's multiple comparison test (1955 and 1964) was performed using the migration areas in arbitrary different from that of the control. The symbols used are as follows; I: $P>0.05$, II: $0.05>P>0.01$, III:

plete Freund's adjuvant and PEC was taken 6 weeks after immunization. The results are shown in Tables 11 and 12.

The responses of individual guinea pigs to P_{17N} varied more than the responses to HL, but in general the MMI responses to HL tended to be weaker, whereas responses to P_{17i} increased.

P_{17m}, derived from the middle portion of P_{17}, was also tested at a concentration of 10^5 ng per ml on 11 guinea pigs immunized with P_{17N} 6 weeks previously. None of them showed any reaction with P_{17m}, and hence the data are omitted from Tables 11 and 12.

P_8 constitutes a completely different portion of the HL molecule and it is reported to be another immunodominant group of HL in delayed hypersensitivity (Miyagawa et al., 1975). Therefore, if there is any cell population of PEC in animals immunized with P_{17N} which is sensitized by HL or if a single clone of lymphocytes responds exclusively to HL and its immunodominant split peptides, these cells should also respond to P_8 in MMI test. However, these possibilities were disproved, by the finding that the PEC of 8 guinea pigs did not show more than 30% MMI with P_8, even at a concentration of 100 μg per ml of antigen. Therefore, these data are also not listed in Tables 11 and 12.

Sera from 11 guinea pigs including the animals listed in Table 11 were also tested for circulating antibody to HL by passive hemagglutination. Again none of them showed a $\log_2$ titer of more than 2. Therefore, the observed cross reaction of PEC from guinea pigs immunized with P_{17N} does not seem to be due to slight contamination of P_{17N} with intact HL.

5. *Comparisons of groups immunized with different antigens*

The reactivities to various antigens in groups of animals immunized with different antigens, were compared by calculating the mean migration areas of the groups and results are listed in Table 13. First, the reactivities to various

(6 weeks previously) by HL and P_{17} derivative peptides

concentrations[a] of various antigens

P_{17N} ng/ml					P_{17t} ng/ml				P_{17i} ng/ml			
10	10^2	10^3	10^4	10^5	10^2	10^3	10^4	10^5	10^2	10^3	10^4	10^5
		96.2 (I)	71.2 (III)	61.5 (III)		88.5 (I)	92.3 (I)	75.0 (II)		92.3 (I)	83.1 (I)	86.9 (I)
92.9 (I)	53.6 (III)	42.9 (III)	46.4 (III)	39.3 (III)		75.0 (III)	64.3 (III)	46.4 (III)		64.3 (III)	50.0 (III)	64.3 (III)
81.1 (I)	75.7 (I)	64.9 (III)	64.9 (III)	70.3 (II)	81.1 (I)	56.8 (III)	54.1 (III)	43.2 (III)		97.3 (I)	75.7 (I)	81.1 (I)
79.6 (I)	81.0 (I)	80.5 (I)	65.9 (III)	48.2 (III)	91.2 (I)	91.2 (I)	86.3 (I)	61.9 (III)		86.7 (I)	91.2 (I)	86.3 (I)
	73.8 (III)	73.8 (III)	52.4 (III)	59.5 (III)	78.6 (II)	71.4 (III)	57.1 (III)	61.9 (III)	95.2 (I)	71.4 (III)	78.6 (II)	
	81.8 (I)	63.6 (III)	45.5 (III)	36.4 (III)	95.5 (I)	100 (I)	81.8 (I)	72.7 (I)		90.9 (I)	90.9 (I)	100 (I)
85.2 (I)	68.5 (III)	63.0 (III)	72.2 (III)	55.6 (III)	96.3 (I)	77.8 (II)	80.0 (I)	74.1 (I)		87.4 (I)	92.6 (I)	66.7 (III)
91.3 (I)	86.5 (I)	81.7 (I)	64.9 (III)	48.1 (III)	96.2 (I)	101 (I)	91.3 (I)	67.3 (III)		76.9 (II)	67.3 (III)	62.5 (III)

units and the symbols in parentheses are the probabilities that the migration areas with antigens are not $P<0.01$.

test antigens within a group were compared. In groups immunized with HL or P_{17} the MMI activities of P_{17}, P_{17N} and P_{17t} were similar but the MMI activity of P_{17i} was clearly weaker. However, the MMI activity of P_{17i} was similar to those of other peptides using PEC from animals immunized with P_{17N}. Second the MMI activities of each peptide were compared in groups of guinea pigs, which had been immunized with HL, P_{17} and P_{17N} respectively. The cells of animals immunized with P_{17} or P_{17N} in general reacted more to P_{17} itself or its derivative peptides than to HL, in contrast to cells of animals immunized with HL. In the former the increase in reactivity to P_{17i} was especially remarkable. These results show that the immunogenicities of P_{17} and P_{17N} are clearly different from that of HL. On the other hand, a fair degree of cross reaction of PEC from guinea pigs immunized by P_{17} or P_{17N} with HL was observed.

6. *Skin reactions of guinea pigs immunized with HL or its derivative peptides*

First a series of experiments was carried out on the skin reactions to P_{17} and its derivative peptides in guinea pigs immunized wit 100 μg of HL. Various antigens in 0.05 ml of saline were injected into animals subcutaneously 3 weeks after immunization. The sites of injection were observed 4 and 24 hr after the subcutaneous injection and the results are shown

TABLE 12. *Molar ratios of HL and P_{17} derivatives to P_{17} inducing 30% MMI in PEC of guinea pigs immunized with P_{17N} 6 weeks previously*

Animal number	Range of molar ratios inducing 30% MMI[a]			
	HL/P_{17}	P_{17N}/P_{17}	P_{17t}/P_{17}	P_{17i}/P_{17}
213	[b]>0.28	0.13 -13	>1.9	>2.3
215	0.28-28	0.13 -13	19-1,900	2.3 -230
216	>28	0.13 -13	0.19- 19	>230
221	>0.28	0.013 -1.3	0.19- 19	>2.3
222	0.28-28	1.3 -130	1.9 - 190	>230
224	>2.8	0.013 -1.3	>19	> 23
225	>2.8	0.0013-0.13	>19	2.3 -230
228	0.28-28	0.13 -13	1.9 - 190	0.23- 23

[a] Molar ratios were calculated from the results of Table 11.

[b] Where the symbol>is used more than 30% MMI could not be attained within the antigen concentration used.

TABLE 14. *Skin reactions of guinea pigs 3 weeks*

Dose of test antigen[b]	Mean diameters (mm) and standard errors of skin reactions[a]					
	HL		P_{17}		P_{17N}	
	4 hr	24 hr	4 hr	24 hr	4 hr	24 hr
0.5 μg	1.8±2.3	6.5±1.4	0[c]	2.4±0.6	0	0
5.0	5.7±4.4	12.0±2.5	0	4.9±1.1	0	0.9±1.3
50.0	9.8±3.2	22.5±3.9	0	8.7±1.3	0	5.8±1.6

[a] Mean diameters and standard errors were calculated from results on 10 guinea pigs.

[b] A given amount of antigen in 0.05 ml of saline was injected subacutaneously.

[c] A skin reaction of less than 2 mm diameter was recorded as 0.

in Table 14.

As can be seen, only HL showed a positive, immediate type reascion whereas the other peptides did not cause any reaction within 4 hr. On the other hand, not only HL but also P_{17}, P_{17N} and P_{17t} gave positive delayed hypersensitivity reactions.

The high reactivity of HL is understandable, because it must contain more determinants than the peptides. The reactivity of P_{17N} was rather similar to that of P_{17}, but the reactivity of P_{17t} was less.

Six normal guinea pigs were tested with HL and the derivative peptides of P_{17} in the same way as animals immunized with HL, but the diameters of the reaction sites after injections of the various antigens were in all cases less than 2 mm.

A second group of guinea pigs was immunized with 100 μg of P_{17} in complete Freund's

TABLE 13. *Difference between MMI activities of various antigens within groups of animals immunized with HL, P_{17} and P_{17N}*

Immunized with	Mean migration areas±SE[a] in the presence of various antigens				
	P_{17}	HL	P_{17N}	P_{17t}	P_{17i}
HL	9.97±3.19	8.41±3.56 (I)[b]		11.4±3.38 (I)[b]	15.3±4.07 (II)[b]
HL	9.41±3.64	7.69±3.08 (I)[b]	9.30±3.97 (I)[b]		
P_{17}	6.28±1.98 (II)[c]	8.38±2.69 (I)[c] (I)[b]	7.38±2.28 (I)[c] (I)[b]	7.97±2.51 (I)[c] (I)[b]	9.94±2.96 (II)[c] (II)[b]
P_{17N}	6.43±2.16 (II)[c]	8.23±2.77 (I)[c] (I)[b]	6.31±2.41 (I)[c] (I)[b]	8.09±3.34 (I)[c] (I)[b]	8.17±2.66 (II)[c] (I)[b]

[a] The means and standard errors were calculated from the mean migration areas (weights of cut paper) of 8 animals at antigen concentrations of 10^4 ng per ml. The values used for calculation were taken from the results in Tables 2, 3, 8 and 11.

[b] The multiple comparison test (Dunnett, 1955 and 1964) was performed taking the response by P_{17} as a control, the symbols in parentheses are the probabilities that the migration areas with various antigens are not different from that with P_{17}. The symbols used are as follows; I: $P>0.05$, II: $0.05>P>0.01$, III: $P<0.01$.

[c] The multiple comparison test was performed taking the response of animals immunized with HL as a control. The symbols in parentheses are the probabilities that the migration areas of PEC from animals immunized with the various antigens are not different from that of animals immunized with HL. The symbols used are the same as in b.

after immunization with 100 μg of HL

with various antigens at the indicated times after injections					
P_{17t}		P_{17i}		P_{17m}	
4 hr	24 hr	4 hr	24 hr	4 hr	24 hr
0	0	0	0	0	0
0	1.6±1.7	0	0	0	0
0	4.5±1.9	0	1.3±1.0	0	0

adjuvant and the results of skin reactions are listed in Table 15.

Again, P_{17}, P_{17N} and P_{17t} showed definitely positive delayed hypersensitivity reactions. The reactivity of P_{17t} was rather similar to that of P_{17}, whereas the reactivity of P_{17N} is weaker. In addition, HL showed a fair degree of cross reaction.

P_8, which is derived from a different portion of the HL molecule from P_{17}, gave no reaction. This fact is also evidence that the observed skin reactions are not due to contamination of the peptide preparations used for immunization with intact HL and that different clones of lymphocytes respond to respective immunodominant groups. None of the antigens injected except HL gave any skin reaction within 4 hr after their subcutaneous injections.

A third group of guinea pigs was immunized with 100 μg of P_{17N} in complete Freund's adjuvant and skin reactions were measured 6 weeks later. The results are shown in Table 16. The same peptides as in the previous experiment gave a positive delayed hypersensitivity reactions. The reactivity of P_{17N} was similar to that of P_{17} whereas that of P_{17t} was weaker. HL gave a positive delayed hypersensitivity reaction, but the reactions of individual animals to HL varied greatly. Two guinea pigs gave strong positive reactions to P_{17N} but scarcely any reaction to HL whereas in 8 guinea pigs the reactivities to the two antigens were similar. Again P_{17i}, P_{17m} and P_8 gave no reaction.

DISCUSSION

The antigenic determinants for circulating antibody are often different from those for cellular immunity or for delayed hypersensitivity. An example of a natural antigen which showed these characters is bovine glucagon as reported by Senyk et al. (1971a, b).

In the present study, the terminal half of

TABLE 15. *Skin reactions of guinea pigs 4 weeks after immunization by 100 μg of P_{17}*

Dose of test antigen[b]	Mean diameters (mm) and standard errors of skin reactions[a] by various antigens at 24 hr after injection						
	HL	P_{17}	P_{17N}	P_{17t}	P_{17i}	P_{17m}	P_8
0.5 μg	1.2±1.1	1.8±1.7	0.3±0.7	0.3±0.7	0[c]	0	0
5.0	2.9±1.7	5.3±1.6	0.8±1.3	3.1±1.8	0	0	0
50.0	6.9±2.4	8.9±1.5	4.5±1.2	6.1±1.4	1.0±1.2	0.6±0.9	0

[a] Seven guinea pigs were used.
[b] The amounts of antigen indicated in 0.05 ml of saline were injected intracutaneously.
[c] Skin reactions of less than 2 mm diameter were recorded as 0.

TABLE 16. *Skin reactions of guinea pigs 6 weeks after immunization with 100 μg of P_{17N}*

Dose of test antigen[b]	Mean diameters (mm) and standard errors of skin[a] reactions by various antigens at 24 hr after injection						
	HL	P_{17}	P_{17N}	P_{17t}	P_{17i}	P_{17m}	P_8
0.5 μg	1.2±1.3	0.6±0.9	0.4±0.8	0.5±1.0	0[c]	0	0
5.0	2.1±1.7	2.5±1.9	1.8±1.5	1.2±1.3	0.6±0.9	0	0
50.0	3.8±3.1	6.8±1.8	6.4±2.0	3.6±2.1	1.1±1.3	0	0

[a] Ten guinea pigs were used.
[b] The indicated amounts of antigen in 0.05 ml of saline were injected intracutaneously.
[c] Skin reactions of less than 2 mm diameter were recorded as 0.

P_{17} (P_{17t}; sequence 1-[Cys·6]-Homoser·12, 123-[Cys·127]-129) was reactive with both circulating antibody to HL and with cells sensitized with HL in delayed hypersensitivity. But when P_{17} was reduced and alkylated, the resultant N-terminal peptide (P_{17N}) had lost its reactivity to circulating antibody to HL (Ha et al., 1975) but still retained its ability to induce delayed hypersensitivity in cells sensitized with HL. These results indicate that the sequence 1-[Cys–6]-12 of HL is the immunodominant group in delayed hypersensitivity.

On the other hand, when the peptides were used as immunogen, cells sensitized to these peptides still retained a fair degree of cross reactivity with undegraded antigen (HL). A problem in this kind of experiment is to ensure that very slight contamination of the peptide preparation with undegraded antigen did not induce delayed hypersensitivity, because the reaction has extraordinarly high sensitivity. Therefore, contamination of the peptide preparations with HL was especially carefully checked, and this possibility was ruled out. On the other hand, Komatsu et al. (1975) reported that when rabbits are immunized with P_{17} they produce antibody which reacts with P_{17} but which shows almost no cross reactivity with HL. In this respect, the specificity of the delayed hypersensitivity seems to be different from that of the circulating antibody.

When peptides were used as immunogens to induce delayed hypersensitivity the maximal reactive state seemed to be reached at a different time from that using HL. Therefore, the times of maximal reactivity with the different peptide antigens were determined in preliminary experiments and delayed hypersensitivity reactions were measured at the time of maximal reactivity.

As described in the introduction of this paper, results using the hapten-carrier system indicate that the specificity of delayed hypersensitivity is in general more strict than that of circulating antibody. Thus it seems unlikely that reduced and alkylated P_{17} retains the ability to induce delayed hypersensitivity in animals sensitized with HL. But it seems possible that as in the case of the p-azobenzenearsanilate system a relatively small portion of immunizing antigen can act as a determinant for delayed hypersensitivity and the antigenic determinant responsible for delayed hypersensitivity found in peptide may be similar to that of the p-azobenzenearsanilate system (Leskowitz, 1963 and 1971).

It was not possible to measure the reactivity to HL of cell populations which only reacted to the P_{17} determinant involved in HL-sensitized cells. Thus it is possible that among the cells sensitized to HL some cells only reacted with the conformational determinant of HL.

Actually the reactivities of cells which had been sensitized to intact HL, P_{17} or P_{17N} differed somewhat in their susceptibilities to the MMI activity of P_{17}. That is, the susceptibilities to P_{17} of cell populations sensitized with the peptides were greater than those of cells sensitized with intact HL. Moreover, the susceptibility of cells immunized with P_{17} or P_{17N} to the P_{17i} portion of P_{17} was also greater than that of cells immunized with intact HL.

Although Ichiki and Parish (1971) and Parish (1971) suggested that degraded antigens might actually act as determinants in delayed hypersensitivity, there is another possible explanations for the augmented sensitizing ability of degraded antigens. That is, when the intact antigen is degraded a suitable sized peptide, the numbers of determinant groups involved in the delayed hypersensitivity could be greater than that on immunization with intact antigens. Nevertheless our finding that the degraded peptides can still induce cell populations which can cross react with the undegraded antigen in delayed hypersensitivity are completely consistent with that of Ichiki and Parish (1971).

The results of MMI reactions were roughly, but not completely paralled with those of the skin reaction. One reason for this may be that the rate of elimination of antigen from the injection site in the skin reaction may influence

to result in addition to the reactivity of the cells to peptide antigens.

The correlation of MMI with delayed hypersensitivity but not with antibody production seems to be well established (Bloom and Benett, 1966; George and Vaughan 1962; David et al., 1964). Delayed hypersensitivity reactions have generally been assumed to be mediated by thymus dependent lymphocytes, but recently David et al. (1975) reported that the cells responsible for MMI are T-lymphocytes and some subpopulation of B-lymphocytes in humans. However, Yoshida et al. (1973) reported that B-lymphocytes of guinea pigs can also produce migration inhibition factor (MIF) when purified protein derivatives are used as antigens, but that they do not produce MIF against protein antigen or hapten-protein conjugates. In our system, the lymphocytes eluted from the HL-immunoplate with HL solution showed MMI when mixed with HL again. According to Rocklin et al. (1974) the lymphocyte propulation which still shows MMI after preliminary treatment with antigen seems to represent B-lymphocytes. Therefore, the lymphocyte population responding to MMI activity in our system need to be clarified as T- or B-lymphocytes. Experiments on this are now in progress.

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