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Inhibition, No-effect or Enhancement of Immune Responses following Injection of Mixtures of Immunogenic and Non-Immunogenic Synthetic Polypeptides

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Summary. The phenomenon of antigenic competition has been investigated by the use of synthetic compounds and guinea-pigs of inbred strain 2 as experimental animals. The effect of addition to the immunizing mixture of immunogenic and non-immunogenic synthetic compounds has been studied.

Antigenic competition has been demonstrated between the synthetic antigen 252,p(Tyr,Glu,Lys) and DNP-p(Lys) or 28,pDLAla-p(Tyr,Glu)--pLys.

The non-immunogenic compounds 251,p(DTyr,DGlu,DLys), 33,pTyr-pDLAla--pLys, and p(Tyr) have been found respectively to inhibit, enhance, or not affect the immune response to 252,p(Tyr,Glu,Lys). Two weak antigens, namely 509,p(Tyr, Glu,)-pDLAla--pLys and p(Lys) also enhanced the response to 252,p(Tyr,Glu, Lys). Possible explanations for these findings are discussed.

INTRODUCTION

The phenomenon of antigenic competition was first described by Glenny and Waddington (1926) and studied in more detail in recent years (for review, see Adler, 1964); it involves inhibition of the immune response to an antigen by previous or simultaneous injection of another unrelated antigen. To classify this competition as an immunological phenomenon, it is necessary to relate it to a certain stage of the immune response. An intimate knowledge of the process of antigenic competition may thus be useful in the understanding of the immune response in general.

The study of antigenic competition will be greatly advanced if the composition of the immune determinants involved is well known and will thus ensure work with completely unrelated antigens. The use of synthetic antigens of known chemical composition and optical configuration should meet this requirement. Such synthetic antigens have been described recently by various workers (for reviews, see Maurer, 1964; Sela, 1966).

The present report extends previous work (Ben-Efraim and Liacopoulos, 1966) on antigenic competition between synthetic antigens and deals also with the effect of non-immunogenic synthetic polypeptides on the immune response towards synthetic antigens.

MATERIALS AND METHODS

Animals

Guinea-pigs weighing 250–400 g of the highly inbred strain 2 (Sewall Wright) were used, because 100 per cent of the animals responded to the synthetic antigens employed.

All the animals were from the breeding colony kept at the National Institutes of Health, Bethesda, Maryland.

Antigens

The substances used are listed in Table 1. The convention for the nomenclature of the polymers has been given previously (Sela, Fuchs and Arnon, 1962).

TABLE 1

SYNTHETIC COMPOUNDS USED FOR IMMUNIZATION*

No. and designation of sample	Molar ratio of amino acid residues				Average molecular weight
	Lys	Tyr	Glu	DL-Ala	
Conjugate					
DNP-p(Lys)†	1·0	–	–	–	
Linear					
p(Lys)$\overline{1250}$‡	1·0	–	–	–	
252,p(Tyr,Glu,Lys)	6·0	1·0	10·4	–	61,000
251,p(DTyr,DGlu,DLys)	6·0	1·0	10·4	–	44,000
p(Tyr)	–	1·0	–	–	10,000
Multichain					
509,p(Tyr,Glu)-pDL-Ala--pLys	1·0	2·1	4·1	19·6	232,000
28, pDL-Ala-p(Tyr,Glu)--pLys	1·0	1·2	2·0	15·7	
33,p(TyrDL-Ala)--pLys	1·0	1·7	–	24·7	39,800

* No optical configuration implies L-amino acids.
† Approximately 3 molecules DNP/1 mole p(Lys).
‡ Average degree of polymerization.

Two antigens described previously (Ben-Efraim and Liacopoulos, 1966) as inducing a response in 100 per cent of the animals of inbred strain 2 were used for immunization: poly-L lysine conjugated with dinitrophenyl (DNP-p(Lys) in a ratio of approximately 3 molecules DNP/1 mole lysine and a linear copolymer, 252,pTyr,Glu,Lys) containing tyrosine, glutamic acid and lysine as characterized by Borek, Stupp, Fuchs and Sela (1965).

The polymers tested for their effect on the immune response to 252,p(Tyr,Glu,Lys) and DNP-p(Lys) included: the multichain copolymers 28,pDLAla-p(Tyr,Glu)--pLys and 33,pTyr-pDLAla--pLys as described by Sela *et al.* (1962), the multichain copolymer 509,p(Tyr,Glu)-pDLAla--pLys as described by McDevitt and Sela (1965); the D polymer 251,p(DTyr,DGlu,DLys) as characterized by Borek *et al.* (1965). The poly-L-lysine preparation (Lys)$\overline{1250}$ was derived from a high molecular weight poly-ε, N-benziloxycarbonylamine-L-lysine prepared with triethylamine as the initiator (Schlossman, Ben-Efraim, Yaron and Sober, 1966). The poly-L-tyrosine, p(Tyr) preparation of an average molecular weight of 10,000 was obtained from Yeda, Rehovoth, Israel.

Immunization

Antigen solutions in 0·15 M NaCl–0·15 M phosphate (buffered saline), pH 7·2, were emulsified with an equal volume of Freund's complete adjuvant containing 10 mg/ml of killed *Mycobacterium tuberculosis* H37 Rv. A total of 0·4 ml of this emulsion was injected into the two hind footpads. The polymers tested for their effect on the immune response were included in the immunizing mixture as described previously for protein antigens (Neveu, 1964).

In some cases one or two more injections were given, in two places behind the neck, 3 weeks after the first one, in complete adjuvant and 6 weeks after the first one, in incomplete adjuvant.

Collection of blood samples

The animals were bled before immunization, and 10 days after the first injection, before the intradermal tests. Additional bleedings were performed in animals receiving more than one immunizing injection at days 42 and 70 after the beginning of immunization.

Intradermal tests

Skin tests were performed with 10 μg in 0·1 ml of DNP-p(Lys) and 50 μg in 0·1 ml of various copolymers, 10 days after the first immunizing injection. Animals receiving more than one immunizing injection were again tested at days 20 and 70 after the beginning of immunization.

Reactions were read at 3, 6, 24 and 48 hours. The reactions observed were of delayed type only, appeared at 24 hours and remained still strong at 48 hours after injection. The diameters of the skin reactions (erythema) were recorded.

The significance of skin reactions was ascertained by injecting intradermally the various substances in guinea-pigs of strain 2 sensitized with an emulsion of buffered saline in Freund's complete adjuvant. These controls were invariably negative with all substances tested with the exception of p(Lys) when a faint reaction of about 5 mm in diameter was observed.

Passive cutaneous anaphylaxis reactions (PCA)

The PCA test was performed in Hartley guinea-pigs as described previously (Ben-Efraim, Fuchs and Sela, 1964). Three intradermal injections were made with: non-diluted sera, 1:10 and 1:20 dilutions. Eighteen hours later, the animals were challenged with intracardiac injections of 250 μg of substance and 1 per cent solution of Evans blue in 0·15 M sodium chloride. Normal sera obtained from guinea-pigs before immunization were used as controls and found invariably negative. In the case of positive PCA reactions all three concentrations of sera tested were positive, i.e. more than 10 mm in diameter.

RESULTS

INTERACTION BETWEEN DNP-p(Lys) AND 252,p(Tyr,Glu,Lys) ANTIGENS

The results of DNP-p(Lys) and 252,p(Tyr,Glu,Lys) immunizations are presented in Table 2. The minimum immunizing dose found to induce a delayed response in 100 per cent of guinea-pigs of inbred strain 2 was 10 μg for both antigens. Some guinea-pigs responded also to 1 μg of DNP-p(Lys).

Addition of 600 μg DNP-p(Lys) per guinea-pig to the immunizing mixture containing a dose of 10 μg 252,p(Tyr,Glu,Lys) inhibited almost completely the reaction to the copolymer. On the other hand, only partial inhibition of the immune response to 10 μg DNP-p(Lys) was observed, when a dose of 600 μg 252,p(Tyr,Glu,Lys) was added to the immunizing mixture. It seems also that the delayed reaction induced by injection of 600 μg 252,p(Tyr,Glu,Lys) was reduced if a dose of 10 μg DNP-p(Lys) was injected simultaneously.

TABLE 2

INTERACTION BETWEEN DNP-p(Lys) AND 252p(Tyr,Glu,Lys) ANTIGENS IN GUINEA-PIGS OF INBRED STRAIN 2

Group No.	μg DNP-p(Lys) per guinea-pig	μg 252,p(Tyr, Glu,Lys) per guinea-pig	Delayed cutaneous reactions			PCA
			Product injected*	No. of positive animals	Mean diameter (mm)	
1	600		DNP-p(Lys)	5/5		
2	100		DNP-p(Lys)	5/5	16·6	
3	10		DNP-p(Lys)	5/5	11·3	0/5
4	1		DNP-p(Lys)	8/10	4·0	
5		100	p(Tyr,Glu,Lys)	11/11	14·5	5/11
6		10	p(Tyr,Glu,Lys)	10/10	9·0	0/5
7		1	p(Tyr,Glu,Lys)	0/5	0·0	
8	600	10	p(Tyr,Glu,Lys)	1/5	1·4	
			DNP-p(Lys)	5/5	13·9	
9	10	600	p(Tyr,Glu,Lys)	4/5	10·0	
			DNP-p(Lys)	4/5	4·4	

* DNP-p(Lys): 10 μg/site; 252,p(Tyr,Glu,Lys): 50 μg/site.

EFFECT OF VARIOUS POLYMERS ON THE IMMUNE RESPONSE TO DNP-p(Lys) AND 252,p(Tyr, Glu,Lys)

A number of polymers were tested for their effect on immunization with DNP-p(Lys) or with 252,p(Tyr,Glu,Lys). The immune responses to these polymers when injected alone are shown in Table 3.

TABLE 3

IMMUNE RESPONSE IN GUINEA-PIGS OF INBRED STRAIN 2 TO SUBSTANCES ADDED TO DNP--p(Lys) OR 252,p(Tyr,Glu,Lys) ANTIGENS

Group No.	Immunizing substance	Total amount injected (μg)	No. of immunizing injections	Delayed cutaneous reactions		PCA
				No. of positive animals*	Mean diameter (mm)	
10	509,p(Tyr,Glu)-pDLAla--pLys	100	1	0/5	0	
		260	2	0/4	0	0/5
11	509,p(Tyr,Glu)-pDLAla--pLys	600	1	0/5	0	0/5
		1260	2	0/5	0	5/5
12	28,pDLAla-p(Tyr,Glu)--pLys	100	1	0/5	0	
		260	2	0/5	0	0/5
13	28,pDLAla-p(Tyr,Glu)--pLys	600	1	10/10	12·0	2/10
		1260	2			5/5
14	33,pTyr-pDLAla--pLys	600	1	0/5	0	0/5
		1260	2	0/5	0	0/5
		1920	3	0/5	0	0/5
15	251,p(DTyr,DGlu,DLys)	600	1	0/10	0	0/10
		760	2	0/10	0	0/10
		920	3	0/5	0	0/5
16	$(Lys)\overline{1250}$	600	1	5/5	6·5 weak	0/5
		710	2	5/5	7·0 weak	0/5
17	p(Tyr)	600	1	ND		0/5
		1210	2	ND		0/5
		1320	3	ND		0/5
18	Freund's complete adjuvant	4000	2	0/5†		

ND, Not done.

* (Lys): 10 μg/skin site; other polymers: 50 μg/skin site.

† No skin reactions detected with the various substances.

The multichain copolymer 509,p(Tyr,Glu)-pDLAla--pLys did not induce an immune response if two doses of 100 μg each, or if only one large dose of 600 μg were injected. However, if two doses of 600 μg each were injected, an immune response was observed which was detectable only in PCA tests.

A positive response in strain 2 was also obtained with a large dose of the multichain copolymer 28,pDLAla-p(Tyr,Glu)--pLys.

Injection of $(Lys)_{\overline{1250}}$ induced a weak delayed reaction of 6–7 mm which was only slightly higher as compared with the skin reactions observed in normal control animals (approximately 5 mm). No immune response was detected after immunization with 33,pTyr-pDLAla--pLys, 251,p(DTyr,DGlu,DLys) and p(Tyr). The check of immune response to p(Tyr) was done in PCA tests only because alkaline solutions of p(Tyr) gave non specific skin reactions in control guinea-pigs.

The results obtained with immunizing mixtures containing one of these polymers and DNP-p(Lys) or 252,p(Tyr,Glu,Lys) antigens are presented in Table 4.

TABLE 4

EFFECT OF VARIOUS POLYMERS ON THE IMMUNE RESPONSE TOWARDS DNP--p(Lys) AND 252,p(Tyr,Glu,Lys)* IN GUINEA-PIGS IN INBRED STRAIN 2

Group No.	μg antigen per animal	μg of polymer per animal		Delayed cutaneous reactions			PCA
		Polymer	μg	Product injected†	No of positive animals	Mean diameter (mm)	
DNP-p(Lys) antigen							
19	10	251,p(DTyr,DGlu,DLys)	600	DNP-p(Lys)	5/5	11·4	
				251	0/5	0·0	
20	1	509,p(Tyr,Glu)-pDLAla--pLys	600	DNP-p(Lys)	3/5	3·8	0/5
				509	0/5	0	0/5
252,p(Tyr,Glu,Lys) antigen							
21	100	251,p(DTyr,DGlu,DLys)	600	252	5/5	10·4	
				251	0/5	0	
22	10	251,p(DTyr,DGlu,DLys)	600	252	0/5	0	0/5
				251	0/5	0	
23	10	28,pDLAla-p(Tyr,Glu)--pLys	600	252	4/5	5·1 weak	1/5
				28	0/5	0	
24	10	p(Tyr)	600	252	5/5	9·5	1/5
25	10	$(Lys)_{\overline{1250}}$	600	252	4/5	7·0	5/5
				$(Lys)_{\overline{1250}}$	5/5	6·2	
26	10	33,pTyr-pDLAla--pLys	600	252	4/4	14·2	5/5
				33	0/4	0	0/4
27	10	509,p(Tyr,Glu)-pDLAla--pLys	600	252	5/5	14·8	5/5
				509	0/5	0	1/5
28	1	509,p(Tyr,Glu)-pDLAla--pLys	600	252	0/5	0	0/5
				509	0/5	0	0/4

* See Table 2 for results in groups injected with DNP--p(Lys) or 252,p(Tyr,Glu,Lys) only.
† DNP--p(Lys): 10 μg/skin site; other products: 50 μg/skin site.

Injection of 600 μg 251,p(DTyr,DGlu,DLys) simultaneously with 252,p(Tyr,Glu,Lys) inhibited completely the immune reaction to 10 μg 252 copolymer and reduced the skin reaction to 100 μg of the same copolymer. On the other hand, 600 μg of the D copolymer had no effect on the immune response to 10 μg DNP-p(Lys).

A mutual inhibitory effect was observed with the mixture containing 600 μg 28,DLAla-p(Tyr,Glu)--pLys and 10 μg of 252,p(Tyr,Glu,Lys). The response to 252,p(Tyr,Glu,Lys) was markedly inhibited, while the response to 28,pDLAla-p(Tyr,Glu)--pLys was completely abolished.

Addition of doses of 600 μg 509,p(Tyr,Glu)-pDLAla--pLys or 33,pTyr-pDLAla--pLys enhanced the response to 10 μg 252,p(Tyr,Glu,Lys) as expressed by marked increase in the diameter of delayed skin reactions and positive PCA tests.

The same dose of 600 μg 509 copolymer had no effect on the immune response to 1 μg 252,p(Tyr,Glu,Lys) or 1 μg DNP-p(Lys).

Some enhancing effect on immunization with 10 μg of 252,p(Tyr,Glu,Lys) was observed with 600 μg p(Lys) as expressed in positive PCA reactions with antisera from all the animals tested. Addition of a dose of 600 μg p(Tyr) had no effect on immunization with 10 μg or 252,p(Tyr,Glu,Lys) copolymer.

DISCUSSION

Our results, which regularly show an inhibition of immune response towards one antigen when two synthetic antigens are injected simultaneously, confirm previous work with protein antigens and suggest that the phenomenon of antigenic competition may well have a general significance.

Moreover, the use of fully immunogenic and non-immunogenic synthetic compounds as competitors gives an insight into the mechanism of this phenomenon.

The results obtained in guinea-pigs of inbred strain 2 with mixtures of two fully immunogenic substances injected simultaneously, DNP-p(Lys) and 252,p(Tyr,Glu,Lys), show inhibition of delayed response towards the antigen injected in a single minimum immunizing dose by a large excess of the other (Table 2). The inhibitory effect of DNP-p(Lys) seems more marked than the inhibitory effect of 252,p(Tyr,Glu,Lys).

It has been shown that competition of antigens has a quantitative as well as a qualitative basis (Amkraut, Garvey and Campbell, 1964; Schechter, 1965; Stiffel, Ben-Efraim, Perramant and Liacopoulos, 1966). Similarly, in the present work, DNP-p(Lys) which proved to be more antigenic than 252,p(Tyr,Glu,Lys) on the basis of a better delayed response to the same immunizing dose and a lower minimal immunizing dose (Table 2, groups 3, 4 and 6) was also more competitive than the latter. It appears therefore that degree of immunogenicity is also important in competition of antigens. An additional argument for the importance of antigenic strength in competition experiments is the finding that 28,pDLAla-p(Tyr,Glu)--pLys which is a weak antigen (Table 3) only partially inhibits reactivity to 10 μg of 252,p(Tyr,Glu,Lys) polymer. However, the presence in the immunizing mixture of 10 μg of the 252,p(Tyr,Glu,Lys) polymer is sufficient to completely inhibit immunization by 600 μg of the 28,pDLAla-p(Tyr,Glu)--pLys polymer (Table 4, group 23).

Of special interest are the results obtained by immunizing mixtures containing 251,p (DTyr,DGlu,DLys) (Table 4, groups 19, 21, 22). This compound repeatedly tested alone (Table 3, group 15) was completely non-immunogenic, but when mixed with a dose of 10 μg of 252,p(Tyr,Glu,Lys) copolymer (Table 4, group 22) totally inhibited the sensitization towards this antigen.

It is already known (Sage, Deutsch, Fasman and Levine, 1964) that immunologically competent cells are able to recognize D haptens as immune determinants. The present finding that 251,p(DTyr,DGlu,DLys) polymer efficiently competes with the homologous 252 L polymer would indicate that immunologically competent cells recognize both polymers as antigens.

The D polymer may thus be able, in contrast to the L polymer, to act as a sort of 'abortive

antigen' and induce only the first stage of immune response characterized by its recognition as an immune determinant. This assumption would imply that the competition of antigen takes place during the phase of antigenic recognition by the competent cells.

It is of interest to mention that according to Schechter and Sela (1965), the D-amino acid residue acted as a stronger immune determinant than the L-amino acid residue upon injection of rabbits with a poly-DL-alanyl protein conjugate. In our hands, the 251,p (DTyr,DGlu,DLys) polymer acted as a 'weak antigen'. Partial inhibition was still observed when the dose of the D polymer was only 6 times higher than that of the 252,p(Tyr,Glu,Lys) polymer (Table 4, group 4) and no inhibition was detected towards the stronger antigen DNP-p(Lys) (Table 4, group 19).

Some support of the assumption that the 251,p(DTyr,DGlu,DLys) acts as an 'abortive antigen' comes from results obtained with the other non-immunogenic compounds. Among them, poly-L-tyrosine (Table 3, group 17) failed to compete with the 252,p(Tyr,Glu,Lys) copolymer (Table 4, group 24). On the other hand the non-immunogenic copolymer 33,p-Tyr-DLAla--pLys (Table 3, group 14), enhanced the delayed response as well as antibody production towards the p(Tyr,Glu,Lys) compound (Table 4, group 26).

The question arises why the 33,pTyr-DLAla--pLys copolymer enhances the immune response to 252,p(Tyr,Glu,Lys) antigen. Among various possibilities, two seem to us worthy to be taken into consideration: (a) the non-immunogenic compound 33,p(Tyr-DLAla--pLys) contains components in common to the part of 252,p(Tyr,Glu,Lys) antigen which determines its antigenic specificity or its immunogenic capacity. Addition of such components will increase the stimulation of competent cells; and (b) non-immunogenic compounds may act as adjuvants synergistic to Freund's complete adjuvant.

The enhancement of the immune response to 252,p(Tyr,Glu,Lys) copolymer observed with the 509,p(Tyr,Glu)-pDLAla--pLys copolymer (Table 4, group 27) and to a less extent with $(Lys)_{\overline{1250}}$ (Table 4, group 25) may be also explained either by addition of components present in the immune determinant of 252,p(Tyr,Glu,Lys) antigen or by adjuvant action. The 509,p(Tyr, Glu)-pDLAla--pLys copolymer did not induce a detectable immune response under the same conditions employed for the immunizing mixture containing 252,p(Tyr,Glu,Lys). It did, however, induce formation of antibodies after one more immunizing injection. The $(Lys)_{\overline{1250}}$ induced a weak delayed response not followed in our case by production of antibodies. The antigenicity of p(Lys) has been also reported recently by Green, Paul and Benacerraf (1966).

It was possible therefore to prove antigenic competition between fully immunogenic synthetic compounds. However, when an immunogenic compound is mixed with a non-immunogenic one, either inhibition, enhancement or no effect could be observed. Experiments are under way in order to explain these various effects.

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