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The Competitive Effect of DNP–Poly-L-Lysine in Responder and Non-Responder Guinea-Pigs

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Summary. The synthetic antigen DNP–p(Lys), which it is known to induce an immune response only in a certain percentage of randomly bred guinea-pigs, inhibited the immune response towards p(Tyr, Glu, Ala) and p(Tyr, Glu) synthetic antigens in all the guinea-pigs tested. The extent of the competitive effect was related to the quantity of DNP–p(Lys) injected.

The hapten moiety DNP–mono-lysine, had no inhibitory effect, while the p(Lys) carrier interfered with the response to p(Tyr, Glu), although to a lesser extent than did the complete conjugate.

INTRODUCTION

It has been shown (Ben-Efraim and Liacopoulos, 1967) than an immunogenic conjugate dinitrophenyl–poly-L-lysine (DNP–p(Lys)) can interfere and regularly inhibit the immune response to another synthetic antigen. These experiments were performed in guinea-pigs of inbred strain 2, in which all individuals are known to respond to DNP–p(Lys) (Levine, Ojeda and Benacerraf, 1963a). In the case of randomly bred guinea-pigs, it was shown by Kantor, Ojeda and Benacerraf (1963), that this population includes both responder and non-responder animals to DNP–p(Lys). It was concluded by Levine *et al.* (1963a), that for genetic reasons non-responder guinea-pigs fail to recognize the DNP–p(Lys) as an immunogen.

For understanding antigenic competition it is important to know whether such interference is confined to immunogenic substances. The occurrence within a random bred population of guinea-pigs of responders and non-responders to DNP–p(Lys) may provide a useful tool for investigating this problem. We have therefore tested, in animals of each kind, the effect of DNP–p(Lys) on the immune response towards other synthetic antigens which regularly induce an immune response in all the guinea-pigs (Stupp, Borek and Sela, 1966).

The results obtained indicate that the DNP–p(Lys) interferes with the immune response to other antigens in responders as well as in non-responders. This poses the question, whether non-responder guinea-pigs are really unable to recognize DNP–p(Lys) as an immunogen.

MATERIALS AND METHODS

Animals

Randomly bred albino guinea-pigs weighing 250–400 g were employed. All the animals were from a local breeding colony.

Compounds

The substances used, and their source and characterization are listed in Table 1. The convention for the nomenclature of the polymers has been given previously by Sela, Fuchs and Arnon (1962). The DNP–p(Lys) conjugate was prepared from the sample p(Lys) LY 90 of average molecular weight 2960. A conjugate of DNP–bovine serum albumin (DNP–BSA) was used for detection of DNP antibodies in PCA tests.

TABLE 1

SYNTHETIC COMPOUNDS USED*

No. and designation of sample	Molar ratio of amino acid residues				Average molecular weight	Source and characterization
	Lys	Tyr	Glu	Ala		
Conjugate						
DNP–p(Lys)†	1·0	–	–	–		
Polymers						
44, p(Tyr, Glu, Ala)	–	1·0	1·2	1·1	17,400	Sela *et al.* (1962)
102, p(Tyr, Glu)		1·0	1·0	–	12,500	Sela *et al.* (1962)
138, p(Tyr, Glu)	–	1·0	1·16	–	21,850	Miles-Yeda, Israel
LY90, p(Lys)	1·0	–	–	–	2,960	Miles-Yeda
LY74, pD(Lys)	1·0	–	–	–	161,000	Miles-Yeda
Haptens						
N-ε-DNP-mono-Lys						Sigma, U.S.A.
ε-Amino-n-caproic acid						Sigma, U.S.A.

* L-Amino acids were present except where the D-form is specified.
† Approximately 3–4 DNP groups/average number prepared from LY90, p(Lys).

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 (FCA) containing 10 mg/ml of killed *Mycobacterium tuberculosis* strain H 37 Rv. A total of 0·4 ml of this emulsion was injected at four sites into the hind footpads and behind the neck. The effect of DNP–p(Lys) and of other products on the immune response was tested usually by including them in the immunizing mixtures. In order to determine whether the effect obtained was due to some physico-chemical interactions between the compounds injected, additional experiments were carried out in which each compound was injected at separate sites. In such experiments, the compound tested for interference was injected in two sites (100 μg/site) on 3 consecutive days, in order to administer the same quantity as employed for simultaneous injections (600 μg). The emulsion incorporating the test antigen was injected on the 3rd day, separately from that containing the interfering compound. In some cases a second injection of the test antigen was performed 27–30 days after the first one.

Tests

The immune responses of the animals towards the interfering compound and the test antigen were checked by direct intradermal injections to detect delayed type reactions, and by passive cutaneous anaphylaxis (PCA) tests with their sera. Blood was taken and

TABLE 2

INTERACTION BETWEEN DNP–p(Lys) AND EITHER 44, p(Tyr, Glu, Ala) OR 102, p(Tyr, Glu) IN RANDOMLY BRED GUINEA-PIGS

Group No. (No. of animals)	No. of immunizing injections	μg DNP–p(Lys) per guinea-pig	μg of 44, p(Tyr, Glu, Ala) or 102, p(Tyr, Glu) per guinea-pig	Tests: Material injected*	Tests: Delayed cutaneous reactions: No. of positive animals	Tests: Delayed cutaneous reactions: Mean diameter (mm) of positive animals	Tests: PCA†
1 (7)	1st	600	–	DNP–p(Lys)	4/7	15	4/7
2 (18)	1st	100	–	DNP–p(Lys)	3/18	16·5	3/18
	2nd	100	–				3/16
3 (9)	1st	10	–	DNP–p(Lys)	5/9	8·7	
	2nd	10	–		4/7	9·6	3/6
4 (14)	1st	0	p(Tyr, Glu, Ala) 100	p(Tyr, Glu, Ala)	12/14	8·0	0/14
	2nd	0	p(Tyr, Glu, Ala) 100		8/8	10·0	
5 (22)	1st	600	p(Tyr, Glu, Ala) 100	DNP–p(Lys)	10/22	15·3	10/22
				p(Tyr, Glu, Ala)	0/22	0	
	2nd	0	p(Tyr, Glu, Ala) 100	DNP–p(Lys)	0/12‡	0	
				p(Tyr, Glu, Ala)	10/12	6·0	
6 (12)	1st	0	p(Tyr, Glu) 100	p(Tyr, Glu)	12/12	9·8	
	2nd	0	p(Tyr, Glu) 100	p(Tyr, Glu)	7/8	10·4	4/6
7 (7)	1st	600	p(Tyr, Glu) 100	DNP–p(Lys)	3/7	10·5	4/8
				p(Tyr, Glu)	0/7	0	
	2nd	0	p(Tyr, Glu) 100	DNP–p(Lys)	3/7	12·3	
				p(Tyr, Glu)	5/7	6·3	
8 (6)	1st	100	p(Tyr, Glu) 100	DNP–p(Lys)	1/6	12·0	
				p(Tyr, Glu)	3/6	6·0	
9 (10)	1st	10	p(Tyr, Glu) 100	DNP–p(Lys)	6/10	10·6	
				p(Tyr, Glu)	5/10	5·6	

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

† PCA: 1 mg/animal; DNP–BSA was used for check of DNP antibodies; only animals found positive to DNP–p(Lys) were positive in PCA tests with DNP–BSA.

‡ These animals did not respond to DNP–p(Lys) after the 1st injection.

intradermal tests were performed 14 days after the first immunizing injection and, in some cases, at 10 days after the second immunizing injection. The procedures employed for performing and evaluating the response, were described previously (Ben-Efraim and Liacopoulos, 1967).

RESULTS

INTERACTION BETWEEN DNP–p(Lys) AND 44, p(Tyr, Glu, Ala) OR 102, p(Tyr, Glu) ANTIGENS

In preliminary experiments the immunogenicity of these three products was determined: DNP–p(Lys) was found to be immunogenic in doses of 600, 100 and 10 μg. 44, p(Tyr, Glu, Ala) regularly produced delayed hypersensitivity only when a dose of 100 μg was injected. Lower doses either failed to induce an immune response (10 μg/animal—0/5 animals, 20 μg/animal—0/4 animals), or did so only in some animals (40 μg/animal—2/4 animals). Similarly 102, p(Tyr, Glu) was regularly immunogenic in doses of at least 100 μg/animal.

The results of immunizing with DNP–p (Lys), alone or with 44, p(Tyr, Glu, Ala) or 102, p(Tyr, Glu) are presented in Table 2 (groups of guinea-pigs) and Table 3 (individual animals).

Injection of DNP–p(Lys) alone induced delayed type responses and circulating antibodies, as reflected by PCA reactions, in only a proportion of guinea-pigs. However, all the guinea-pigs responded to immunizations with either p(Tyr, Glu, Ala) or p(Tyr, Glu) polymers. The response to these polymers was completely inhibited when the immunizing mixture also contained 600 μg of DNP–p(Lys) per animal. This inhibition was evident whether the guinea-pigs were responders or non-responders to DNP–p(Lys), as may be seen by comparing the reactions to DNP–p(Lys) and to p(Tyr, Glu) in individual animals (Table 3).

TABLE 3

INTERACTION BETWEEN DNP–p(Lys) CONJUGATE AND 102, p(Tyr, Glu) ANTIGEN IN INDIVIDUAL RESPONDERS AND NON-RESPONDERS

Guinea-pig No.	Immunizing mixtures					
	DNP–p(Lys) + p(Tyr, Glu) 600 μg / 100 μg		DNP–p(Lys) + p(Tyr, Glu) 10 μg / 100 μg		p(Tyr, Glu) 100 μg	DNP–p(Lys) 10 μg
	Delayed cutaneous reactions*					
	DNP–p(Lys)	p(Tyr, Glu)	DNP–p(Lys)	p(Tyr, Glu)	p(Tyr, Glu)	DNP–p(Lys)
1	10·0	0	11·0	5·0	10·0	10·0
2	0	0	5·0	5·0	9·0	0
3	11·5	0	13·5	0	13·0	7·0
4	0	0	0	8·0	11·0	8·0
5	0	0	0	5·0	10·5	0
6	0	0	10·0	0	11·5	9·0
7	10·0	0	11·0	5·0	12·0	0
8			0	0	11·5	0
9			12·5	0	9·5	9·5
10			6·0	0		
Positive/total	3/7	0/7	7/10	5/10	9/9	5/9
Mean + diameter	10·5†	0	9·6†	5·6†	10·9†	8·7†

* DNP–p(Lys): 10 μg/skin site; p(Tyr, Glu): 50 μg/skin site.
† Mean of positive animals only.

Lower concentrations of DNP–p(Lys) (100 or 10 μg/animal) also had an inhibitory effect on the response to 100 μg of p(Tyr, Glu) antigen (groups 8 and 9, Table 2). This is shown by the smaller proportion of animals reacting, as well as by the decreased size of skin reactions to p(Tyr, Glu) in comparison with those of the control group injected with p(Tyr, Glu) only (group 6, Table 2). Even with lower doses of DNP–p(Lys), its effect did not depend upon whether the individual animals were responders to DNP–p(Lys) or not (Table 3).

Neither addition of 100 μg of p(Tyr, Glu, Ala) to 600 μg of DNP–p (Lys), nor of 100 μg of p(Tyr, Glu) to various doses of DNP-p(Lys) (600, 100 and 10 μg), interfered significantly with the immune response to DNP–p(Lys) (Table 2).

INTERACTION BETWEEN DNP–p(Lys) AND 138, p(Tyr, Glu) INJECTED AT SEPARATE SITES

In order to exclude the possibility that the inhibition is due to some physico-chemical interaction between DNP–p(Lys) and the other synthetic antigens, experiments were carried out in which the DNP–p(Lys) and 138, p(Tyr, Glu) were injected at separate sites. A constant dose of 200 μg/animal of the new batch 138, p(Tyr, Glu) was chosen, because previous tests had shown that this was the minimal effective dose for the majority of animals. Animals injected with Freund's complete adjuvant without DNP–p(Lys) were used as controls. The results (Table 4), show that also under these conditions, DNP–p (Lys) inhibited the immune response towards p(Tyr, Glu), while injections of adjuvant alone did not. The inhibitory effect of DNP–p(Lys) was observed, as in the previous experiments, in responders as well as in non-responders to this conjugate.

TABLE 4

INTERACTION BETWEEN DNP–p(Lys) AND 138, p(Tyr, Glu) ANTIGENS IN RANDOMLY BRED GUINEA-PIGS WHEN INJECTED AT SEPARATE SITES

Group No. (No. of animals)	Immunizing injections				Tests			
	Day	No. of injection	μg DNP–p(Lys) per guinea-pig (FCA was given at each injection)	μg p(Tyr, Glu) per guinea-pig	Material injected*	Delayed reactions: No. of positive animals	Delayed reactions: Mean diameter (mm) of positive animals	PCA
1 (8)	1	1		200				
	2	2		200				
	3	3		200				
	3	4	FCA‡	0	DNP–p(Lys)	1/8	12·0	1/8
2 (19)	1	1	FCA	0	0			
	2	2	FCA	0	0			
	3	3	FCA	0	0			
	3	4			200	p(Tyr, Glu)	16/19	8·2
3 (10)	1	1	FCA	0				
	2	2	FCA	0				
	3	3		200				
	3	4			200	DNP–p(Lys)	0/10†	0
						p(Tyr, Glu)	2/10	8·0
4 (10)	1	1		200				
	2	2		200				
	3	3		200				
	3	4			200	DNP–p(Lys)	5/10	12·5	5/10
						p(Tyr, Glu)	3/10	6·8	

* See footnote to Table 2.
† This group included only animals which did not respond to DNP–p(Lys).
‡ FCA alone.

EFFECT OF p(Lys) CARRIER AND OF DNP–Lys HAPTEN ON THE IMMUNE RESPONSE TO 138, p(Tyr, Glu)

It has been postulated by Levine *et al.* (1963b) that the ability of guinea-pigs to respond to hapten poly-lysine conjugates is related to the immune 'recognition' of the lysine carrier moiety. It was shown later by Green, Paul and Benacerraf (1966), that an immune response to poly-L-lysine alone can be detected in some guinea-pigs. It was, therefore, of interest to test the effect of poly-δ-lysine itself on the immune response to p(Tyr, Glu) (Table 5).

TABLE 5

INTERACTION BETWEEN EITHER POLY-LYSINE OR HAPTENS AND 138, p(Tyr, Glu) ANTIGEN IN RANDOMLY BRED GUINEA-PIGS*

Group No. (No. of animals)	Immunizing injections				Tests			
	Day	No. of injection	Material and amount (μg) injected	μg p(Tyr, Glu) per guinea-pig	Material injected	Delayed reactions: No. of positive animals	Delayed reactions: Mean diameter (mm) of positive animals	PCA
1 (18)	1	1st	p(Lys) 600	–	p(Lys)	0/18	0	0/18
	29	2nd	600	–		0/7	0	0/7
2 (14)	1	1st	–	200	p(Tyr, Glu)	12/14	10·1	0/14
	31	2nd	–	200				2/7
3 (8)	1	1	p(Lys) 600	200	p(Lys)	1/8	10·5	0/8
					p(Tyr, Glu)	2/8	6·7	0/8
4 (10)	1	1st	pD(Lys) 600	200	pD(Lys)	3/10	6·3	
					p(Tyr, Glu)	2/10	9·75	
	27	2nd	–	200	p(Tyr, Glu)	8/10	10·6	
5 (10)	1	1st	DNP–monoLys 600	200	DNP–BSA			0/7
					p(Tyr, Glu)	10/10	17·1	6/10
	30	2nd	–	200	p(Tyr, Glu)			8/9
6 (10)	1	1st	ε ACA† 600	200	p(Tyr, Glu)	10/10	12·9	5/10
	30	2nd	–	200	p(Tyr, Glu)			9/9

* See footnote to Table 2; p(Lys) and pD(Lys): 10 μg/skin sites.
† ε-Amino-n-caproic acid.

We could not detect, under our conditions, a significant response to poly-L-lysine in animals injected with this compound (group 1, Table 5), except for one animal out of the eight of group 3 (Table 5). Nevertheless, simultaneous injections of poly-L-lysine inhibited the immune response towards p(Tyr, Glu) in the majority of animals tested (group 3, Table 5) although to a lesser extent than did DNP–p(Lys). Subsequent injection of DNP–p(Lys) into animals which had already received p(Lys) and p(Tyr, Glu) was performed in some cases and induced positive delayed type response to DNP–p(Lys) in only some of the animals. This implies that the competitive effect of p(Lys) towards p(Tyr, Glu) occurs in responders as well as in non-responders to DNP–p(Lys). An inhibitory effect on the immune response to p(Tyr, Glu) was also observed with poly-D-lysine (group 4, Table 5). The inhibition of the immune response to p(Tyr, Glu) was shown to be due to interference with simultaneous stimulation by poly-D(Lys), because a second later injection of p(Tyr, Glu) alone (group 4, Table 5) induced an immune response similar to that of control animals which received one injection of p(Tyr, Glu) alone. Injections of either p-Lys or p-D-Lys and of p(Tyr, Glu) at separate sites again produced a clear inhibition of the responses to the latter antigen.

Addition of DNP–mono-Lys or ε-amino-n-caproic acid haptens to the immunizing mixture containing p(Tyr, Glu) did not inhibit the immune response towards this antigen. An intriguing enhancing effect on the immune response towards p(Tyr, Glu) was recorded: the size of delayed reactions to p(Tyr, Glu) of the animals sensitized with these mixtures was increased and circulating antibodies to p(Tyr, Glu) were detected even after the first immunizing injection (compare results of group 2 and groups 5 and 6, Table 5). No antibody reacting with DNP–BSA was found in the animals injected with the mixture DNP–mono-Lys+p(Tyr, Glu) (group 5, Table 5), showing that no immunogenic complex of these two compounds had been formed in the immunizing mixture.

DISCUSSION

We have shown previously (Ben-Efraim and Liacopoulos, 1967) that DNP–p(Lys) has a strong inhibitory effect on the immune response to an unrelated synthetic antigen in guinea-pigs of inbred strain 2. Our present results demonstrate that similar material regularly inhibited the immune response in randomly bred guinea-pigs towards two unrelated synthetic antigens namely p(Tyr, Glu, Ala) and p(Tyr, Glu). Such inhibition was observed in all the guinea-pigs tested, independently of their capacity to respond to DNP–p(Lys). The finding that competition could be produced when DNP–p(Lys) and p(Tyr, Glu) were injected in separate sites, shows that this effect was not due to some physico-chemical interaction occurring between the two compounds in the immunizing mixture.

The competitive effect of DNP–p(Lys) towards p(Tyr, Glu) was found to be related to the quantity of conjugate used. However, even when smaller quantities (100 or 10 μg) of DNP–p(Lys) were used, no relation was found between the response to the conjugate itself and its inhibitory effect on p(Tyr, Glu) antigen in the animals tested.

We did not find in our experiments any competitive effect of p(Tyr, Glu, Ala) or p(Tyr, Glu) on the immune response towards DNP–p(Lys). This finding may indicate that DNP–p(Lys) is a stronger antigen than the polymers tested. A competitive effect was reported previously (Ben-Efraim and Liacopoulos, 1967) when 600 μg of p(Tyr, Glu, Lys) were injected together with 10 μg of DNP–p(Lys). It is possible that larger amounts of p(Tyr, Glu, Ala) or p(Tyr, Glu) would compete with minimal doses of DNP–p(Lys).

Attempts were made to relate the competitive property of DNP–p(Lys) either to the hapten or to the carrier moiety of the molecule. Our results indicate that, as expected, the hapten itself (DNP–mono-Lys) did not interfere with the immune response towards p(Tyr, Glu) antigen. However the carrier moiety (poly-L-lysine) could inhibit the immune response to p(Tyr, Glu) although, at comparable doses, to a lesser extent than did the complete conjugate DNP–p(Lys). These results are in agreement with previous statements (Levine *et al.*, 1963b) on the role played by poly-L-lysine in determining the immunogenicity of DNP–p(Lys) conjugates.

A similar competitive effect was also observed of poly-D(Lys) on the immune response towards p(Tyr, Glu). This finding indicates again that D-polymers are not immunologically inert. It was shown already by Gill, Kunz, Gould and Doty (1964) that some D-amino acid polymers can elicit an immune response, and by our previous results (Ben-Efraim and Liacopoulos, 1967) that some apparently non-immunogenic D-amino acid polymers can interfere with the immune response towards another antigen.

The reason for testing the effect of DNP–p(Lys) on the response to other synthetic

antigens in randomly bred guinea-pigs was to define the competitive role of DNP–p(Lys) in relation to the ability of animals to respond to it immunologically. Our finding that DNP–p(Lys) can inhibit the immune response towards other synthetic antigens in responders as well as in non-responders can be explained by either one of two assumptions: (a) DNP–p(Lys) interferes with the second antigen at some non-specific step of the immunization process; and (b) DNP–p(Lys) is also recognized as a complete antigen by what are commonly called 'non-responder' guinea-pigs in spite of the absence of delayed type reactions and of PCA inducing antibodies (7S γ_1 antibodies: White, Jenkins and Wilkinson, 1963; Ovary, Benacerraf and Block, 1963). The investigations reported in the following paper (Liacopoulos, Ben-Efraim, Harel and Gille, 1969) were undertaken in order to choose between these alternatives.

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