The Nature of Antigen–Antibody Complexes Initiating PCA Reactions in the Guinea Pig[1] (34674)

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Several investigations have been carried out relating to the molecular weight of antigens and their abilities to elicit anaphylactic reactions (1–5). With a group of protein antigens of differing molecular size it was found that the weight of antigen needed to elicit passive cutaneous anaphylactic reactions (PCA) increased with the molecular weight of the antigens (1). In these studies antibodies with different specificities were used. With antihapten antibodies it is possible to use the same antibody preparations with antigenic molecules of different size. Levine *et al.* studied the effectiveness of a homologous series of benzylpenicilloyl conjugates of poly-L-lysine of widely differing molecular sizes (2–5). They found that the different sized haptens were equally effective in eliciting PCA reactions (2, 3) as well as wheal-and-flare reactions in humans (4, 5) when compared in an equimolecular concentration basis, but not when compared in an equal weight concentration basis. The former studies were performed in guinea pigs passively sensitized by intravenous injection of antibody with elicitation by intradermal injection of hapten dilutions after a suitable sensitization period. Since PCA reactions are usually performed by intradermal sensitization with antibody and subsequent intravenous challenge with antigen [PCA-I in Ref. (7)] it was of interest to determine whether the quantitative findings using intravenous sensitization would also hold true for intradermal sensitization. It is conceivable that the diffusion rates of antigen and antibody molecules through tissues could differ in the two procedures.

The results presented below show that similar findings were obtained for both methods and the theoretical implications with regard to the nature of the antigen–antibody complexes initiating such reactions are discussed.

Materials and Methods. Haptens. Bisbenzylpenicilloyl-hexamethylenediamine (BPO_2HMD) was prepared as described previously (2). The multivalent haptens were prepared by alkaline coupling of the different sized poly-L-lysines and purified and assayed as described previously (3). PLL_{184} and PLL_{999} were purchased from Pilot Laboratories, Watertown, Mass.; their average degree of polymerization was calculated from specific viscosity measurements in 0.2 *M* NaCl, pH 3.0 (manufacturer's analysis). PLL_7 was a preparation synthetized by Cyclo Chemical Corporation, Los Angeles, Calif. (lot M-2307). Paper chromatography using the Waley and Watson solvent system (8) modified as described previously (5), show it to be composed mainly of $lysine_6$, $lysine_7$ and $lysine_8$, and to contain a small amount of $lysine_5$ and a trace of high molecular weight polylysine. BPO-hapten solutions were made and stored and diluted in Tris–buffered saline (TBS), pH 8.2.

Antisera. Hyperimmune pooled rabbit anti-BPO sera prepared against BPO_{33} -bovine gamma globulin or BPO_{97} -bovine fibrinogen were used for passive sensitization of guinea pigs (5). These sera were prepared in rabbits as follows: 0.5 mg of conjugate emulsified in Freund's complete adjuvant was injected into

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the four foot pads as the primary dose and followed by a booster of 5 mg of conjugate intravenously on day 30. The animals were bled 7, 8, and 9 days after the booster and the sera from several animals were pooled. BPO-specific antibody was assayed by quantitative precipitin reactions using BPO_{200} - $PLL_{999}S$ as the antigen (5).

Passive cutaneous anaphylactic (PCA) reactions. PCA reactions were performed as described in Refs. (6) and (7) (PCA-I). Briefly, saline dilutions of antisera containing known quantities of antibody were injected intradermally into the freshly shaved skin of the back. Groups of four guinea pigs were used for each challenge dose of antigen. Each antibody dilution was injected in two sites in each animal. After a sensitization period of 2 to 4 hr the animals were challenged by intravenous injection of 1 ml of TBS, pH 8.2, containing the hapten and 0.5% Evans blue dye. Results with two different rabbit antisera were identical and were pooled. The reactions were read 20 min after challenge. The animals were killed, the skin was reflected and the reaction was measured on the internal side of the skin. The magnitude of the reaction was graded by the average diameter of the blue spots as described in Ref. (6).

Results. In the first experiments BPO_6 - PLL_7 and BPO_{465} -PLL $_{999}$ were compared quantitatively in eliciting PCA. Guinea pigs were passively sensitized by intradermal injections of 6 doses of rabbit anti-BPO antibody as described in Methods and challenged

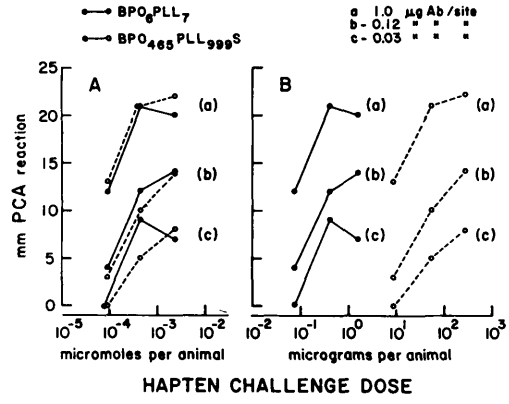

FIG. 1. PCA reactions in guinea pigs (means of 8 sites) sensitized with rabbit anti-BPO antisera. Comparative efficiency of BPO_6PLL_7 and $BPO_{465}P_{999}S$ haptens in challenge. The same data are shown either in a molar concentration basis (A) or in a weight concentration basis (B); see also Table I.

by intravenous injection of one of the two haptens in one of 3 doses. The results are shown in Table I. Equimolar doses of the two different sized haptens were virtually equally effective in eliciting PCA reactions throughout the dose response curve. As may be seen graphically in Fig. 1, the two different sized haptens are equally effective when compared at equimolar concentrations but not when compared at equal weight concentrations. In the following experiments the divalent hapten, BPO_2 -HMD, was compared with the multivalent hapten BPO_{125} PLL $_{184}S$. Groups of 4 guinea pigs were passively sensitized as above and after a 4-hr sensitization period, the animals were challenged by

TABLE I. PCA in Guinea Pigs with Rabbit Anti-BPO Antisera.
Comparative efficiency of BPO_6PLL_7 and $BPO_{465}PLL_{999}S$ for challenge.

Antibody (μg/skin site)	Hapten injected (μmoles)					
	8×10^{-5}		4×10^{-4}		2×10^{-3}	
	BPO_6PLL_7	$BPO_{465}PLL_{999}S$	BPO_6PLL_7	$BPO_{465}PLL_{999}S$	BPO_6PLL_7	$BPO_{465}PLL_{999}S$
0.03	0[a]	0	9	5	7	8
0.06	0	2	12	9	12	11
0.12	4	3	12	10	14	14
0.25	7	6	13	11	13	16
0.50	12	10	17	15	16	17
1.00	12	13	21	21	20	22

[a] Mean PCA reaction (mm) in 8 sites.

TABLE II. PCA in Guinea Pigs with Rabbit Anti-BPO Antisera.
Comparative efficiency of BPO_2HMD and $BPO_{125}PLL_{184}S$ for challenge.

Antibody (μg/skin site)	Hapten injected (μmoles)					
	8×10^{-5}		4×10^{-4}		2×10^{-3}	
	BPO_2HMD	$BPO_{125}PLL_{184}S$	BPO_2HMD	$BPO_{125}PLL_{184}S$	BPO_2HMD	$BPO_{125}PLL_{184}S$
0.03	1[a]	5	4	7	4	5
0.06	3	7	8	9	7	7
0.12	3	9	9	10	9	9
0.25	5	13	13	13	13	13
0.50	6	15	15	15	15	16
1.00	7	16	17	17	17	17

[a] Mean PCA reaction (mm) in 8 sites.

intravenous injections of the divalent or the multivalent haptens in one of 3 doses. Table II shows that multivalent and divalent haptens were equally effective at the higher equimolar doses, but that the divalent hapten was less effective at the lowest dose.

Discussion. The foregoing results show that bivalent and multivalent BPO haptens differing in molecular size by a factor of 100 or more were virtually equally effective in eliciting PCA reactions in the guinea pig when compared at equimolar doses, throughout the dose–response curve. The lesser effectiveness of the bivalent hapten at the low concentration cannot readily be explained, but may be due to the degradation or rearrangement of one of the BPO groups in the molecule. This would convert it into a univalent hapten and thus to an inhibitor rather than an elicitor of PCA reactions (2, 9). It could also be that the lesser effectiveness of the bivalent hapten might be due to the affinities of the antibody populations. In fact, bivalent haptens are not always effective for elicitation of PCA reactions. If the affinity of the antibody population is relatively low, bivalent haptens may be ineffective (10–12, 2, 5).

Different antibody–antigen systems have already been used to show that in PCA reactions by intradermal sensitization and intravenous challenge (PCA-I) there is an inverse relationship between the amount of antigen required and its molecular weight (1). These investigations were done with native protein antigens and, therefore, antibodies of different specificities and antigens which differ in terms of the distribution of antigenic determinants as well as in molecular weight. The advantage of hapten-conjugates is that the same antibody preparations are used, as well as carrier molecules which have the same basic structure, such as poly-L-lysines of different length and thus differing only in molecular size. The present results are in accord with previous experiments using the system of intravenous sensitization and intradermal challenge by the hapten (2, 3).

Concerning the reaction of the antibody with the bi- or multivalent haptens or antigens, two possibilities may be considered: (i) that the antigen reacts with the antibody in solution, and (ii) that the antigen reacts with the antibody fixed to a cell membrane or some tissue constituent. In the first case, multivalent haptens of different sizes should be equally effective on a weight concentration basis, especially where the antibody limits the intensity of the reaction, and the bivalent hapten should be relatively ineffective. This is, in fact, the case in the precipitin reaction (3, 11) and in the elicitation of the Arthus reaction (3, 5). In the second case, the different sized bivalent and multivalent haptens should be equally effective on an equimolar concentration basis throughout a wide range of antigen-to-antibody ratios, which is the case in the present experiments.

It seems that bridging of two antibody molecules by one molecule of antigen with two determinants (10, 11, 13, 2) is sufficient

to elicit PCA reactions mediated by high affinity antibodies, whereas, at least in this system (anti-BPO antibodies and BPO-poly-L-lysine) the bridging of more than two and less than 4 antibody molecules is necessary to elicit these reactions mediated by rabbit antibodies of low affinities (3, 5). Thus, the present studies show that equimolar concentrations were needed throughout the dose–response curve for $BPO_{465}PLL_{999}S$ and BPO_6PLL_7 to elicit the same intensity of PCA reaction, in spite of the fact that the larger molecule is more than 100 times larger and has about 75 times more determinants than the smaller one. This fact is a strong argument in favor of the hypothesis that on one antigenic molecule only two, or a very small number of determinants participate in the elicitation of the PCA reaction and the remainder of the determinant groups are not active.

However, it is alternatively possible that the molecular size of the hapten significantly affects its rate of diffusion through tissues. Thus, less effective, but more readily diffusible small molecules would be as efficient as more effective but less readily diffusible large molecules. This point could be further investigated with *in vitro* systems employing sensitized cell suspensions, such as mast cells or leukocyte suspensions.

In some instances, however, certain univalent haptens seem capable of eliciting PCA reactions (14–17). The mechanism whereby these univalent haptens elicit PCA reactions is not yet clear. In one case (2) much higher concentrations of a toxic univalent hapten than the divalent hapten was needed to elicit equally intense PCA reactions. These observations (14–17) would not negate the possibility that the majority of protein antigens may elicit anaphylactic reactions by the bridging mechanism rather than by other hypothetical mechanisms involving certain univalent haptens.

Summary. Benzylpenicilloyl (BPO) haptens of widely different molecular size were compared for their efficiency in eliciting PCA reactions in guinea pigs sensitized with rabbit anti-BPO antibodies. Equimolar doses of different sized haptens were found to be equipotent in eliciting the PCA reactions. Therefore, in this system the elicitation of PCA reactions by multivalent haptens could also be compatible with a simple bridging hypothesis.

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