Pottery dating to Pueblo III, as well as other items, accompanied it. A second complete cradleboard with a bark hood was found in Moqui Canyon in southern Utah (*7*); it was poorly preserved but still held the remains of an infant. Pottery from the site, although not directly associated, suggested a Pueblo III time of placement. A third cradleboard with a bark hood (Fig. 1) was found in 1924, associated with an infant burial at Tseahatso, a large, dry, rock shelter in Canyon del Muerto in northeastern Arizona. This find has not previously been published. The hood had become detached from the backrest. Associated pottery indicated a Pueblo III date. Small clay effigies of infants in cradleboards with hoods of this shape have been found at Waterfall Ruin in northeastern Arizona (*8*) and near Flagstaff, Arizona (*9*); the latter effigies were parts of handles of ceramic dippers. The contexts clearly demonstrate the true function of these curved bands of bark. The earlier identification of these cradleboard hoods as corsets on the basis of a specimen lacking known context demonstrates the difficulties faced in attempting to interpret archeological materials out of context.

Calvin Wells wrote (*10*) that "it is most unwise for anthropologists who lack clinical training to venture into the infinitely subtle field of ancient disease." The specific corollary is obvious. To it we would like to add another: the cooperation of specialists in many professions is required to reconstruct the subtle aspects of prehistoric cultures.

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Antigen-Antibody Reaction: Nature of Complex Initiating Delayed Hypersensitivity

Abstract. *Two homologous lightly coupled dinitrophenyl conjugates of poly-L-lysine of differing average molecular sizes were compared with regard to their abilities to elicit in guinea pigs specific delayed hypersensitivity skin reactions, passive cutaneous anaphylaxis, and active Arthus reactions. Equal concentrations by weight (but not equimolar concentrations) of the two conjugates elicited equally intense delayed hypersensitivity reactions and Arthus reactions, whereas equimolar concentrations (but not equal weight-concentrations) elicited equally intense passive cutaneous anaphylaxis reactions. These results suggest that delayed hypersensitivity reactions are initiated by the reaction of antigen with antibody molecules in true solution, and not by the simple bridging by antigen of a small number of antibody molecules firmly fixed to cell membrane surfaces. Whether "sensitized cells" or circulating "delayed hypersensitivity antibodies" are the specific mediators of the delayed hypersensitivity reactions is discussed.*

Delayed hypersensitivity reactions (DHR) are characterized by their slow evolution, their histological appearance (mononuclear cell infiltrate), and their ability to be passively transferred by lymphoid cells, but not by serum, from hypersensitive donors. On the basis of these observations and other evidence, these reactions have been widely viewed as being mediated by "sensitized mononuclear cells" and not by freely circulating antibodies (*1*). The nature of "sensitized cells" has not been defined, but a classical possibility is that "sensitized cells" may be sensitized by having antibody molecules (*2*) firmly bound to their cell membranes. According to this model, DHR may be visualized as resulting from the following sequence: (i) Antigen reacts with and bridges a small number of antibody molecules firmly fixed on sensitized cell membranes. (ii) This simple bridging of the cell membrane in some way interferes with membrane function, and results in the release of toxic intracellular materials into extracellular environment causing tissue damage. A classical example of this kind of antigen-antibody reaction occurs in anaphylaxis (*3, 4*). Sensitized cells may conceivably become so in other ways (*5*), and this consideration will be taken up below.

More recently, Karush and Eisen (*6*) have argued that the available experimental evidence does not provide an adequate logical basis for the "sensitized cell" hypothesis. Based on the known heterogeneity of the immune response and on other considerations, they have hypothesized that DHR may be mediated by freely circulating antibodies which have high antigen-binding affinities and which are present in serum in extremely low concentrations (*6*). According to this model, DHR may be visualized as resulting from the following sequence: (i) Antigen reacts with soluble (unrestricted in mobility) antibody molecules to form comparatively large complexes. (ii) These complexes (with or without substances bound from serum) chemotactically attract mononuclear cells. (iii) The interaction of preformed complexes and cells cause the release of toxic materials from the cells into the extracellular environment, causing tissue damage. A classical example of this kind of antigen-antibody reaction occurs in the Arthus reaction (*4*). Indirect experimental evidence supporting the notion that antibodies meditating DHR are of comparatively high binding affinities has recently been obtained (*7*).

I have attempted to choose between the two general kinds of antigen-antibody reactions already mentioned by considering them as different physical-chemical situations, that is, the interaction of antigen with a reactant which is restricted in mobility (tissue-fixed antibody) in comparison to its interaction with a reactant which is freely mobile (antibody in true solution). Experiments were set up comparing the abilities of two homologous and structurally well-defined antigens of different molecular sizes to elicit specific DHR.

In the first situation (restricted antibody), the two antigens should be precisely equally effective when their molar concentrations are the same, and in the second situation (freely mobile antibody), the two antigens should be equally effective when their concentrations by weight are equal (*8*), provided the following requirements are met: (i) Delayed hypersensitivity is specific for the same simple antigenic unit contained in the two antigens. (ii) The number of antigenic combining sites per unit weight conjugate is the same

for both antigens. (iii) Antigens are compared in the part of the dose-response curve where DHR intensities are limited by antigen concentration. For the restricted antibody model, antigen is visualized as being required to bridge only a comparatively small number of fixed antibody molecules on "sensitized cells" in order to produce the postulated membrane changes leading to DHR (*9*). This argument has been developed in more detail in relation to passive cutaneous anaphylaxis (PCA) and Arthus reactions (*3, 4*). The results of these experiments are now reported.

Table 1. Effect of average molecular size of DNP-PLL conjugates on intensity of delayed hypersensitivity skin reactions in the guinea pig. Each guinea pig was immunized with 100 μg of conjugate in complete adjuvant; animals 1, 3, and 5 with $DNP_{6.6}$-PLL_{96} and animals 2, 4, and 6 with DNP_{63}-PLL_{999}. The delayed reaction intensity was tested by intradermal injection of 0.1 ml of conjugate solutions on the 16th day. Reactions read at 24 hours were graded according to extent of tissue damage: 1+, a nodule 7 to 11 mm in diameter; 2+, an indurated nodule with superficial skin necrosis 10 to 15 mm; 3+, a nodule 12 to 17 mm, with central punched-out ulcer; the numbers 96 and 999 refer to $DNP_{6.6}$-PLL_{96} and DNP_{63}-PLL_{999}, respectively; Neg, negative reaction; Tr, trace reaction. Controls were immunized with crystalline ovalbumin. When given skin tests on the 16th day with the PLL conjugates (100 μg/ml), the control animals showed flat papules 4 to 7 mm in diameter, that is, a negative reaction.

Guinea pig No.	Weight concentrations of conjugates (μg/ml)							
	3.0		10		30		100	
	96	999	96	999	96	999	96	999
1	Neg	Neg	Tr	Tr	1+	1+	2+	2+
2	1+	1+	2+	2+	2+	2+	3+	3+
3	Tr	Tr	1+	1+	2+	2+	3+	3+
	Molar concentrations of conjugates (mole/liter)							
	1×10^{-7}		3×10^{-7}		1×10^{-6}			
	96	999	96	999	96	999		
4	Neg	Tr	Tr	1+	1+	2+		
5	Neg	Tr	Tr	1+	1+	2+		
6	Neg	1+	1+	2+	2+	3+		

Table 2. Effect of average molecular size of DNP-PLL conjugates on intensity of passive cutaneous anaphylaxis reactions in the guinea pig. All animals were passively sensitized by intravenous injection of 0.5 mg of purified rabbit antibody to DNP. After a latent period of 48 hours, 0.5 ml of 1-percent Evans blue was injected intravenously, and 0.1 ml of antigen solutions was injected intradermally (*3, 4*). The passive cutaneous anaphylaxis reactions were at maximum intensity at 15 minutes and were graded according to intensity of blue color: P, pale; PM, pale to moderate; M, moderate; MS, moderate to strong; S, strong; for explanation of the numbers 96 and 999, see Table 1; Neg, negative reaction. Conjugate solutions at these concentrations gave negative reactions in nonsensitized, blue-treated animals.

Guinea pig No.	Weight concentrations of conjugates (μg/ml)					
	0.10		1.0		10.0	
	96	999	96	999	96	999
1	M	P	MS	M	S	MS
2	M	P	MS	M	S	MS
3	Neg	Neg	P	Neg	M	P
	Molar concentrations of conjugates (mole/liter)					
	1×10^{-9}		1×10^{-8}		1×10^{-7}	
	96	999	96	999	96	999
4	P	P	M	M	MS	MS
5	PM	PM	M	M	S	S
6	Neg	Neg	P	P	PM	PM

Table 3. Effect of average molecular size of DNP-PLL conjugates on intensity of active Arthus reactions in the guinea pig. Each guinea pig was immunized with 100 μg of $DNP_{6.6}$-PLL_{96} conjugate in complete adjuvant. Animals 1, 2, 4, and 5 were strain 2; animals 3 and 6 were random-bred responders. The animals were tested on the 16th day by intradermal injection of 0.1 ml of antigen solutions. Arthus reactions were read at 2 hours and graded according to the extent of hemorrhage: 1+, 2-mm-diameter hemorrhage; 2+, 2- to 5-mm-diameter hemorrhage; 3+, more than 5-mm-diameter hemorrhage; for explanation of the numbers 96 and 999, see Table 1; Neg, negative reaction; Tr, trace reaction (here determined by a trace of a stippled hemorrhage). All positive reactions showed 15-mm diameter edema as well. Conjugates (100 μg/ml) gave 4- to 7-mm edema without hemorrhage in ovalbumin immunized controls, that is, a negative reaction.

Guinea pig No.	Weight concentrations of conjugates (μg/ml)							
	3.0		10		30		100	
	96	999	96	999	96	999	96	999
1	Neg†	Neg	1+	1+	2+	2+	3+	3+
2	Neg	Neg	1+	Tr	1+	1+	2+	2+
3	Neg	Neg	Tr	Tr	1+	1+	2+	2+
	Molar concentrations of conjugates (mole/liter)							
	1×10^{-7}		3×10^{-7}		1×10^{-6}			
	96	999	96	999	96	999		
4	Neg	Tr	Neg	Tr	Tr	1+		
5	Neg	Tr	Neg	1+	Tr	1+		
6	Neg	Tr	Neg	Tr	Tr	2+		

The two antigens used were homologous dinitrophenyl (DNP) conjugates of poly-L-lysine (PLL) preparations of average degrees of polymerization of 96 and 999 lysine residues. The PLL_{96} and PLL_{999} (*10*) (lots 24 and 27) were obtained as the hydrogen bromide salts (Pilot Laboratories). Average degrees of polymerization were calculated from specific viscosity measurements in 0.2*M* NaCl, *p*H 3.0 (*10*). The conjugates used, $DNP_{6.6}$-PLL_{96} (*10*) and DNP_{63}-PLL_{999} were coupled to an equal extent, and accordingly contain (*11*) equal numbers of combining sites per unit weight, fulfilling the second requirement mentioned above. The conjugates were prepared by reaction of the PLL preparations with 0.08 molar equivalents of dinitrofluorobenzene in aqueous solution, 1 hour, 25°C with *p*H maintained at 10 $\pm$ 0.2 in a *p*H stat. Conjugates were purified by exhaustive dialysis and assayed by spectrophotometric and microkjeldahl analyses (*12, 13*). Purified rabbit antibodies to DNP were provided by G. Siskind (*14*). Random-bred albino Hartley and strain-2 inbred guinea pigs (400 to 500 g) were actively immunized. Albino Hartley guinea pigs (270 to 300 g) were used for passive cutaneous anaphylaxis experiments. Strain-2 animals were offspring of the strain-2 guinea pigs obtained from M. Brandriss of the National Institutes of Health.

In the first experiment, two groups of three strain-2 guinea pigs were immunized with a total dose of 100 μg of conjugates emulsified in 0.2 ml saline and 0.2 ml complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) injected into the footpads of the hind feet (*15*). One group was immunized with $DNP_{6.6}$-PLL_{96} and the other with DNP_{63}-PLL_{999}. On the 16th day after immunization, some animals from each group were skin-tested with the two conjugates at four equal-weight concentrations, and the other animals were skin-tested with the two conjugates at three equimolar concentrations. The two conjugates elicited equally intense specific DHR when they were compared

on an equal weight-concentration basis and not when they were compared on an equimolar concentration basis (Table 1). This was true at all doses producing either faint or intense reactions; thus the third requirement for this system is fulfilled. In addition, guinea pigs immunized with $DNP_{6.6}$-PLL_{96} or with DNP_{63}-PLL_{999} reacted identically to the two conjugates (Table 1). Accordingly, this system for studying delayed hypersensitivity did not reveal antigenic differences between $DNP_{6.6}$-PLL_{96} and DNP_{63}-PLL_{999}; that is, the two conjugates are "antigenically equivalent" (*16*). This observation is consistent with the known structural simplicity of hapten conjugates of the random-coil polylysine and with the view that the antigenic specificity of hypersensitivity induced by lightly coupled DNP-PLL conjugates is toward the same structural unit consisting of the DNP group and a PLL peptide of unknown length (*7*), the first requirement for this system thus being fulfilled. The prior Arthus reactions manifested by these guinea pigs would not be expected to interfere with quantitation of the delayed reactions, as has been discussed (*7, 13*), since Arthus reactions in guinea pigs are at their peak at 2 to 3 hours and generally leave insignificant residue at 24 hours, when delayed reactions are read. However, to completely eliminate such interference (and also to repeat this experiment in random-bred animals), ten random-bred guinea pigs were immunized with a total dose of 20 μg of $DNP_{6.6}$-PLL_{96} conjugate emulsified in complete adjuvant, which was injected in the hind footpads; the animals were given skin tests on the 6th day with the four equal weight-concentrations of $DNP_{6.6}$-PLL_{96} and DNP_{63}-PLL_{999} conjugates. Under these conditions, the three responder animals (*15*) did not manifest Arthus reactions. In these animals also, equal weight-concentrations of the two conjugates elicited equally intense delayed reactions throughout the dose-response curve, the results of the first experiment thus being confirmed.

In the next experiments, the same dilutions of the two conjugates which had been used in the previous experiments were compared with regard to their abilities to elicit passive cutaneous anaphylaxis reactions in guinea pigs passively sensitized by intravenous injections of rabbit antibodies to DNP, and to elicit active Arthus reactions in strain-2 and random-bred responder guinea pigs. Both $DNP_{6.6}$-PLL_{96} and DNP_{63}-PLL_{999} elicited precisely equally intense specific PCA reactions when compared on the basis of equimolar concentration, whereas they elicited equally intense specific Arthus reactions when compared on the basis of equal weight-concentration (Tables 2 and 3). This was true at all doses administered. These results with DNP conjugates are in agreement with previous results on PLL conjugated with the larger and more polar benzylpenicilloyl hapten (*4*). The results support the view that passive cutaneous anaphylaxis reactions in the guinea pig are initiated by the simple bridging by antigen of a small number of membrane-fixed antibody molecules, whereas Arthus reactions are initiated by the binding of antigen with antibody molecules in true solution (*3, 4*).

The foregoing results with DHR demonstrate that equal weight-concentrations (and not equimolar concentrations) of two homologous DNP-PLL conjugates of widely different molecular sizes elicit precisely equally intense specific delayed hypersensitivity reactions under conditions where (i) hypersensitivity is specific for the same simple antigenic unit contained in the two conjugates; (ii) the number of antigenic-combining sites per unit weight conjugate is the same for both conjugates; and (iii) the conjugates were compared at concentrations where intensity of the elicited reactions are limited by low concentrations of conjugates. According to the foregoing argument, these results are consistent with the possibility that DHR are initiated by the reaction of antigen with freely mobile antibody molecules, that is, antibody molecules in true solution. This conclusion would tend to provide some experimental support for the view that DHR are mediated by the reaction of antigen with circulating "delayed hypersensitivity antibodies" (*2, 6*), that is, antibody molecules in solution in extracellular fluid, and not by the direct reaction of antigen with "sensitized cells." However, results of the experiments described cannot exclude the possibility that the pertinent antigen-antibody reaction may take place in intracellular fluid within "sensitized cells," nor the possibility that the antigen may be required to be either degraded or metabolized (or both) within "sensitized cells" prior to its reaction with antibody (*5*). My results exclude the possibility that DHR are mediated by "sensitized cells" by an anaphylaxis-like mechanism, that is, the simple bridging by antigen of a small number of antibody molecules which are firmly fixed onto cell membranes of "sensitized cells."

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8. Antibodies may also exist loosely bound to a membrane surface, thus existing, at any given time, in part membrane-fixed and in part in true solution. In this situation, either the antigen may react predominantly with one or the other antibody species, in which case these conclusions would still follow, or antigen might react with both species, in which case the two different-sized antigens would not be precisely equivalent at equimolar nor at equal weight-concentrations.
9. Theoretically this number should be 6.6 (average) or smaller in order for one molecule of the 6.6-valent antigen ($DNP_{6.6}$-PLL_{96}) to be as effective a bridging reagent as one molecule of the 63-valent antigen (DNP_{63}-PLL_{999}). If this number is significantly larger than 6.6 (if the intensity of DHR is a function of the total number of "fixed" antibody molecules bridged rather than of simple bridging), the two conjugates should not be precisely equivalent on an equimolar or on an equal weight-concentration basis.
10. Subscripts refer to average numbers of residues (DNP or lysyl) per molecule; for example, DNP_{63}-PLL_{999} has an average chain length of 999 lysyl residues, 63 (average) of which are coupled with DNP groups. Assays of average degrees of polymerization of PLL preparations were performed by Pilot Laboratories, Watertown, Mass.
11. A benzylpenicilloyl-polylysine conjugate (BPO_{242}-PLL_{999}) was capable of specifically binding up to 190 antibody molecules per molecule of conjugate from a rabbit antiserum (*4*). Accordingly, for the DNP-PLL conjugates used in this study, their average numbers of combining sites per mole should be equal to their average extents of conjugation; that is, steric repulsion between antibody molecules should not limit the numbers of antibody molecules bound by these antigens.
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17. I thank Miss V. Levytska for assistance. Supported by the Health Research Council of the City of New York under contracts 1-240 and U-1297.

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