SPECIFIC INHIBITION OF WHEAL-AND-ERYTHEMA RESPONSES WITH UNIVALENT HAPTENS AND UNIVALENT ANTIBODY FRAGMENTS*, ‡

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According to current immunologic doctrine, the specificity of allergic wheal-and-erythema responses in human skin is determined by soluble antibodies; *e.g.*, those which occur in serum and in extravascular fluid. Not all antigen-antibody systems appear to be competent, however, to incite this response and the basis for the competency of some systems, and for the incompetency of others, has yet to be established. To a great extent the difficulties which have been encountered in attempts to elucidate the basis for competency have arisen from the multiplicity of antibody specificities in antisera produced to protein antigens, even those which are exceedingly pure by usual standards. As a consequence, correlations between skin test responses and the amounts and characteristics of serum antibodies in test subjects have been difficult to interpret with confidence; *i.e.*, because of uncertainty as to whether the specific determinants in skin tests are identical with those of the serum antibodies assayed. In passive transfer skin tests the use of whole serum, or γ-globulin fractions of antiserum, leads, for the same reasons, to similar ambiguity. Under these circumstances the immune systems which offer the greatest promise for analysis of the immunologic requirements for wheal-and-erythema production are those for which highly purified antibodies can be obtained, and those in which the antigenic determinants are defined. On these grounds, certain naturally occurring polysaccharides and synthetic organic molecules, and their homologous antibodies, qualify as particularly suitable systems.

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In the present work, wheal-and-erythema responses were studied in a human subject who had been actively sensitized with 2,4-dinitrophenyl antigens, and also in a number of other human subjects whose skin sites were passively sensitized with purified rabbit antibodies specific for the dinitrophenyl group. The determinant involved in the cutaneous reaction, both in the passively prepared sites and in the actively sensitized subject, was verified as being the dinitrophenyl group by means of specific inhibition with appropriate DNP-containing substances.[1] The effects of unifunctional haptens and univalent antibody fragments, produced by papain digestion of purified antibodies, suggest that the formation of multimolecular aggregates involving mutually multivalent antibody and antigen is a necessary condition for development of the wheal-and-erythema response in human skin.

Materials and Methods

Antigens.—The methods used to prepare DNP-protein conjugates and to estimate the number of DNP substituents per mole protein have been described previously (1). Crystallized ovalbumin was obtained from the Pentex Corp., Kankakee, Illinois. Human serum albumin was kindly furnished by the American Red Cross, and bovine γ-globulin was fraction II of bovine plasma supplied by Armour and Co., Chicago, Illinois.

Haptens.—Most of the low molecular weight dinitrobenzenes were obtained from commercial sources. Except for 2,4-dinitrofluorobenzene, they were recrystallized one to four times. Their melting points were in agreement with data in the literature. Previously described methods were used to prepare ε-DNP lysine (2) and ε-DNP aminocaproic acid (3).[1]

Antisera.—Rabbits were immunized by a single injection of 5 mg. EA or 5 mg. DNP-BγG in Freund's complete adjuvant (0.2 to 0.4 ml. per footpad), and were usually bled 1 month later (4).[1] Anti-EA sera from about ten rabbits were pooled, and antibody was concentrated from the pool as a γ-globulin fraction prepared by precipitation at 1.75 M ammonium sulfate, pH 7–8. Anti-EA antibody concentrations in the γ-globulin fraction and anti-DNP antibody concentrations in pooled sera were determined by quantitative precipitin analyses (4–6).

Purified Antibodies.—Purified anti-DNP antibody was isolated from pooled anti-DNP antisera by the method described in the accompanying report (4). The purified antibody was generally lyophilized and stored at about 4°. For use in skin testing, it was dissolved in 0.01 M phosphate, pH 7.4–0. 15 M NaCl and sterilized by passage through millipore[2] or Seitz filters, which had been first flushed with phosphate-saline. The protein content of the sterilized solution was determined from absorbance at 278 mμ (4). DNP-protein conjugates and haptens were similarly sterilized, and concentrations, in most cases, were measured by absorbance at 360 mμ.

Papain Digestion of Purified Antibody and Rabbit γ-Globulin.—Purified anti-DNP antibodies and the γ-globulin fraction of rabbit anti-EA sera, were digested with crystalline papain (Worthington Biochemical Corp., Freehold, New Jersey) under the conditions described by

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[1] Abbreviations used: DNP is used generically for the 2,4-dinitrophenyl group. Hence, ε-DNP lysine and ε-DNP aminocaproate are, respectively, ε-*N*-2,4-dinitrophenyl lysine and ε-*N*-2,4-dinitrophenyl aminocaproic acid. EA is hens' egg albumin. BγG is bovine γ-globulin (fraction II of bovine plasma). DNP-BγG is bovine γ-globulin whose lysine residues are almost maximally substituted with 2,4-dinitrophenyl groups.

[2] Millipore filter, type HA. Millipore Filter Corp., Bedford, Massachusetts.

Porter (7). When the digest was dialyzed in the cold against 10^{-3} M phosphate, pH 6–7, fraction III was obtained as flat, crystalline plates (7). After centrifugation to remove this crystalline fraction, the supernatant was considered to be a mixture of fractions I and II (Porter's terminology). Subsequent experience with chromatography of this supernatant on carboxymethyl cellulose[3] columns has yielded two fractions, essentially as described by Porter (7).

I^{131}-Labeling of Proteins.—Purified antibody and the fractions isolated from it by papain digestion and dialysis (fractions I and II, and fraction III) were iodinated with I^{131} (9, 10). Uncoupled I^{131}-iodide ion was removed with an anion exchange resin (amberlite, IRA-400, Cl^- form). More than 95 per cent of I^{131} in the iodinated protein was precipitated with 5 per cent trichloracetic acid. In the case of fractions I and II, precipitation with trichloracetic acid

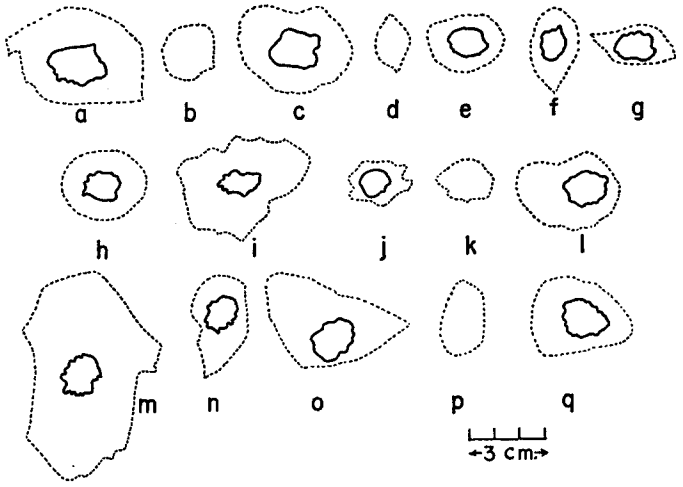

FIG. 1. Direct tracings of some of the wheal-and-erythema responses given in Tables I to III.

was unusually slow, and often required standing overnight in the cold for completion. I^{131} was counted in a well-type scintillation counter.

Skin Tests.—All injections were made intradermally with 26 gauge needles in volumes of either 0.05 or 0.1 ml. In passive transfer experiments, purified anti-DNP antibody or antibody in the form of whole serum or γ-globulin fraction was injected in normal human volunteers, the sites were marked, and 3½ to 4 hours later the same sites were challenged by intradermal injection of DNP proteins or haptens, singly or together, or mixed with the papain-split antibody fragments. For control purposes, identical injections of antigen were made at the same time in adjacent normal skin; *i.e.*, not prepared with preceding injection of antibody. In a few experiments, sites injected with antibody were not challenged until 1 to 5 days later. 15 minutes after injection of test antigen, the margins of the response were outlined in ink using a solid line for the wheal and a broken line for the erythema. A record of the response was made either by direct measurement of the major and minor axes of the wheal and of the erythema, or by applying transparent tape[4] to the area. When the tape was removed, it carried with it the tracing on the skin and furnished a permanent record of the response. The results were

[3] Type 40 (reagent grade), Brown Paper Co., Berlin, New Hampshire.

[4] "Scotch tape." Minnesota Mining and Manufacturing Co.

scored as follows: 0, a slightly raised bleb (no pseudopodia), corresponding in size to the original injection. In an occasional subject, such blebs were surrounded by a narrow zone of erythema (at most 2 to 3 mm.). ±, a bleb as in 0, but with slightly more erythema. +, a typical wheal with pseudopodia up to 15 mm. average diameter with surrounding erythema. ++, a wheal of average diameter greater than 15 mm., with the surrounding erythematous zone being two to three times, or more, greater than the wheal. The actual tracings of some critical responses in Tables I to III are given in Fig. 1, and the responses in these tables are recorded only in terms of the arbitrary scoring units, 0 to ++. For the responses in Tables IV to VI, the actual measurements of the wheal and erythema are given.

RESULTS

The present study became feasible when it was realized that one of us (HNE), who has been exposed over a period of years to protein-reactive 2,4-dinitrobenzenes, gives wheal-and-erythema reactions on intradermal injection of DNP-protein conjugates. Systematic studies of the responses of this subject to a number of DNP-containing substances are summarized in Table I. Low molecular weight dinitrobenzenes did not evoke a response although a DNP-protein conjugate, having as little as 1 mμeq. DNP, did.[5] Although the low molecular weight substances did not elicit responses, they were immunologically reactive in the skin, their activity being demonstrated by inhibition of the response to DNP-HSA. The order of inhibitory effectiveness was ε-DNP lysine > ε-DNP aminocaproate > 2,4-dinitrophenol.

Porter has shown that under certain conditions rabbit γ-globulins are split by papain into three nearly equal pieces, designated as fractions I, II, and III (7). Fractions I and II each bear one of the two antigen-reactive regions of the intact antibody molecules, while fraction III, which is readily obtainable as a crystalline protein, lacks these reactive regions (7, 8). When an unresolved mixture of I, II, and III (prepared by papain digestion of purified rabbit anti-DNP antibody) was mixed with DNP-HSA and injected, the wheal-and-erythema response was inhibited. When III was separated from the digestion mixture by crystallization, the supernatant which consisted primarily of I + II, was shown to account for the inhibitory activity of the total digest. Crystalline fraction III had no inhibitory activity (Table I).

The specificity of inhibition by DNP-haptens and by fraction I + II of papain-digested anti-DNP antibody is apparent from their failure to influence wheal-and-erythema responses to ragweed, elicited in the same subject (Table I).

The responses obtained in the actively sensitized subject were duplicated in all respects in normal human subjects in whom skin sites were sensitized by intradermal injection of 5 to 140 μg. of purified rabbit anti-DNP antibody. The passively conditioned sites gave wheal-and-erythema responses to DNP-HSA.

[5] A solution of a DNP-protein conjugate has 10 mμeq. DNP/ml. when its absorbance at 360 mμ is 0.174.

TABLE I

*Wheal-and-Erythema Responses of a Human Subject Sensitive to the 2,4-Dinitrophenyl Group**

Test No.	Substance injected	Quantity		Response	Reference to tracing in Fig. 1
		As DNP	As protein		
		mμeq.	*μg.*		
1	ϵ-DNP lysine	410	–	0	
2	ϵ-DNP-aminocaproate	420	–	0	
3	2,4-Dinitrophenol	4600	–	0	
4	2,4-Dinitrobenzene sulfonate	480	–	0	
5	2,4-Dinitrochlorobenzene	4	–	0	
6	2,4-Dinitrofluorobenzene	6	–	0	
7	DNP-HSA	1.5	4	++	a
8	DNP-HSA +	1.5	4	0	b
	ϵ-DNP lysine	460	–		
9	DNP-HSA +	1.5	4	±	
	2,4-dinitrophenol	4600	–		
10	DNP-HSA	5	10	++	c
11	DNP-HSA +	5	10	0	
	ϵ-DNP lysine	110	–		
12	DNP-HSA +	5	10	±	d
	ϵ-DNP-aminocaproate	450	–		
13	DNP-HSA +	2.5	5	0	
	total papain digest of purified anti-DNP antibody	–	280		
14	DNP-HSA	1	2	+	
15	DNP-HSA +	1	2	0	
	fractions I + II of anti-DNP antibody	–	175		
16	DNP-HSA +	1	2	+	
	fraction III of anti-DNP antibody	–	111		
17	Ragweed extract (100 units)‡	–	–	+	e
18	Ragweed extract (100 units) +	–	–	+	f
	ϵ-DNP lysine	190	–		
19	Ragweed extract (100 units) +	–	–	+	
	ϵ-DNP-aminocaproate	450	–		
20	Ragweed extract (100 units) +	–	–	+	g
	fractions I + II of anti-DNP antibody	–	436		

* All injections were in 0.1 ml. volumes. Subject: H. N. E. Mixtures of DNP-HSA (or ragweed extract) and papain digestion products of anti-DNP antibody were incubated for 30 minutes at 37° before injection.

‡ Extract of ragweed pollen, 1000 protein N units/ml. The failure of haptens or papain-split antibody fragments to inhibit responses to ragweed was confirmed by testing another subject (R. O.) who gave large wheals on injection of 10 units of ragweed extract.

TABLE II

*Wheal-and-Erythema Responses in Normal Human Skin Produced with Rabbit Anti-2,4-Dinitrophenyl Antibodies and Their Specific Inhibition with Haptens**

Subject	Test No.	Skin site prepared with*, ‡	Tested with			Result	Reference in Fig. 1§
			Substance	Quantity			
				As DNP	As protein		
				mμeq.	*μg.*		
MK	1	Rabbit antiserum,‖ 50 μl.	DNP-HSA	5	10	0	
"	2	Anti-DNP antibody, 170 μg.	DNP-HSA	5	10	+	h
"	3	" " " , 170 "	DNP-HSA + ε-DNP lysine	5 240	10 —	0	
FSF	4	Rabbit antiserum,‖ 50 μl.	DNP-HSA	5	10	0	
"	5	Anti-DNP antibody, 170 μg.	DNP-HSA	5	10	+	i
"	6	" " " , 170 "	DNP-HSA + ε-DNP lysine	5 240	10 —	0	
WJ	7	Anti-DNP antibody, 56 μg.	DNP-HSA	5	10	+	j
"	8	" " " , 56 "	DNP-HSA + ε-DNP lysine	5 110	10 —	0	
"	9	" " " , 56 "	DNP-HSA + ε-DNP aminocaproate	5 450	10 —	±	k
AK	10	Anti-DNP antibody, 23 μg.	DNP-HSA	5	10	++	l
"	11	" " " , 23 "	DNP-HSA + ε-DNP lysine	5 110	10 —	0	
"	12	" " " , 23 "	DNP-HSA + 2,4-dinitrophenol	5 440	10 —	+	
"	13	γ-Globulin fraction of rabbit anti-EA, 22 μg.	DNP-HSA	5	10	0	

* Sites were prepared by intradermal injection with 0.05 to 0.1 ml. anti-DNP antibody or a γ-globulin fraction of rabbit anti-EA serum. 3 to 4 hours later 0.1 ml. of a test substance was injected into each site and, for control purposes, into an unprepared site in the same recipient.

‡ Anti-DNP antibody refers to purified antibody, about 90 per cent specifically precipitable (see reference 4). The purified antibody used in tests 2, 3, 5, and 6 had been prepared from the same antiserum (pool VI) used in tests 1 and 4.

§ Actual tracings of the responses are given above the corresponding letter in Fig. 1.

‖ The antiserum (pool VI) contained 1.87 mg. antibody/ml., precipitated with DNP-BγG. Hence 50 μl. had 89 μg. anti-DNP antibody (*i.e.* 95 per cent of the antibody precipitated with this antigen is anti-DNP; see reference 4). Other sites prepared with 50 μl. of this pool were tested 21 hours later with DNP-HSA, and were also negative.

These reactions were inhibited in descending order of effectiveness by ε-DNP lysine, ε-DNP aminocaproate, and by 2,4-dinitrophenol (Table II). They were also inhibited by a papain digest of purified rabbit anti-DNP antibody, and by the same digest from which fraction III had been removed (by crystallization at

TABLE III

*Specific Inhibition by Univalent Antibody Fragments of Wheal-and-Erythema Responses in Human Skin Produced with Rabbit Anti-2,4-Dinitrophenyl Antibodies**

Subject	Test No.	Skin sites prepared with	Tested with			Result	Reference to tracing in Fig. 1‡
			Substance	Quantity			
				As DNP	As protein		
				mμeq.	*μg.*		
AdeW	1	Anti-DNP antibody, 140 μg.	DNP-HSA	5	10	++	m
"	2	Total papain digest of anti-DNP antibody, 120 μg.	DNP-HSA	5	10	0	
"	3	Anti-DNP antibody, 140 μg.	DNP-HSA + total papain digest of anti-DNP antibody	2.5 —	5 280	+	n
WW	4	Anti-DNP antibody, 110 μg.	DNP-HSA	5	10	++	o
"	5	" " " , 110 "	DNP-HSA + fractions I + II of anti-DNP antibody	5 —	10 218	±	p
"	6	" " . " , 110 "	DNP-HSA + fractions I + II of rabbit anti-EA γ-globulin	5 —	10 210	++	q

* Sites were prepared initially by intradermal injection of 0.1 ml. purified rabbit anti-DNP antibody or a papain hydrolysate of the same antibody. 3 to 4 hours later the sites were reinjected with 0.1 ml. of antigen (DNP-HSA), or antigen mixed with papain-digested anti-DNP antibody, or anti-EA γ-globulin fraction, or fractions I + II of the corresponding papain hydrolysates.

‡ Tracings of the responses are given above the corresponding letter in Fig. 1.

pH 6-7). The specificity of the inhibition by papain-split products is evident from the absence of inhibition by fractions I + II obtained by papain digestion of the γ-globulin fraction of rabbit anti-EA (Table III).

As expected from the foregoing results, sites injected with the γ-globulin fraction of rabbit anti-EA serum did not respond to subsequent injection of DNP-

HSA (Table II). What is especially interesting, however, is that sites injected with a papain digest of anti-DNP antibody did not respond to DNP-HSA (Table III). In view of the latter observation, comparison was made of the rates at which purified antibody and the fragments split from it by papain disappeared from skin sites. For this purpose, two normal human volunteers were injected intradermally with the several proteins labeled with I^{131} (see Methods), and I^{131} at the skin sites was measured, with time, using a collimated probe-type scintil-

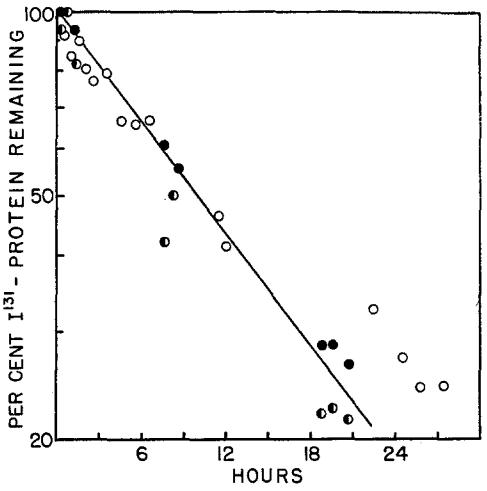

FIG. 2. Disappearance from human skin of purified rabbit anti-DNP antibody and the fragments split from this antibody by papain. One volunteer (R. P.) was injected intradermally with 150 μg. I^{131}–antibody (~4 μc.). A second volunteer (W. G.) was injected in one site with 250 μg. I^{131}–fractions I + II (~1 μc.) and in another site with 80 μg. I^{131}–fraction III (~1 μc.). The zero time values at the injected sites were 30,000, 5400, and 1800 C.P.M., respectively. A standard was counted at each time point to correct for radioactive decay and instrument fluctuation. The curve is drawn for a 1st order process with half-life 10 hours. Purified antibody (○). Fractions I + II (●). Fraction III (◐).

lation counter. The results, shown in Fig. 2, demonstrate little, if any, difference in the rates at which the several I^{131}-proteins disappear from inoculated intradermal sites over the period of interest in these experiments.

It is important to note that in two subjects skin sites which were prepared with unfractionated anti-DNP serum (0.05 ml. containing about 90 μg. anti-DNP antibody) did not react to DNP-HSA. In these same subjects, however, purified anti-DNP antibody isolated from the same serum was competent in preparing sites for the wheal-and-erythema response (Table II). The incompetence of whole antiserum could arise from competition between anti-DNP antibodies and other γ-globulins in serum for some unknown reactant in skin. This possibility was evaluated by comparing the capabilities of mixtures which

contained a constant amount of purified anti-DNP antibody and varying amounts of a γ-globulin fraction prepared from rabbit anti-EA serum. The results are given in Table IV.

It has been suggested that non-precipitating antibodies are uniquely competent to mediate wheal-and-erythema responses (11). With this suggestion in mind, anti-DNP antibody purified from a specific precipitate made in the antibody excess region of a precipitin curve was compared with antibody purified from an equivalence region precipitate. The latter preparation is expected to be contaminated with non-precipitating anti-DNP antibodies, if they exist in the

TABLE IV

*Interference by Indifferent Rabbit γ-Globulins in Wheal-and-Erythema Responses Produced by DNP-Protein and Purified Rabbit Anti-DNP Antibody**

Skin sites prepared with		Responses‡		
Purified anti-DNP antibody	γ-Globulin fraction of rabbit anti-EA serum§	Wheal	Erythema	Rating
μg.	*μg.*	*mm.*	*mm.*	
56	0	12 × 10	25 × 18	+
56	56	15 × 12	35 × 19	+
56	380	0	10 × 16	0
56	760	0	10 × 12	0

* Subject: WJ.

‡ 15 minutes after injection of 0.1 ml. DNP-HSA (5 mμeq. DNP, 10 μg. protein) intradermally in skin sites which had been injected 4 hours previously with 0.1 ml. containing the substances shown in the first two columns.

§ For inability of γ-globulin of rabbit anti-EA serum to prepare sites for specific response to DNP-HSA, see Table II, test 13.

present anti-DNP sera. As shown in Table V, both preparations were equally competent in preparing skin for wheal-and-erythema responses.

One further attempt was made to test the possibility that something other than precipitating anti-DNP antibody prepares skin sites for DNP-specific wheal-and-erythema responses. In this experiment purified antibody was precipitated with DNP-BγG, and the antibody was reisolated from the precipitate by the procedure previously described (4). This twice purified antibody was similarly precipitated, and from the precipitate a three times purified antibody was obtained. The once, twice, and three times purified preparations were found to be essentially indistinguishable (see Table V), although 90 per cent of the protein present in the once purified antibody was sacrificed in obtaining from it the twice and three times purified proteins.

The persistence of responsiveness in passively prepared skin sites was examined in four subjects in whom comparison was made of the responses to DNP-

HSA 4 hours and 1 to 5 days after injecting sites with anti-DNP antibody. The results of this comparison, summarized in Table VI, are compatible with the decay data of Fig. 2 and with the fact that sites given as little as 5 μg. purified antibody react (3 to 4 hours later) to DNP-HSA (Table V).

TABLE V

Comparison of Skin-Sensitizing Ability of Rabbit Anti-2,4-Dinitrophenyl Antibody Isolated from Precipitates of Varying Antigen-Antibody Composition and Subjected to Successive Purification Cycles

Antibody injected*		Response‡		Antibody injected§		Response‡	
Preparation	Amount	Wheal	Erythema	Preparation	Amount	Wheal	Erythema
	μg.	mm.	mm.		μg.	mm.	mm.
Isolated from precipitate in antibody excess	140	14 × 15	43 × 40	Once purified	23	12 × 17	25 × 40
	70	13 × 12	65 × 25		12	15 × 13	35 × 23
	26	12 × 11	30 × 30		4.6	12 × 11	15 × 18
	13	10 × 15	40 × 35		2.3	0	16 × 10
	7	12 × 18	32 × 16				
				Twice purified	28	17 × 12	19 × 42
Isolated from precipitate in equivalence region	130	15 × 13	27 × 32		14	16 × 14	40 × 28
	65	15 × 15	37 × 31		5.6	17 × 11	25 × 12
	24	14 × 15	46 × 24		2.8	0	16 × 11
	12	16 × 10	50 × 30				
	7	14 × 11	44 × 15	Three times purified	24	18 × 14	35 × 22
					12	10 × 10	13 × 16
					4.8	0	15 × 15
					2.4	0	13 × 12

* The precipitate in "antibody excess" contained one-fourth the amount of antibody maximally precipitable at equivalence region. The precipitate in equivalence region contained the maximal amount of antibody that could be precipitated from the given volume of antiserum. Subject: JW.

‡ 4 hours after injecting the indicated amounts of purified antibody intradermally each site was reinjected with DNP-HSA (2.5 mμeq. DNP, 5 μg. protein).

§ For the distinction between once, twice, and three times purified antibody see text. Subject: AK.

In one preliminary titration, the magnitude of the urticarial response varied only slightly with the amount of antigen injected into passively prepared sites. Several skin sites of a normal subject (E.H.) were first injected intradermally, each with 130 μg. purified anti-DNP antibody. 4 hours later 0.1 ml. aliquots containing varying amounts of DNP-HSA (1 mμeq. DNP = 2 μg. protein) were injected into the sites. The results (in millimeters; W is wheal and E is erythema) were as follows:—1 mμeq. DNP (12 × 10W; 32 × 30E), 5 mμeq. DNP (13 × 11W; 33 × 34E), 25 mμeq. DNP (17 × 17W; 44 × 38E), 50 mμeq. DNP (16 × 14W; 44 × 31E).

Serum from the actively sensitized human subject (Table I) did not precipitate with DNP-protein added to it in widely varying amount and kept at near 4° for up to 1 month. This serum did not, moreover, passively sensitize the skin of normal recipients (to DNP-protein) when injected undiluted (0.1 ml.). A

TABLE VI

Persistence of Wheal-and-Erythema Reactivity in Sites Passively Sensitized with Purified Rabbit Anti-2,4-Dinitrophenyl Antibody

Subject	Amount anti-DNP antibody injected	Time of test injection*	Response	
			Wheal	Erythema
	μg.		mm.	mm.
MK	170	4 hrs.‡	14 × 11	27 × 38
"	170	1 day	13 × 8	43 × 20
FSF	170	4 hrs.§	15 × 10	50 × 40
"	170	1 day	15 × 10	42 × 42
AdeW	140	4 hrs.‖	17 × 16	82 × 50
"	140	2 days	15 × 10	67 × 35
EH	130	5 hrs.	13 × 11	33 × 34
"	130	3 days	10 × 8	19 × 19
"	130	5 "	0	0

* Tests made with 0.1 ml. DNP-HSA (5 mμeq. DNP, 10 μg. protein) injected intradermally, after the indicated period, into sites previously injected with antibody.

‡ Test 2, Table II.

§ Test 5, Table II.

‖ Test 1, Table III.

crude attempt was made, therefore, to isolate and concentrate anti-DNP antibody from this serum by the following procedure:

60 ml. serum was mixed with 60 mg. of DNP-BγG (60 moles DNP per 160,000 gm. protein) that had been made virtually insoluble by treatment with a detergent (12). 35 mg. of streptomycin sulfate was added (4) and the mixture was kept for 60 minutes at 4°. After centrifugation the DNP-BγG sediment was washed and extracted at 37° with 0.1 M 2,4-dinitrophenol in 0.01 M phosphate, pH 7.4–0.15 M NaCl (4). The extract was passed through a Dowex-1 column (Cl^- form), and the protein-containing effluent (3 ml.) was dialyzed overnight at 4° against phosphate-NaCl. Absorbance at 278 mμ indicated that the dialyzed material contained about 1 mg. protein/ml., but only 30 μg. protein/ml. was maximally precipitated in a precipitin analysis using DNP-BγG as test antigen. 0.1 ml. aliquots were injected into skin sites of six human recipients. Three of these gave a small wheal-and-erythema response on subsequent injection of DNP-HSA, but three other recipients failed to react. Assuming that the recovery of antibody from serum was about 40 per cent (4), we tentatively estimate that this subject's serum contained about 3 μg. anti-DNP antibody/ml.

DISCUSSION

The most direct and unambiguous means for identifying the antigenic determinant of an antibody-dependent reaction is through inhibition with chemically defined substances that are structurally related to the immunizing agent (hapten inhibition). The present work demonstrates the applicability of this general method to wheal-and-erythema responses. Hapten inhibition of the response in human skin sites which had been prepared by local injection of purified rabbit antibody is not surprising since monovalent haptens inhibit anaphylaxis and a number of related immediate type allergic reactions (13–15). In view of the obscurity and controversy surrounding the human antibody responsible for wheal-and erythema response (16), it could not have been anticipated with certainty, however, that in the actively sensitized human subject, whose wheal-and-erythema reaction to DNP-protein conjugates was classic in morphology and time course, that hapten inhibition could here also be clearly demonstrable. Aside from its pathogenetic significance, hapten inhibition furnishes the only means for identifying the determinant involved in allergic skin reactions.

The order of inhibitory effectiveness of the haptens tested was: ϵ-DNP lysine > ϵ-DNP aminocaproic acid > 2,4-dinitrophenol. This order is consistent with the affinities of these haptens for anti-DNP antibodies (ϵ-DNP lysine = ϵ-DNP aminocaproate ≫ 2,4-dinitrophenol, see references 4 and 8), except that ϵ-DNP aminocaproate was less effective than ϵ-DNP lysine (Tables I and II). The decreased inhibitory activity of ϵ-DNP aminocaproate *in vivo* may be ascribed to its reversible binding by serum albumin (3), which is present in the skin (17).

It is generally believed that a fundamental property of immediate type allergic reactions is their amenability to transfer (to insensitive recipients) with serum derived from sensitive donors. This property is not, however, always demonstrable. For example, with serum from human beings who gave wheal-and-erythema responses to dextran, Kabat was unable to obtain positive transfer responses on intradermal injections of quantities of serum containing up to 50 μg. anti-dextran antibody (16). Likewise, in the present work, undiluted serum from the subject who reacted to DNP-HSA did not transfer reactivity to normal recipients. These failures with whole serum are readily accounted for by the observation that 0.05 ml. of a whole (unfractionated) rabbit antiserum pool, containing 90 μg. precipitating anti-DNP antibody, was incapable of preparing skin sites to react to DNP-HSA, yet 5 μg. of purified antibody, isolated from the same antiserum, was competent (Table V). Provisionally, we ascribe the unequal effectiveness of antibody in serum and of purified antibody to competition between antibodies of a given specificity and indifferent γ-globulins, possibly for restricted skin sites. Similar competition has been observed in passive cutaneous anaphylaxis (18, 19). It is necessary, therefore, in evaluating the competency of a given antigen-antibody system to take into account not only the amount of antibody of appropriate specificity, but, at the same time, the

amount of indifferent γ-globulin in the test solution. Purified antibodies become, in this respect, virtually necessary for assessing the competency of a given system.

On the foregoing grounds it is possible to reconcile the incompetence of the present rabbit anti-DNP sera (unfractionated) with Wood's successful transfers using rabbit antipneumococcal polysaccharide sera (20). In our sera, anti-DNP antibody is generally about 1 to 3 mg./ml., and amounts to not more than 10 to 15 per cent of the total γ-globulin. In Wood's sera, which had been prepared for therapeutic use, there were 10,000 units of antibody/ml. and it is reasonable to assume that in such preparations perhaps 30 per cent of the total γ-globulin was antibody of the designated specificity. The minimal effective amount of unfractionated serum was found by Wood to be 0.0001 ml.; *i.e.*, 1 unit or 5.7 μg. antibody[6] in a total of about 18 μg. rabbit γ-globulin. With the DNP system, positive reactions have been obtained with 5 μg. purified antibody and significant inhibition was noted when the weight ratio of indifferent γ-globlulin:anti-DNP antibody was about 6. The results obtained with rabbit antipneumococcal polysaccharide antibody and with rabbit anti-DNP antibody are, therefore, in remarkably good agreement. Quantitative comparison between these systems is of interest also in respect to the concentrations of circulating serum antibodies associated with skin responsiveness. Wood found that human beings treated intravenously with rabbit antipneumococcal polysaccharide sera gave wheal-and-erythema responses to the corresponding polysaccharide when free antibody levels in serum exceeded 0.1 unit (0.5 μg.) antibody/ml. (20). In the present study it is estimated that there was about 3 μg. anti-DNP antibody/ml. serum of the actively sensitized human subject (Table I).

Of the three distinguishable components in papain digests of rabbit γ-globulin, fractions I and II are of particular interest. On the basis of their ability to inhibit precipitation specifically, Porter (7) inferred that these fragments have 1 antigen-combining region per mole (55,000 molecular weight). Titration with hapten of the fractions isolated from papain split anti-DNP antibody has confirmed the univalence of fractions I and II, and demonstrated that the sites on these fragments interact just as strongly with homologous haptens as do the corresponding sites of intact, bivalent, antibody (8). It is especially interesting to note, therefore, that these fractions (I + II) were not capable of preparing skin sites for wheal-and-erythema responses. These fragments could only, in fact, specifically inhibit the response in otherwise competent sites. From the equal persistence in skin sites of I^{131}-labeled antibody and I^{131}-labeled fractions I + II, the incompetency of the split products cannot be ascribed to their rapid disappearance from injected skin sites (Fig. 2).

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[6] In the case of rabbit antipneumococcal polysaccharide, type I, 1 mg. antibody N, isolated in a precipitin reaction, corresponds to 1100 mouse protective units (21).

The only activity exhibited by univalent haptens and univalent antibody fragments was inhibition of both active and passive wheal-and-erythema responses. We conclude, therefore, that mutually multivalent antibody and antigen are necessary for this allergic reaction in human skin. By analogy with the precipitin reaction, the requirement for mutual multivalency indicates that rather large multimolecular complexes, having as a minimum 2 antigen and 2 antibody molecules ($G_{>2} \cdot B_{>2}$), are essential for this response. These findings are in agreement with Ishizaka and Campbell who found that passive cutaneous anaphylaxis (in guinea pigs) is dependent on the composition of antigen-antibody complexes: those composed of 2 antigen molecules and 1 antibody molecule ($G_2 \cdot B$) did not have activity, whereas $G_3 \cdot B_2$ and more complicated complexes did (22).

The prompt and transient inflammatory response produced with the aid of rabbit antibody in human skin (wheal-and-erythema) resembles closely the corresponding response of rat and guinea pig skin (passive cutaneous anaphylaxis). Both responses are characterized by transient increases in capillary permeability, and in both immunologically indifferent serum proteins (γ-globulins) reduce the effectiveness of a given amount of antibody (Table IV and references 18, 19). Both responses can, moreover, be specifically inhibited by unifunctional haptens (Table II and reference 15), and require large antigen-antibody complexes in which each reactant is represented by more than 1 molecule (see above, and reference 22). The latter requirement has been correlated with the ability of competent complexes to bind complement (23, 24).

While there is a high degree of consistency in the immunologic characteristics of passive cutaneous anaphylaxis and of human wheal-and-erythema responses to certain determinants (pneumococcal polysaccharides, 2,4-dinitrophenyl group, and probably dextran), these systems do not yet afford a satisfactory explanation for the peculiar and striking skin-reactive properties of certain human "reaginic" sera, the case of sera from ragweed-sensitive individuals being the best known example. Intradermal injection of normal human beings with unfractionated human serum of this type transfers wheal-and-erythema reactivity to ragweed extracts. But these sera give no precipitation on addition of antigens which are effective in eliciting the wheal-and-erythema response. While a number of possible explanations may be visualized as accounting for the differences between ragweed and related systems and the more thoroughly defined systems mentioned above, it seems likely that a satisfactory reconciliation of the discrepancies will require the availability of purified and characterized reactants of reaginic systems.

SUMMARY

Wheal-and-erythema responses were studied in normal human volunteers and in a single human subject who is sensitive to the 2,4-dinitrophenyl group. In the normal subjects, reactive skin sites were established by intradermal

injection of purified rabbit antibody specific for the 2,4-dinitrophenyl group. In both the active and passively sensitized subjects, wheal-and-erythema was elicited by intradermal injection of a 2,4-dinitrophenyl protein, but not by injection of the same conjugate mixed with certain low molecular weight 2,4-dinitrophenyl haptens or with univalent fragments split by papain from anti-2,4-dinitrophenyl antibody. The latter fragments, unlike intact, bivalent, antibody, do not sensitize normal human skin sites. From these and other observations it is concluded that the wheal-and-erythema response in human skin requires mutually multivalent antigen and antibody. This requirement suggests that multimolecular complexes, containing at least 2 antigen and 2 antibody molecules, are essential in the pathogenesis of this allergic response.

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BIBLIOGRAPHY

1. Eisen, H. N., Kern, M., Newton, W. T., and Helmreich, E., A study of the distribution of 2,4-dinitrobenzene sensitizers between isolated lymph node cells and extracellular medium in relation to induction of contact skin sensitivity, *J. Exp. Med.*, 1959, **110,** 187.
2. Porter, R. R., Use of 1:2:4 fluorodinitrobenzene in studies of protein structure, *Methods Med. Research*, 1950, **3,** 256.
3. Carsten, M., and Eisen, H. N., The interaction of dinitrobenzene derivatives with bovine serum albumin, *J. Am. Chem. Soc.*, 1953, **75,** 4451.
4. Farah, F. S., Kern, M. and Eisen, H. N., The preparation and some properties of purified antibody specific for the 2,4-dinitrophenyl group, *J. Exp. Med.*, 1960, **112,** 1195.
5. Gitlin, D., Use of ultraviolet absorption spectroscopy in the quantitative precipitin reaction, *J. Immunol.*, 1949, **62,** 437.
6. McDuffie, F. C., and Kabat, E. A., A comparative study of methods used for analysis of specific precipitates in quantitative immunochemistry, *J. Immunol.*, 1956, **77,** 193.
7. Porter, R. R. The hydrolysis of rabbit γ-globulin and antibodies with crystalline papain, *Biochem. J.*, 1959, **73,** 119.
8. Velick, S. F., Parker, C. W., and Eisen, H. N., Excitation energy transfer in the study of specific antibody reactions, *Proc. Nat. Acad. Sc.*, 1960, in press.
9. Eisen, H. N., and Keston, A. S., The immunologic reactivity of bovine serum albumin labeled with trace-amounts of radioactive iodine (I^{131}), *J. Immunol.*, 1949, **63,** 71.
10. Kern, M., Helmreich, E., and Eisen, H. N., A demonstration of antibody activity on microsomes, *Proc. Nat. Acad. Sc.*, 1959, **45,** 862.
11. Kuhns, W. J., Certain forms of hypersensitivity in man mediated by antigen-antibody reactions, *in* Cellular and Humoral Aspects of the Hypersensitive States, (H. S. Lawrence, editor), New York, P. B. Harper, Inc., 1959, 535.
12. Lundgren, H. P., Synthetic protein fibers from protein-detergent complexes, *Textile Research J.*, 1945, **15,** 335.
13. Tillett, W. S., Avery, O. T., and Goebel, W. F., Chemoimmunological studies on

conjugated carbohydrate-proteins. III. Active and passive anaphyalxis with synthetic sugar-proteins, *J. Exp. Med.*, 1929, **50,** 551.

14. Gell, P. G. H., Sensitization to "tetryl," *Brit. J. Exp. Path.*, 1944, **25,** 174.
15. Ovary, Z., and Karush, F., Antibody-hapten interactions studied by passive cutaneous anaphylaxis in the guinea pig, *J. Immunol.*, 1960, **84,** 409.
16. Kabat, E. A., Problems in the study of allergic antibodies, *Proc. 3rd Internat. Congr. Allergology*, 1958, 167.
17. Rothschild, M. A., Bauman, A., Yalow, R. S., and Berson, S. A., Tissue distribution of I^{131}-labeled human serum albumin following intravenous administration, *J. Clin. Inv.*, 1955, **34,** 1354.
18. Ovary, Z., and Bier, O. G., Action empêchante du serum normal de lapin sur l'anaphylaxie cutanée passive du cobaye, *Ann. Inst. Pasteur*, 1953, **84,** 443.
19. Ishizaka, K., and Campbell, D. H., Biological activity of soluble antigen-antibody complexes. IV. The inhibition of the skin reactivity of soluble complexes and the PCA reaction by heterologous complexes, *J. Immunol.*, 1959, **83,** 116.
20. Wood, W. B., Jr., The control of the dosage of antiserum in the treatment of pneumococcal pneumonia. I. A study of the mechanism of the skin reaction to type specific polysaccharide, *J. Clin. Inv.*, 1940, **19,** 95.
21. Gerlough, T. D., Palmer, J. W., and Blumenthal, R. R., Studies on antipneumococcic rabbit serum. II. The relation between mouse protective units and precipitin-nitrogen, *J. Immunol.*, 1940, **39,** 163.
22. Ishizaka, K., Ishizaka, T., and Campbell, D. H., The biological activity of soluble antigen-antibody complexes. II. Physical properties of soluble complexes DAving skin-irritating activity, *J. Exp. Med.*, 1959, **109,** 127.
23. Ishizaka, K., and Campbell, D. H., Biological activity of soluble antigen-antibody complexes. III. Various antigen-antibody systems and the probable role of complement, *J. Immunol.*, 1959, **83,** 105.
24. Osler, A. G., Hawrisiak, M. M., Ovary, Z., Siqueira, M., and Bier, O. G., Studies on the mechanism of hypersensitivity phenomena. II. The participation of complement in passive cutaneous anaphylaxis of the albino rat, *J. Exp. Med.*, 1957, **106,** 811.