THE SPECIFICITY OF ALLERGIC REACTIONS

I. Delayed versus Arthus Hypersensitivity

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Injection of foreign protein into a guinea pig may induce delayed hypersensitivity, followed by circulating antibody and Arthus-type hypersensitivity (1, 2). If the antigenic dose is sufficiently minute, delayed hypersensitivity is not followed by the appearance of detectable amounts of circulating antibody (3). If a guinea pig with delayed hypersensitivity is, however, again stimulated with the specific homologous antigen, an anamnestic response appears, wherein circulating antibody and Arthus-type hypersensitivity develop.

The suggestion has been made that delayed hypersensitivity is a step in the formation of circulating antibody (2). The question then arises whether delayed hypersensitivity has a more primitive type of specificity than Arthus reactions and circulating antibody. The present paper describes experiments which indicate that the specificity governing delayed hypersensitivity is different from that governing circulating antibody. The delayed response, as illustrated by experiments with protein conjugates and avian albumins as antigens, is produced in response to a broad general area of the antigen molecule, whereas the reactions of circulating antibody are controlled by small, specific groupings of the antigen. These studies also indicate that delayed hypersensitivity is an intermediate stage in the formation of circulating antibody.

Materials and Methods

Animals.—Guinea pigs of the Hartley strain weighing 400 to 500 gm. were used for studies on sensitization and immunization. White or albino guinea pigs weighing from 300 to 400 gm. were employed for studies on passive cutaneous anaphylaxis (PCA).

Antigens.—

Hen egg albumin (HEA): Five times recrystallized hen egg albumin was obtained from the K & K Laboratories, Inc., Jamaica, New York.

Duck egg albumin (DEA): Whites of four dozen duck eggs were separated, diluted with an equal volume of water, and strained through cheese cloth. Globulins were precipitated with an equal volume of saturated (room temperature) ammonium sulfate and filtered out. Albumin was then precipitated by acidification of the supernatant to pH 4.7. Repeated attempts at crystallization were unsuccessful.

The albumin was redissolved, dialyzed against acetate buffer of 0.02 ionic strength and pH 5.9, and the resulting solution chromatographed on a 1 liter column containing about 40 gm. diethylaminoethyl cellulose (DEAE). The DEAE was regenerated with 0.1 M NaH_2PO_4, followed by 0.1 M NaOH + 0.5 M sodium acetate, then by acetate buffer pH 5.9 ionic strength 0.2, and finally by acetate buffer pH 5.9 at ionic strength 0.02. 250 ml. of 1 per cent protein solution were poured into the column and eluted with stepwise additions of 200 ml. each of acetate buffer 0.04 ionic strength, pH 5.9 with no NaCl, 0.06 M NaCl, and 0.12 M NaCl. The material eluted by the 0.12 M NaCl was dialyzed free of salt and lyophilized.

Goose egg albumin (GEA): Goose eggs were procured locally and the albumin purified in the same way as duck egg albumin.

Bovine gamma globulin (BGG): Armour purified bovine gamma globulin was used without further treatment.

Criteria of Purity.—The 3 egg albumins were examined by ultracentrifugation, by Tiselius moving-boundary electrophoresis, and by Ouchterlony agar diffusion techniques against guinea pig anti-HEA and anti-GEA sera.

Ultracentrifuge patterns of the proteins in phosphate buffer pH 7.1, ionic strength 0.02 and acetate pH 5.0, ionic strength = 0.02, all showed a single symmetrical peak.

Electrophoresis patterns in phosphate buffer pH 7.0, ionic strength 0.1, all showed single peaks but were not absolutely symmetrical. The HEA and GEA were slightly asymmetrical, while the DEA had a slightly more pronounced shoulder on the leading edge of the descending peak.

In Ouchterlony agar diffusion plates, anti-HEA serum produced single bands of precipitate with HEA, GEA, or DEA.

Conjugates.—1-fluoro,2,4-dinitrobenzene (DFB) or picryl chloride (PiCl) were conjugated with HEA or BGG (4). After dialysis, with stirring, in the cold against many changes of distilled water, the conjugates were centrifuged to remove insoluble material and analyzed (*a*) by evaporation of measured aliquots to dryness and subsequent weight determination and (*b*) by micro-Kjeldahl technique. The two methods agreed within 3 per cent in spite of the high content of nitrate nitrogen in the conjugates.

Sensitization.—Antigens were dissolved in physiologic saline plus 1 per cent normal guinea pig serum or in 1 per cent Difco peptone water, and then emulsified with an equal volume of Freund's adjuvant (Difco), without mycobacteria. Guinea pigs were sensitized with 5 μg. albumin or bovine gamma globulin, or 15 μg. conjugate in oil-water emulsion by injection of 0.5 ml. intracutaneously into the digits of the feet.

Skin Tests.—Guinea pigs were tested intradermally on the sides with 0.1 ml. of antigen containing 50 μg/ml. protein or protein-conjugate. Reactions were observed and diameters of areas of induration measured at intervals for the first 4 hours after injection and at 18 to 24 hours.

Antibody Determination.—Guinea pigs were bled just prior to skin testing, and the sera were assayed for antibody. The passive cutaneous anaphylaxis (PCA) reaction was used primarily for this purpose (5), although the hemagglutination test was used in some cases (6). In the PCA tests, 0.1 ml. test serum was injected intradermally in the flank. Three to 4 hours later, 350 μg. protein in 0.5 ml. physiologic saline and 0.5 ml. 1 per cent Evans blue in physiologic saline were introduced intravenously. Fifteen to 30 minutes later, areas of pigmentation in the skin were measured and recorded.

RESULTS

Delayed versus Arthus Reactions in Guinea Pigs Sensitized with a Single Dose of Hen Egg Albumin, Duck Egg Albumin or Goose Egg Albumin.—Guinea pigs

sensitized by injection into the foot-pads of 5.0 μg. hen egg albumin (HEA) in Freund's adjuvant showed delayed hypersensitivity to the homologous antigen on the 5th day after injection and Arthus-type hypersensitivity on the 8th to 9th day (Table I). Such animals, when skin-tested with heterologous duck egg

TABLE I

Cross-Reactions of Avian Albumins in Guinea Pigs Sensitized with 5.0 μg. Hen Egg Albumin in Adjuvant

No. guinea pigs	Day tested	Skin-testing antigen (5.0 μg.)								
		HEA			DEA			GEA		
		A	Ab	D	A	Ab	D	A	Ab	D
3	5	0	N	++	0	N	++	0	N	+
3	6	0	N	++	0	N	++	0	N	+
6	7	0	N	+++	0	N	++	0	N	+
8	8	++	3/8	++	0	N	±	0	N	0
8	9	+++	P	.	++	1/8	+	0	N	±
5	10	+++	P	.	++	1/5	±	+	1/5	0
4	11	+++	P	.	++	2/4	+	+	3/4	+
2	12	+++	P	.	+	0	+	+	1/2	+
6	13	+++	P	.	++	5/6	++	++	5/6	++
6	16	+++	P	.	+++	5/6	.	+++	P	.
2	19	++	P	.	++	P	.	++	P	.
3	21	++	P	.	++	P	.	++	P	.

Numerator of fraction indicates number of guinea pigs showing antibody by PCA test. Difference between denominator and numerator indicates animals possibly showing delayed hypersensitivity.

. = Reaction not determinable.

A, Arthus-type hypersensitivity.

AB, Circulating antibody (by PCA test (4)).

D, Delayed hypersensitivity.

P, Circulating antibody present; N, circulating antibody not detected.

0, Mean diameter of induration < 10 mm. (in animals showing particular reaction).

±, Mean diameter of induration about 10 mm.

+, Mean diameter of induration 10 to 14 mm.

++, Mean diameter of induration 15 to 19 mm.

+++, Mean diameter of induration 20 to 24 mm.

albumin (DEA) or goose egg albumin (GEA), showed different types of responses. Guinea pigs, skin-tested with 5 μg. DEA, had variable and sometimes questionable delayed hypersensitivity but did show Arthus reactions and circulating antibody to heterologous DEA. Circulating antibody to heterologous DEA appeared in some animals later than did antibody to homologous HEA, but was variable from the 9th to the 16th day following sensitization. Guinea

pigs sensitized with HEA did not show striking delayed hypersensitivity to GEA. However, during the period from the 10th to the 13th day after sensitization, Arthus reactions and circulating antibody became detectable with greater frequency and by the 16th day all animals displayed Arthus reactions and had circulating antibody. When HEA-sensitized guinea pigs were skin-tested with heterologous albumins, HEA and DEA seemed more closely related to each other than HEA and GEA. Although the delayed reactions were weak, questionable, or lacking, Arthus reactions and circulating antibody to heterologous GEA did eventually appear in all animals immunized with HEA.

This phenomenon was further exemplified in guinea pigs sensitized with

TABLE II

Cross-Reactions of Avian Albumins in Guinea Pigs Sensitized with 5.0 μg. Goose Egg Albumin in Adjuvant

No. of guinea pigs	Day tested	Skin-testing antigen (5 μg.)								
		HEA			DEA			GEA		
		A	Ab	D	A	Ab	D	A	Ab	D
7	6	0	N	+	0	N	++	0	N	++
3	7	0	N	+	0	N	+	0	N	++
7	8	0	N	+	0	N	++	++	1/7	++
6	9	0	N	+	++	1/6	+	++	2/6	++
4	10	0	N	±	++	2/4	+++	+++	P	.
4	11	0	N	0	+++	P	.	+++	P	.
2	12	0	N	+	+++	P	.	+++	P	.
5	13	++	1/5	+	+++	P	.	+++	P	.
3	16	±	N	±	+++	P	.	+++	P	.
3	19	++	2/3	±	++	P	.	++	P	.
3	21	+	1/3	0	++	P	.	++	P	.

GEA and skin-tested with homologous and heterologous antigens (Table II). Delayed responses to DEA were weaker than to homologous GEA, and Arthus reactions appeared about a day later than they did to homologous GEA. Delayed hypersensitivity to heterologous HEA was weak, questionable, or absent, and detectable antibody did not appear until the 13th to 19th day after sensitization.

Guinea pigs sensitized to DEA showed typical skin responses to the homologous antigen, with delayed responses being replaced by Arthus reactions on the 8th to 9th day (Table III). The responses to heterologous GEA were somewhat similar to those with DEA, but antibody against HEA was not produced until the 14th day after sensitization.

Delayed versus Arthus Reactions in Guinea Pigs Sensitized with Hen Egg

Albumin, Bovine Gamma Globulin, or Hapten-Protein Conjugates.—Guinea pigs were sensitized by injection into the foot-pads of 15 μg. of a protein, such as HEA or BGG, or of a hapten-protein conjugate, such as picryl bovine gamma globulin (Pi·BGG) or 1-fluoro-2,4-dinitrobenzene ovalbumin (DFB·HEA), emulsified in Freund's adjuvant without mycobacteria. In one group of experiments with guinea pigs (Table IV), animals sensitized with HEA showed on the 9th day a typical replacement of delayed hypersensitivity to the homologous protein by Arthus-type hypersensitivity. Such animals, skin-tested on the 5th to 8th days with the hapten-protein conjugate Pi·HEA, showed only weak delayed reactions, but had Arthus reactions and circulating antibody (by PCA

TABLE III

Cross-Reactions of Avian Albumins in Guinea Pigs Sensitized with 5.0 μg. Duck Egg Albumin in Adjuvant

No. of guinea pigs	Day tested	Skin-testing antigen (5 μg.)								
		HEA			DEA			GEA		
		A	Ab	D	A	Ab	D	A	Ab	D
6	6	0	N	++	0	N	++	0	N	++
3	7	0	N	++	0	N	++	0	N	++
7	8	0	N	+	++	1/7	++	++	1/7	++
4	9	0	N	+	++	2/4	++	0	N	++
5	10	0	N	+	+++	P	.	+++	4/5	.
5	11	+	2/5	±	+++	P	.	+++	P	.
4	13	±	N	±	+++	P	.	+++	P	.
3	14	+++	P	.	+++	P	.	+++	P	.
3	16	+++	P	.	+++	P	.	+++	P	.
4	21	++	P	.	++	P	.	++	P	.

test) on the 9th day. The same animals skin-tested with the conjugate DFB·HEA did not show perceptible delayed hypersensitivity, but had weak Arthus reactions to DFB·HEA starting on the 12th day and detectable antibodies on the 19th day. The inability to detect delayed hypersensitivity with DFB·HEA may be due to changes in the HEA molecule produced in the process of conjugation. No skin reactions of any kind were elicited when the guinea pigs were skin-tested with Pi·BGG or DFB·BGG conjugates or BGG alone. This tendency, in guinea pigs sensitized with whole proteins, of showing delayed hypersensitivity followed by Arthus hypersensitivity to homologous antigen, but only Arthus reactions to conjugated protein, was further illustrated when BGG was used as the sensitizing antigen, and the animals were skin-tested with BGG, Pi·BGG, DFB·BGG, and the completely heterologous Pi·HEA (Table V).

TABLE IV

Cross-Reactions in Guinea Pigs Sensitized with 5–15 μg. Hen Egg Albumin in Adjuvant

No. of guinea pigs	Day tested	Skin-testing antigen (5 μg.)*								
		HEA			Pi·HEA			DFB·HEA		
		A	Ab	D	A	Ab	D	A	Ab	D
3	5	0	N	+++	0	N	+	0	N	0
7	6	0	N	+++	0	N	+	0	N	0
2	7	0	N	+				0	N	0
4	8	0	N	++	0	N	+	0	N	0
9	9	+++	P	.	++	P	.	0	N	0
3	10	+++	P	.	++	P	.	0	N	0
3	11	+++	P	.	++	P	.	0	N	0
6	12	+++	P	.	++	P	.	±	N	.
3	13	+++	P	.	+++	P	.	±	N	.
2	15	++	P	.	++	P	.	+	N	.
3	16	++	P	.	++	P	.	+	N	.
4	19	++	P	.	++	P	.	+	P	.
2	22	++	P	.	++	P	.	±	N	±

* Guinea pigs showed neither delayed nor Arthus reactions to skin tests with 5 μg. DFB·BGG, Pi·BGG, or BGG.

TABLE V

Cross-Reactions in Guinea Pigs Sensitized with 5 μg. Bovine Gamma Globulin in Adjuvant

No. of guinea pigs	Day tested	Skin-testing antigen (5 μg.)*								
		BGG			Pi·BGG			DFB·BGG		
		A	Ab	D	A	Ab	D	A	Ab	D
2	5	0	N	0	0	N	0	0	N	0
5	6	0	N	++	0	N	±	0	N	0
3	7	0	N	+	0	N	0	0	N	0
4	8	+	2/4	++	0	1/4	0	0	N	0
3	9	++	P	.	±	2/3	0	0	N	0
3	10	++	P	.	0	N	0	0	N	0
3	12	+++	P	.	±	N	0	±	N	0
2	13	+++	P	.	+	P	.	±	N	0
4	16	++	P	.	+	3/4	0	±	N	0
3	19	++	P	.	+	P	.	+	1/3	0
3	22	+	P	.	0	N	0	0	N	0
2	27	.	P	.	.	N	.	.	N	.

* Guinea pigs showed neither delayed nor Arthus reactions to skin tests with 5 μg. HEA or Pi·HEA.

Further clarification of the specificities of delayed *versus* Arthus reactions appeared when guinea pigs were sensitized with a hapten-protein conjugate. Animals sensitized with either Pi·HEA (Table VI), DFB·HEA (Table VII), or Pi·BGG (Table VIII) developed delayed hypersensitivity followed by Arthus hypersensitivity to the homologous conjugate. Only delayed reactions developed when guinea pigs sensitized with conjugated DFB·HEA or conjugated Pi·BGG were skin-tested with the homologous unconjugated protein. In guinea pigs sensitized with Pi·HEA, antibodies and Arthus reactions ultimately appeared against HEA on the 12th day. Only Arthus reactions

TABLE VI

Cross-Reactions in Guinea Pigs Sensitized with 15 μg. Pi·HEA in Adjuvant

No. of guinea pigs	Day tested	Skin-testing antigen (5 μg.)*											
		HEA			Pi·HEA			DFB·HEA			Pi·BGG		
		A	Ab	D	A	Ab	D	A	Ab	D	A	Ab	D
2	5		N		0	N	++		N		0	N	0
5	6	0	N	++	0	N	+	0	N	±	0	N	0
3	7		N		++	2/3	.	.	2/3	.	0	1/3	0
4	8	0	N	+	+++	P	.	+++	P	.	++	1/2	0
3	9	0	N	+	+++	P	.	++	P	.	+++	P	.
5	10	0	N	+	+++	P	.	+++	P	.	++	P	.
3	12	++	P	.	+++	P	.	+++	P	.	+++	P	.
3	13	+++	P	.	+++	P	.	+++	P	.	+++	P	.
3	15	+++	P	.	+++	P	.	+++	P	.	+++	P	.
2	16	+++	P	.	++	P	.	+++	P	.	++	P	.
2	17	+	P	.	++	P	.	++	P	.	++	P	.
3	19	+	P	.	++	P	.	++	P	.	++	P	.
4	22	+	P	.	++	P	.	++	P	.	++	P	.

* Guinea pigs sensitized with 15 μg. PiCl or DFB in adjuvant showed neither Arthus reactions nor detectable circulating antibody to Pi·HEA, Pi·BGG, or DFB·HEA.

developed in animals sensitized with a conjugate and tested against another conjugate consisting of homologous hapten-heterologous protein. When a heterologous hapten-homologous protein was used to provoke reactions, delayed hypersensitivity was detected. The reactions, however, were at times weak. Arthus reactions were also elicited, but these varied somewhat in severity depending on the particular hapten-protein conjugate.

Anamnestic Response with Albumins as Antigens.—Guinea pigs given a primary dose of 0.5 μg. HEA in saline developed only delayed reactions against the antigen. When such animals were injected 8 to 10 days later with 5.0 μg. of the homologous antigen in adjuvant, an anamnestic response occurred with consequent production of antibodies and hypersensitive state of the Arthus

type (3). Since animals sensitized with 5.0 μg. HEA developed only weak delayed reactions, if any, to GEA, and *vice versa*, the question arose as to

TABLE VII

Cross-Reactions in Guinea Pigs Sensitized with 15 μg. DFB·HEA in Adjuvant

No. of guinea pigs	Day tested	Skin-testing antigen (5 μg.)											
		HEA			Pi·HEA			DFB·HEA			DFB·BGG		
		A	Ab	D	A	Ab	D	A	Ab	D	A	Ab	D
2	4	0	N	++	0	N	+	0	N	+	0	N	0
3	5	0	N	+++				0	N	+	0	N	0
4	6	0	N	++	0	N	+	0	N	+	0	N	0
2	8	0	N	++	0	N	+	0	N	+	0	N	0
3	9	0	N	+	+	2/3	.	+	2/3	.	0	2/3	0
4	10	0	N	+	++	P	.	++	P	.	+	P	.
3	12	0	N	+	++	P	.	+	P	.	+	P	.
3	19	0	N	±	++	P	.	+	P	.	+	P	.

TABLE VIII

Cross-Reactions in Guinea Pigs Sensitized with 15 μg. Pi·BGG in Adjuvant

No. of guinea pigs	Day tested	Skin-testing Antigen (5 μg.)											
		BGG			Pi·BGG			DFB·BGG			Pi·HEA		
		A	Ab	D	A	Ab	D	A	Ab	D	A	Ab	D
2	5	0	N	+	0	N	+	0	N	±	0	N	0
5	6	0	N	++	0	N	+	0	N	±	0	N	0
3	7	0	N	++	0	N	+	0	N	±	0	N	0
4	8	0	N	+	++	2/4	+	0	N	±	0	N	0
3	9	0	N	+	++	P	.	+	P	.	+	P	.
3	10	0	N	++	+	P	.	+	P	.	+	P	.
3	12	0	N	+	++	P	.	++	P	.	++	P	.
2	13	0	N	±	++	P	.	++	P	.	++	P	.
4	16	0	N	±	++	P	.	++	P	.	++	P	.
3	19	0	N	+	++	P	.	++	P	.	++	P	.
3	22	0	N	+	+	P	.	±	N	0	±	N	.

whether heterologous antigens could induce anamnestic responses to one another.

Guinea pigs were injected into the foot-pads with 0.5 μg. HEA, DEA, or GEA in saline. Eight to 10 days later, they were given a secondary dose of 5.0 μg. HEA in Freund's adjuvant, without mycobacteria. Groups of animals receiving a single dose of 0.5 or 5.0 μg. of avian albumin served as controls.

TABLE IX

Cross-Reactions in Guinea Pigs Sensitized with a Primary Dose of HEA, DEA, or GEA and a Secondary Dose of Hen Egg Albumin

Primary antigen and dose	Secondary antigen and dose	No. of guinea pigs	Day tested	HEA Ab	DEA Ab	GEA Ab
0.5 μg. HEA in saline	5.0 μg. HEA in adjuvant	4	2	0	0	0
		6	3	1/6	0	0
		12	4	7/12	0	0
		7	5	6/7	0	0
		7	6	+	2/7	2/7
		10	7	+	2/10	0
		8	8	+	1/8	0
		4	9	+	1/4	0
		13	10	+	3/13	3/13
		4	11	+	1/4	0
		2	12	+	0	0
		4	14	+	2/4	0
		7	15	+	6/7	3/7
		4	16	+	+	+
		3	17	+	+	+
		4	18	+	+	+
		4	20	+	+	+
0.5 μg. DEA in saline	5.0 μg. HEA in adjuvant	4	3	0	0	0
		12	4	0	0	0
		6	5	0	0	0
		7	6	0	0	0
		10	7	7/10	3/10	3/10
		7	8	+	3/7	1/7
		3	9	+	2/3	1/3
		11	10	+	7/11	2/11
		3	11	+	2/3	0
		2	12	+	+	+
		3	14	+	+	+
		3	15	+	+	+
		3	16	+	+	+
0.5 μg. GEA in saline	5.0 μg. HEA in adjuvant	12	4	0	0	0
		6	5	0	0	0
		7	6	0	0	0
		10	7	4/10	0	0
		7	8	4/7	0	0
		3	9	+	0	0
		11	10	10/11	6/11	4/11
		3	11	+	1/3	0
		2	12	+	1/2	0
		3	14	+	+	0
		3	15	+	2/3	0
		3	16	+	1/3	2/3
		3	18	+	+	+
		3	19	+	+	+

30 guinea pigs sensitized with 0.5 μg. of HEA, DEA, or GEA in saline had no detectable antibody. Guinea pigs sensitized with 5.0 μg. HEA in adjuvant developed antibody on the 9th day (*cf.* Table IV).

Most of the guinea pigs receiving only 0.5 μg. in saline of avian albumin showed demonstrable delayed reactions to the homologous antigen, but none showed circulating antibody. Those receiving 5.0 μg. of HEA in adjuvant developed circulating antibody to the homologous antigen on the 9th day. Guinea pigs administered both primary and secondary injections of HEA developed Arthus reactions on the 3rd to 5th day following the secondary dose. Arthus-type responses to skin tests with DEA and GEA similarly showed a reduction in the length of the induction period (Tables I, IX).

GEA, which does not induce a strong delayed hypersensitivity to HEA, was used as a primary antigen, before a secondary injection with HEA (Table IX). Arthus reactions and circulating antibody to HEA (by PCA test) became apparent from the 7th to the 10th day following administration of the HEA. Antibody to DEA and GEA did not appear until the 10th to 16th day. Thus, GEA, which produces a questionable delayed hypersensitivity to HEA, likewise causes little anamnestic effect when used as a primary antigen to a secondary HEA injection. Injection of DEA as a primary antigen before HEA produced little enhancement of the antibody response (Table IX).

Anamnestic Response with Hapten-Protein Conjugates.—Hapten-protein conjugates, such as DFB·HEA or Pi·BGG, when injected into guinea pigs, cause delayed hypersensitivity, followed by Arthus-type hypersensitivity, to the whole conjugate. However, the tendency exists for guinea pigs to manifest delayed reactions to the protein and Arthus reactions to the hapten portion of the conjugate (Tables VI, VII, and VIII). If delayed hypersensitivity is an early stage in antibody formation, then the protein moiety should induce a strong anamnestic response to the conjugate, while the hapten should induce little, if any, anamnestic response.

In one set of experiments (Table X), secondary injection of 15.0 μg. Pi·HEA in adjuvant was preceded 8 to 10 days by a primary injection in saline of either (*a*) homologous conjugate (Pi·HEA), (*b*) homologous protein alone (HEA), (*c*) heterologous hapten-homologous protein (DFB·HEA), or (*d*) homologous hapten-heterologous protein (Pi·BGG). Appropriate controls of primary or secondary antigens alone were included.

Arthus reactions and circulating antibody were detectable in animals on the 8th day after sensitization with 15.0 μg. of Pi·HEA only in adjuvant. A primary injection in saline of either the homologous conjugate Pi·HEA or the heterologous hapten-homologous protein conjugate DFB·HEA prior to an injection of Pi·HEA in adjuvant resulted in the appearance of circulating antibody against the Pi hapten on the 4th day after the secondary sensitization. A primary injection of HEA prior to administration of Pi·HEA led to the production of antibody by the 5th day. A primary injection of the homologous hapten-heterologous protein (Pi·BGG) followed by Pi·HEA resulted in circulating antibody to Pi·HEA being demonstrable by the PCA test on about the 8th day

TABLE X

Anamnestic Response in Guinea Pigs to Picryl Chloride—Hen Egg Albumin (Pi·HEA) and Its Variants, with 10 Days between Primary and Secondary Doses

Primary antigen and dose	Secondary antigen and dose	No. of guinea pigs	Day tested	HEA Ab	Pi·HEA Ab	DFB·HEA Ab	Pi·BGG Ab
	15 μg. Pi·HEA in adjuvant	7	6	0	0	0	0
		4	7	0	0	0	0
		6	8	0	+	+	+
		4	10	0	+	+	+
0.5 μg. HEA in saline	15 μg. Pi·HEA in adjuvant	4	3	0	0	0	0
		6	4	2/6	2/6	0	0
		2	5	1/2	1/2	1/2	1/2
		7	6	+	+	+	+
		3	7	+	+	+	+
1.0 μg. Pi·HEA in saline	15 μg. Pi·HEA in adjuvant	4	3	0	2/4	0	2/4
		4	4	0	+	2/4	+
		4	5	0	+	2/4	+
		4	6	0	+	2/4	+
		4	7	0	+	3/4	+
		4	8	0	+	3/4	+
		4	10	+	+	2/4	+
		4	11	3/4	+	+	+
		4	12	+	+	+	+
		4	13	+	+	+	+
1.0 μg. DFB·HEA in saline	15 μg Pi·HEA in adjuvant	4	3	0	0	0	0
		7	4	0	+	+	+
		4	5	0	+	0	+
		8	6	0	+	5/8	+
		4	7	0	+	+	+
		4	8	0	+	+	+
		6	10	5/6	+	+	+
		4	11	+	+	+	+
1.0 μg. Pi·BGG in saline	15 μg. Pi·HEA in adjuvant	2	3	0	0	0	0
		3	4	0	0	0	0
		4	5	0	0	0	0
		3	6	0	0	0	0
		3	7	0	1/3	1/3	1/3
		3	8	0	+	+	+
		3	9	0	+	+	+
		2	11	1/2	+	+	+
		3	12	2/3	+	+	+
		3	13	+	+	+	+

Controls of a primary injection of HEA, Pi·HEA, or DFB·HEA in saline did not produce any antibody detectable by the PCA test.

DFB·BGG in saline + Pi·HEA in adjuvant induced antibody formation to Pi·HEA on the 8th day after the second injection.

TABLE XI

Anamnestic Response in Guinea Pigs to 1,Fluoro-2,4-Dinitrobenzene-Hen Egg Albumin (DFB·HEA) and Its Variants, with 10 Days between Primary and Secondary Doses

Primary antigen and dose	Secondary antigen and dose	No. of guinea pigs	Day tested	HEA Ab	DFB·HEA Ab	Pi·HEA Ab	DFB·BGG Ab
None	15 μg. DFB·HEA in adjuvant	4	6	0	0	0	0
		3	7	0	0	0	0
		5	8	0	0	0	0
		7	10	0	5/7	+	3/7
		5	11	0	+	+	3/5
		3	13	0	+	+	+
		4	14	0	+	+	+
1.0 μg. DFB·HEA in saline	15 μg. DFB·HEA in adjuvant	5	3	0	0	0	0
		6	4	0	4/6	2/6	4/6
		2	5	0	1/2	0	0
		4	6	0	+	+	+
		3	7	0	2/3	0	0
		3	8	0	2/3	0	0
		3	10	0	2/3	0	0
		3	11	0	+	+	+
		3	13	0	+	+	2/3
1.0 μg. Pi·HEA in saline	15 μg. DFB·HEA in adjuvant	3	3	0	0	0	0
		4	4	0	1/4	1/4	0
		3	5	0	1/3	0	1/3
		2	6	0	1/2	1/2	1/2
		3	7	0	2/3	1/3	1/3
		3	8	0	2/3	2/3	2/3
		3	9	0	+	+	2/3
		3	10	0	+	+	—
0.5 μg. HEA in saline	15 μg. DFB·HEA in adjvant	5	3	2/5	0	2/5	0
		6	4	3/6	0	2/6	0
		3	5	2/3	0	1/3	0
		6	6	3/6	0	3/6	0
		3	7	1/3	0	1/3	0
		3	8	+	0	+	0
		3	9	+	+	+	0
		3	10	1/3	+	+	+
1.0 μg. DFB·BGG in saline	15 μg. DFB·HEA in adjuvant	3	3	0	0	0	0
		3	4	0	0	0	0
		3	5	0	0	0	0
		3	6	0	0	0	0
		3	7	0	0	0	1/3
		3	8	0	2/3	2/3	2/3
		3	9	0	2/3	2/3	2/3

(Table X). Thus, administration of protein contained in a conjugate induces an anamnestic response in animals previously sensitized with homologous protein or protein conjugate. The hapten portion of such a conjugate, however, fails to induce an anamnestic response in animals previously sensitized with homologous hapten-heterologous protein.

Similar data were obtained when Pi·BGG or DFB·HEA was used as the secondary antigen (Tables XI and XII). The results with DFB·HEA as secondary antigen were not as striking as with the Pi conjugates, possibly because of the change in the HEA molecule produced by the DFB (Table XI). Evidence for this belief lies in the fact that DFB·HEA and Pi·HEA as primary antigens did not induce as striking an anamnestic effect for DFB·HEA as they did for Pi·HEA. Also, no antibody response was detected to the HEA protein itself. The use of HEA as a primary antigen to DFB·HEA had a greater effect on the antibody response to HEA and Pi·HEA than to DFB·HEA. The foregoing responses were still much greater than those following injections of DFB·BGG and DFB·HEA as primary and secondary doses.

DISCUSSION

Delayed hypersensitivity has been shown to precede circulating antibody after the intradermal administration of foreign proteins to guinea pigs (1, 2, 7–9). This delayed response is typical, for it does not become apparent until about 6 to 8 hours after administration of the sensitizing dose of antigen and does not reach a maximum in guinea pigs until about 18 to 24 hours. The response is primarily mononuclear and can be transferred passively by washed cells from lymph nodes. Circulating antibody (diphtheria antitoxin) cannot be detected during this phase by the rabbit intracutaneous test, which can detect as little as 0.0024 μg. AbN.

The delayed response follows injection of a wide variety of proteins and hapten-protein conjugates. Thus far, however, it has not been demonstrated with polysaccharides. The hypothesis has been presented that delayed hypersensitivity is an early, immature step in the development of conventional circulating antibody. The data in this paper are consistent with this view-point.

Cross-reactions have been demonstrated between proteins of related animal sera. For example, a pattern of determinant groupings has been demonstrated for the ovalbumins of hen, turkey, guinea hen, duck, and goose (10). The assumption can be made that every normal serum contains many proteins identical with those of other species, and that their presence and relative abundance is governed by the extent of the animals' phylogenetic relationships. A more likely explanation lies in the assumption that the action of antibodies extends to structures that are chemically similar to those of the homologous antigen (11). The latter belief has been substantiated by investigations with azoproteins.

The extent of cross-reactions noted in guinea pigs sensitized with goose, duck, or hen ovalbumin and tested for immunologic response with heterologous

TABLE XII

Anamnestic Response to Picryl Chloride—Bovine Gamma Globulin and Its Variants, with 8 Days between Primary and Secondary Injections

Primary injection	Secondary injection	No. of guinea pigs	Day after sensitization	Antibody to			
				BGG	Pi·BGG	DFB·BGG	Pi·HEA
None	15 μg. Pi·BGG in adjuvant	3	3	0	0	0	0
		3	4	0	0	0	0
		3	5	0	0	0	0
		3	6	0	0	0	0
		3	7	0	0	0	0
		3	8	0	0	0	0
		3	9	0	+	0	+
		3	10	0	+	0	+
		3	11	0	+	2/3	+
		3	12	0	+	0/3	+
		3	13	0	+	1/3	+
0.5 μg. BGG in saline	15 μg. Pi·BGG in adjuvant	3	3	0	0	0	0
		3	4	0	0	0	0
		3	5	0	0	0	0
		3	6	0	0	0	0
		3	7	0	+	2/3	0
		3	8	0	2/3	2/3	2/3
		3	9	1/3	2/3	2/3	2/3
		3	10	1/3	+	+	+
		3	11	+	+	1/3	+
		3	12	+	+	2/3	+
1.0 μg. Pi·BGG in saline	15 μg. Pi·BGG in adjuvant	3	3	0	0	0	0
		3	4	0	2/3	1/3	1/3
		3	5	0	2/3	2/3	2/3
		3	6	0	+	+	+
		3	7	0	+	+	+
		3	10	0	+	+	+
1.0 μg. DFB·BGG in saline	15 μg. Pi·BGG in adjuvant	3	3	0	1/3	1/3	0
		3	4	0	2/3	+	1/3
		3	5	0	2/3	+	2/3
		3	6	0	+	+	+
		3	7	0	+	+	+
		3	10	0	+	+	+
1.0 μg. Pi·HEA in saline	15 μg. Pi·BGG in adjuvant	3	3	0	0	0	0
		3	4	0	0	0	0
		3	5	0	1/3	0	0
		3	6	0	0	0	0
		3	7	0	0	0	0

TABLE XII—(*Continued*)

Primary Injection	Secondary Injection	No. of guinea pigs	Day after sensitization	Antibody to			
				BGG	Pi·BGG	DFB·BGG	Pi·HEA
1.0 μg. Pi·HEA in saline	15 μg. Pi·BGG in adjustment	3	8	0	0	0	0
		3	9	0	2/3	0	0
		3	10	0	2/3	0	2/3
		3	11	0	2/3	2/3	1/3
		3	12	0	2/3	2/3	2/3
		3	13	0	+	+	+
1.0 μg. DFB·HEA in saline	15 μg. Pi·BGG in adjuvant	3	4	0	0	0	0
		3	5	0	0	0	0
		3	6	0	0	0	0
		3	7	0	0	0	0
		3	8	0	0	0	0
		3	9	0	0	0	0
		3	10	0	0	0	0
		3	11	0	0	0	2/3
		3	12	0	+	0	+
		3	13	0	+	0	+

and homologous antigens differs with respect to delayed and Arthus-type hypersensitivity (Tables I to III). For example, in guinea pigs sensitized with HEA, delayed hypersensitivity to GEA is either weak, doubtful, or lacking. Yet, circulating antibodies appear which combine with both homologous and heterologous antigens in PCA tests. Similar observations were recorded in guinea pigs sensitized with GEA and tested with the homologous GEA and heterologous HEA. It may be inferred that the determinant groups responsible for antigen-antibody reactions are different from those groups responsible for delayed hypersensitivity.

At first, this information may seem to be evidence that delayed hypersensitivity is a qualitatively different process from that involved in Arthus reactions and circulating antibody. The two hypersensitivities may appear related on a temporal basis, but there is actually no biochemical continuity between them. This suggestion was emphasized recently (9, 12) in some studies with proteins conjugated with picryl, acetyl, and ethoxymethylenephenyloxazolone groups. Immunization with conjugates was followed by the appearance of delayed hypersensitivity to the protein in the absence of detectable antibodies against it, although antibodies were formed at that time against the hapten itself. Delayed hypersensitivity to the haptenic group was not detected.

Further examination of the data in Tables VI, VII, and VIII, however, indicates that because delayed hypersensitivity is a step in the production of

circulating antibody and is an immature stage of the immune process, it is associated with a different part of the antigen molecule than are the more mature stages of immunity. When a guinea pig is sensitized with a hapten-protein conjugate, the delayed response seems directed toward some broad determinant in the protein molecule itself. Thus, there are delayed responses with homologous hapten-homologous protein conjugates, homologous protein, heterologous hapten-homologous protein conjugates, but not with homologous hapten-heterologous protein conjugates. As the immune process evolves and the basis for circulating antibody is laid, the determinant factor in the antigen molecule becomes more limited, finite, and specific and changes to the small surface groupings. Thus, animals sensitized with a hapten-protein conjugate eventually develop Arthus-type reactions to the hapten even though the hapten used for testing is contained in a homologous or heterologous protein conjugate.

That the delayed reaction serves as the basis for later derivation of circulating antibody is established by experiments on anamnestic responses. Guinea pigs were sensitized with a part of a conjugate in such a manner as to produce delayed hypersensitivity only. A second injection of conjugate followed. Maximal anamnestic response occurred when the same hapten-protein conjugate was used for both the primary and secondary doses of antigen, and minimal responses occurred when a heterologous protein-homologous hapten conjugate was used as the primary antigen (Pi·HEA prior to Pi·BGG). When a homologous protein-heterologous hapten conjugate was injected as the primary antigen, maximal anamnestic effect on the whole conjugate resulted (Table X). Only a minimal anamnestic response is produced by a secondary injection of HEA into guinea pigs previously given GEA. The latter protein likewise usually fails to induce striking delayed hypersensitivity against HEA. These findings seem especially significant since the moiety that induces delayed hypersensitivity is also the one responsible for maximum anamnestic response to the whole conjugate. These studies of the anamnestic response show that delayed and Arthus-type hypersensitivities are associated with different portions of a hapten-protein conjugate. Consequently, the idea that the types of reaction are basically different does not appear tenable.

The absence of a striking anamnestic response to HEA after a primary injection of GEA indicates a greater specificity amongst the animal egg or serum albumins than previously reported in experiments by Dixon and Maurer (13), wherein an anamnestic response occurred in animals given a primary dose of HEA and a secondary of BSA. The contrasting results, however, may be due to the following differences in experimental conditions. In the present experiments, guinea pigs were used instead of rabbits; a single primary injection of 0.5 μg. protein was administered, instead of two courses of antigen, each lasting 4 days and each containing 388 mg. protein; the antigens were injected intradermally instead of intravenously.

Two contradictions may seem to invalidate the hypothesis that delayed hypersensitivity is a stage in the production of circulating antibody, (*a*) the difficulty of producing circulating antibodies to denatured proteins, such as gelatin, although delayed hypersensitivity develops; and (*b*) the inability to detect delayed hypersensitivity to purified polysaccharides, although circulating antibody may occur. Both of these facts may be explained on the basis that recognition of antibody by antigen in delayed hypersensitivity is directed toward a broad area of the antigen molecule and in Arthus reactions and circulating antibody toward a more narrow, finite area. In the case of gelatin, the denaturation process may alter the antigen molecule in such a way that the small areas of the molecule on which antigen-antibody reactions depend are obscured, thus making the detection of antibody difficult. In the case of polysaccharides, the production of hypersensitivity and circulating antibody may depend on the polysaccharide behaving as a hapten and combining with a host protein. Then, for recognition of the delayed component the protein portion of the conjugate would have to be used. Identification of the circulating antibody, however, would be readily made by the polysaccharide itself. Experiments are now being carried out to test this hypothesis.

Much effort was spent to purify antigens before their incorporation in the foregoing experiments. Physico-chemical analyses indicated the absence of detectable impurities in the protein solutions. The antigens, nevertheless, may still be impure, for manipulation of the solutions may cause partial alteration of the molecule and produce the effect of additional antigenic alteration. This possibility must be borne in mind in interpretation of experimental results.

The administration of the antimetabolite 6-mercaptopurine to rabbits has been shown to inhibit the development of circulating antibody (14). The compound has been tested in guinea pigs for its efficacy in eliminating delayed hypersensitivity and circulating antibody (15), with the hope that it would inhibit circulating antibody and thereby isolate delayed hypersensitivity for further study. Daily intraperitoneal injection of 6-mercaptopurine into guinea pigs in quantities up to 75 mg./kg., however, did not prevent the appearance of delayed or Arthus types of hypersensitivity after injection of 1 Lf diphtheria toxoid in adjuvant (incomplete) into the foot-pads.

The presence of an intermediate phase in antibody production which has a broad basis for its specificity may be of assistance to the host animal. The animal would be primed for an anamnestic response to a family of antigens after exposure to only one of the group.

Whether delayed hypersensitivity is an early, immature and essential phase in the development of circulating antibody or whether delayed hypersensitivity is a distinct and qualitatively separate immunologic response from circulating antibody has been in doubt. Present data favor the first hypothesis: (*a*) Delayed reactions to a foreign protein occur prior to appearance of circulating

antibody. (*b*) Antigens that are most effective in inducing delayed hypersensitivity in the guinea pig are good antibody producers. Conversely, antigens that are ineffective in inducing delayed hypersensitivity are poor antibody producers. (*c*) Guinea pigs in which delayed hypersensitivity has been induced by injection of minute amounts of antigen in saline develop distinct anamnestic responses. The closer the time of the secondary injection to the peak of delayed hypersensitivity, the greater is the anamnestic reaction. (*d*) Injection of a whole homologous protein, such as HEA, prior to the administration of a conjugate composed of the same protein plus a hapten (Pi·HEA) induces an anamnestic response to both portions of the conjugate. Primary injection of a conjugate with homologous hapten and heterologous protein (Pi·BGG), which does *not* induce delayed hypersensitivity to the hapten, does not induce a distinct anamnestic reaction to the secondary injection of a conjugate which contains the same hapten but a heterologous protein (Pi·HEA).

SUMMARY

Guinea pigs sensitized with either hen, duck, or goose egg albumin showed delayed hypersensitivity followed by Arthus reactions to the homologous antigen, but tended to have much weaker delayed responses and slower antibody formation to heterologous antigens. Guinea pigs with delayed hypersensitivity to one of the avian antigens had a slower antibody response to a secondary injection of heterologous antigen than to one of the homologous antigen.

Sensitization with a protein conjugated with a hapten such as picryl chloride (Pi) or dinitrofluorobenzene (DFB) resulted in delayed hypersensitivity to the homologous conjugate, the homologous protein, and the homologous protein with a heterologous hapten. Circulating antibody and Arthus reactions occurred subsequently to the homologous conjugate, as well as to the homologous hapten attached to a heterologous protein. Delayed hypersensitivity thus seemed associated with the protein moiety, and Arthus responses with the hapten.

Anamnestic responses followed injection of an antigen causing delayed hypersensitivity, but not of a hapten not causing delayed reactions. Thus, animals sensitized initially with Pi·HEA, DFB·HEA, or HEA produced antibodies sooner after a secondary injection of Pi·HEA than did unsensitized animals. No anamnestic response resulted when animals sensitized to Pi·BGG were injected with Pi·HEA.

Thus, delayed hypersensitivity is indicated to be a preliminary and immature step in the immune process, with specificity directed against broad, more general features of the protein antigen. This intermediate step is followed by production of circulating antibody to any antigen having a similar basic structure, with the specificity of the antibody also directed against smaller immunologically active sites on the antigen molecule.

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