The Specificity of Allergic Reactions. IV. The Cornea. (26662)

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Intracorneal injection of foreign protein into animals with delayed hypersensitivity to that protein produces damage to the cornea. This allergic reaction consists of edema, swelling, and necrosis of the corneal fibers; heavy polymorphonuclear leucocyte infiltration; and occasional necrosis of overlying epithelium. This reaction, occurring in the avascular cornea, might represent an antigen-specific cytotoxic response, independent of both blood vessels and circulating antibody. Moreover, Arthus reactions presumably could not occur in the cornea, even when reactions of this type are pronounced in the skin, since an Arthus reaction is associated with endothelial injury.

Marked changes in scarified corneas after instillation of nonhemolytic streptococci were described by Derick and Swift(1) in rabbits sensitized previously to this bacterial antigen (1). Julianelle(2,3) provoked similar corneal reactions in rabbits after sensitization with intact pneumococci, but he could not detect such responses in rabbits sensitized with pneumococcal nucleoprotein and having Arthus reactions to this antigen. He further showed that serum from rabbits hypersensitive to either egg albumin or pneumococcus nucleoprotein conferred Arthus reactions, but not corneal reactions, to normal recipients. Corneal reactions were then shown to be allied to tuberculin hypersensitivity(4,5), in that (a) the response required 24-48 hours to develop; (b) tuberculoprotein had a markedly toxic action on connective tissue of corneas of tuberculous guinea pigs, and led to inflammation and partial degeneration; and (c) histologically, the cornea showed changes characteristic of allergic responses.

A more recent work by Raffel *et al.*(6) describes the appearance of corneal reactions in guinea pigs after sensitization with a pure protein (*i.e.*, egg albumin) as long as tubercle bacillus "wax" was added to the sensitizing medium. Leskowitz and Waksman(7) have shown definite intracutaneous delayed reactions in rabbits after sensitization to bovine serum albumin in complete Freund's adjuvant as well as simultaneous corneal reactions to the same antigen. Schlossman and Stetson(8) have described vascularization of the cornea after intracorneal injections of antigen in guinea pigs sensitized with ovalbumin-anti-ovalbumin specific precipitates in complete Freund's adjuvant.

In the course of immunization of guinea pigs with purified proteins, delayed hypersensitivity preceded in time Arthus hypersensitivity and circulating antibody(9). As the animal progressed from a state of delayed allergy to one of antibody formation, a change in specificity to the antigen appeared, with delayed hypersensitivity oriented toward a broad portion of the antigenic molecule and Arthus reactions directed toward small, finite groupings(10,11,12). This observation suggests a method for determining whether corneal reactions are purely of the delayed type, whether they are influenced by circulating antibody, and to what portion of the antigenic molecule their specificity is directed. Accordingly, investigations were initiated in guinea pigs to elucidate further the exact relationship between corneal response, delayed hypersensitivity and circulating antibody.

Materials and methods. Animals. Guinea pigs were of the Hartley strain and weighed between 350-450 g.

Antigens. Five times recrystallized hen egg albumin (HEA) was obtained from K & K Laboratories, Inc., Jamaica, N. Y. Purified bovine gamma globulin (BGG) from Armour Laboratories, Chicago, Ill., was used without further treatment. Conjugated proteins were made as previously described(12).

Sensitization. The antigen was dissolved in physiologic saline containing 1% guinea pig serum and emulsified in a syringe with

an equal volume of Freund's adjuvant (Difco), with mycobacteria (2 mg per animal). Except where specified, the animals were sensitized by injection of 0.5 ml of the oil-water emulsion into the footpads.

Skin tests. Guinea pigs were tested intradermally on the sides with 0.1 ml of antigen containing 50 μg/ml protein. Reactions were observed and diameters of areas of induration measured at 4 hours and at 18 to 24 hours after injection.

Corneal tests. Two to 3 drops of 0.5% Pontocaine hydrochloride (Winthrop Laboratories, New York City) were placed on the corneal surface of the guinea pig eye several minutes before testing. 0.01 ml of the antigenic solution containing 500 μg/ml protein was injected intracorneally with a 30-gauge needle attached to a ¼-ml tuberculin syringe. The injections resulted in a temporary clouding of the cornea which cleared in about 2 hours. Reactions were observed 4, 24 and 48 hours after injection. Several animals were sacrificed by intraperitoneal injection of Nembutal, the eyes were removed, fixed in Zenker's solution, and sections of the cornea stained with Harris's hematoxylin and eosin.

Antibody production. Guinea pigs were bled just prior to skin testing, and the sera assayed for antibody by the passive cutaneous anaphylaxis (PCA) reaction(13). In the PCA tests, 0.1 ml test serum was injected intradermally in the flank. Three hours later, 350 μg protein in 0.5 ml physiologic saline and 0.5 ml 1% Evans blue in physiologic saline were introduced intravenously. Fifteen to 30 minutes later, areas of pigmentation in the skin were measured and recorded.

Results. Corneal reactions to purified proteins. Guinea pigs were sensitized by injection in the footpads with 50 μg bovine gamma globulin (BGG) in Freund's adjuvant containing 2 mg mycobacteria. Animals were bled, skin-tested, and tested intracorneally daily from the 5th to the 21st day after sensitization, and irregularly thereafter. No guinea pig was tested on more than one occasion. At this dose level, delayed hypersensitivity to BGG was present on the 5th day after sensitization, and circulating antibody on the 7th day (Table I). Corneal reactions, on the other hand, did not appear consistently until the 12th day, well after delayed hypersensitivity first could be detected and at a time when delayed reactions in the skin of the flanks were masked by Arthus reactivity. This delay in time of manifestation of the corneal reactions may be explained on the basis (a) that additional time was required for delayed hypersensitivity to reach a degree of intensity wherein corneal responses could be observed, or (b) that circulating antibody may in some way be associated with the expression of corneal hypersensitivity.

TABLE I. Corneal, Delayed and Arthus Responses after Sensitization with 50 μg BGG in Complete Freund's Adjuvant.

Time after sensitization (days)	No. of animals	Corneal response	Delayed intracut. response	Arthus response
5	2	0/2†	2/2	0/2
6	3	0/3	3/3	
7	6	1/6	2*/6	3/6
8	4	0/4	1*/4	2/4
9	4	2/4		4/4
10	2	0/2		2/2
11	5	0/5		5/5
12	8	4/8		8/8
13	6	3/6		6/6
14	8	2/8		8/8
15	6	2/6		6/6
16	4	3/4		4/4
17	1	1/1		1/1
18	4	2/4		4/4
19	3	3/3		3/3
20	2	2/2		2/2
21	4	3/4		4/4
25	3	3/3		3/3
28	5	5/5		5/5

* These guinea pigs did not have circulating antibody.

† Numerator indicates No. of guinea pigs with positive reaction; denominator, No. of guinea pigs tested.

Guinea pigs were then sensitized with 5 μg hen egg albumin (HEA) in Freund's adjuvant plus 2 mg mycobacteria (Table II). Herein, both delayed intracutaneous and corneal reactions appeared on days 4-5, whereas circulating antibody could not be detected until days 8-9. Thus, corneal reactions were not associated with the appearance of circulating antibody, as determined by PCA tests which can detect 0.001-0.002 μg antibody nitrogen.

TABLE II. Corneal, Delayed and Arthus Responses after Sensitization with 5 μg HEA in Complete Freund's Adjuvant.

Time after sensitization (days)	No. of animals	Corneal response	Delayed response	Arthus response
4	4	4/4	4/4	
5	5	4/5	5/5	
6	5	4/5	5/5	
7	5	5/5	5/5	
8	3	2/3		3/3
9	3	3/3		3/3
10	2	2/2		2/2
11	4	3/4		4/4
13	2	2/2		2/2
18	3	3/3		3/3
19	4	1/4		4/4
20	2	2/2		2/2

The corneas from the hypersensitive animals in experiments with both BGG and HEA were markedly thickened and opaque. Histological sections showed that the bands of sensitized corneal fibers had thickened to about twice the cross-section of the bands of normal fibers, and that the corneas had extensive cellular exudation and striking polymorphonuclear infiltration (Fig. 1-4).

The animal that tended to show a stronger intradermal delayed response also tended to have the more pronounced corneal reaction. Frequently, however, guinea pigs with weak to moderate delayed intradermal responses on the sides did not develop visible corneal opacities. Thus, if both corneal and intracutaneous delayed reactions are essentially caused by the same mechanism, the corneal tissue shows allergic changes less readily.

Twenty-four guinea pigs were sensitized in the footpads with 50 μg BGG in Freund's adjuvant without mycobacteria, and tested periodically up to 21 days postsensitization for cutaneous delayed hypersensitivity and for corneal hypersensitivity. Delayed reactions occurred after intradermal injections, but allergic reactions could not be detected after intracorneal testing. Twenty-one additional guinea pigs were sensitized with 1.0 μg HEA in saline in the footpads and 10 days later administered a second dose of 5.0 μg HEA in Freund's adjuvant (without mycobacteria)(14). Periodic testing up to 23 days postsensitization again produced no evidence of corneal hypersensitivity. The tubercle bacillus apparently increases the hypersensitivity sufficiently so that the cornea can be affected or adds a substance to the tissues which enables them to react. The former concept seems more logical.

Specificity. Guinea pigs sensitized with para-amino benzoic acid diazotized to hen egg albumin (PABA • HEA) develop initially a delayed state (as indicated by intracutaneous testing) which is oriented in its specificity primarily toward the HEA part of the antigen molecule, whereas the later-appearing Arthus phase is directed primarily toward the PABA moiety(12). Since the corneas, from 4 to 45 days after sensitization, required from 24 to 48 hours for the allergic opacity to become macroscopically visible, delayed hypersensitivity seemed to be the basis for this immune reaction. No change in the appearance of the reaction occurred in the cornea during this 4- to 45-day period, although the skin was undergoing a change from delayed to Arthus reactions. To determine whether this consistency in the corneal reaction was apparent or real, corneal reactions were examined in guinea pigs sensitized to PABA • HEA.

Guinea pigs were sensitized in the footpads by injection of 15 μg PABA • HEA in Freund's adjuvant plus 2 mg tubercle bacillus. Pairs of guinea pigs were then tested in the cornea with either PABA • HEA and HEA, PABA • HEA and PAAA • HEA (p-amino arsonic acid diazotized to HEA) or PABA • HEA and PABA • BGG (p-amino benzoic acid diazotized to bovine gamma globulin) and at the same time intradermally with PABA • HEA (Table III). The specificity pattern of delayed hypersensitivity in the cornea consistently followed that typical of the skin. For as long as 45 days after sensitization, a reaction followed intracorneal injection of PABA • HEA, HEA or PAAA • HEA. At no time, however, did a corneal reaction appear after introduction of the homologous hapten-heterologous protein conjugate, PABA • BGG, even though circulating antibody was present and Arthus reactions could be elicited to PABA • BGG. Thus, for 45 days after sensitization, a pure delayed reaction can develop in the cornea,

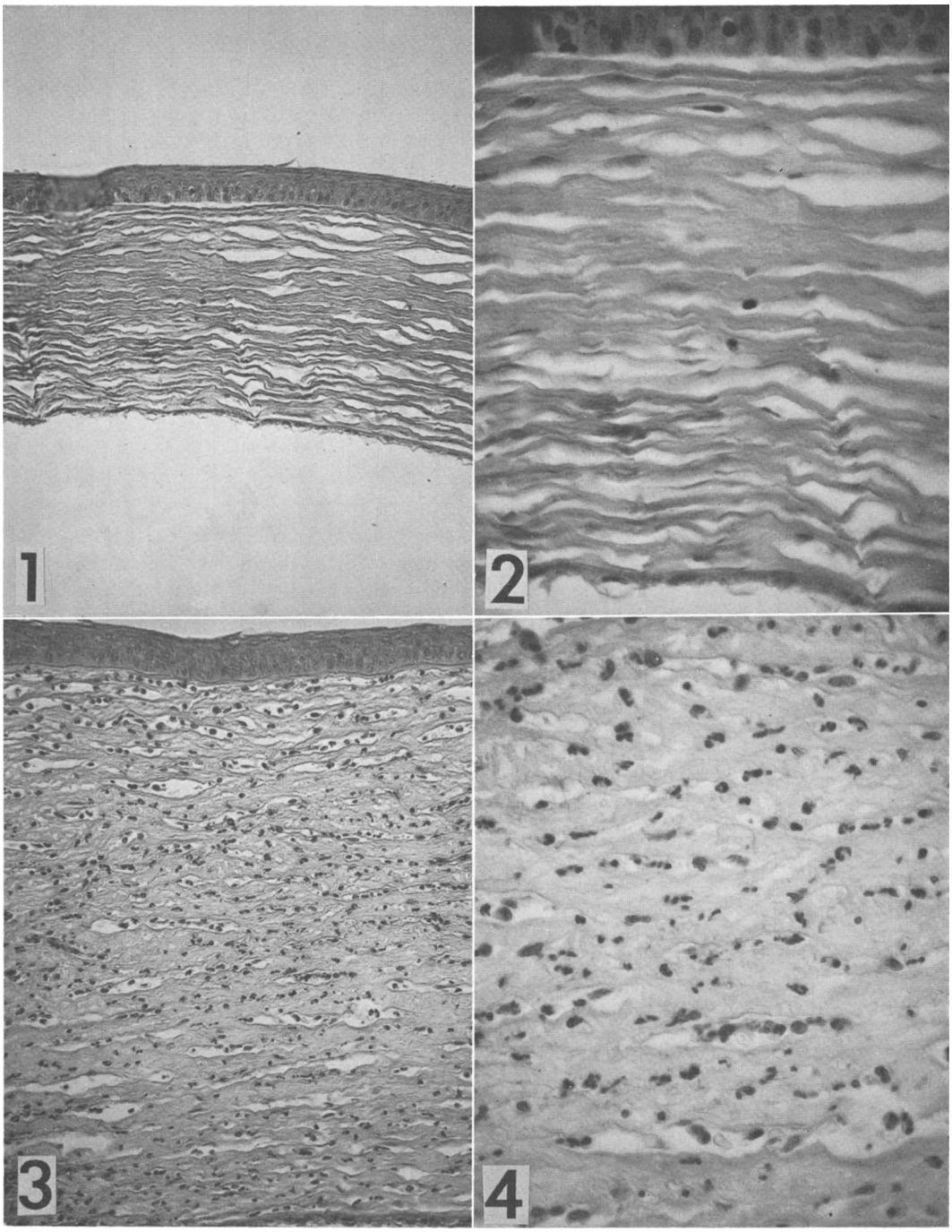

FIG. 1. Section of cornea from a nonsensitized guinea pig 24 hr after intracorneal inj. of 5 μg hen egg albumin. × 187.

FIG. 2. Higher magnification of same section of cornea as in Fig. 1. × 461.

FIG. 3. Section of cornea from a guinea pig sensitized 19 days previously with 5 μg HEA in complete Freund's adjuvant 24 hr after intracorneal inj. of 5 μg HEA. × 187.

FIG. 4. Higher magnification of same section of cornea as in Fig. 3. × 461.

TABLE III. Corneal, Delayed and Arthus Reactions after Sensitization with 15 μg PABA • HEA in Complete Freund's Adjuvant.

Time after sensitization (days)	No. of animals	Corneal response to PABA • HEA	Corneal response to PABA • BGG	Corneal response to HEA	Corneal response to PAAA • HEA	Intradermal response to PABA • HEA Delayed	Intradermal response to PABA • HEA Arthus
6	2	2/2		2/2		2/2	
7	1	1/1		1/1		1/1	
8	7	6/7	0/3	3/4		7/7	
9	2	2/2		1/2		2/2	
10	4	1/4	0/2	1/2		4/4	
12	7	6/7	0/2	4/5		4/7	3/7
13	4	3/4	0/2	1/2		2/4	2/4
14	4	1/4	0/2	1/2		3/4	1/4
15	4	3/4	0/2	1/2		2/4	2/4
16	3	3/3	0/1	0/2		1/3	2/3
19	7	4/7	0/3	0/4			7/7
26	4	3/4	0/2	2/2			3/4
29	2	2/2	0/1	0/1			2/2
32	4	2/4	0/2	0/2			4/4
39	6	5/6	0/3		3/3		6/6
45	4	4/4	0/2		2/2		4/4

in spite of the possible presence of circulating antibody, as judged by the foregoing experiments with protein conjugates.

Discussion. Introduction of minute quantities of antigen in Freund's adjuvant into the footpads of guinea pigs results in delayed hypersensitivity, followed by appearance of circulating antibody. The delayed hypersensitivity may also be observed after transfer of washed lymph node cells from an allergic animal to a normal one and after subsequent intradermal testing of the recipient (9). During this period of delayed allergy, circulating antibody to the allergen cannot be detected.

During much of the time in which delayed allergy can be demonstrated in the skin and also during the subsequent period when circulating antibody can be demonstrated by PCA tests and Arthus reactions, injection of the cornea with specific antigen induces a reaction. This response seems to be basically controlled by the mechanism associated with delayed hypersensitivity. Even though circulating antibody and Arthus reactions may be present, and even though some vascularization may develop in the fibrous layer of the cornea, the specificity of the response is oriented toward the protein moiety of a conjugated antigen. The corneal tissue does not respond to circulating antibody, or possibly the concentration of gamma globulin in the cornea does not reach or exceed threshhold values. That the former hypothesis seems to be the true one is further indicated by the fact that guinea pigs with delayed hypersensitivity to pure proteins (HEA) or tuberculin showed a delayed polymorphonuclear response in the cornea after intravitreal challenge, although other ocular tissues such as the choroid, sclera, and uvea, show predominantly a mononuclear infiltration(15). Injection of antibody intravitreally followed by intracorneal testing causes an iridocyclitis more readily than a corneal opacity(16). Passive transfer of sensitized cells intravenously into normal recipients produces a delayed hypersensitivity which can be detected by corneal injections(17).

The corneal response, in contrast to the intradermal delayed reaction, seems to be primarily polymorphonuclear in cell type. Whether this is actually so, or whether the polymorphonuclear invasion follows one of mononuclear elements(18), requires further investigation. A similar polymorphonuclear infiltration was demonstrated on subarachnoid injection of "purified protein derivative" of the tubercle bacillus into tuberculin positive humans(19). Also, explants of cornea from a sensitized guinea pig do not show a cytotoxic response when they are exposed to specific antigen in tissue culture (20). This observation seems to point

toward invading leucocytes as the cells responding chemotactically to injection of antigen in the cornea. The possibility arises, therefore, that the cells of the cornea are merely the seat of the allergic response, and play either a minor role or none at all in actually eliciting it.

Summary. Injection of protein antigens in complete Freund's adjuvant into the footpads of guinea pigs produces a hypersensitivity which induces allergic reactions in the cornea both during the delayed and Arthus phases of the allergic response. This corneal reaction is similar to the intracutaneous delayed type in that its specificity is oriented toward the protein portion of a conjugate and not toward the hapten.

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Fatty Acids of Human Platelet Phosphatides.* (26663)

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The development of gas-liquid chromatography (GLC) provides a highly sensitive method for separation of fatty acid mixtures and identification of individual components (1,2). Studies of the fatty acid composition of a wide variety of lipids have been reported (3,4). Since platelet phosphatides exhibit specialized activity in the generation of blood thromboplastin(5) it is of interest to determine their constituent fatty acids by means of GLC.

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Materials and methods. Blood was collected(5) for platelet isolation from 25 subjects without apparent hematologic disorder. The phospholipids were extracted(5) and pooled. One hundred fifty-nine mg of crude phospholipid were chromatographed on a silicic acid column and the 150 individual fractions identified by paper chromatography(5). Each fraction was esterified by transmethylation in superdry methanol followed by sublimation according to Stoffel *et al.*(6). The yield of methyl esters from the sublimation cold finger was at least 95%