# THE IN VITRO PRODUCTION OF ANTIBODY BY DELAYED PHASE CORNEAL LIMBAL LYMPHOID FOCI*

By HOWARD M. I. LEIBOWITZ,‡ M.D. AND JAMES J. PARKS,§ M.D.

*(From the Wilmer Ophthalmological Institute of the Johns Hopkins Hospital and University, Baltimore)*

PLATES 2 TO 4

Since the pioneer work of Wessely (1) in 1911 and of von Szily and Arisawa (2) three years later, it has been well established that the cornea may be the site of a specific, antibody-mediated hypersensitivity reaction. This is generally manifested by a macroscopic ring of opacification in the cornea, appearing 13 or more days after the intralamellar inoculation of a soluble protein antigen. The intracorneal ring is composed of precipitated antigen-antibody complexes and inflammatory cells (3–5).

In a previous study, the present authors demonstrated that if a sufficient quantity of antigen is introduced intracorneally in rabbits, one obtains a biphasic reaction in that cornea (6). The initial response is characterized by a sudden diffuse corneal clouding and a dilatation of the limbal vessels 3 to 5 days after the introduction of the antigen. It thus occurs during the early induction phase of the antibody-mediated reaction described above. At the time of the early corneal response, no humoral antibodies are demonstrable by either serological or histological techniques. In addition, the animals react to intradermal inoculation of small amounts of the specific sensitizing antigen with a delayed type of skin response and this sensitivity can be passively transferred to normal guinea pigs with the cells of pooled lymph nodes or buffy coats. Based on these observations, the earlier stage of the biphasic corneal reaction was considered a manifestation of delayed hypersensitivity.

During the course of this biphasic hypersensitivity reaction, we were able to demonstrate a cellular infiltrate at the limbus of each experimental eye. Histologic study of the cellular elements composing this limbal infiltrate revealed a very definite evolutionary sequence. At the time of the delayed phase response, the inflammatory focus consisted predominantly of elements of the lymphocytic-mononuclear series—the cells generally incriminated in delayed hypersensitivity. In contrast, during the later, antibody-mediated corneal ring response, the limbal infiltrate consisted overwhelmingly of plasma cells. It is generally agreed that these cells are a major source of antibody at the cellular level and as such, are responsible for the latter type of immune phenomenon. In the interval between the two phases of corneal activity, the

197

sensitized eye appears clinically essentially normal. However, cellular forms, intermediate between lymphocytes and plasma cells (*i.e.*, preplasma cells), make their appearance at the limbus.

Many workers in the field of immunology have suggested that delayed hypersensitivity is a stage in the development of antibodies. The evidence for this hypothesis is generally based on experiments similar to that described above, in which there is demonstrated such a stage of reaction preceding the appearance of humoral antibodies. With the establishment of the sequential relationship of the two types of hypersensitivity, and the demonstration of the apparent maturation of the cells involved in the earlier delayed stage to those held responsible for the later anaphylactic type of reaction, the question which naturally arises is: Is delayed hypersensitivity engendered by the very cells which produce humoral antibodies? The present communication is a report of our approach to the problem regarding the relationship of the cytologic elements mediating the two forms of immune response.

## Materials and Methods

*Animals.*—Albino rabbits of the New Zealand strain, weighing 2 to 3 kg, were utilized in this study. For the passive cutaneous anaphylaxis experiment, albino guinea pigs, weighing approximately 250 gm, were used.

*Antigen.*—Crystalline bovine serum albumin (BSA: Mann Research Labs., New York, Lot F3830) was reconstituted in isotonic saline to give a stock solution with a protein concentration of 80.4 mg per ml. The solution was aseptically prepared, Seitz-filtered and tested for sterility.

*Corneal inoculation.*—The rabbit was immobilized and several drops of proparacaine hydrochloride (Ophthaine, Squibb) were instilled topically into the cul-de-sac of the right eye. The eye was then proptosed and, with the aid of a binocular loupe and a strong light, 0.05 ml of stock BSA (representing 4.02 mg) was introduced intralamellarly into the cornea with a half-inch 27 gauge needle attached to a 0.25 ml tuberculin syringe. The animals were individually caged and observed daily.

### PROCEDURES AND RESULTS

As previously stated, on about the 3rd day after the intracorneal inoculation of 4 mg of bovine serum albumin into the eye of a rabbit, a delayed type of hypersensitivity response, manifested by a diffuse clouding of the cornea, was observed (Fig. 1). Microscopic examination of the experimental eye at this time revealed a heavy infiltration of the limbal area by lymphoid elements (Fig. 2).

Accordingly, at times varying between 3 and 5 days postinoculation, coinciding with the peak of the delayed phase corneal response, the animals were sacrificed and the experimental eye immediately enucleated. The latter was rinsed in cool running tap water and soaked in aqueous merthiolate 1:1000 for several minutes. The eye was then immersed in sterile balanced salt solution at pH 7.4. While immersed, circular explants measuring 1 mm in diameter were circumferentially removed, under operative conditions, from the corneal-scleral junction by

means of a corneal trephine (Text-fig. 1). The explants were placed in a small volume of Cultur STAT Medium 199-Hank's Base (Cappel Laboratories, West Chester, Pennsylvania) containing 10 per cent rabbit serum (made up on special order), amino acids, vitamins, glutamine, and 100 units of penicillin and streptomycin per milliliter. Several drops of rabbit plasma were placed in a 16 × 150 mm roller tube and the tube rotated until its bottom half was coated by the plasma. Then with a Pasteur pipette, four to five explants were placed on the uncoated area near the top of the tube and the excess nutrient solution removed from around the explants. Again using a Pasteur pipette, the individual explants were transferred to the plasma-coated surface at the lower half of the tube. Approximately 10 minutes later the tubes were
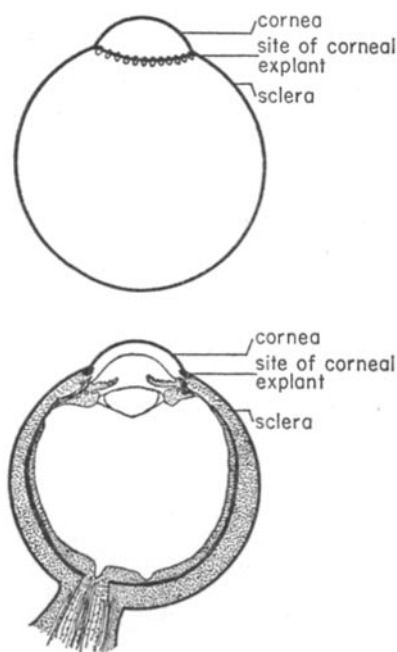


TEXT-FIG. 1. Schematic drawings illustrating site of removal of corneal explants

capped, placed in a roller drum and incubated at 37°C for 2 hours to allow adherence of the explants to the walls of the tube. At the termination of this 2 hour period, 0.5 ml of Cultur STAT 199-Hank's Base with 10 per cent rabbit serum was added to the tube and the latter returned to the incubator and maintained at 37°C.

In addition to the treatment described above, random explants from each experimental eye were fixed in formalin immediately after their excision and were subjected to routine paraffin sectioning and staining with hematoxylin and eosin. The experimental eye itself was also fixed in formalin and was sectioned and stained for histological study of the limbal areas remaining between the perforations left by trephination.

The explants maintained in tissue culture were divided into three groups, consisting respectively of those removed on the 3rd, 4th, and 5th postinoculation day. On the 14th and 21st days and, in some instances, also on the 28th day after the introduction of antigen into the cornea, the overlay fluid, in which the explants of limbal tissue were being maintained, was

tested for the presence of specific humoral antibodies by the passive cutaneous anaphylaxis technique of Ovary (7). Each guinea pig was inoculated intradermally with 0.1 cc of the overlay fluid from each of three roller tubes. All such tests were performed in duplicate. In addition, each animal received 0.1 cc of high titer, anti-BSA serum intradermally for the purpose of

TABLE I

*Results of the Passive Cutaneous Anaphylaxis Test on the Overlay Fluid of Explants Removed on the Third Postinoculation Day*

| Animal No. | Average area of positive test site | | | |
| --- | --- | --- | --- | --- |
| | 14th postinoculation day | | 21st postinoculation day | |
| | *cm* | *cm* | *cm* | *cm* |
| 4-01 A | 1.5 × 1.5 | 1.0 × 1.5 | 2.0 × 2.5 | 1.5 × 2.5 |
| 4-01 B | 3.0 × 3.5* | 2.5 × 2.5 | 2.0 × 1.0 | 2.5 × 2.0 |
| 4-02 A | 2.0 × 1.5 | 2.0 × 2.5 | 2.5 × 2.5* | 3.0 × 2.0 |
| 4-02 B | 1.5 × 1.0 | 0 | 2.0 × 1.5 | 1.5 × 2.5 |
| 4-02 C | 2.0 × 2.5* | 2.5 × 3.5* | 3.0 × 2.5* | 2.5 × 4.0* |
| 4-03 A | 0 | 1.0 × 2.0 | 1.5 × 1.0 | 0 |
| 4-03 B | 2.5 × 3.0 | 1.5 × 2.5 | 2.0 × 2.0 | 2.5 × 2.0 |
| 4-04 A | 3.0 × 1.5 | 2.5 × 2.5 | 2.5 × 3.0 | 2.0 × 2.5 |
| 4-04 B | 2.0 × 2.0 | 2.0 × 3.0 | 0 | 0 |
| 4-04 C | 2.5 × 1.5* | 2.0 × 2.0* | 3.0 × 2.5* | 2.5 × 3.5* |
| 4-05 A | 1.0 × 1.5 | 2.0 × 1.5 | 1.0 × 2.0 | 2.0 × 1.5 |
| 4-05 B | 2.0 × 1.5* | 2.0 × 2.5 | 2.5 × 1.5 | 2.0 × 2.5 |
| 4-05 C | 2.5 × 3.0* | 2.5 × 2.5* | 3.5 × 3.5* | 2.0 × 2.5* |
| 4-06 A | 3.0 × 3.5 | 2.5 × 2.0 | 2.0 × 2.0 | 2.0 × 1.5 |
| 4-06 B | 0 | 1.5 × 1.5 | 1.0 × 1.0 | 0 |
| 4-07 A | 2.0 × 1.5 | 2.0 × 2.0 | 1.5 × 1.5 | 1.5 × 1.0 |
| 4-07 B | 3.0 × 2.5* | 2.0 × 2.5* | 2.5 × 2.0* | 3.5 × 3.0* |
| 4-07 C | 0 | 1.0 × 1.0 | 0 | 0 |
| Control A | 0 | 0 | 0 | 0 |
| Control A | 0 | 0 | 0 | 0 |
| Control B | 0 | 0 | 0 | 0 |
| Control B | 0 | 0 | 0 | 0 |

* Deep blue positive PCA, see text (Procedures and Results)
Control A, overlay fluid incubated without explants in tube.
Control B, overlay fluid incubated with explants taken from normal eyes.

verifying that the later antigen-dye injection was in fact intravenous. In all cases, this control measure yielded a highly positive response.

Microscopic examination of random explants which had been formalin-fixed and prepared for routine histologic study revealed an overwhelming predominance of cells of the lymphocytic-mononuclear series infiltrating the underlying homogeneous collagenous tissue (Fig. 3). Histologic study of the limbal areas remaining between the puncture sites left by trephination of the experimental

eye revealed an identical infiltration by lymphoid elements. It is therefore reasonable to assume that the *in vitro* explants contained large numbers of the delayed-phase, lymphocytic-mononuclear cells in relatively "pure culture."

The results of the passive cutaneous anaphylaxis experiments for the detection of humoral antibody in the overlay fluid are tabulated (Tables I to III). It

TABLE II

*Results of the Passive Cutaneous Anaphylaxis Test on the Overlay Fluid of Explants Removed on the Fourth Postinoculation Day*

| Animal No. | Average area of positive test site | | | | | |
|---|---|---|---|---|---|---|
| | 14th postinoculation day | | 21st postinoculation day | | 28th postinoculation day | |
| | *cm* | *cm* | *cm* | *cm* | *cm* | *cm* |
| 2-05 A | 1.0 × 1.0 | 0 | 0.75 × 1.25 | 0 | 0 | 0 |
| 2-05 B | 2.0 × 1.5 | 2.5 × 2.5 | 2.0 × 2.5 | 2.0 × 3.0 | 2.5 × 2.5 | 2.5 × 2.0 |
| 2-07 B | 2.5 × 3.0 | 2.5 × 2.5 | 2.0 × 2.5 | 0.75 × 0.75 | 0 | 0 |
| 2-08 A | 2.5 × 2.5* | 3.0 × 2.5* | 0 | 1.0 × 2.0 | 1.5 × 1.5 | 2.0 × 1.5 |
| 2-09 A | 0 | 0 | 0 | 0 | 0 | 0 |
| 2-09 B | 2.5 × 2.0* | 2.5 × 3.5* | 2.0 × 2.0 | 2.0 × 3.5 | 2.5 × 2.0 | 2.0 × 2.0 |
| 2-10 A | 2.0 × 2.0 | 2.0 × 1.5 | 2.0 × 2.0 | 1.5 × 2.0 | 0 | 0 |
| 2-10 B | 1.5 × 2.5 | 0 | 2.0 × 2.5 | 2.5 × 1.5 | 0 | 0 |
| | | | | | | |
| 3-01 A | 1.5 × 2.0* | 0 | 2.0 × 2.5 | 1.5 × 1.5 | | |
| 3-01 B | 2.5 × 2.0* | 2.0 × 1.5 | 0 | 0 | | |
| 3-02 A | 1.5 × 1.5* | 2.0 × 2.0 | 1.5 × 2.0 | 1.5 × 1.5 | | |
| 3-02 B | 1.5 × 2.0* | 2.0 × 3.0* | 2.0 × 2.0 | 2.0 × 2.5 | | |
| 3-02 C | 2.5 × 2.0* | 2.5 × 2.5* | 2.0 × 2.5 | 2.0 × 1.5 | | |
| 3-03 A | 2.0 × 2.0 | 1.5 × 2.0 | 2.0 × 2.0" | 3.0 × 1.5* | | |
| 3-03 B | 1.0 × 1.0 | 0 | 2.0 × 2.0" | 3.0 × 1.5* | | |
| 3-04 A | 1.5 × 1.5 | 0 | 3.0 × 2.5* | 2.5 × 2.5* | | |
| 3-04 B | 2.0 × 2.0* | 1.0 × 1.5 | 2.0 × 1.0* | 2.0 × 3.0* | | |
| 3-04 C | 2.0 × 2.0 | 1.5 × 2.0 | 2.0 × 2.0 | 2.0 × 2.5 | | |
| 3-05 A | 2.0 × 1.5 | 1.0 × 1.0 | 0 | 2.0 × 1.0 | | |
| 3-05 B | 0 | 2.0 × 1.5 | 2.0 × 0.75 | 1.0 × 1.0 | | |
| 3-05 C | 0 | 2.5 × 1.5 | 2.0 × 1.5 | 3.0 × 2.5 | | |
| | | | | | | |
| Control A | 0 | 0 | 0 | 0 | | |
| Control A | 0 | 0 | 0 | 0 | | |
| Control B | 0 | 0 | 0 | 0 | | |
| Control B | 0 | 0 | 0 | 0 | | |

* Deep blue positive PCA, see text (Procedures and Results)
Control A, overlay fluid incubated without explants in tube.
Control B, overlay fluid incubated with explants taken from normal eyes.

may be noted from these tables that the findings of occasional duplicate tests are not in accord. Failure to obtain similar results in all duplicate tests is attributed to several, quite understandable technical difficulties. Injection of the toe vein of the guinea pig is a rather difficult procedure; it is therefore not always possible to control the precise quantity of antigen-dye mixture introduced into the animal's general circulation. In addition, the skin of the guinea pig is very thin, with the resulting difficulty that one cannot always be certain

that he is, in fact, introducing the precise quantity of overlay fluid intradermally on each occasion. Leakage of some of the fluid in retrograde fashion along the needle tract further complicates this situation. Consequently, dealing as we are with what are probably very minute quantities of antibodies, it is

TABLE III

*Results of the Passive Cutaneous Anaphylaxis Test on the Overlay Fluid of Explants Removed on the Fifth Postinoculation Day*

| Animal No. | Average area of positive test site | | | | | |
|---|---|---|---|---|---|---|
| | 14th postinoculation day | | 21st postinoculation day | | 28th postinoculation day | |
| | cm | cm | cm | cm | cm | cm |
| 2-01 A | 1.0 × 1.0 | 1.5 × 2.0 | 2.0 × 1.5 | 0.5 × 1.25 | 0 | 0 |
| 2-01 B | 1.5 × 2.5 | 2.0 × 1.5 | 2.0 × 2.0 | 2.0 × 2.5 | 2.0 × 2.5* | 2.0 × 2.0* |
| 2-02 A | 2.5 × 3.0 | 2.5 × 2.5 | 1.5 × 2.5 | 2.0 × 2.5 | 1.5 × 2.0* | 1.5 × 1.5 |
| 2-02 B | 1.5 × 2.0 | 2.0 × 2.0 | 0 | 0 | 2.0 × 2.5* | 2.0 × 2.0* |
| 2-03 A | 3.5 × 2.5 | 2.0 × 3.0 | 1.0 × 1.5 | 0 | 1.5 × 1.5 | 1.0 × 1.0 |
| 2-04 A | 1.5 × 0.75 | 0 | 1.5 × 1.5 | 1.75 × 1.75* | 2.0 × 3.0* | 1.5 × 2.0* |
| 2-06 A | 1.5 × 1.5 | 2.0 × 2.5 | 1.0 × 1.0 | 1.5 × 1.5* | 0 | 1.5 × 2.0 |
| 2-06 B | 1.5 × 1.0 | 1.5 × 1.5 | 1.0 × 1.0 | 1.25 × 1.0* | 2.0 × 1.5 | 2.0 × 2.0 |
| | | | | | | |
| 3-06 A | 1.5 × 2.5 | 2.0 × 2.0 | 2.3 × 1.5* | 2.0 × 2.0 | | |
| 3-06 B | 0 | 0 | 1.5 × 1.75 | 2.0 × 2.75* | | |
| 3-06 C | 0 | 0 | 2.5 × 2.5* | 2.0 × 3.0* | | |
| 3-07 A | 2.0 × 2.0 | 1.0 × 1.5 | 2.5 × 3.0* | 2.25 × 2.5* | | |
| 3-07 B | 1.5 × 3.5* | 3.0 × 2.5* | 2.0 × 2.0* | 2.5 × 1.5* | | |
| 3-07 C | 3.0 × 2.0* | 1.25 × 1.0* | 2.0 × 1.75* | 2.0 × 2.0* | | |
| 3-08 A | 1.0 × 1.0 | 1.5 × 1.0 | 2.0 × 2.5* | 1.5 × 2.5 | | |
| 3-08 B | 1.0 × 0.5 | 1.0 × 1.5 | 3.0 × 3.5* | 2.0 × 2.5* | | |
| 3-09 A | 2.0 × 3.0 | 1.5 × 2.0 | 3.0 × 2.5* | 3.0 × 3.0* | | |
| 3-09 B | 1.5 × 2.0* | 2.0 × 2.5* | 1.5 × 2.0 | 3.0 × 2.5* | | |
| 3-09 C | 2.25 × 2.5 | 2.0 × 2.0 | .2.5 × 2.5* | 3.0 × 3.0* | | |
| 3-10 A | 1.5 × 1.5 | 3.5 × 3.5* | 1.5 × 1.5 | 1.5 × 2.0* | | |
| 3-10 B | 2.5 × 1.0 | 2.0 × 2.0 | 2.5 × 2.0* | 2.5 × 3.0* | | |
| 3-10 C | 2.0 × 2.0 | 2.0 × 2.5 | 2.0 × 2.5* | 2.0 × 2.0 | | |
| | | | | | | |
| Control A | 0 | 0 | 0 | 0 | | |
| Control A | 0 | 0 | 0 | 0 | | |
| Control B | 0 | 0 | 0 | 0 | | |
| Control B | 0 | 0 | 0 | 0 | | |

* Deep blue positive PCA, see text (Procedures and Results)
Control A, overlay fluid incubated without explants in tube.
Control B, overlay fluid incubated with explants taken from normal eyes.

entirely possible that these slight discrepancies mean the difference between a positive and a negative result; most certainly they explain the difference in size of the response.

It is also to be noted that many of the values indicating the size of the positive response, as listed in the three tables, are followed by an asterisk. This signifies that the response in question was of a very deep blue color in contrast to the light blue reaction characteristic of the other positive results. A typical reac-

tion of the former type is illustrated (Fig. 4). We interpret this to indicate a greater degree of local binding of the antigen-dye (Evans blue) mixture in the case of the darker reactions, in all probability secondary to the presence of a greater quantity of antibodies in the intradermal overlay inoculum.

The results of the passive cutaneous anaphylaxis experiments demonstrate the presence of humoral antibody in the overlay fluid of all three groups of explants. No attempt was made to quantitate the amount of antibody present. We have thus shown that at least a portion of the lymphoid elements, present at the limbus during the delayed phase corneal response, is capable of producing antibodies at a later date.

A brief histologic examination was performed on the 21st postinoculation day on explants maintained *in vitro*. Our limited study of this aspect of the problem allows only the mention of two broad observations: (*a*) that the total number of infiltrating cytologic elements is markedly decreased with respect to the number present at the time the explants were initially removed from the eye and (*b*) of the infiltrating cells still remaining, the majority were noted to be plasma cells.

## DISCUSSION

Beginning with the work of Dienes and his collaborators in the 1930's, many investigators in the field of immunology (8–16) have suggested that delayed hypersensitivity is a stage in the production of antibodies. This hypothesis is based predominantly on the demonstration of a phase of delayed-type skin hypersensitivity early in the course of sensitization to a foreign protein, prior to the expected development of immediate or Arthus-type sensitivity.

These observations remained largely forgotten until 1954 when Gell and Hinde (17), on the basis of their studies of the microscopic appearance of the Arthus reaction, suggested that it consists of two components: (*a*) a delayed proliferative mononuclear reaction, similar in pattern and in the type of cells nvolved to a delayed hypersensitivity reaction of the tuberculin type, and (*b*) the acute, exudative, polymorphonuclear component responsible for the macroscopic appearance and associated with the presence of anaphylactic sensitivity. These workers also noted that during the course of immunization by repeated iintradermal injections of small doses of antigen, the mononuclear component referred to above was evident before the immediate anaphylactic hypersensitivity stage appeared. Uhr, Salvin, and Pappenheimer (18) then demonstrated that a delayed hypersensitivity state, directed against a single protein antigen, could be induced by the intradermal inoculation of minute amounts of washed immune precipitates containing the antigen in question. If the specific immune precipitates were formed in the region of antibody excess, maximum sensitivity of the delayed type developed in guinea pigs at least 2 to 3 weeks prior to the time when demonstrable circulating antibodies against the sensitizing antigen appeared. Salvin (19), Raffel and Newell (20), and Sell and Weigle (21)

all added similar observations, showing that during the course of sensitization by injection of immune precipitates in adjuvants, the delayed hypersensitivity state develops first and then gives way to the Arthus type of sensitivity as antibody production becomes manifest. Leskowitz and Waksman (22) took this observation one step further and showed that the height of the anamnestic antibody response in rabbits was correlated, to a certain degree, with the intensity of delayed hypersensitivity present before the anamnestic stimulus.

The earlier studies cited above all illustrate the point that in the absence of mycobacteria or an equivalent substitute, one encounters great difficulty producing delayed sensitivity following injection of antigen by any route other than the intradermal one. The most recent observations on the induction of delayed hypersensitivity by means of immune precipitates serve to further emphasize the importance of the intradermal route. It was therefore with much excitement that the present authors added their observation of an experimentally induced, biphasic corneal reaction, representing a transient stage of delayed hypersensitivity prior to the appearance of the antibody mediated "Wessely Phenomenon," to the literature (6).

Immediately following the introduction of a soluble protein antigen (bovine serum albumin or bovine gamma globulin) directly into the corneal lamellae, one observes an opaque bleb in the center of the cornea. This bleb merely represents the inoculum which has mechanically separated the corneal lamellae. It is either no longer visible or just barely perceptible with the unaided eye eight hours after the injection of the antigen. The cornea then remains clear during the following 2 to 3 days, at which time it suddenly develops a ground glass opacity. The opacification increases and the cornea frequently becomes so cloudy that only its surface layers can be visualized with the slit lamp. This corneal response is preceded and accompanied by a dilatation of the circumlimbal blood vessels. The reaction persists for several days; the cornea then gradually clears. This clearing phase is terminated by the development of a second circumlimbal flare, followed in a matter of a day or so by the development of an intracorneal ring of opacification which is visible in the gross. The rings so formed are dependent upon the presence of circulating antibodies and persist for a variable number of days, rarely exceeding 2 weeks. Finally, the cornea once again clears and remains normal in the gross.

The early phase of corneal clouding, occurring approximately 3 to 5 days after the introduction of antigen into the cornea, is distinct from the later appearing, antibody mediated ring response. At the time it is manifested, no humoral antibodies are demonstrable by either serological or histological techniques. It is reproducible neither with non-allergic reagents nor with homologous or autologous proteins. A concomitant delayed skin hypersensitivity to the specific antigen is generally present; this reactivity can be passively transferred with cells but not with serum or cell-free products. Based on these

observations, the authors concluded that the primary corneal response is a manifestation of delayed sensitivity of what Raffel terms the "Jones-Mote" type (23).

The primary response in the cornea has been shown to be dose-dependent. In direct contrast to previously described biphasic immune reactions in which minute quantities of the sensitizing antigen were utilized, our primary reaction was not obtained unless a rather large dose of antigenic protein was inoculated into the cornea. In our experience, it was never demonstrated when less than 1.9 mg of BSA were used and was not reliably reproducible until the inoculum was increased to the order of 4 mg of protein. Again in contrast to several studies cited, neither adjuvant nor mycobacteria were utilized. It is well established that killed acid-fast organisms, such as mycobacteria, when mixed in adjuvants with protein antigens, greatly enhance both the degree of delayed sensitivity as well as the extent and duration of the antibody response. In addition, the incorporation of the sensitizing antigen into Freund's complete adjuvant greatly decreases the significance of the intradermal route in the determination of the degree of delayed type sensitization. This manipulation renders other routes, the subcutaneous or intramuscular for example, equally effective in the induction of the delayed hypersensitive state. It has been suggested that the stimulation of the sensitization provoked by mycobacteria is the result of the type of cellular reaction they call forth. We have previously presented evidence indicating that comparable titers of circulating antibodies are induced following the injection of identical quantities of antigen into the avascular ocular tissues (i.e., cornea or vitreous body) or intramuscularly in adjuvant (24). It was suggested that the introduction of antigen into the avascular cornea results in the slow diffusion of the antigen from the depot thus formed. This results in the prolonged stimulation of antibody production—much the same mechanism postulated for the effectiveness of adjuvant in the induction of antibody formation. It may well be that this identical local retention of antigen, with the subsequent formation of a focal granuloma, is also responsible for the effectiveness of the intracorneal route of immunization in the induction of the delayed hypersensitive state.

Dienes and Mallory (10) long ago showed that the delayed type of skin reaction, demonstrable prior to anaphylactic sensitivity, was characterized by a marked infiltration of leukocytic elements, predominantly of the mononuclear type; they were among the earliest investigators to stress that this mononuclear cell infiltration was a definite and specific part of delayed hypersensitivity. They further stressed that the later anaphylactic type of reaction was characterized by a polymorphonuclear infiltration, and were perhaps the first to suspect the linkage between the cellular infiltrate of the two modes of hypersensitivity. It remained for Gell and Hinde (17), however, to emphasize that active Arthus reactions are, in reality, a two-component system. This system is made up of

(a) a "delayed" component, characterized by mononuclear cell infiltration very similar to that found in tuberculin-type sensitivity, and (b) the generally recognized "immediate" polymorphonuclear component, responsible for the macroscopic appearance and associated with the presence of anaphylactic sensitivity. These authors also identified a stage of extensive maturation of the proliferated mononuclear cells to elements of the plasma-cell series. In doing so, they described all transitions between immature plasma cells and mature plasma cells on the one hand and histiocytes and undifferentiated mesenchyme cells on the other. They suggested that this stage is connected with the production of "free" antibody.

Our microscopic observations of the biphasic corneal reaction leave us very much in accord with Gell's views. During the early, delayed type corneal reaction, a marked infiltrate of lymphocytic-mononuclear elements was noted at the limbus. This localized collection of lymphoid elements is not present in the normal cornea and is a product of the sensitization of this tissue with soluble protein antigens. Histologic examination of the experimental eye during the later antibody-mediated ring response again reveals a marked infiltrate at the limbus, but with a significant change in the component cytologic elements. The limbal focus in this case is made up overwhelmingly of plasma cells. In the interval between the two corneal reactions, the limbal infiltrate is characterized by transitional forms, the maturation of the plasma cell series paralleling the appearance of humoral antibody.

Based on the sequential relationship of the two types of immune phenomena as well as the histologic evidence described above, Gell and Hinde suggested, as a working hypothesis, that a course of immunization induces all the cells of the reticulo-endothelial system to develop a specific reaction capacity to antigen. In support of this, they cited the infiltration and proliferation of "delayed" reactions. The course of immunization subsequently leads to the maturation of these cells to plasma cells and to the production of free antibody. These authors regarded the classical tuberculin reaction as an incomplete immunization response that has failed to progress to the stage of the formation of plasma cells and free antibody, with both proliferation and cell differentiation ceasing prematurely. Somewhat later, Gell cautiously modified this view. In studies utilizing proteins conjugated with picryl, acetyl, and ethoxymethylenephenyloxazolone groups, he demonstrated delayed hypersensitivity to the protein in the absence of detectable antibodies against it, despite the concomitant formation of antibodies against the hapten itself. Delayed hypersensitivity to the haptenic group was not demonstrable. On the basis of these results, the authors offered the possibility that the two modes of hypersensitivity might, despite their apparent temporal relationships, be qualitatively different processes with no actual continuity at the biochemical level (25, 26). Salvin and Smith (27), on the other hand, following similar observations with protein conjugates, felt

that delayed hypersensitivity is indeed a step in the production of circulating antibodies. They concluded that it is an immature stage in the immune process and therefore associated with a different, broader part of the antigen molecule than are the more mature stages of immunity. It was their feeling that the delayed response is directed toward some broad determinant in the protein molecule itself. As the immune process evolves and the basis for the circulating antibody is laid, the determinant factor in the antigen molecule becomes more limited, finite, and specific and changes to small surface groupings. In contrast to this theory, that delayed sensitivity represents a state of altered reactivity of the cells of the lymphocytic-mononuclear series and that it is an integral step in the development of circulating antibodies, Waksman and Matoltsy (28) suggested the thesis that delayed hypersensitivity represents an alternative type of response to antibody formation. They postulated that the sensitive cells in question might respond to an antigenic stimulus by proliferation and evolution to histiocytes and/or macrophages in the case of delayed hypersensitivity and into plasma cells in the immediate type of reaction.

Our previous studies seemed to support the hypotheses that (a) delayed hypersensitivity is a stage in antibody production; and (b) it is correlated with the evolution of the lymphocytic-mononuclear cells (the reactive cells in delayed sensitivity) to preplasma and finally to plasma cells with the concurrent formation of circulating antibodies. It was stated earlier that the delayed phase corneal response is dose-dependent, occurring only after a large antigenic stimulus. Histologic studies of the reaction, with the amount of antigenic inoculum as a variable, revealed that the focal infiltration of the limbus by lymphoid-mononuclear elements is also quantitatively directly proportional to the amount of antigen introduced into the cornea. Inoculation of large doses results in an extensive infiltrate at the limbus; lesser doses, in a diminished response. Since x-irradiation as well as the administration of either cortisone or lymphocyte antiserum produce considerable degrees of lymphopenia with the concurrent depression of delayed hypersensitivity reactions, it does not seem at all unreasonable to conclude that lymphocytes and/or closely related mononuclear cells are essential to these reactions. We therefore felt that the next course of action, to further support the linkage theory, was the demonstration that the cells of the lymphocytic-mononuclear series, present at the limbus during the delayed type corneal reaction and obviously in some way a part of it, were capable of going on and producing antibodies. Our experimental system, utilizing the cornea and limbus of the rabbit eye, offered unique advantages for the study of this problem. In it, we were able to induce a localized collection of lymphoid elements which are not present in the normal cornea and which accompany and are part of a corneal hypersensitivity reaction of the delayed type. This lymphoid focus is solely the product of the sensitization of the tissue with a specific soluble protein antigen. Our procedure thus eliminates a problem

incurred with the utilization of lymph nodes, that of insult to the experimental system by multiple antigens. Moreover, our "induced lymph node" lies in a collagenous stroma whose structural simplicity and homogeneity lend it nicely to microscopic study of invasion by foreign elements secondary to an allergic response. We had previously been able to show that a similar lymphoid focus persists in an experimental eye months after sensitization. Further, it had been demonstrated that these lymphoid elements are rapidly converted into plasma cells, accompanying a localized anamnestic response to intravenous challenge with the specific antigen (29). In view of the general agreement that plasma cells are either the sole or, at very least, one of the major cellular sources of antibodies, it seems quite reasonable to equate this stage of maturation with local antibody production.

To the end then, of demonstrating that the cytologic elements present during the delayed corneal reaction possess the capability of producing specific humoral antibodies which are responsible for the later immediate type ring response, we enucleated the right eye of rabbits during the height of the delayed phase response, 3 to 5 days after the intracorneal inoculation of bovine serum albumin. Immediately thereafter, 1 mm buttons of limbal tissue, containing large numbers of the infiltrating cells, were removed under aseptic conditions utilizing a corneal trephine and the explants thus obtained maintained in tissue culture. By means of the passive cutaneous anaphylaxis technique, it was shown that the overlay fluid contained specific antibodies to bovine serum albumin.

Thus we have demonstrated that at least some of the lymphocytic-mononuclear elements which infiltrate the limbus at the time of the delayed type corneal response are capable of then going on to produce humoral antibodies. This would seem to indicate, at least with regard to our biphasic corneal reaction, that the two types of hypersensitivity are clearly related, and indeed suggests, with a high degree of probability, that they are interdependent. We feel that our present experimental results strongly suggest that the delayed hypersensitivity response arises in the very cells or in cells closely related to those which eventually differentiate into plasma cells, produce humoral antibodies, and are the basis for the later, anaphylactic type of sensitivity. Unfortunately, the observations presented fail to determine which of the possibilities prevails—whether delayed hypersensitivity and circulating antibodies have their origins in different, closely related cells both of which are present during the earlier delayed phase response or, alternatively, whether the two types of immune phenomena indeed represent either distinct or interrelated physiologic processes occurring in the identical cell.

## SUMMARY

The intracorneal inoculation of a sufficient quantity of a soluble protein antigen into the eye of a rabbit produces a biphasic allergic reaction in that

cornea. The earlier stage, characterized by a diffuse corneal clouding, is a manifestation of delayed hypersensitivity and is accompanied by a limbal infiltrate composed predominantly of lymphocytic-mononuclear elements. The later response, known as the Wessely Phenomenon, is a ring of opacification in the cornea which is visible in the gross. This reaction is dependent upon the presence of specific circulating antibodies and is therefore classified among the immediate types of hypersensitivity. It is accompanied by a dense limbal infiltration of plasma cells. Intervening between the two reactions is a period of several days during which the eye appears relatively normal.

Explants containing large numbers of infiltrating lymphocytic-mononuclear elements were removed from the corneal-scleral junction of experimental eyes during the height of the delayed type hypersensitivity reaction and maintained *in vitro* in tissue culture. At a later date the overlay fluid in which the explants were maintained was shown to contain specific humoral antibodies, demonstrating the capability of cells present at a delayed reaction for the later production of antibodies. The possible linkage of the two modes of immune phenomena is discussed.

## BIBLIOGRAPHY

1. Wessely, K., Ueber anaphylaktische Erscheinungen an der Hornhaut (Experimentelle Erzeugung einer parenchymatösen Keratitis durch artfremdes Serum), *Münch. med. Woch.* 1911, **58,** 1713.

2. Von Szily, A., and Arisawa, U., Die Anaphylaxie in der Augenheilkunde, Stuttgart, Ferdinand Enke, 1914.

3. Germuth, F. G., Maumenee, A. E., Pratt-Johnson, J., Senterfit, L. B., Van Arnam, C. E., and Pollack, A. B., Observations on the site and mechanisms of antigen-antibody interaction in anaphylactic hypersensitivity. *Am. J. Ophth.,* 1958, **46,** 282.

4. Breebart, A. C., and James-Witte, J., Studies on experimental corneal allergy, *Am. J. Ophth.,* 1959, **48,** 37.

5. Schwab, F., Untersuchungen über den Antikörpergehalt (Pracipitine) der Hornhaut nach gleichzeitiger Einverleibung verschiedener Antigene. Ein Beitrag zur Frage der lokalen Antikörperbildung in der Hornhaut. Graefe *Arch. Ophth.,* 1959, **160,** 592.

6. Parks, J. J., Leibowitz, H. M. I., and Maumenee, A. E., A transient stage of suspected delayed sensitivity during the early induction phase of immediate corneal sensitivity, *J. Exp. Med.,* 1962, **115,** 867.

7. Ovary, Z., Immediate reactions in the skin of experimental animals provoked by antigen-antibody interaction, *Progr. Allergy,* 1958, **5,** 459.

8. Dienes, L., The first manifestation of the developing hypersensitiveness, *Proc. Soc. Exp. Biol. and Med.,* 1930, **28,** 75.

9. Dienes, L., Comparative study of the anaphylactic and tuberculin types of hypersensitiveness. II. The influence exerted by the nature of the antigen on

the development of the different types of hypersensitiveness, *J. Immunol.*, 1931, **20**, 333.

10. Dienes, L., and Mallory, T. B., Histologic studies of hypersensitive reactions, *Am. J. Path.*, 1932, **8**, 689.

11. Dienes, L., and Simon, F. A., The flaring up of injection sites in allergic guinea pigs, *J. Immunol.*, 1935, **28**, 321.

12. Dienes, L., The specific immunity response and the healing of infectious diseases. Significance of active immunity and the connections between the immunity response and the anatomic lesions, *Arch. Path.*, 1936, **21**, 357.

13. Redfern, W. W., Skin reactions produced by antihuman serum, *J. Immunol.*, 1930, **18**, 109.

14. Jones, T. D., and Mote, J. R., The phases of foreign protein sensitization in human beings, *New England J. Med.*, 1934, **210**, 120.

15. Mote, J. R., and Jones, T. D., The development of foreign protein sensitization in human beings, *J. Immunol.*, 1936, **30**, 149.

16. Simon, F. A., and Rackemann, F. M., Development of foreign protein sensitization in man following intradermal injection of antigen, *J. Allergy*, 1934, **5**, 439.

17. Gell, P. G. H., and Hinde, I. T., Observations on the histology of the Arthus reaction and its relation to other known types of hypersensitivity, *Int. Arch. Allergy*, 1954, **5**, 23.

18. Uhr, J. W., Salvin, S. B., and Pappenheimer, A. M., Induction of delayed hypersensitivity in guinea pigs by means of antigen-antibody complexes, *J. Exp. Med.*, 1957, **105**, 11.

19. Salvin, S. B., Occurrence of delayed hypersensitivity during development of Arthus type hypersensitivity, *J. Exp. Med.*, 1958, **107**, 109.

20. Raffel, S., and Newell, S. M., The "delayed hypersensitivity" induced by antigen-antibody complexes, *J. Exp. Med.*, 1958, **108**, 823.

21. Sell, S., and Weigle, W. O., The relationship between delayed hypersensitivity and circulating antibody induced by protein antigens in guinea pigs, *J. Immunol.*, 1959, **83**, 257.

22. Leskowitz, S., and Waksman, B. H., The effect of route of injection of BSA in Freund adjuvant on the production of circulating antibody and delayed hypersensitivity, *J. Immunol.*, 1960, **84**, 58.

23. Raffel, S., *Immunity*, New York, Appleton-Century-Crofts, Inc., 2nd Edition, 1961, 349.

24 Parks, J. J., Leibowitz, H. M. I., and Maumenee, A. E., The effect of route of inoculation upon development of antibody in rabbits, *J. Immunol.*, 1961, **87**, 199.

25. Benacerraf, B., and Gell, P. G. H., Studies on hypersensitivity. I. Delayed and Arthus-type skin reactivity to protein conjugates in guinea pigs, *Immunology*, 1959, **2**, 53.

26. Gell, P. G. H., and Benacerraf, B., Studies on hypersensitivity. II. Delayed hypersensitivity to denatured proteins in guinea pigs, *Immunology*, 1959, **2**, 64.

27. Salvin, S. B., and Smith, R. F., The specificity of allergic reactions. I. Delayed versus Arthus sensitivity, *J. Exp. Med.*, 1960, **111**, 465.
28. Waksman, B. H., and Matoltsy, M., The effect of tuberculin on peritoneal exudate cells of sensitized guinea pigs in surviving cell cultures, *J. Immunol.*, 1958, **81**, 220.
29. Leibowitz, H. M. I., Parks, J. J., and Maumenee, A. E., Manifestations of localized hypersensitivity in a previously sensitized tissue, *Arch. Ophth.*, 1962, **68**, 66.

EXPLANATION OF PLATES

PLATE 2

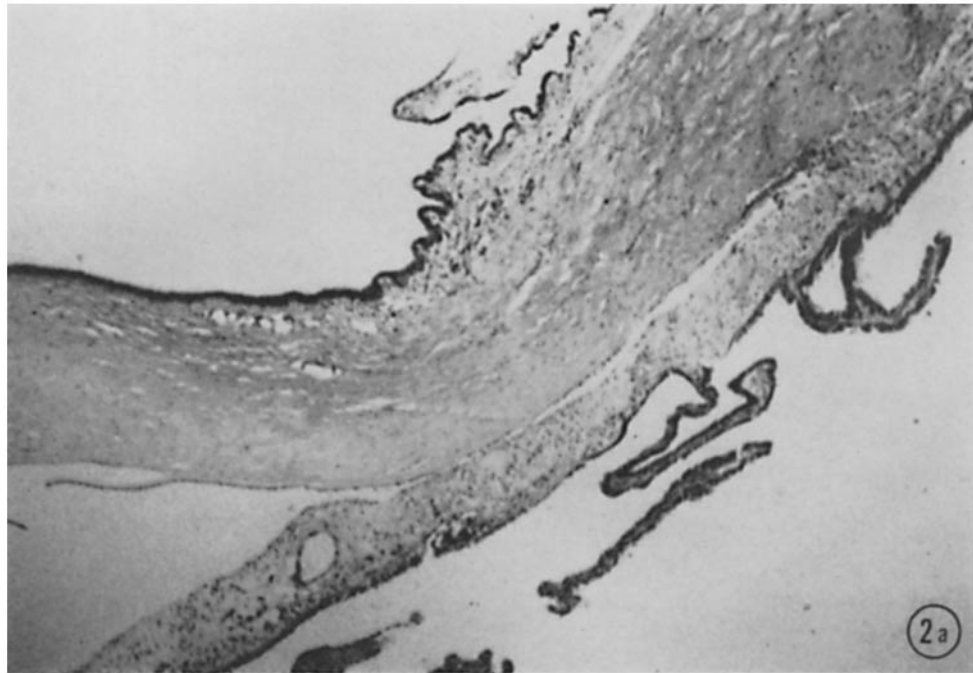FIG. 1. Diffuse corneal clouding during the "delayed" type hypersensitivity reaction. × 5.
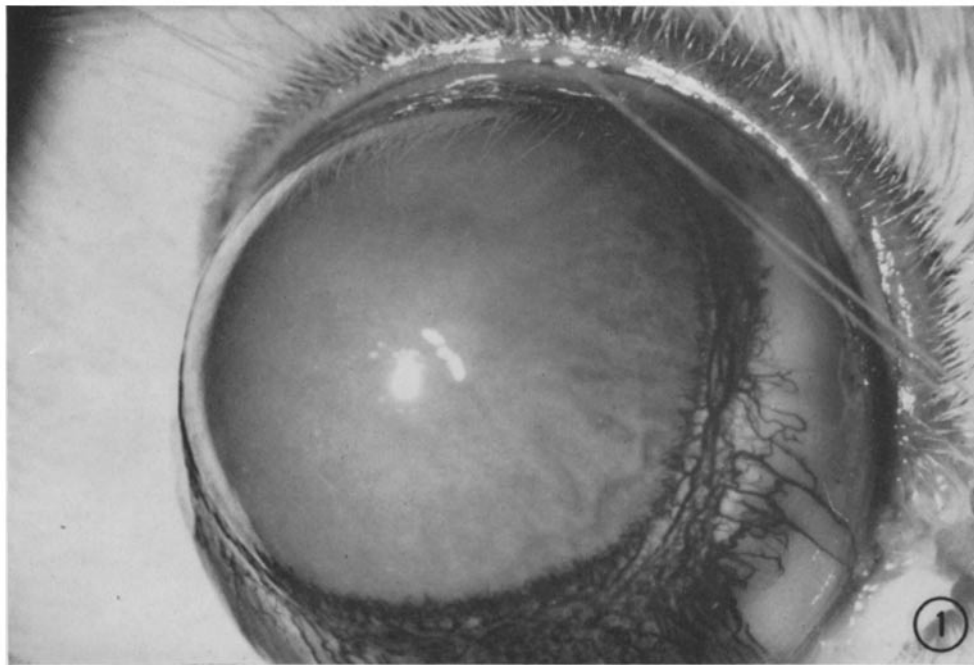
(Leibowitz and Parks: Corneal limbal lymphoid foci)

PLATE 3

FIGS. 2a and 2b. 2a, Histologic section of the limbus of a normal rabbit eye. 2b, Similar section of an experimental eye illustrating the dense focal infiltration by lymphoid elements. × 25.
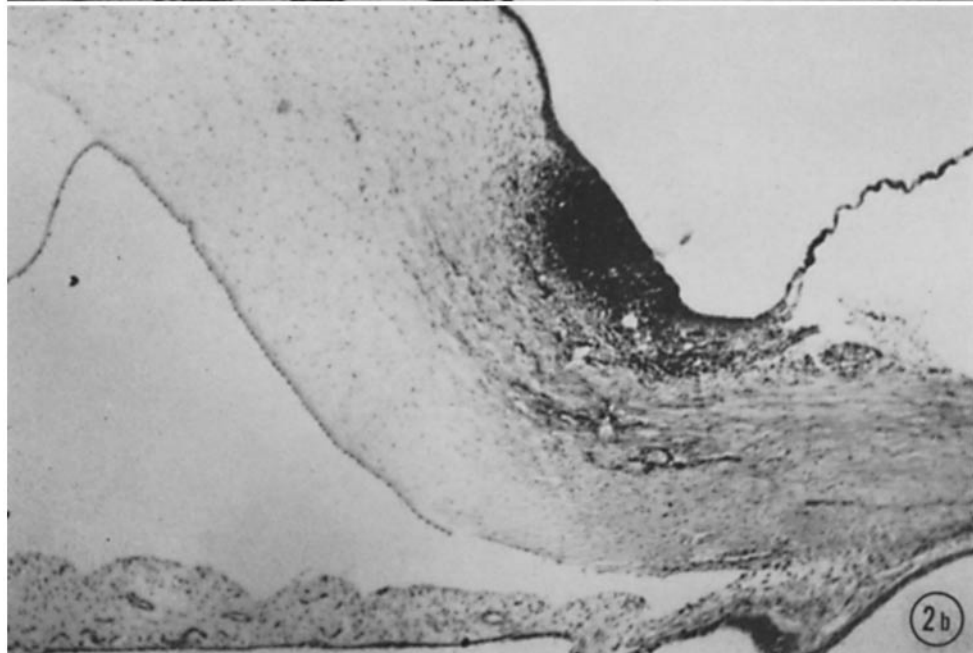
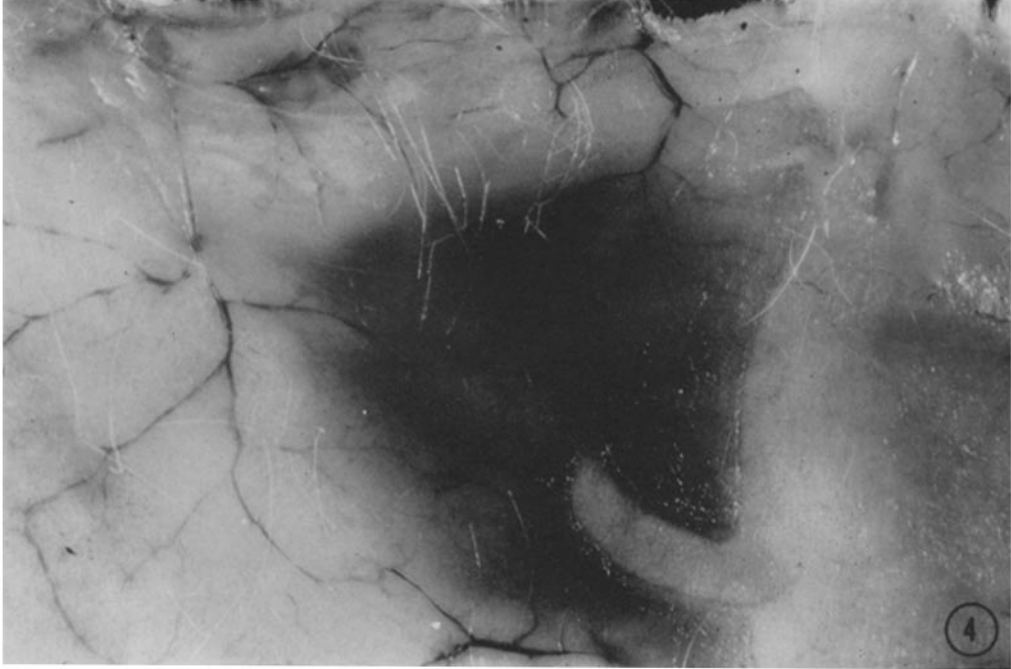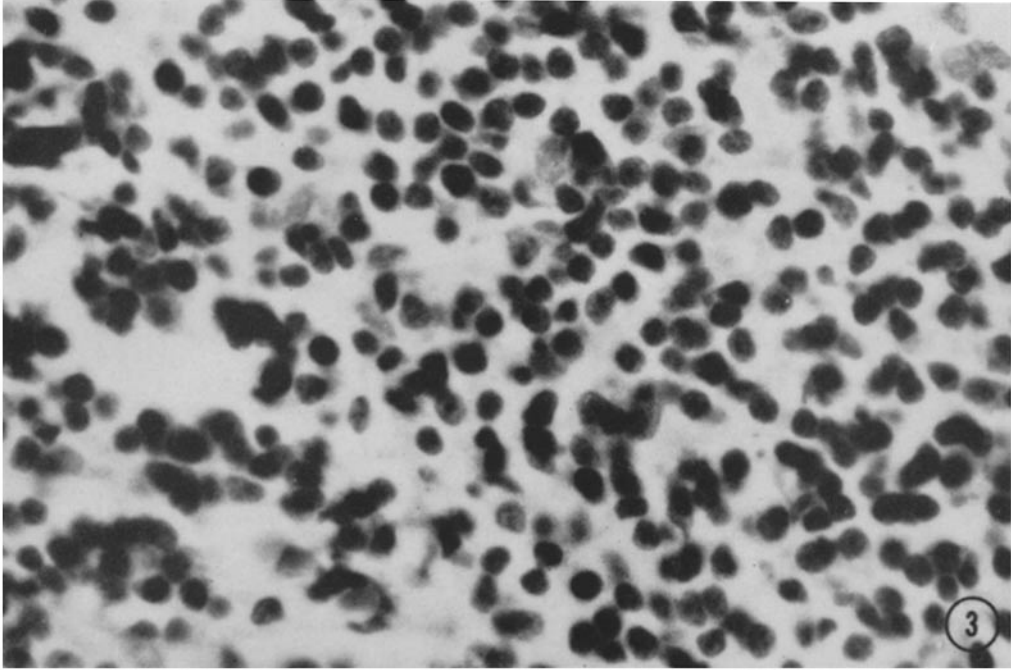FIG. 3. High power view of a section of an explant fixed in formalin and stained with hematoxylin and eosin illustrating heavy infiltration of the tissue by lymphocytic elements. × 1000.

FIG. 4. Positive PCA. × 3.

(Leibowitz and Parks: Corneal limbal lymphoid foci)