Antibody formation by single cells during experimental immunogenic uveitis

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The Jerne plaquing technique for the demonstration of single antibody-forming cells was employed to study the kinetics of the antibody response that accompanies experimental immunogenic uveitis in rabbits produced by intravitreal injection of sheep erythrocyte stromas. Prior to the onset of uveitis, a modest increase in antibody formation in the spleen can be detected. With the appearance of uveitis, the proportion in the uveal tract of inflammatory cells that form antibody rapidly increases, reaches a peak at about the tenth day, and then slowly declines; 150 days after clinical uveitis has subsided, no antibody-forming cells can be found in the uveal tract. The role of ocular antibody formation in the pathogenesis of recurrent uveitis is discussed.

Key words: Allergic uveitis, antibody formation, antigens, red blood cells, intravitreal injection, neutralizing antibody titers, antigen-antibody reactions, spleen, uvea, aqueous humor, time factors, pathogenesis.
in uveal tissue,\textsuperscript{17,19} and even in the cornea.\textsuperscript{20,21} Of great practical interest was the demonstration\textsuperscript{16,22} that antibody formed within the eye might be extremely useful in the establishment of the etiologic diagnosis of uveitis.

The fundamental significance of antibody formation within the eye is still unclear. Many investigators appear to consider, at least implicitly, that antibody is formed within the eye during uveitis as the silent witness of an earlier and more significant immunologic event, and is thus not importantly implicated in the initiation of the disease process. On the other hand, it has recently been suggested\textsuperscript{23} that the cellular events associated with the act of antibody formation may play a significant role in the pathogenesis of uveitis, especially the recurrent anterior form of the disease.

One of the barriers to further investigation of the significance of local ocular antibody formation has been the technical difficulty in detecting minute amounts of antibody in tissue suspensions. It has thus been difficult to characterize quantitatively the temporal events, both cellular and molecular, that occur during the development of experimental immunogenic uveitis. The introduction by Jerne and co-workers\textsuperscript{24} of a technique sensitive enough to detect antibody formation by individual cells has opened the door to further investigation of this problem. In the present study, this technique was adopted for use with ocular tissue in an effort to assess its usefulness in sorting out some of the complex cellular events that occur during immunogenic ocular inflammation. Preliminary data are presented on the cellular kinetics of antibody formation within the uveal tract (and spleen) in response to antigenic stimulus within the eye.

Materials and methods

\textbf{Antigens.} Reagents were sterilized by Seitz filtration. Sheep red blood cells collected in Alsever's solution were washed three times in normal saline and then lysed in five times their own volume of 0.1M sodium acetate buffer, pH 4.6. The stroma was collected and washed repeatedly by centrifugation with the acetate buffer until it was only slightly pink. The sediment was then suspended in an equal volume of phosphate-saline buffer, pH 7.4, and stored at -20\degree C, without preservative.

In order to assure that the data obtained in this study with sheep erythrocyte stroma are generally applicable, and not peculiar to this particular antigen, the adaptation of the plaquing technique proposed by Merchant and Hrabai\textsuperscript{25} was also employed. Following their procedure, arsanilic acid, diazotized and coupled to bovine serum albumin (Ars-BSA), was employed for intravitreal sensitization of rabbits; and arsanilic acid diazotized and coupled to sheep erythrocytes was employed as the indicator in the plaque-assay procedure described below. Since difficulty was experienced in obtaining reproducible preparations of this type, the study of antibody formation to hapten antigens will be described only briefly in this paper, insofar as it parallels the results obtained with the sheep erythrocyte stroma system.

\textbf{Animals.} New Zealand Giant albino rabbits weighing 7 to 9 pounds were used. They were given Purina rabbit chow and water ad libitum. Before use all eyes were examined with the slit lamp for the presence of pre-existing disease.

\textbf{Intravitreal injections.} Injections were performed after anesthesia with 0.5 per cent proparacaine HCl. The eye was fixed by gently grasping the sclera with forceps, and the eye rotated downward. A sharp 27 gauge needle was then inserted at a slight angle backward, 2 mm. behind the corneoscleral junction, avoiding the lens. A 0.1 ml. quantity of the stroma preparation (equivalent to about 10\textsuperscript{6} RBC) or of a 1 per cent solution of Ars-BSA was injected into the center of the vitreous. The amount of material that exuded from the injection site was slight, and on only a few occasions was a transient subconjunctival bleb observed.

\textbf{Examination.} The animals were observed daily, grossly and with the slit lamp, for perlimbal injection, iris hyperemia, anterior chamber flare and cells, precipitates and fibrin in the anterior chamber, and distortions of the pupillary margin of the iris due to posterior synechiae. The uveitis was graded on a 4+ scale. Vitreous veils were observed after injection of the stroma suspension.

\textbf{Preparation of cell suspension.} Following intravitreal inoculation, rabbits were painlessly put to death with intravenous air injections at intervals before and after the onset of uveitis. The eyes were removed and the uveal tract was dissected out by inserting a narrow, sharp corneal scalpel through the limbus and into the anterior chamber, and then sharply dissecting away the cornea with a few millimeters of sclera. The iris could then be grasped with fine, smooth forceps and gently
pulled away with the ciliary body. It was often necessary to scrape the adherent iris from the lens. The intact iris and ciliary body were placed in 2 c.c. of chilled Hank's medium. A suspension of single cells was produced by scraping the uveal tract over a 40-mesh stainless steel screen into the Hank's medium, until no large aggregates remained. For further separation of clumps, this suspension was aspirated into a small bulb pipette and was forced through the screen several times. The suspension was then stored on ice and, after gross sediment was separated by decantation, the cells were centrifuged and resuspended in fresh Hank's medium to eliminate any free antibody which had been observed in some cases to cause complete hemolysis on the agar plates. Occasionally, aqueous humor samples were obtained prior to dissection and held in small tubes for plating.

The spleen was also removed, and cell suspensions were prepared for assay using the same technique. Cell counts were performed on these suspensions using Giemsa stain and standard white-cell counting chambers. Smears of the suspensions, stained with Giemsa or Wright's stain, were examined in an unsuccessful attempt to determine the proportions of various cell types present.

Antibody plaquing. The technique of Jerne and associates24 for the assay of antibody formation by single cells was utilized. Petri dishes (2 inch diameter) plated with 1.5 per cent agar in 6 c.c. of Eagle's Tris culture medium (pH 7.3 to 7.4) were stored at 4° C. On these dishes the following mixture was plated: 1.2 c.c. of 0.75 per cent agar in Eagle's Tris culture medium, pH 7.3 to 7.4 (kept at 45° C.); 0.05 c.c. of 1 per cent diethy laminoethyl Dextran; 0.05 c.c. of 30 per cent washed sheep red blood cells or arsanilic acid azo-erythrocytes in saline; and a precisely measured volume (0.1 to 0.2 c.c.) of the uveal-cell or spleen-cell suspension. Each plate was prepared in duplicate. The plates were incubated at 37° C. for one hour, after which 1 c.c. of 20 per cent guinea pig complement in Tris buffer was added. Each plate was prepared in duplicate. The plates were reincubated for one hour, the complement was poured off, and the surface was rinsed gently with saline. Plaques were counted with the use of a dissection microscope. Results were expressed as numbers of plaque-forming cells (PFC's) per million cells plated.

Results

Rabbits were killed and the plaquing technique applied to uvea and spleen at 3, 5, and 7 days after the intravitreal injection of the sheep red blood cell preparation; most of the animals developed uveitis on day 9. One to three rabbits were also killed on the day of onset of uveitis (day zero), almost daily for the next two weeks, and at intervals thereafter through day 46. Ocular antibody formation was also assessed 150 days after the initial onset of uveitis. In addition, two uninjected control animals were killed. Fig. 1 illustrates these results in graphic form, and permits a ready comparison of the uveal and spleen response. A representative portion of the raw data is presented in Table I, so that an appreciation may be obtained of the numbers of cells employed in this approach.

Controls. Uninjected animals had clinically normal eyes, with no PFC's detectable in the uvea. The control spleens did have a few "background" cells, at a level of 0.12 to 0.16 PFC's per million spleen cells. In no case did the uninjected fellow eye of a test animal become inflamed, nor were any antibody-forming cells (PFC's) found in the uninjected eye.

Injected animals, prior to onset of uveitis. Clinically, these animals had shown only a mild, transient reaction to the trauma of injection. None of these animals was found to have any PFC's in the uveal tract. However, there was a progressive tenfold increase in the proportion of PFC's in the spleen, rising to a level of 1.5 PFC's per million spleen cells by the seventh day after intravitreal injection, prior to the onset of clinical ocular disease.

At onset of uveitis. This was the first day on which PFC's were found in the uvea, at a level of 0.12 to 0.16 PFC's per million cells plated. At this point, the activity in the spleen had reached 2.3 PFC's per million.

Days 1 to 3 of uveitis. Clinically, these animals exhibited a full-blown uveitis, with 3 to 4+ aqueous flare and cells, perilimbal injection, and iris hyperemia. The proportion of PFC's in the uvea rose to about 200 PFC's per million, whereas the level in the spleen reached its maximum of just below 100 PFC's per million in some animals.

Days 4 to 9 of uveitis. During this period, the clinical uveitis slowly resolved,
Fig. 1. Cellular kinetics of uveal and spleen antibody formation following intravitreal injection of sheep erythrocyte stroma. Numbers of antibody (plaque)-forming cells (PFC's) per million cells plated are plotted against time, using the day of onset of uveitis as the principal reference time.

Table I. Plaque assay of antibody formation by single cells in the uveal tract and spleen of sensitized rabbits: representative data

<table>
<thead>
<tr>
<th>Day of uveitis</th>
<th>Cells plated (thousands)</th>
<th>Plaques per plate</th>
<th>PFC's per million</th>
<th>Cells plated (millions)</th>
<th>Plaques per plate</th>
<th>PFC's per million</th>
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<td>Control</td>
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<td>0</td>
<td>0</td>
<td>19</td>
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<td>0.16</td>
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<tr>
<td>Control</td>
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*Average of 2 plates per tissue.
presenting only a mild aqueous flare and perilimbal injection. However, the proportion of antibody-forming cells in the uveal tract continued to rise, reaching a level of 1,500 PFC's per million by day 9. The spleen, on the other hand, had reached a plateau at this time, with values ranging up to 40 PFC's per million.

After clinical uveitis. After day 9, the injected eyes were essentially normal. However, on the tenth day after the onset of uveitis, when clinically apparent inflammation had subsided, the peak level of PFC's was found in the uveal tract, the maximum reaching 7,000 PFC's per million cells plated. The spleen at this time remained at a level averaging 20 PFC's per million. After day 10, the level of PFC's in the uveal tract slowly decreased over a period of 1 to 2 months, 10 PFC's per million being found on the thirty-fourth day after the onset of uveitis. At 150 days, PFC's could no longer be found in the uvea of the injected eye. Likewise, after day 10, the level of PFC's in the spleen began to decline at a slow rate, reaching a level that approached "background" activity at 150 days.

Aqueous humor studies. Aqueous humor samples obtained in several animals immediately after they were put to death contained an occasional antibody-forming cell. No quantitation was possible in terms of PFC's per million in this system, because of the paucity of material available; but more important was the ability to detect even a single antibody-forming cell. Studies of aqueous were further complicated by the large amounts of free antibody often present in the fluid, frequently leading to general hemolysis of the red cells on the assay plate.

Antihapten studies. The technique of Merchant and Hrabá permitting the plaque-assay of the formation of antihapten antibodies by single cells was investigated in a preliminary manner. Uveitis could be induced routinely by intravitreal injection of 1 mg. of bovine serum albumin-azo-arsanilic acid, and the antihapten antibody hemolytic plaques were assayed by using the same hapten diazotized and coupled to sheep erythrocytes. In view of the difficulty experienced in obtaining reproducible preparations of the azo-erythrocytes, complete cell kinetic curves of antihapten antibody formation associated with this type of immunogenic uveitis could not be obtained. However, sufficient data were forthcoming to demonstrate the essential similarity between antierythrocyte and antihapten response in uveitis induced by the respective antigen.

Discussion

In the present study, a plaquing technique permitting the enumeration of single antibody-forming cells has been applied to the study of experimental immunogenic uveitis. A quantitative comparison of antibody formed locally in the eye with that formed systemically in the spleen provides a clearer picture of the cellular kinetics of the immunogenic inflammatory reaction in the uveal tract, and of the role of intraocular antibody formation in this immunopathologic process.

Following intravitreal injection of antigen, a depot is formed within the eye from which antigen leaks only slowly out into the circulation. The escaping antigen stimulates the spleen and other lymphoid tissue, resulting in systemic sensitization to the foreign protein. Abrogation of the systemic response, such as may be accomplished by whole-body irradiation (even with the eyes shielded), has been shown to inhibit the subsequent development of uveitis in the injected eye. Further confirmation of the systemic sensitization prior to the development of uveitis was demonstrated by the findings recorded here of a tenfold increase in the number of antibody-forming cells in the spleen. Only the extreme sensitivity of the plaque-assay technique employed in this study permitted detection of this early response in the spleen, amounting to about one antibody-forming cell per million lymphoid cells. This confirms the earlier
finding on the absence of significant titers of circulating antibody at the time of onset of uveitis.

As the result of the continuing leakage of antigen from the eye, systemic sensitization progresses, so that, 7 to 10 days following intraocular injection, the antigen remaining in the eye becomes the specific target of the organism's stimulated immune mechanisms. The interaction that then takes place assumes the form of a marked clinical uveitis. The cytology of the early inflammatory response and the absence of detectable circulating antibody at the time of onset of this inflammation support the conjecture that antigen-activated immunologically competent cells from the circulation mediate the uveitis, rather than free antibody. The proliferation and differentiation of these cells lead to the expanding population of antibody-forming cells detectable in the uveal tract in this study (Fig. 1).

Even after the clinical reaction has passed its maximum, the proportion of antibody-forming cells in the uveal tract continues to increase. This is undoubtedly due to the continued persistence in the inflammatory site of the highly differentiated antibody-forming cells and their precursors, in conjunction with the departure from the site of other, immunologically "uninvolved" inflammatory cells. The presence of plaque-forming cells as long as a month or more after clinically apparent uveitis has disappeared confirms the earlier morphologic demonstration of the persistence of plasma cells in the anterior uvea for equally long periods of time. Preliminary studies of the antihapten antibody response accompanying uveitis induced by the hapten-protein conjugate (arsanilic acid-azo-bovine serum albumin) show the two systems to have similar kinetics, and thus confirm the general nature of this uveal response to intraocular antigen.

It is interesting to compare the level of antibody activity within the eye to that in the spleen, in a situation in which the antigenic stimulus is initially intraocular. In this instance, the specific activity of the spleen is significantly lower than that found in the eye. The response in the spleen is an early gradual increase in the proportion of antibody-forming cells, reaching a level of about 50 PFC's per million lymphoid cells, or roughly 1/100 of that found in the eye. This is followed by a gradual diminution of activity, to control levels. This is a pattern differing from that found in the adult in response to an intravenous or intraperitoneal injection of antigen. The latter response is marked by a peak of activity on the fourth to sixth day following injection of antigen, with a subsequent rapid decline in activity.

This result is explicable on the basis of the slow leakage of antigen from the eye, resulting in a more continuous stimulation of extraocular lymphoid tissues over a greater period of time. It should be further pointed out that the large number of cells (probably both epithelial and stromal) found in preparations from control eyes suggests that the proportions of antibody-forming cells found during uveitis represent underestimates.

Of special interest in a consideration of the pathogenesis of recurrent immunogenic uveitis has been the repeated demonstration of a long-term persistence within the eye of immunologic memory of the antigen responsible for the earlier inflammatory response. Thus, many investigators have been able to induce an exacerbation of uveitis only within the previously involved eye by systemic challenge with homologous antigen, many months after the initial episode. Preliminary efforts along the same lines were also successful with the sheep erythrocyte antigen system employed here, despite the fact that at the time of systemic challenge (6 months after immunogenic uveitis induced by stroma), no antibody-forming cells could be found within the uveal tract. These data implicate further the interaction of antigen with immunologically-competent lymphoid cells directly, rather than through the mediation of conventional circulating...
antibody. They lend further support to the earlier suggestion that intraocular antibody formation per se may be an important pathogenetic factor in the development of recurrent uveitis. However, the demonstration that local antibody formation may continue in the uveal tract longer than does clinically visible inflammatory disease points up the importance of antigen in this process. Not only is antigen required to initiate cellular proliferation and differentiation, but it serves also to perpetuate the clinically important components of the response. Presumably, when antigen is exhausted locally, no further recruitment to the inflammatory reaction takes place, and those cells previously activated quietly complete their commitment to antibody formation.

It should be noted, finally, that the finding of occasional antibody-forming cells in the aqueous humor suggests a possible tool in the etiologic diagnosis of recurrent uveitis. The more frequent demonstration of gross hemolysis produced by aqueous humor on the assay plates indicates the presence of free antibody in the anterior chamber, and confirms the observations of others who have detected elevated antibody titers in aqueous samples, employing other techniques.15,10

REFERENCES


