Studies on intravitreal initiation of the immune response

James F. Pribnow and Joan M. Hall

Injection of 1.5 mg. of bovine gamma globulin (BGG) intravitreally into New Zealand white rabbits resulted in the appearance of antibody within 7 to 10 days. Specific antibody-forming cells were found in suspensions of uveal tract cells from rabbits killed several days following the onset of uveitis. Challenge of the contralateral eye several months later resulted in an accelerated onset of uveitis and rapid rise in antibody titer. Plaque-forming cells were found in the uveal tract as early as 5 days after challenge. Footpad or intravenous injection of 1.5 mg. of BGG primed rabbits for a secondary ocular reaction, although no primary antibody response was detected. Injection of peritoneal macrophages containing BGG (referred to as macrophage-BGG) into the footpad resulted in development of moderately high antibody titers in the recipients. Intravitreal injection of macrophage-BGG neither precipitated a primary antibody response nor primed for a secondary response. Following intravitreal challenge of these recipients, antibody titers rose only to levels observed in rabbits which had received a primary injection of BGG. Plaque-forming cells were found in uveal tract suspensions only if the plaque assays were carried out more than 12 days after challenge.

Key words: immune response, macrophage, plaque-forming cells, intravitreal immunization, serum antibody.
bovine gamma globulin-coated erythrocytes.

Pribnow and Silverman\(^6\) demonstrated that footpad injection of peritoneal macrophages incubated in vitro with bovine gamma globulin (macrophage-BGG) resulted in the appearance of circulating antibody to BGG in the recipients. Studies using \(^131^1\)-labeled BGG showed that the amount of antigen injected under these circumstances was very small and had undergone some degradation.

The present experiments were undertaken to determine whether intravitreal injection of macrophage-BGG would be effective in inducing antibody formation to BGG. Because of the small amount of antigen injected and its intracellular nature, it was felt that such a technique might prove useful when using substances available in small amounts or substances which might be poorly antigenic in their native form.

Contrary to expectation, intravitreal injection of macrophage-BGG failed to elicit antibody production or to prime the host for a secondary response. The reasons for this failure and their relationship to ocular immunity are discussed.

Materials and Methods

Immunization procedure. The method used for obtaining peritoneal cells has been described previously.\(^6\) The average differential cell count of oil-induced peritoneal exudates was 89 per cent macrophages, 7 per cent lymphocytes, 3 per cent polymorphonuclear neutrophiles, and 1 per cent eosinophiles.

Two hundred milligrams of heat-aggregated BGG were added to approximately \(1.5 \times 10^6\) washed peritoneal cells in Medium 199 containing 20 per cent normal rabbit serum. The mixture was rotated at 10 r.p.m. at 37° C. for 2 hours. The cells were then washed 4 times in Medium 199. Experiments with labeled BGG indicated that negligible radioactivity remained in the supernatant fluid after the third washing.

Results

Clinical observations. Control rabbits which received 1.5 mg of BGG intravitreally showed the typical response described previously. The eyes were normal for approximately one week, at which time the injected eye developed uveitis. The onset of the inflammatory reaction was accompanied by the appearance of circulating antibody. Challenge of the con-
tralateral eye of these rabbits several months later with 1.5 mg. of BGG resulted in accelerated onset of uveitis. In most cases antibody was no longer detectable at the time of challenge. Rabbits immunized either intravenously or via the footpad with 1.5 mg. of BGG did not develop circulating antibody detectable by passive hemagglutination. However, these rabbits showed an accelerated response following intravitreal challenge.

In contrast to the often severe uveitis that followed intravitreal injection of BGG, rabbits that received macrophage-BGG showed only a transient inflammation which might well have been due to trauma. The iris seemed normal at all periods following injection. The only notable change was that the eye developed a somewhat "glassy" appearance, which persisted until the rabbits were killed. When the rabbits were challenged in the contralateral eye with 1.5 mg. of BGG, an accelerated inflammatory response did not take place. While rabbits immunized initially with BGG developed uveitis within 1 to 2 days following intravitreal challenge, those which had received macrophage-BGG did not show a reaction until the seventh to tenth postchallenge day. In this respect, the response resembled a primary response to intravitreal injection of BGG. Rabbits which initially received antigen-containing cells via the footpad route did, however, develop uveitis by the second day following intravitreal challenge with BGG.

**Antibody production and plaque assay.** Table I presents the results of plaque assays on cells from rabbits immunized intra-

### Table I. Immune response of rabbits injected with 1.5 Mg. of BGG either intravitreally or via the footpad

<table>
<thead>
<tr>
<th>Rabbit No</th>
<th>Route of injection</th>
<th>Primary titer log₂</th>
<th>Day of intravitreal challenge*</th>
<th>Secondary titer log₂</th>
<th>Plaques per 10^6 uveal tract cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>222</td>
<td>Intravitreal</td>
<td>2</td>
<td>36</td>
<td>7</td>
<td>1,480</td>
</tr>
<tr>
<td>196</td>
<td>Intravitreal</td>
<td>3</td>
<td>105</td>
<td>7</td>
<td>1,615</td>
</tr>
<tr>
<td>194</td>
<td>Intravitreal</td>
<td>5</td>
<td>128</td>
<td>7</td>
<td>2,397</td>
</tr>
<tr>
<td>201</td>
<td>Footpad</td>
<td>1</td>
<td>116</td>
<td>3</td>
<td>2,658</td>
</tr>
<tr>
<td>202</td>
<td>Footpad</td>
<td>1</td>
<td>115</td>
<td>7</td>
<td>427</td>
</tr>
</tbody>
</table>

*Challenge injection given in left eye. Primary injection given in right eye.

fTiter represents that of serum drawn on day of plaque assay, 5 to 7 days after challenge.

Plaque numbers represent those found in uveal tract cells of challenged eye.

### Table II. Immune response of rabbits injected intravitreally with 1.5 Mg. of BGG-containing macrophages

<table>
<thead>
<tr>
<th>Rabbit No</th>
<th>Number of cells injected</th>
<th>Primary titer log₂</th>
<th>Day of intravitreal challenge</th>
<th>Secondary titer log₂</th>
<th>Plaques per 10^6 uveal tract cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>109</td>
<td>3.2 × 10⁷</td>
<td>&lt;1</td>
<td>16</td>
<td>&lt;1</td>
<td>0f</td>
</tr>
<tr>
<td>110</td>
<td>3.2 × 10⁷</td>
<td>&lt;1</td>
<td>49</td>
<td>5</td>
<td>0f</td>
</tr>
<tr>
<td>132</td>
<td>3.2 × 10⁷</td>
<td>&lt;1</td>
<td>49</td>
<td>5</td>
<td>0f</td>
</tr>
<tr>
<td>154</td>
<td>1.6 × 10⁷</td>
<td>&lt;1</td>
<td>28</td>
<td>3</td>
<td>151§</td>
</tr>
<tr>
<td>155</td>
<td>1.6 × 10⁷</td>
<td>&lt;1</td>
<td>28</td>
<td>5</td>
<td>890§</td>
</tr>
<tr>
<td>156</td>
<td>1.6 × 10⁷</td>
<td>&lt;1</td>
<td>28</td>
<td>5</td>
<td>0j</td>
</tr>
<tr>
<td>A29</td>
<td>1.4 × 10⁷</td>
<td>&lt;1</td>
<td>51</td>
<td>&lt;1</td>
<td>243§</td>
</tr>
<tr>
<td>A41</td>
<td>1.4 × 10⁷</td>
<td>&lt;1</td>
<td>51</td>
<td>5</td>
<td>1586§</td>
</tr>
<tr>
<td>A53</td>
<td>1.4 × 10⁷</td>
<td>&lt;1</td>
<td>51</td>
<td>5</td>
<td>957†</td>
</tr>
</tbody>
</table>

*Plaque numbers represent those found in uveal tract cells from challenged eye.

fRabbits killed on the sixth day after challenge. No uveitis seen on gross examination.

fRabbit killed on the fifteenth day after challenge. Very mild uveitis had developed.

†Killed on day 15 after challenge. Severe uveitis had developed.

§Killed on day 15 after challenge. No uveitis had developed.

†Killed on day 13 after challenge. Uveitis developed by day 8.
vitreally with BCG and challenged in the contralateral eye. All rabbits in this series developed uveitis by the third day after challenge, and all showed a rapid rise in antibody titer and many plaque-forming cells in the uveal tract of the challenged eye. Cells from the spleen and preauricular nodes did not produce a significant number of plaques, nor did uveal tract cells from the eye which had received the primary injection. It is felt that this represents a true secondary response, because few or no plaque-forming cells are found in the uveal tract when the plaque assay is performed only 6 days following primary injection with BCG. Sections of the challenged eyes showed a dense infiltrate of mononuclear cells in the uveal tract. The methyl green-pyronin stain revealed that many of these cells were pyroninophilic. Only a few scattered pyroninophilic cells and no mononuclear infiltrate were observed in the eye initially injected.

Table II presents the results of plaque assays and serum antibody determinations on rabbits that had received a primary intraocular injection of macrophage-BCG and a challenge with BCG. No serum antibody was produced following the injection of antigen-containing macrophages. Plaque-forming cells were absent or few in number in rabbits 109, 110, and 152 which were killed 6 days following challenge. These rabbits showed almost no inflammatory response in the challenged eye at the time they were killed. Plaque-forming cells were found only in those rabbits killed 13 to 15 days after challenge, when uveitis had been present for several days. Mononuclear infiltrates were found in the sections of challenged eyes from these rabbits, but not in those from rabbits killed 6 days after challenge. Figs. 1 and 2 present Giemsa-stained smears of the exudate removed at the time of the plaque assay from the eye initially injected. Many of the cells seen were suggestive of macrophages, and some appear to contain droplets of the oil used to induce the peritoneal exudate.

No plaque-forming cells were found in the preauricular nodes or spleens. The secondary titers listed are those from serum

Fig. 1. Giemsa-stained smear of vitreous exudate removed 2 months following intravitreal injection of macrophage-BCG. Large macrophage-like cell resembling peritoneal macrophages originally injected. (×1,200.)
obtained on the day the plaque assay was performed. The titers are lower than the secondary titers shown in Table I, and, in fact, are closer to the primary titers obtained after intravitreal injection of BGG.

In contrast, rabbits injected initially via the footpad with macrophage-BGG were able to form appreciable amounts of antibody. Table III presents the serum antibody titrations of several rabbits which received varying numbers of antigen-containing cells in the footpads. It is evident that injection of as few as \(1.7 \times 10^7\) BGG-containing macrophages can result in the production of high-titer antibody. The table also shows the immune response of some of these rabbits after intravitreal challenge. The antibody titer rose rapidly, as it did in rabbits immunized with native BGG, and large numbers of plaque-forming cells were found in the uveal tract tissue.

### Table III. Immune response of rabbits injected via the footpad with BGG-containing macrophages

<table>
<thead>
<tr>
<th>Rabbit No.</th>
<th>Number of cells injected</th>
<th>Primary titer log₂</th>
<th>Day of intravitreal challenge</th>
<th>Secondary titer log₂</th>
<th>Plaques per 10⁶ uveal tract cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>111</td>
<td>(3.2 \times 10^7)</td>
<td>6</td>
<td>49</td>
<td>10</td>
<td>1,000</td>
</tr>
<tr>
<td>161</td>
<td>(1.5 \times 10^8)</td>
<td>6</td>
<td>55</td>
<td>9</td>
<td>3,116</td>
</tr>
<tr>
<td>160</td>
<td>(1.5 \times 10^8)</td>
<td>4</td>
<td>41</td>
<td>9</td>
<td>4,000</td>
</tr>
<tr>
<td>A51</td>
<td>(4.2 \times 10^8)</td>
<td>6</td>
<td>66</td>
<td>9</td>
<td>1,105</td>
</tr>
<tr>
<td>84</td>
<td>(1.7 \times 10^7)</td>
<td>7</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
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<tr>
<td>45</td>
<td>(2.2 \times 10^7)</td>
<td>10</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>98</td>
<td>(2.8 \times 10^7)</td>
<td>9</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>74</td>
<td>(6.5 \times 10^7)</td>
<td>7</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>7</td>
<td>(6.5 \times 10^7)</td>
<td>9</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
</tbody>
</table>

NC = Rabbits not challenged.

*Plaque numbers represent those found in uveal tract suspensions from challenged eye. Plaque assay carried out on sixth day post challenge.

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**Fig. 2.** Giemsa-stained smear of vitreous exudate removed 2 months following intravitreal injection of macrophage-BGG. Phagocytic cells containing oil droplets. (×1,300.)
Discussion

The response of rabbits to intravitreal injection of protein antigens has been well documented. These investigations have shown that injection of small amounts of protein antigens by the intravitreal route results in the production of serum antibody. Antibody production can be elicited by amounts of antigen which do not produce a detectable response when injected by the more conventional methods. Previous investigations have shown that a secondary response can be elicited several months after the first injection by injecting the contralateral eye with the same antigen. The characteristic rapid rise in antibody titer and rapid onset of uveitis in the challenged eye were attributed to the fact that circulating "memory" cells were present in that eye or migrated there following challenge. This method of challenge did not cause a recurrence of uveitis in the eye initially injected, as did intravenous challenge. Priming for the accelerated ocular reaction was also accomplished by injection of small amounts of antigen, either intravenously or in the footpad, even though these injections did not elicit a primary antibody response. It was postulated that conversion of X to Y cells had taken place, according to the scheme outlined by Sterzl and Silverstein and Sercarz and Coons. However, in those instances, differentiation into the Z or antibody-forming cells did not take place. The challenge injection caused migration of circulating Y or "memory" cells into the eye, where they proliferated and differentiated into Z cells.

The eye has been compared to an auxiliary lymph node, which has the capacity to produce antibody locally. If sufficient antibody is produced, it would appear in the serum. Until recently, however, direct evidence that lymphoid cells are actually producing antibody has been relatively scarce. The experiments of Smith and associates and those of Hall and O'Connor have shown that dispersed cells from the uveal tract are indeed capable of produc-
unable to elicit a response in irradiated recipients unless lymphoid cells were injected along with the macrophages. Unanue also showed that macrophage antigens placed in diffusion chambers in the peritoneal cavities of mice were less effective in priming for the secondary response than were those merely injected intraperitoneally. In the present experiments, it seems conceivable that macrophage-BGG injected intravitreally, unlike that injected into the footpad, is not able to come into contact with immunocompetent lymphoid cells. The eye could then be considered as a sort of "natural diffusion chamber." Although intravitreal injection of macrophage-BGG did not elicit the typical inflammatory reaction associated with similar injections of protein antigens, a white exudate was noted in the vitreous of all rabbits. This exudate could possibly represent the residuum of the injected macrophages, which were unable, perhaps because of their size, to leave the eye. Some evidence is available which supports this hypothesis. Stained smears of the exudate removed when the rabbits were killed 2 months after injection of macrophages showed typical large macrophages. Many of these cells still contained drops of the oil used to elicit the peritoneal exudate in the donor rabbits. The footpad has a more efficient lymphatic drainage, and macrophage-BGG injected via this route would have adequate contact with the lymphoid system of the normal rabbit.

The peritoneal cavities of animals contain several types of cells, the percentage of each depending on the method and time that the cells are harvested. It is possible that contaminating lymphoid cells could be responsible for the antibody production when peritoneal cells containing antigen were injected. However, the experiments of Prinbnow and Silverman and those of Unanue showed that the lymphocytes transferred along with the macrophages were not sufficient to produce antibody. Additional lymphoid cells had to be supplied when macrophage-rich exudates were given to irradiated animals. Also, injection of large numbers of lymphocytes, treated in the same manner as the macrophages, were found by Gallily and Feldman and Prinbnow and Silverman to be ineffective in inducing antibody formation. The numbers of lymphocytes which may have been injected intravitreally in our experiments were clearly not sufficient to initiate a response.

The possibility that some antigen remains on the surface of the macrophages, even after repeated washing, cannot be entirely eliminated. However, even if this were the case, the sequestered position of the eye would make it difficult for the antigen to make the necessary contact with immunocompetent cells. Antigen bound to the surface of macrophages injected in the footpad would, of course, reach the lymph nodes and spleen.

The eye remains a rather unique tool for immunologic study. It can act in some instances like an adjuvant, permitting slow release of antigen and thus making possible the use of small amounts of antigen to produce a significant serum antibody response. The eye can also be considered as a lymph node, producing antibody locally after initial recruitment of immunocompetent cells from extraocular sources. The present experiments would seem to indicate that the eye can also act as a diffusion chamber, preventing antigen from coming into contact with lymphoid cells when it is presented in the form of macrophages containing antigen.

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Erratum
In the May, 1970, issue of the JOURNAL, in the article entitled “The effect of bretylium 
on the degeneration mydriasis and intraocular pressure decrease in the conscious rabbit after 
unilateral cervical ganglionectomy” by Giora Treister and Ernst H. Bárany, line 2 in the 
legend of Fig. 6 should be interchanged with line 2 in the legend of Fig. 7.