Kinetics and Immunoglobulin Nature of the Antibody Response to Mycobacterium tuberculosis

J. E. O'CONNOR AND I. S. SNYDER

Department of Microbiology, College of Medicine, University of Iowa, Iowa City, Iowa 52240

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Antibody production after injection of Mycobacterium tuberculosis was studied by use of the single cell plaque assay technique. Maximal numbers of antibody-forming cells were found 9 days after a single injection of M. tuberculosis, about 1 day before the appearance of maximal circulating antibody. Immunelectrophoretic studies and 2-ME studies on 19S and 7S globulin fractions obtained by gel filtration show that the hemagglutination and hemagglutination-lysis antibody activity is associated with 19S immunoglobulin.

The plaque assay system of Jerne, Nordin, and Henry (8) has been widely used to study the kinetics of the antibody response to numerous antigens (3, 4, 12, 17). However, this technique has not been used to study the immunological response after injection of mycobacterial antigens.

Little information on the immunoglobulins responsible for hemagglutination (HA) and hemagglutination-lysis (HL) of old tuberculin-coated cells is available. Daniel (1, 2) has reported that injection of soluble old tuberculin or alum-precipitated purified protein derivative elicits 19S antibody production, whereas injection of old tuberculin in adjuvant results in production of 19S and 7S antibody. Injection of Mycobacterium tuberculosis or live BCG caused production of both 19S and 7S antibody. Turcotte, Freedman, and Sehon (18) found both 19S and 7S antibodies in patients with tuberculosis. Patients with active disease had low levels of 19S antibody but high levels of 7S antibody. Both Daniel (2) and Turcotte et al. (18) feel that 7S antibody is associated with injection of live organisms or the active stage of tuberculosis.

This study was designed to determine whether the plaque assay system of Jerne et al. (8) could be used to measure the antibody response to mycobacterial antigens, and to identify the immunoglobulin class of antibody responsible for HA and HL of old tuberculin-coated erythrocytes.

Materials and Methods

Organisms. M. tuberculosis (H37RV) was grown in Kirchner's medium, containing 0.05% Tween 80, for 12 days. The turbidity was adjusted on a Bausch & Lomb Spectronic-20 colorimeter to give an optical density equivalent to 10⁶ organisms/ml.

Animals. Male white New Zealand rabbits weighing 4 to 5 lb (1.8 to 2.3 kg) were used throughout the study. Blood was obtained from the ear artery before injection of organisms and at the desired time after injection.

Determination of plaque-forming cells. The method of Jerne et al. (8) was used with the following modifications. Diethyldithiothreitol dextran was not used to remove the anticomplementary activity of agar, and undiluted complement was used to develop plaques. The viability of the splenic cells was determined through use of trypan blue by a method similar to that of Gray et al. (5).

The method described by Zaalberg, van der Meul, and van Twisk (20) was used in an attempt to measure hemagglutinating antibody by cluster formation.

HA and HL tests. The "drop" modification of the Middlebrook-Dubos test described by Rheins et al. (16) was used. Old tuberculin (Lederle Laboratories, Pearl River, N.Y.) concentrated 4X the international standard was used for coating of the cells. Complement was obtained commercially (BBL).

Gel filtration. Separation of 19S and 7S globulin was done by the method of Möller (12). A 1.5-ml amount of rabbit anti-M. tuberculosis serum, inactivated and absorbed three times with sheep red blood cells was applied to the column. Fractions of 3 ml were collected. The protein concentration was determined by adsorption at a wavelength of 280 μm.

Dissociation of 19S antibody. Serum or serum fractions were treated with an equal volume of 0.2 M 2-mercaptoethanol (2-ME; Eastman Kodak Co., Roberts, N.Y.). After incubation at 37 C for 30 min, the sample was dialyzed against repeated changes of 0.15 M phosphate-buffered NaCl (pH 7.3) containing 0.02 M iodoacetamide.

Concentration of 19S and 7S immunoglobulins.
The peak fractions of both the 19S and 7S immuno-
globulins showing the greatest absorbance at 280 m, were precipitated with an equal volume of cold saturated (NH₄)₂SO₄. The precipitates were allowed to stand at 3 C for 1 hr and were collected by centrifuga-
tion at 11,000 X g for 15 min. The precipitates were dissolved in water and were dialyzed against two changes of phosphate-buffered NaCl (1,000 ml) and then overnight in 1,500 ml. The final concentration was ninefold for the 19S fraction and eightfold for the 7S fractions. Immuno-electrophoretic studies were done on the concentrated fractions.

**RESULTS**

Plaque assay studies. Spleens were removed from the rabbits at several time intervals after a single intravenous injection of 10⁷ viable *M. tuberculosis* and were assayed for plaque-forming cells. Even though considerable variation is observed between counts from different animals (Fig. 1), some information can be obtained. A low number of plaques was obtained on the fifth day. By the 9th day after injection of the organisms, the number of plaque-forming cells reached a maximum, returning by the 16th day to a number comparable to that found on day 5. In general, the curve obtained by use of the mean values for each time interval was similar to that obtained by Jerne et al. (8) and by Möller (12).

The mean HA and HL titer of the animal sera collected on the day of sacrifice are also shown in Fig. 1. The circulating antibody titer and the number of plaque-forming cells appear to peak at about the same time. In an attempt to explain the variation in the antibody-forming spleen cells,
rabbits were injected and bled for serological studies at several time intervals. Figure 2 contains the results of this study and shows various rates of antibody formation by the animals. However, all rabbits with the exception of rabbit 4 produced the same titer by day 10. Thus, it would appear that the variations in plaque counts are due to variation in onset and rate of antibody formation.

Table 1. Antibody activity of rabbit serum and Sephadex fractions after treatment with 2-mercaptoethanol

<table>
<thead>
<tr>
<th>Sample</th>
<th>HL units</th>
<th>HA units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole serum + buffer</td>
<td>8,192</td>
<td>1,024</td>
</tr>
<tr>
<td>Whole serum + 2-mercaptoethanol</td>
<td>&lt;2</td>
<td>4</td>
</tr>
<tr>
<td>19S fraction + buffer</td>
<td>512</td>
<td>32</td>
</tr>
<tr>
<td>19S fraction + 2-mercaptoethanol</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>7S fraction + buffer</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>7S fraction + 2-mercaptoethanol</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Buffer + 2-mercaptoethanol</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

Table 2. 2-Mercaptoethanol treatment of rabbit serum obtained 9 weeks after injection of Mycobacterium tuberculosis

<table>
<thead>
<tr>
<th>Rabbit no.</th>
<th>Serum + buffer</th>
<th>Serum + 2-mercaptoethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HL units</td>
<td>HA units</td>
</tr>
<tr>
<td>48</td>
<td>512</td>
<td>16</td>
</tr>
<tr>
<td>49</td>
<td>1,024</td>
<td>32</td>
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<td>50</td>
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<td>16</td>
</tr>
<tr>
<td>52</td>
<td>256</td>
<td>2</td>
</tr>
</tbody>
</table>

Immunoglobulin nature of the antibody. Rabbits were given two injections of $10^7$ viable M. tuberculosis 1 week apart in the marginal ear vein. Serum was obtained 5 days after the last injection. Separation of the serum globulins by gel filtration (Fig. 3) showed that most of the HL and HA activity was found in the first protein peak (19S antibody). A small amount of antibody activity was found in the second peak (7S immunoglobulin).

Because the slight activity in the 7S immunoglobulin nature of the antibody.

Table 4. Immunoelectrophoretic studies on untreated and 2-ME treated rabbit 19S fractions obtained by gel filtration. The troughs contain goat antirabbit total serum globulin. Top slide: Upper well contains 19S fraction, and bottom well contains 7S fraction. Bottom slide: Upper well contains 19S fraction, and bottom well contains 2-ME treated 19S.
globulin peak could be due to retardation of 19S antibody as it filtered through the column, serum as well as the 19S and 7S fractions showing the highest protein and antibody concentration was treated with 2-ME. As can be seen in Table 1, 2-ME abolished the HA and HL activity in the serum and in the 19S and the 7S immunoglobulin fractions. A small amount of HA activity in serum (4 units) was not affected by 2-ME.

Since Daniels (1) reported the presence of 7S immunoglobulin activity in rabbits injected with killed *M. tuberculosis*, serum obtained from rabbits 9 weeks after injection was treated with 2-ME. The results (Table 2) show that a small amount of antibody resistant to 2-ME was found in one of four rabbits.

**Immunoelectrophoretic studies.** Immunoelectrophoresis of the 19S fraction and development of precipitin lines with goat antirabbit total serum globulin resulted in formation of two precipitin

**Fig. 5.** Immunoelectrophoretic studies of untreated and 2-ME treated fractions using goat antirabbit 7S globulin. Top slide: Upper well contains 19S fraction and bottom well contains 7S fraction. Middle slide: Upper well contains untreated 7S fraction, and bottom well contains 2-ME treated 7S fraction. Bottom slide: Upper well contains 19S fraction, and bottom well contains 2-ME treated 19S fraction.
bands in the 19S region extending from the well to the right (Fig. 4, top slide). A 7S immunoglobulin was not detected in the 19S fraction, indicating the absence of detectable 7S globulin in the 19S fraction. The 7S fraction in the bottom well gave a 7S precipitin band. Treatment of the 19S fraction with 2-ME resulted in a loss of one precipitin band and an increase in the diffusion rate of the other precipitin band (Fig. 4, bottom slide). Immunoelectrophoresis of the 19S and 7S fractions and development of precipitin lines with goat antirabbit 7S globulin showed a single 19S precipitin band (Fig. 5, top slide, top well) and a 7S precipitin band (bottom well). The absence of an effect by 2-ME on the immunoelectrophoretic pattern of the 7S fraction is shown in Fig. 5, middle slide. Immunoelectrophoresis of the 2-ME treated 19S fraction and development of precipitin lines with goat antirabbit 7S globulin show the formation of two precipitin lines with increased rates of diffusion (Fig. 5, bottom slide).

**DISCUSSION**

The data presented here show that mycobacterial antigens present in old tuberculin can be used to study the kinetics of antibody formation by the single cell antibody plaque method of Jerne et al. (8). However, considerable variation was found in the number of plaque-forming cells obtained from rabbits sacrificed at the same time intervals after injection of *M. tuberculosis*. Variability in plaque counts among individual animals has also been shown by other investigators (12, 13, 19). However, greater variability in our study might be due to use of animals which were not highly inbred. One other factor which might account for the variability is the use of live microorganisms in immunization.

Attempts to show antibody production by the cluster formation method of Zaalberg et al. (20) were unsuccessful, although this technique was successfully employed in studies with other antigens.

The effect of 2-ME on rabbit serum indicated that HA and HL activity was due to a 19S immunoglobulin or a 2-ME sensitive 7S globulin. Daniel (1) has reported the presence of a 2-ME sensitive 7S globulin in rabbits. However, the presence of antibody in the 19S fraction isolated by Sephadex G-200 chromatography shows that the antibody is a 19S immunoglobulin. Whether the immunoglobulin responsible for antibody activity is the well-described 19S γ M or the 19S γ G described by Kim, Bradley, and Watson (9–11) is open to speculation. Immunoelectrophoresis of both 19S and 7S fractions does not show contamination of the 19S fraction with 7S globulin or vice versa. However, two precipitin lines are formed with the 19S fraction and the goat antirabbit total serum globulin. The position of the lines indicates the presence of both 19S γ M and 19S γ G immunoglobulin. After treatment of the 19S fraction with 2-ME, a precipitin band with an increased rate of diffusion is formed, suggesting reduction of 19S γ M into subunits. This has also been shown by Onoue et al. (15). However, the other band disappears after 2-ME treatment. Kim et al. (9) have shown that the 19S γ G precipitin band is unaffected by 2-ME treatment.

Daniel (1) has shown the presence of small amounts of 2-ME sensitive 7S antibody in rabbits 2 weeks after injection of killed *M. tuberculosis*. This 2-ME sensitive 7S antibody persisted until the 6th or 7th week after injection at which time most of the antibody activity was due to 2-ME resistant antibody. Our studies on serum obtained 9 weeks after injection with viable *M. tuberculosis* show the presence of a small amount of 2-ME resistant antibody (4 HL and 8 HA units) in one of four sera.

Thus, it appears that the antibody responsible for the HA and HL reaction is a 19S antibody. However, studies with antisera from animals with a prolonged infection might yield evidence for other immunoglobulin types. Finally, it must be noted that the techniques for detecting antibody activity in this study are more sensitive for 19S immunoglobulin than for 7S immunoglobulin (6, 7, 14).

**LITERATURE CITED**

7. ISHIZAKA, K., T. ISHIZAKA, E. H. LEE, AND H. H.