Rosette-Forming Cells During Immune Response to
Toxoplasma gondii in Mice

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The rosette-forming cell (RFC) response of mice immunized with varying doses of Toxoplasma gondii was studied by immunocytoadherence (ICA). The specificity of ICA in the present system was tested by passive sensitization with hyperimmune serum in vivo and in vitro. A slight increase in RFC was observed with the latter. Prior treatment of spleen cells from immunized animals with rabbit anti-mouse immunoglobulin resulted in total inhibition of ICA. During the primary and the secondary response after 10 days, the number of RFC rose rapidly to reach the peak on the 3rd day. With secondary immunization 30 days later, the peak shifted to the 2nd day. Mice infected with a lower dose of Toxoplasma had a greater number of RFC during the secondary response after 10 days than with a larger dose.

The production of antibody at the cellular level can be studied by immunocytoadherence (ICA). This phenomenon is based on adherence in a cluster or "rosette" formation of a particulate antigen due to its binding the receptor sites around a lymphoid cell. The method of localized hemolysis in gel (19) can also be used for this. However, a comparison of these two techniques has shown ICA to be more sensitive (40). It was introduced for detecting cells producing anti-erythrocyte antibody, and the term ICA was coined for it (25). It is of interest to note that similar adherence had reportedly been observed earlier in this century with protozoan parasites, Trypanosoma, and Leishmania (24), and the occurrence of ICA has been demonstrated more recently for trypanosomes (11).

The many applications of ICA include cyto-dynamic studies of the immune response (6, 35), evaluation of drugs used as immunosuppressants (2), studies of time of transplantation rejection (3), determining drug hypersensitivity (28), as a diagnostic tool in sporotrichosis and histoplasmosis (30, 31), and in correlating cell-mediated immunity estimated as delayed-type hypersensitivity (34).

The immune response to Toxoplasma gondii, a coccidian protozoan, has been studied in depth at the humoral level by measurement of circulating serum antibody. However, there has been no attempt to investigate antibody production of this parasite at a basic cellular level. The present report deals with this aspect by applying ICA.

MATERIALS AND METHODS

Preparation of Toxoplasma suspensions. The BK strain, maintained by regular intraperitoneal passage in mice was used. Parasites were harvested by washing out the peritoneal cavity with 2 ml of phosphate-buffered saline (PBS), pH 7.2. Exudate was collected on the 3rd day of infection or on the 5th day if brain homogenate was injected. Toxoplasmas were separated from the mouse peritoneal cells by a simple paper filtration method (22). Briefly, the exudate was diluted to contain 5 × 10⁶ organisms per ml. Two milliliters of this was filtered through a fluted no. 589 filter paper (Schleicher and Schull, Dassel, West Germany) and washed with an equal volume of PBS. Each filter was used twice, and the parasites from pooled filtrates were pelleted down by centrifugation. After overnight treatment with 1% formalinized PBS, which inactivated the organisms, the suspension was kept in 0.2% formalinized PBS at 4 C.

Immunization. NMRI female mice weighing 18 to 20 g were immunized intravenously with 10⁴, 10⁵, or 10⁶ formalinized Toxoplasma, using the tail vein. For preparation of hyperimmune serum, the low-virulence, cyst-forming Alt strain, which was isolated from a human case of toxoplasmosis and is being maintained in this laboratory, was used. Mice were injected with approximately 10 brain cysts and serum obtained after 90 days.

Preparation of cell suspension. Animals were sacrificed by exsanguination at selected time points. Their spleens were placed in PBS, pH 7.2, and gently cut with scissors into a fine pulp. Cells were further dissociated by being drawn three times into an unattached 2-ml plastic syringe. The suspension was filtered through two layers of gauze to remove larger debris and centrifuged for 8 min at 500 × g. The erythrocytes present were lysed by osmotic...
shock, adding cold distilled water followed by PBS. To prevent loss in activity by release of antibody from the RFC surface and to fix the antibody already present, the cell suspension was treated for 30 min at 4°C with 5% formalinized PBS (5), washed twice, and adjusted to 10^7 splenocytes per ml.

**ICA test procedure.** Equal volumes of cell suspension and *Toxoplasma* standardized to 10^8 organisms per ml were thoroughly mixed (1:10 ratio) and incubated at room temperature for 20 min with occasional shaking.

The microscopic reading was performed in a hemocytometer. Replicate counts were made with each suspension. Cells exhibiting adherence with at least three toxoplasmas were regarded as RFC and enumerated. They were expressed as the number per million spleenocytes.

**Specificity tests.** (i) Passive sensitization. For in vivo sensitization, normal mice were injected intravenously with 0.3 ml of hyperimmune serum. Animals were killed 6 and 24 h later, and ICA and RFC of circulating antibody were determined.

For in vitro sensitization, cells from spleens of unimmunized mice were incubated at 4°C for 1 h with hyperimmune serum (1:4,000 Sabin-Feldman dye test [SFT] titer) and washed twice before testing for ICA. Normal mouse serum was used as control in both experiments.

(ii) Inhibition of ICA. Spleen cells from immune mice were incubated in equal volumes with rabbit anti-mouse immunoglobulin diluted 1:50 at 4°C for 30 min, and the ICA test was performed. An untreated cell suspension of the same pool was also tested.

**Antibody titration.** Serum antibody levels were determined by using a modification (8) of the SFT (33).

**RESULTS**

The pattern of immune response as measured by the number of RFC in the spleen and the titer of circulating serum antibody detected by SFT is presented graphically in Fig. 1 to 6. Each value was obtained with pooled spleen cells or sera from at least three mice. A spleenocyte exhibiting ICA is shown in Fig. 7. There was a relative paucity of cells with adherence of *Toxoplasma* on the entire surface as compared with the frequency of cells observed with only portions of the periphery covered.

**Primary immune response.** Animals received varying *Toxoplasma* doses as indicated in Fig. 1. Day 0 shows the background value of RFC before immunization. A progressive increase in the number of RFC was produced soon after injection, with the peak being attained on the 3rd day. Thereafter, a rapid decline of RFC set in. The RFC response preceded the humoral one by a couple of days. Serum antibody was not discernible until 5 to 7 days postinjection and could not be detected within this period with a 10^6 dose. The increase in number of spleen cells obtained after immunization was generally maintained during the ascending phase of ICA but became variable in the descending stage.

**Secondary immune response.** Mice were challenged 10 or 30 days after priming with the same dose and route as was used for primary antigen injection. The resulting secondary responses are plotted in Fig. 3 to 6. Both cellular and humoral effects were still present from priming at the time of secondary immunization. The response after 10 days revealed little
difference in the pattern at the cellular level with a 10⁴ dose and a slightly higher peak with the 10⁵ dose. A considerably lower peak was obtained with the 10⁶ dose. Antibody was present at higher titer, and the peak was reached on the 3rd day in all cases. The secondary response 30 days later was markedly different. There was a very rapid increase in the number of RFC, resulting in the peak being reached earlier, on the 2nd day. At the humoral level, a relatively elevated SFT titer was obtained.

**Specificity tests.** Spleen cells from nonimmunized mice were sensitized in vivo by passive administration and in vitro by addition of hyperimmune serum with an SFT titer of 1:4,000 (Table 1). The number of RFC encountered after in vivo sensitization was near the background range. After in vitro exposure to antibody, there was an increase in the number of RFC. In the presence of rabbit anti-mouse immunoglobulin, the RFC from immunized...
mice were inhibited, contrasting with RFC observed in untreated controls from the same pool.

**DISCUSSION**

Sequential studies of the interaction between cells during immune response have shown that the initiation of antibody formation involves synergism between thymus-derived (T) and bone marrow-derived (B) lymphocytes, with the latter differentiating into antibody-forming cells (AFC) (23). In addition to these, macrophages have been shown to be of importance in immune induction (38). All these cell types can form rosettes in immunized animals (6, 36, 39), depending on temperature conditions (12). In some cases rosette formation can be nonimmunological and nonspecific (10, 20). The specificity of adherence in the present system was, therefore, tested by experiments utilizing in vitro and in vivo passive sensitization of normal mouse spleen cells with hyperimmune and normal serum. The number of RFC after in vivo sensitization was near the background range, but an increase was observed after in vitro exposure. The ICA performed using the described conditions would seem in part to be nonspecific and may explain to some extent the high numbers of RFC observed. However, the complete inhibition of RFC obtained with rab-

bit anti-mouse immunoglobulin affirms to the immunological nature and involvement of antibody in ICA described.

The significance of RFC, known to be mostly small lymphocytes and macrophages (29), in the lymphoid tissues of nonimmunized animals has not been completely resolved. In the described system, $2 \times 10^2$ to $3 \times 10^3$ spontaneous RFC per $10^6$ spleen cells were present. Presence of a preconditioned cell population has been indicated in the selective theory of antibody formation (9). When spontaneous RFC are removed from an immunologically competent population, further reactivity to that specific antigen is also depleted (4). It has been suggested that in immunized animals RFC could be natural AFC (7, 21), and most of the precursors of AFC have been shown to be present among these spontaneous RFC (15, 27).

A feature common to all the immune responses described was a rapid rise in the number of RFC followed by a sudden decrease, in contrast at this stage to increasing circulating antibody. This could imply induction of a process for regulation of immune response. Experiments using dinitrophenylated *Ascaris* extract (26) and other studies (13) have indicated T cell-mediated regulation during transition from immunoglobulin M to immunoglobulin G antibody production (14). Extrasplenic migration of AFC and the possibility that active antibody production could be intrinsically only for a limited period (18) may also contribute to this decrease in AFC numbers.

The primary and secondary responses showed that subsequent challenge with antigen in mice primed 10 days earlier with a smaller dose of $10^7$ *Toxoplasma* resulted in greater numbers of RFC than did a $10^8$ dose. This suppression of cellular response using a larger dose may in part be due to specific antibody produced during the primary immunization acting on the primed cells in an inhibitory feedback mechanism. Animals injected with a $10^7$ dose had a circulating antibody titer of 1:6, whereas those given a $10^8$ dose had an SPT titer of 1:256 at the time of secondary immunization (Fig. 4). Passively administered antibody has been shown to inhibit antibody production (32, 37) including the *Toxoplasma* system (1), and similar higher secondary responses with lower antigen doses have been reported (16, 17). With the lapse of a longer period of time, memory cells having relatively high-affinity antibody as receptors are generated and the immune mechanism becomes less suppressible (37). The secondary response 30 days after the first immunization was indeed found to be greater in magnitude.
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LITERATURE CITED


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Volume 13, no. 6, p. 1678, column 2, line 16: Change "overweight" to read "overnight."

Page 1681, column 2, last paragraph, line 11: Change "1:6" to read "1:16."