Proliferative Responses of Rabbit Lymphocytes to Pasteurella multocida Decrease with Prolonged Immunization

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Peripheral blood and splenic lymphocytes from rabbits immunized with Pasteurella multocida by various schedules were stimulated to undergo blast transformation in vitro in response to this antigen. Repeated immunizations suppressed this response.

Many established procedures for the production of antibacterial antibodies in laboratory rabbits recommend a series of closely spaced, intravenous injections of bacterial cells or components (2, 3, 6). Although this approach generally elicits a strong humoral response in the animals, it may be inappropriate for inducing optimal cellular responses. The results of the present study indicate that lymphocytes from animals that have received repeated, closely spaced intravenous immunizations with Pasteurella multocida will lose cellular reactivity to this antigen.

Female New Zealand White rabbits, approximately 3 kg in weight, were obtained from a local breeder and were used in all experiments. Rabbits were housed individually and fed standard laboratory chow and unlimited quantities of water.

For this study, rabbits were prebled, and only rabbits with anti-P. multocida immunoglobulin G (IgG) titers of 1,000 or less were used. Titers were determined by an enzyme-linked immunosorbent assay (ELISA) which was performed as follows. P. multocida cells were boiled in saline for 2.5 h, washed, and then suspended in phosphate-buffered saline to 75% transmission at 610 nm and incubated overnight in Gilford cuvettes. The cells were then washed, incubated in 10% fetal calf serum buffer to bind nonspecific binding sites, and then washed again. Sera to be tested were diluted in this buffer, added to the cuvettes, and incubated. The cells were washed and peroxidase-labeled antirabbit IgG (H- and L-chain specific) was added. Finally, after extensive washes, O-phenylenediamine substrate was added. Readings were taken at 30, 60, and 120 min at 450 nm. The titer was taken as the last dilution of serum with an optical density greater than the mean of 10 control samples plus two standard deviations.

P. multocida used in the enzyme-linked immunosorbent assay and for immunizations was originally isolated from nasal cultures of two rabbits at this facility. Cells were cultivated on rabbit blood agar plates. For immunization, cells were transferred from the plates to saline, boiled for 2.5 h and suspended to approximately 10^9 P. multocida per ml.

Immunized animals received intravenous injections of boiled P. multocida as per the following schedules.

All preimmune sera were collected 10 days before the injection regimen began. Group I received one injection on day 0 and was assayed on day 8. Group II received injections on days 0 and 4 and was assayed on day 8. Group III received injections on days 0, 2, and 4 and was assayed on day 8. Group VI received injections on days 0, 2, 4, 7, 9, and 11 and was assayed on day 15. Group VI-rest also received injections on days 0, 2, 4, 7, 9, and 11, but was rested for 1 week before assay (day 22). Groups were numbered according to the number of injections received. e.g., Group I received 1 injection, Group VI received 6 injections. The volume of the first injection of P. multocida was 0.5 ml, the remaining injections were 1.0 ml.

On each day of assay, the animals (two to four per group) were bled by cardiac puncture and then sacrificed with an intracardiac or intravenous injection of 1.5 ml of Euthanol. Blood was defibrinated, a portion was removed for the determination of antibody titer, and peripheral blood lymphocytes (PBL) were prepared (5). Spleens were removed, and splenic cell suspensions were prepared as well (5). Both spleen cells and PBL were tested in vitro for blastogenic responses (uptake of [125I]iododeoxyuridine) to P. multocida as well as to concanavalin A (ConA) and anti-immunoglobulin serum. Control animals were age-matched normal female rabbits with anti-P. multocida titers of 1,000 or less. Methods for the culture and assay of rabbit
lymphocytes have previously been established in this laboratory (5).

Figure 1a shows blastogenic responses of spleen cells from control and immunized animals to *P. multocida* in vitro. The concentration of *P. multocida* cells that induced maximal lymphocyte stimulation (10^5 *P. multocida* per culture) was determined by dose titration. Spleen cells from groups II, III, and VI-rest responded vigorously to *P. multocida* in culture (10,000 to 15,000 cpm per culture). Although the cells from group VI were more reactive to *P. multocida* than were the normal control cells, the level of responses from group VI was less than half of that of the other reactive groups. Similarly, group VI gave the lowest responses of all groups to ConA (a T-cell mitogen).

Figure 1b shows blastogenic responses of PBL from each group of immunized animals as well as from unimmunized controls. The addition of *P. multocida* in vitro stimulated proliferation of PBL from groups II and III only (approximately 1,000 cpm per culture). The maximum proliferative responses of PBL to *P. multocida* were 10-fold less than the responses of spleen cells to this antigen. Animals that had received one or six injections, or six injections plus a 1 week rest, did not have antigen-reactive cells in their peripheral blood. Responses of PBL to ConA were equivalent for all groups except group VI. The responses of this group to ConA were approximately three- to fourfold lower. In addition, responses to anti-immunoglobulin (a B-cell mitogen) were 5- to 10-fold lower for PBL of group VI than for other groups.

In contrast to the decline in lymphocyte responses that was observed for animals that received more than three injections of *P. multocida*, the levels of anti-*P. multocida* IgG in the sera of all of the immunized animals increased dramatically, regardless of the number of immunizations received (Table 1). Thus, the depressed blastogenic responses of lymphocytes from group VI were not associated with a concomitant depression of circulating antibody titers.

In the present study, we demonstrated that extensive, systemic immunization of rabbits with large doses of boiled *P. multocida* can lead to suppression of specific antigen-reactive lymphocytes as well as diminished nonspecific cellular responses to T- and B-cell mitogens. Whereas two or three immunizations resulted in the presence of antigen-reactive lymphocytes in the blood and spleen, six immunizations reduced antigen-specific responses to the levels observed in unimmunized controls. These results are compatible with the studies reported by Gershon et al. (4) and Bash and Waksman (1) in which nonspecific suppressor T cells were elicited in

![Graph a](image1.png)

![Graph b](image2.png)

**FIG. 1.** (a) Blastogenic responses of spleen cells to *P. multocida* and ConA. Data are expressed as the mean of six replicate cultures from each of two to four animals (background values subtracted) ± the standard error of the mean. The values used were the peak responses observed during a 5-day culture period and represent the counts per minute of [125I]iododeoxyuridine ([125I]UdR) incorporated per culture during a 24-h pulse. (b) Blastogenic responses of PBL to *P. multocida*, ConA, and anti-immunoglobulin (anti-Ig). Legend is the same as for (a).

<table>
<thead>
<tr>
<th>Group</th>
<th>Preimmunization</th>
<th>Postimmunization</th>
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<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td>I</td>
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</tr>
<tr>
<td>II</td>
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<td>III</td>
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<tr>
<td>VI-Rest</td>
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<td>781,250</td>
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a Values (determined by enzyme linked immunosorbent assay) represent the peak titer detected for each group of rabbits.

TABLE 1. Serum levels of anti-*P. multocida* IgG before and after immunization with *P. multocida*
mice and rats given a massive dose of antigen. In these studies, large intravenous doses of antigen (ovalbumin, sheep erythrocytes, bovine gamma globulin, bovine serum albumin) led to suppressed responses in vitro to both specific antigen and mitogen (phytohemagglutinin). This suppression disappeared within 1 week after immunization, presumably due to clearance of antigen. We also observed a reversal of suppression in group VI-rest, which was assayed 1 week after the last immunization.

Although lymphocytes from animals that received more than three injections of *P. multocida* lost reactivity to this antigen, the production of circulating antibody in these animals was not impaired. Since all immunized groups produced similar levels of specific antibody, it is likely that the first immunization triggered the differentiation of antibody-producing B cells. This event, once underway, would have been unaffected in the time frame of these experiments by the subsequent development of a population of suppressor cells that was induced by further immunizations.

Our findings suggest that immunization schedules recommended for the production of high-titer antibacterial antisera can suppress lymphocyte transformation responses to the specific antigens involved. Whereas 1 week of our immunization program led to the development of specifically sensitized lymphocytes in the blood and spleen, additional immunizations eliminated these responses. This information is presented to suggest that caution be exercised when antibacterial immunization regimens are designed. A program intended to establish specific immunity and protection in an animal, although adequate for the production of antibodies, may become suppressive to lymphocyte populations which, at an earlier point in the immunization schedule, could be stimulated to undergo blast transformation by the specific antigen.

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LITERATURE CITED