Effect of Pertussigen on Inflammation Caused by Freund Adjuvant

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Pertussigen, one of the toxins from Bordetella pertussis, greatly increased the inflammatory response produced by complete Freund adjuvant in the footpads of mice. This effect was not produced by pertussigen when the emulsion was made with saline and incomplete Freund adjuvant, but if an antigen was included in incomplete Freund adjuvant, the strong potentiating effect was again demonstrated. As little as 100 ng of pertussigen given intravenously was effective, but 400 ng proved better, and this latter dose was used routinely. The most striking action occurred when pertussigen was injected on the same day or 3 days after complete Freund adjuvant, but an effect was demonstrated when given from 3 days before to 6 days after complete Freund adjuvant. The action of pertussigen was not apparent until about 6 to 8 days after complete Freund adjuvant. The footpad swelling reached its maximum by day 14 and remained undiminished until day 29. Forty days later, a significant effect was still present. Histologically, the cellular infiltrate in the feet of mice injected with complete Freund adjuvant was more intense in animals treated with pertussigen. Nude BALB/c mice receiving an emulsion of complete Freund adjuvant in the footpads did not respond with an increased inflammation after receiving pertussigen, suggesting the possible involvement of T cells in this phenomenon. The intense and prolonged inflammatory response produced in pertussigen-treated mice by Freund adjuvant containing antigenic substances may serve as a useful model to study chronic inflammation.

Pertussigen (Ptx), the crystalline protein toxin purified from Bordetella pertussis cells, is responsible for many of the biological activities of pertussis vaccine (13, 14). In particular, several immunological responses are induced by Ptx. It increases the intensity and duration of delayed-type hypersensitivity (DTH) reactions to protein antigens (18), facilitates the induction of DTH reactions induced by T-cell lines and anti-T-cell line sera (5), promotes the induction of experimental allergic encephalomyelitis in rats (13) and mice (15), promotes the induction of experimental allergic orchitis in mice (8), and increases the levels of specific immunoglobulin E antibody (13).

Pertussis vaccine has also been found effective in augmenting DTH responses to sheep erythrocytes (2), although some workers have found that both pertussis vaccine and the lymphocytosis-promoting factor (another name for Ptx) of B. pertussis suppressed DTH (16) and graft rejection (17), which are also expressions of cellular hypersensitivity. Interestingly, lymphocytosis-promoting factor has also been shown to suppress adjuvant arthritis in rats (16).

While studying the effect of Ptx on the induction of experimental allergic encephalomyelitis (EAE) (15), we noticed that mice that had received Ptx intravenously (i.v.), simultaneously with a footpad injection of spinal cord emulsified in complete Freund adjuvant (CFA), developed a more intense inflammatory response at the site of sensitization than mice that had received only antigen in CFA. This was intriguing in view of the reports that impair preparations from B. pertussis inhibited cell-mediated immune responses (16, 17). Thus, we decided to reinvestigate the effect of Ptx on the inflammatory reaction induced by Freund adjuvant. The results of these studies are reported here.

MATERIALS AND METHODS

Ptx. Ptx was prepared by a combination of previously described methods (4, 12) as indicated before (15). Solutions of the lyophilized Ptx were made in alanine formic acid buffer, pH 3.4 (6.7 g of dt-ala-alanine plus 0.7 g of formic acid in 1 liter of water), and immediately thereafter were diluted with an equal volume of 0.05 M Tris buffer, pH 8, containing 1 M sodium chloride. This solution at a concentration of 200 µg/ml was stored at −15°C for a few months without losing its activity. For inoculation of mice, this stock solution was diluted in phosphate-buffered saline to contain 2 µg/ml, and 0.2 ml was injected i.v. This gave a dose of 400 ng per mouse. In some experiments, Ptx was inactivated by heating the solution containing 2 µg/ml in a boiling-water bath for 20 min.

Antigens. Endotoxin was prepared from B. pertussis cells by the phenol-water method of Westphal et al. (21). Keyhole limpet hemocyanin (KLH) was purchased from (Calbiochem-Behring, La Jolla, Calif.).

Diluents. Phosphate-buffered saline at a concentration of 0.168 M, pH 7.2, was used to make dilutions of Ptx for i.v. injection. NaCl (0.15 M) was used to make the antigen solutions that were emulsified in CFA or incomplete Freund adjuvant (IFA).

Adjuvants. CFA and IFA were purchased from Difco Laboratories, Detroit, Mich.

Mice. BALB/c, AnBradleyWehi mice and the athymic BALB/c nu (nude) mice were raised under specific-pathogen-free conditions at The Walter and Eliza Hall Institute until the age of 7 weeks. The mice were then transferred to a conventional mouse room, where they were given food and water ad libitum. Experiments were started when the mice were 8 to 10 weeks of age.

Inoculations. CFA or IFA was emulsified with an equal volume of 0.15 M NaCl either alone or containing 1 mg of...
KLH per ml. The mice were inoculated subcutaneously with 0.05 ml of the emulsion in the left hind footpad. In one experiment, 0.04 ml of emulsion was given to each hind footpad. Within a few minutes, except as indicated, 400 ng of Ptx was given i.v.

Measurement of feet. The dorsal-to-plantar diameter of the hind feet was measured with a spring-loaded micrometer. (Mitutoyo Mfg. Co., Tokyo, Japan), and the difference between the left and right foot was recorded in millimeters. In one experiment in which both hind feet were injected with the emulsion, the average combined diameters of the hind feet were calculated, and from this measurement the average diameter of the feet of control mice injected with emulsion but no Ptx was subtracted to obtain the increment of swelling produced by Ptx treatment.

Histology. The feet of mice that had been injected with CFA only or with Ptx and CFA were removed 14 days after the treatment and fixed with 10% Formalin. Horizontal sections through the metatarsal region were made and stained with hematoxylin and cosin.

FIG. 1. Enhancement of inflammation by Ptx. The left footpad of each mouse received 0.05 ml of an emulsion made with equal volumes of CFA and saline. One group (●) received 400 ng of pertussigen i.v., and the other (○) received only 0.2 ml of phosphate-buffered saline. The bars at each point give the SEM. *P* values: days 14 and 18, <0.001; days 21 to 29, <0.01; days 31 to 40, <0.05; other days, not significant.

![Graph](http://iai.asm.org/)

FIG. 2. Enhancement of inflammation by Ptx. Experiments were performed as described in the legend to Fig. 1, except that IFA was used instead of CFA. Results were not statistically significant.

![Graph](http://iai.asm.org/)

**TABLE 1. Effect of dose of Ptx on inflammatory response to an emulsion of CFA**

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose of Ptx (ng)</th>
<th>Difference between thicknesses of left and right footpads (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(mean ± SEM)</td>
</tr>
<tr>
<td>1</td>
<td>400</td>
<td>1.78 ± 0.11</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>1.36 ± 0.17</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>0.92 ± 0.09</td>
</tr>
<tr>
<td>4</td>
<td>6.25</td>
<td>0.77 ± 0.09</td>
</tr>
<tr>
<td>5</td>
<td>1.6</td>
<td>0.84 ± 0.07</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0.81 ± 0.06</td>
</tr>
</tbody>
</table>

* Each of five mice per group was inoculated in the left footpad only with 0.05 ml of CFA emulsified in saline. Sixteen days later, the footpads were measured, and the average differences were determined. The doses of Ptx indicated were given i.v. in a total volume of 0.2 ml.

**Statistics.** The data are expressed as arithmetic mean ± standard error of the mean (SEM). Probability (*P*) values were calculated by the Student *t* test.

**RESULTS**

Preliminary experiments showed that mice receiving CFA in the footpads and Ptx i.v. developed an intense inflammation of the footpad that was strikingly greater than in mice that had not received Ptx. To corroborate this finding under more controlled conditions, the following experiment was performed. Two groups of 10 mice each were inoculated in the left footpads with 0.05 ml of an emulsion made with physiological saline and either CFA or IFA. Five mice in each group received 400 ng of Ptx i.v. At different intervals of time, the diameters of the left and right feet of each mouse were measured, and the differences were calculated. The results showed that the group of mice receiving CFA emulsified in saline and Ptx (Fig. 1, solid line) developed a greater footpad swelling than the mice receiving only CFA (Fig. 1, dotted line). When IFA was given, no such difference was found (Fig. 2). When CFA and Ptx were given, there was little or no difference in the increments in foot swelling during the first 4 days (Fig. 1), but by day 8, the swelling in the Ptx-treated animals began to increase. This inflammation increased up to day 14, when it reached a maximal intensity that persisted at this level until day 29. By day 31, there was a sharp drop in the swelling, but even after 40 days, a significant difference was still evident between the two groups (Fig. 1). Throughout day 34 of observation, the thickness of the feet of mice receiving IFA and Ptx was not different from that in mice receiving IFA alone (Fig. 2). The

**TABLE 2. Effect of Ptx on inflammatory response to an emulsion of CFA and IFA containing KLH**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment*</th>
<th>Ptx, 400 ng i.v.</th>
<th>Difference between thicknesses of left and right footpads (mm) (mean ± SEM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CFA-KLH</td>
<td>IFA-KLH</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>−</td>
<td>1.90 ± 0.17</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>−</td>
<td>1.05 ± 0.06</td>
</tr>
<tr>
<td>3</td>
<td>−</td>
<td>+</td>
<td>1.68 ± 0.04</td>
</tr>
<tr>
<td>4</td>
<td>−</td>
<td>+</td>
<td>0.89 ± 0.03</td>
</tr>
</tbody>
</table>

* Each of five mice per group was inoculated in the left footpad only with 0.05 ml of emulsion. Fourteen days later, the left and right footpads were measured, and the differences were determined.

* +, Treated; −, not treated.

* *P* values: group 1 versus group 2, <0.01; group 3 versus group 4, <0.001.
The amount of Ptx required to induce this enhancement of inflammation was determined in groups of five mice each given different fourfold dilutions of Ptx i.v. and 0.05 ml of CFA emulsified in saline in the left footpad. The results, obtained 16 days after CFA, are given in Table 1, in which it can be seen that 400 ng of Ptx induced a significant increase in swelling as shown in Fig. 1, 100 ng induced a significant but much reduced enhancement, and 25 ng or less did not induce a significantly different swelling than was found in the control animals receiving CFA only (Table 1, group 6).

From these experiments, it was clear that antigens from the mycobacterial cells present in CFA appeared to be needed to show the enhancing action of Ptx. In the following experiment, mice were sensitized as in the previous experiment, but either CFA or IFA emulsified with an equal volume of a saline solution containing 1 mg of KLH per ml was used. Each mouse received 0.05 ml of the corresponding emulsion in the left footpad, and shortly thereafter the mice were given 400 ng of Ptx by the i.v. route. The results 14 days after injection (Table 2) indicated that mice injected with KLH emulsified in IFA also developed a swelling that was potentiated by Ptx, as was the case in those mice receiving CFA-KLH. These results clearly showed that the presence of mycobacterial antigens was not necessary for the Ptx effect to occur, so long as the emulsion contained an antigenic material. There was an indication that the addition of KLH to CFA induced slightly greater swelling of the footpad, so this mixture was used in the following experiments.

The influence of the time at which Ptx was given was next studied. In this experiment, CFA was emulsified with an equal volume of saline containing 1 mg of KLH per ml, and 0.04 ml was given into each of the two hind footpads. Groups of five mice each were then given 400 ng of Ptx i.v. on days -7, -3, 0, +3 and +6 with reference to the CFA injection, which was given to all mice on day 0. The footpads were measured 14 days after the injection of CFA. The average footpad thickness of the group receiving CFA-KLH only was subtracted from the average of the Ptx-treated groups. The increments are plotted in Fig. 3. Ptx was effective in increasing the inflammatory response to CFA-KLH when given from 3 days before to 3 days after the injection of CFA, but the greatest effect was noticed when it was given on the day of CFA-KLH or 3 days after. This optimal time coincided with that for the promotion of the DTH reaction (18) and the induction of EAE (15).

Since the crystalline Ptx used was not absolutely pure, it could have had traces of endotoxin at a level difficult to detect but still active in some biological tests. For this reason, the effect of pertussis endotoxin on the inflammatory response to CFA was studied. Two groups of five mice each were included. One group received CFA-KLH by the footpad route and 400 ng of endotoxin i.v.; another group received CFA-KLH only. No statistically significant differences were found between footpad reactions in these two groups at 14 days (data not shown). Further evidence that endotoxin was not involved in these observations comes from experiments with heat-inactivated Ptx, in which this material did not enhance the inflammatory response to CFA (data not shown).

We have shown previously that the effect of Ptx on the enhancement of DTH requires the presence of T lymphocytes (5, 17). Thus, it was of interest to establish whether animals deficient in T lymphocytes such as BALB/c nude mice could respond with an inflammatory response to CFA-KLH and to see whether this response was enhanced by Ptx. To this end, two groups of BALB/c nude mice were inoculated in the left footpads with 0.05 ml of an emulsion made with CFA and an equal volume of saline containing 1 mg of KLH per ml. The footpad increments recorded at 6, 9, and 14 days (Table 3) show that Ptx did not increase the inflammatory response to CFA (group 1), whereas BALB/c mice treated with Ptx (group 3) responded vigorously, as has been shown in previous experiments.

**FIG. 3.** Effect of time of administration of pertussis on inflammatory response to CFA-KLH. Groups of five mice each were given i.v. one dose of 400 ng of pertussis from 7 days before to 6 days after CFA-KLH inoculation. Footpads were measured 14 days after CFA-KLH. From the combined average diameters of the two hind feet of the Ptx-treated animals the average diameter of the mice receiving only CFA-KLH was subtracted to get the increment shown in the figure. The bars at the end of each column indicate SEMs. The P values compared with the group not treated with Ptx were as follows: day -3, <0.05; day 0, <0.001; day +3, <0.001; day +6, not significant.
pared in Fig. 4; a section of an uninoculated foot from a mouse that had received Ptx is also shown. All of these sections were taken from the metatarsal region of the foot. Qualitatively, the cellular responses were essentially the same in the two groups, but it was more intense in mice that had received both CFA and Ptx (Fig. 4A compared with B). Dense accumulations of mononuclear cells (mostly small), granulocytes (mostly neutrophilic and a few eosinophils), and fibroblasts occupied the soft tissue beneath the skin, causing the foot to become greatly swollen. Occasionally, mononuclear macrophages formed focal collections in the inflammatory exudate, but a distinct granulomatous response was not seen in these specimens taken 14 days after inoculation of CFA and Ptx.

**DISCUSSION**

Pertussis vaccine and one of its active substances, Ptx (also known as histamine-sensitizing factor, lymphocytosis-promoting factor, islet-activating protein, and pertussis toxin) are highly effective in enhancing numerous immunological responses, including DTH reactions (2, 5, 18), production of antibodies of the immunoglobulin E class (14), and autoimmune diseases such as EAE in rats (9, 14) and mice (3, 10, 15) and experimental orchitis in mice (8). Despite these findings, some reports have shown an inhibition of cell-mediated reactions such as DTH (16; E. L. Hewlett, C. R. Manclark, and A. A. F. Mahmoud. Clin. Res. 26:327A, 1979), adjuvant arthritis (16), and graft rejection (17). Because the studies of inhibitory effects employed either pertussis vaccine or relatively impure preparations of Ptx, it is possible that substances other than Ptx may have been responsible for these suppressive effects. By contrast, when crystalline Ptx has been used, immunological responses have been enhanced in a variety of systems (8, 13, 18).

The present studies demonstrate another remarkable effect of Ptx, namely the enhancement of the inflammatory reaction associated with Freund adjuvant. The present findings have several similarities to the effects of Ptx on DTH responses (18) and EAE (15). In both instances, Ptx was effective when given close to the time of immunization. Ptx was more effective when given on the day of sensitization or 3 days later, less effective 3 days before or 6 days after, and ineffective 7 or more days before sensitization. As with DTH (18) and EAE (15), the most pronounced reactions occurred when Ptx was given 3 days after footpad injection. Ptx increased markedly the cellular infiltrate in DTH reactions (18) as well as the site of immunization, as shown in the present report. In both instances, mononuclear cells were prominent in the infiltrate. In DTH, the inflammation potentiated by Ptx was dependent on the presence of T lymphocytes (18), and since the T-cell-deficient nude mouse failed to show the enhancing effect of Ptx on footpad swelling produced by CFA, it may be that T cells are also involved in this action of Ptx. These similarities suggest that the effect of Ptx at the site of immunization represents a state of increased cellular sensitivity to an antigen and that this sensitivity persists at the injection site because it is emulsified in Freund adjuvant (7). Another example of chronic inflammation associated with persistence of antigen is the granulomatous reaction produced by *Schistosoma japonicum* eggs in the lungs of certain strains of mice. The inflammation in this condition is also increased by Ptx (11).

The mechanism by which Ptx increased DTH and granulomatous responses is unclear, but Ptx has certain biological actions that may amplify the inflammatory response to antigens retained in a discrete local area. These effects include the well-documented increase in antibody response to antigens, especially stimulation of the immunoglobulin E class of immunoglobulins (14), induction of lymphocytosis with inhibition of lymphocyte homing (20), increased responsiveness of vessels to vasoactive amines (14), and increased number of mast cell precursors in the bone marrow (R.

**FIG. 4.** Photograph of horizontal histological sections of the metatarsal region of the foot. (A) Section of foot 14 days after inoculation with CFA in a Ptx-treated mouse. (B) Section of foot 14 days after inoculation with CFA in a mouse receiving only phosphate-buffered saline. (C) Section of an uninoculated foot from a Ptx-treated animal.
Crapper et al., unpublished work). All of these effects may have some role to play in the altered expression of DTH reactions and in the increased reaction to CFA with Ptx observed in this and other studies (2, 18). In EAE of rats (14) and mice (10), vasoactive amines were implicated in the increased vascular permeability in the nervous system, in which the antigen, myelin basic protein, is located. Similar observations have been made in DTH skin reactions of mice (1, 6). The source of vasoactive substances is most likely mast cells sensitized by antibodies of the immunoglobulin E class that upon contact with the specific antigen degranulate and release vasoactive substances (19).

Since Ptx increases the number of lymphocytes in the blood (13, 14), it is possible that this action may lead to an increased accumulation of lymphocytes in an area of increased vascular permeability.

Ptx may act through one or more of these mechanisms to cause the increase in inflammatory responses as well as in DTH and autoimmune reactions in general. Needless to say, the exact biochemical or enzymatic action of Ptx that leads to these changes is not yet understood and remains a challenging problem for further studies. Nevertheless, the enhancement by Ptx of the inflammatory reaction to CFA is a simple model for the study of chronic inflammation and a useful bioassay for the activity of Ptx in animals immunized with Freund adjuvant.

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LITERATURE CITED


