Induction of Murine Cytolytic T Lymphocytes by 

_Pseudomonas aeruginosa_ Exotoxin A

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_Pseudomonas aeruginosa_ exotoxin A (PA), a potent protein synthesis inhibitor, was found to be a weak T-cell mitogen for murine splenocytes. Maximal stimulation of [3H]thymidine incorporation was obtained with 10 to 100 ng of toxin per ml following a 4-day induction. PA was also shown to be a polyclonal activator of cytolytic T lymphocytes (CTL), effective against concanavalin A-treated target cells. The effective PA dose for CTL induction was the same as that for mitogenic stimulation, only with a prolonged priming time (7 days). In contrast to other mitogens, PA could not reactivate memory CTL into secondary CTL. The stimulation of CTL by subcytotoxic doses of PA may be relevant to its modulatory effect on the immunocellular system.

_Pseudomonas aeruginosa_ secretes a number of exoproteins which affect its ability to cause infection (3, 16). Among these proteins, exotoxin A (PA) is considered to be the most toxic substance produced by this organism and as such may have a significant role in its pathogenicity (16). PA is a highly potent protein synthesis inhibitor (11, 21) and thus acts as a cytotoxic agent on a whole range of mammalian cells, including cells of the immune system (18, 23, 26). A preliminary report by Pavlovsks et al. (22) and two recent studies by Holt and Misfeldt (8, 9) have shown that PA has a modulatory effect on the humoral immune response, mediated through its ability to induce changes in the composition of the lymphocyte population. Many exotoxins produced by various bacterial species have also been shown to exert a modulatory effect on the immune response (4, 6, 7, 13, 14, 17, 19, 25, 29).

To further study the possible effects of PA on the immune system, the ability of this toxin to generate cytolytic T lymphocytes (CTL) from normal, unprimed lymphocytes, as well as from memory-alloimmunized lymphocytes, has been investigated and compared with the reported abilities of staphylococcal enterotoxin B (SEB) and plant mitogens to induce such activity (2, 20, 28). The possibility that PA at subcytotoxic concentrations contains mitogenic activity and through it affects the immune system was investigated as well.

**MATERIALS AND METHODS**

Mice, tumor-derived lines, and spleen cells. BALB/c male mice, 2 to 3 months old, purchased from Jackson Laboratory, Bar Harbor, Maine, were used. Leukemia EL4 of C57BL/6 was maintained as ascites by weekly passage of 25 x 10^6 cells in syngeneic mice. YAC leukemia of A/J was propagated in culture in RPMI 1640 medium supplemented with fetal calf serum (FCS; 10%). HeLa cells and L-929 fibroblasts were cultured in medium 199 supplemented with 10% FCS.

Reagents. PA was purchased from the Swiss Serum and Vaccine Institute, Berne, Switzerland. The purity of the commercial toxin was analyzed on a sodium dodecyl sulfate-polyacrylamide gel, and unless homogeneous, it was rechromatographed on a hydroxyapatite column (15), yielding a product migrating as a single band in electrophoresis (over 99% pure). Concanavalin A (ConA) was purchased from Miles-Yeda Ltd., Rehovot, Israel. Ficoll-Paque was obtained from Pharmacia, Uppsal, Sweden. Neuraminidase was purchased from Behringerwerke AG, Marburg, Federal Republic of Germany. Thy-1.1 and Thy-1.2 antibodies were C3H/eb anti-AKR/J (Thy-1.1 strain) and AKR/J anti-AKR/Cu (Thy-1.2 strain) sera, respectively. These antibodies as well as monoclonal antibodies against Lyt-1 (clone 53-7), Lyt-2 (clone 53-6), and rabbit anti-rat fluorescent immunoglobulin G (IgG) were the gift of A. Peled of the Weizmann Institute, Rehovot, Israel; goat anti-mouse IgG was purchased from Bio-Yeda, Rehovot, Israel.

Mitogenic stimulation of splenocytes. Splenic lymphocytes were prepared by mincing and teasing spleens from normal or alloimmunized mice and filtering the spleens through a 200-mesh stainless steel screen. After being washed with RPMI 1640 medium supplemented with 10% FCS plus antibiotics, 2 mM L-glutamine, and 5 x 10^-5 M β-mercaptoethanol, the cells (2 x 10^6/ml) were incubated with the mitogen ConA or PA for various times. Resultant lymphoblasts were isolated on a Ficoll-Paque density gradient by centrifugation at 450 x g for 20 min. The interface recovered was washed once in phosphate-buffered saline (PBS)-10% FCS (PBS-FCS) and suspended in RPMI 1640 medium supplemented with 10% FCS and 1 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) to a density of 10^6 cells/ml. This suspension was used as a source of lytic effector cells in cytotoxic assays.

[3H]Leucine and [3H]thymidine incorporation by PA-stimulated splenocytes. Normal splenocytes were cultured at 2 x 10^6/ml in 0.15 ml in 96-well flat-bottom microtiter plates (Sterilin, Feltham, England). Cultures supplemented with PA or ConA were maintained at 37°C in a humidified atmosphere of 5% CO_2 and air for various times. At 4 h before harvesting, 1 µCi of [methyl-3H]thymidine (112 Ci/mmol; Radiochemical Centre, Amersham, England) or 1 µCi of [3H]leucine (135 Ci/mmol; Radiochemical Centre) was added in 10 µl of RPMI 1640 medium. Cells labeled with [3H]thymidine were harvested on glass fiber disks with an automatic multiple cell harvester. A 100-µl sample of each cell suspension labeled with [3H]leucine was pipetted onto paper disks (3MM, 25-mm diameter; Whatman, Inc., Clifton, N.J.). The cells were dried for 20 min at room temperature, immersed in 5% trichloroacetic acid, and heated to 85°C for 15 min, followed by two cold trichloroacetic acid washes and one 75% ethyl alcohol-25% ethyl ether wash. The dry glass filter disks as well as the Whatman paper disks...
were monitored in a toluene-based scintillator. All determinations were done in triplicate, and the data are expressed either as mean counts per minute ± the standard deviation of the mean or as percent incorporated activity of control (cultures that do not contain mitogen or PA).

**Elimination of macrophages.** PA-induced blasts from spleen cultures were isolated on Ficoll-Paque and suspended in RPMI 1640 medium plus FCS (10%) to a final concentration of 10^6 cells per ml. Freshly prepared splenocytes were immediately suspended in RPMI 1640 medium and brought to the same concentration. A 5-ml sample of the suspension was placed in a 10-cm petri dish and incubated for 1 h at 37°C. Nonadherent cells were removed and tested for cytolytic activity.

**Cytolytic assay.** The cytolytic activity of PA- and ConA-stimulated splenocytes was assessed against ConA-treated, 51Cr-labeled allogeneic target cells. Effector cells were lymphoblasts isolated from mitogen-transformed splenocytes cultured for various times. The target cells used were EL4 cells pretreated with ConA. Briefly, 20 × 10^6 to 30 × 10^6 target cells were resuspended in a minimal volume (0.1 to 0.2 ml) of PBS-FCS, to which 100 μCi of 51Cr (Radiochemical Centre) was added. Incubation was continued for 30 min at 37°C, followed by a single wash. Chromium-labeled target cells (5 × 10^6 cells per ml) in PBS-FCS were further incubated for 30 min in the presence of 15 μg of ConA per ml and then washed. A cell suspension of 2 × 10^6 cells per ml in RPMI 1640 medium containing 10% FCS and 10 mM HEPES was used as a source of target cells for the cytolytic assay. Effector cells (10⁶) and target cells (10⁵) suspended in 150 μl of the same medium were gently mixed in round-bottom wells, centrifuged at 200 × g for 5 min, and incubated for 90 min at 37°C. To determine 51Cr release, the cells were centrifuged at 450 × g for 10 min at 4°C and 0.1-ml samples were then removed for counting. Percent specific lysis was calculated as follows: % lysis = ([effector-mediated 51Cr release – spontaneous 51Cr release]/[maximal 51Cr release – spontaneous 51Cr release]) × 100. Maximal 51Cr release represented radioactivity released by 1 N HCl from labeled cells. Spontaneous release was the 51Cr released from target cells incubated alone; lytic value represents the mean of triplicate assays.

**Natural killer activity.** Natural killer activity of PA-stimulated splenocytes was assessed against 51Cr-labeled YAC cells that had been pretreated with neuraminidase. Neuraminidase treatment was carried out on a cell suspension (10^7/ml) incubated in the presence of 40 μl of neuraminidase (Behringwerke) at 37°C for 15 min. The treatment was stopped by washing the cells with PBS-FCS and suspending them in the RPMI 1640-FCS-HEPES medium. The cytolytic assay was done essentially as described above except that the number of target cells was 5 × 10^5 cells per well and the time of incubation was 3 h.

**RESULTS**

Mitogenicity of PA for murine splenocytes. The modulatory effect of PA on the immunocellular system could be a result of its mitogenic action on splenocytes at subcytotoxic concentrations. To examine this possibility, nonsensitized spleen cells were cultured with PA for various times and their[^3H]thymidine-incorporating activity was determined. In the first 24 h, no significant stimulation of[^3H]thymidine incorporation could be observed. Upon further incubation of cell cultures, in the presence of PA, DNA synthesis gradually increased, peaking in 4-day cultures (Fig. 1). With ConA, maximal stimulation was already obtained after 2 days. To determine whether PA stimulated T cells or B cells, T cells were eliminated by treating the splenocytes with complement and anti-Thy-1.2 antibodies; their induction with PA was then compared with induction with known T- and B-cell mitogens. The treated splenocytes lost the response to PA as well as to ConA and SEB and retained the response to lipopolysaccharide, indicating that PA is a T-cell mitogen (Table 1). The results of cell surface analysis for splenocytes induced by PA also indicated that PA is a T-cell mitogen (Table 2). However, the high percentage of labeled cells obtained with fluorescein isothiocyanate-conjugated anti-mouse IgG may indicate that PA is also a T-cell-dependent B-cell mitogen. Optimal PA concentrations for stimulating DNA synthesis in 1- to 4-day cultures were 10 to 100 ng/ml. In 7-day cultures, higher doses of PA were required to increase[^3H]thymidine incorporation (Fig. 2A).[^3H]Leucine incorporation in 2- to 7-day cultures supplemented with PA followed the general pattern of[^3H]thymidine incorporation, i.e., at 10 to 100 ng of toxin per ml of culture, protein biosynthesis was markedly enhanced. Pro-
TABLE 1. Proliferative responses of normal spleen cells and a B-cell-enriched population to PA and known B- and T-cell mitogens*

<table>
<thead>
<tr>
<th>Inducer (µg/ml)</th>
<th>[³H]thymidine incorporation (cpm) into splenocytes</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>Anti-Thy-1.2 + complement treated</td>
</tr>
<tr>
<td>None</td>
<td>4,150 ± 193</td>
</tr>
<tr>
<td>PA (0.1)</td>
<td>16,516 ± 1,024</td>
</tr>
<tr>
<td>ConA (2)</td>
<td>43,081 ± 2,236</td>
</tr>
<tr>
<td>SEB (1)</td>
<td>36,047 ± 1,620</td>
</tr>
<tr>
<td>Lipopolysaccharide (10)</td>
<td>40,609 ± 2,036</td>
</tr>
</tbody>
</table>

* Splenocytes (10⁸) were treated with 0.1 ml of Thy-1.2 antibodies for 30 min on ice. The cells were washed and further incubated with complement at 37°C. The control and splenocytes treated with Thy-1.2 antibody plus complement were incubated in the presence of the indicated mitogen for 4 days and suspended in fresh medium. The [³H]thymidine-incorporating activity was determined as described in Materials and Methods. Each value is the mean of triplicate samples ± the standard deviation.

tein synthesis in freshly prepared splenocytes and 24-h cultures of splenocytes was slightly inhibited (Fig. 2B). To verify the low rate of protein synthesis inhibition obtained in splenocytes, the effect of PA on splenocytes was compared with that of PA-sensitive L cells and HeLa cells. It was found that PA at concentrations of 10 to 100 ng/ml extensively inhibited protein biosynthesis in L cells and, to a lesser degree, in HeLa cells (Fig. 3). The same toxin concentrations that extensively inhibited protein synthesis of L cells and HeLa cells slightly affected amino acid incorporation into lymphocytes, lymphoblasts, and YAC cells but only during the first 24 h of incubation (Fig. 3). In conclusion, it appears from these experiments that the inhibition effect of PA on lymphoid cells is marginal and short lived. PA is a weak T-cell mitogen, and its stimulating activity on splenocytes can be observed only upon longer exposure of spleen cells to the toxin.

Induction of CTL by PA as compared with induction by other T-cell mitogens. Splenocytes prepared from BALB/c mice were cultured for various times at two concentrations of PA that were found optimal for mitogenic induction and at optimal concentrations of ConA and SEB (28). The resultant T lymphoblasts were purified and tested for cytolysis against ConA-treated EL4 target cells. Like ConA and SEB, PA induced a primary CTL response, but with entirely different kinetics (Fig. 4). Upon ConA and SEB stimulation,

![Figure 2](http://iai.asm.org/)  
**FIG. 2.** PA dose effect on [³H]thymidine and [¹⁴C]leucine incorporation into lymphoblast cultures incubated for the indicated times. Splenocytes (3 x 10⁶) were incubated in triplicate for the times indicated in the presence of three PA concentrations and in the absence of toxin. Labeled thymidine and leucine were added separately during the last 4 h of incubation, and incorporated radioactivity was determined. The incorporated radioactivity was expressed as a percentage of the control, when the control differed for each indicated time. The data presented are the means of four experiments. Bars represent standard deviations of the means of four experiments.

TABLE 2. Surface phenotype of PA-stimulated effector cells*

<table>
<thead>
<tr>
<th>Days cells in culture</th>
<th>% Labeled cells after treatment with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Thy-1.1</td>
</tr>
<tr>
<td>0</td>
<td>2.9</td>
</tr>
<tr>
<td>7</td>
<td>20.3</td>
</tr>
</tbody>
</table>

* The cells analyzed were isolated from spleen cultures induced with 10 ng of PA per ml. Analysis was carried out with FACS-II (Becton Dickinson and Co., Paramus, N.J.) at gain 16. The experiment presented was repeated with similar results. The percentage of labeled cells in the presence of fluorescein isothiocyanate-labeled anti-rat IgG alone was 2 to 3%. The percentage of labeled cells with fluorescein isothiocyanate-labeled anti-mouse IgG only was over 68% for a 4-day culture and 31% for a 7-day culture.
the cultures reached maximal cytolytic activity after 3 days, with a good correlation between enhanced DNA synthesis and cytolytic activity. In the case of PA-stimulated splenocytes, the cytolytic activity was detected mainly in 7-day cultures in which DNA synthesis had already subsided to normal (Fig. 2 and 4). Optimal PA concentrations for cytolytic induction were in the range of optimal PA concentrations for mitogenic induction (10 to 100 ng/ml). The PA-stimulated cultures (2 to 7 days) did not present any anti-YAC activity, i.e., the lytic activity observed could not be referred to natural killer cells. Removal of adherent cells from PA-induced cultures did not affect the lytic activity (Table 3). However, the removal of adherent cells from splenocytes prior to PA stimulation abolished almost completely the lytic induction (results not shown). PA was also tested for its capability to stimulate memory CTL into secondary CTL. The model for these experiments consisted of splenocytes from BALB/c mice primed 95 days earlier with EL4 and induced in vitro for 1 to 10 days with optimal concentrations of PA or ConA; the activity of the splenocytes was assessed against the original priming target cells (EL4). It appears that PA, as opposed to ConA, could not reactivate memory CTL into secondary CTL in EL4-primed splenocyte cultures (data not shown).

**DISCUSSION**

Staphylococcal enterotoxins as well as pyrogenic toxins produced by the same bacteria and by *Streptococcus pyogenes* have been shown to be highly potent T-cell mitogens (4, 5, 13, 19, 24, 25). As are many other plant mitogens, these toxins are capable of inducing suppressor and helper activity, as well as cytolytic activity, in the immune system (4, 6, 7, 13, 19, 25, 28). Other bacterial protein toxins, like cholera toxin and PA, also enhance or suppress the immune response (8, 9, 14, 17, 22). The observation that PA is, on the one hand, a highly toxic substance for a wide range of mammalian cells and, on the other, an effective immunomo-

**TABLE 3. Properties of cytolytic effector cells activated by PA**

<table>
<thead>
<tr>
<th>Effector cells</th>
<th>Target cells</th>
<th>% Lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonfractionated splenoblasts</td>
<td>ConA-treated EL4</td>
<td>21.3 ± 0.6</td>
</tr>
<tr>
<td>Nonadherent splenoblasts</td>
<td>ConA-treated EL4</td>
<td>19.6 ± 1.1</td>
</tr>
<tr>
<td>Nonfractionated splenoblasts</td>
<td>Neuraminidase-treated YAC</td>
<td>0</td>
</tr>
<tr>
<td>Splenoblasts treated with Thy-1.2 antibody + complement</td>
<td>ConA-treated EL4</td>
<td>3.4 ± 0.4</td>
</tr>
<tr>
<td>Splenoblasts treated with Lyt-2 antibody + complement</td>
<td>ConA-treated EL4</td>
<td>6.5 ± 1.2</td>
</tr>
</tbody>
</table>

* The cells analyzed were isolated from a 7-day culture. Nonadherent splenoblasts were prepared as described in Materials and Methods. Elimination of PA-induced effector cells was as follows. Splenoblasts (10^5) were treated with 0.2 ml of specific antibody on ice. After the cells were washed, complement was added, and the cells were further incubated for 1 h at 37°C. Lytic activity was tested in triplicate against the ^51Cr-labeled target cells indicated. Each value is the mean of triplicate samples ± the standard deviation.

dulator seems primarily paradoxical. The data presented in this study represent an attempt to bridge the gap between these apparently contradictory observations. Since the PA used in this study was highly pure, the presence of a contaminant in the toxin preparation that contained the lymphocyte-stimulating properties was unlikely. First, it was shown that the sensitivity of lymphoid cells to the inhibition of protein synthesis by PA was minor as compared with the sensitivities of other mammalian cells (Fig. 3). This differential sensitivity to PA could be explained on the basis of fast uptake and degradation of the toxin by lymphoid-cell lysosomes, with no active toxin escaping into the cytoplasm. Equally possible explanations may be based on a reduced number of receptors for PA or blockage of toxin-receptor dissociation, preventing release of active toxin to the cytoplasm. Of course, many other explanations are possible. It

**FIG. 3.** PA effect on protein biosynthesis of lymphoid cells as compared to the effect on biosynthesis of epithelium-derived cell lines. A 1.5 × 10^5-amount of splenocytes, SEB-induced lymphoblasts, or YAC cells and 3 × 10^4 HeLa cells or L cells were seeded in a flat-bottom microtiter plate in a final volume of 150 μl. The cells were incubated in the presence of various concentrations of PA for the times indicated. [3H]Leucine was added 4 h before harvesting, and incorporated radioactivity was determined in triplicate as described in Materials and Methods. This experiment was repeated, with essentially the same results.
was also found that PA concentrations which were considered to be inhibitory to other mammalian cells enhanced DNA synthesis concomitantly with protein synthesis in the lymphoid cells; that is, PA was shown to be a weak mitogen for lymphoid cells, and as such, its mode of action as an immunomodulator with effects similar to those of other mitogens can be well understood (1, 13, 25).

Experiments presented here in which T-cell subpopulations were eliminated from splenocyte suspensions before exposure to PA, together with surface phenotype antigen analysis, indicate that PA is a T-cell mitogen (Table 1). The mitogenic characteristics of PA may explain its involvement in the induction of suppression phenomena of the immunocellular system as described by others (8-10, 22). In contrast to other toxin T-cell mitogens (13, 24, 28), PA also stimulated B-cell proliferation, but only in the presence of T cells (Table 2). These results, PA can actually be considered a T-cell-dependent B-cell mitogen, similar to the pokeweed mitogen (27). These data are well in line with the results presented by Holt and Misfeldt (9) describing the effect of PA on the expansion of the B-cell population in the athymic nude mouse.

The present studies also show that PA, like SEB and other mitogenic lectins (2, 20 28), can generate cytotoxic effector cells by stimulating unprimed murine splenocytes (Fig. 4). The PA-stimulated effectors are T cells and not macrophage cytolytic cells, as indicated by their antigenic and adherence properties (Table 3). The induction of the cytolytic cells was, however, accessory-cell dependent, since removal of adherent cells before but not after induction prevented the appearance of cytolytic activity. In contrast to those of SEB and plant lectins (28), the dose response of PA was much lower. The proliferation kinetics of the cytolytic cells induced by PA were also different from the kinetics of those induced by SEB or ConA (peak activity of 7 to 10 days as compared with 3 to 4 days for ConA and SEB). The difference in kinetics of induction may be due to different subpopulations of precursor cells activated by PA as opposed to other lectins, the PA-responsive population being probably initially much smaller. An alternative explanation for this difference may be that interleukin 2 induction by PA is inefficient and therefore is limiting to the expansion process of CTL. In fact, only traces of interleukin 2 were detected in PA-induced splenocyte cultures (results not shown). Another difference between PA and other polyclonal inducers is that they reactivate memory CTL into secondary CTL, whereas in PA induction, only primary CTL activation was observed and no secondary CTL activity was detected in EL4-primed 1-to 7-day cultures (results not shown). Further studies are needed to conclude whether there is a fundamental difference in the mode of splenocyte induction by PA as compared with those of other T-cell mitogens or whether it is only a quantitative matter and under different experimental conditions PA will prove to be a secondary CTL inducer as well.

Lately, a number of microbial products, including exotoxins, have been shown to possess mitogenic activities through which they alter immune responsiveness (4, 10, 12, 14, 17, 19). Results presented in this study clearly show that PA at subcytotoxic concentrations is a T-cell mitogen as well as a T-cell-dependent B-cell mitogen. These mitogenic characteristics of PA, like those of other mitogens, may well explain the suppressive effect that the toxin elicits on the immunocellular system (8-10, 22). The same mode of action also applies to the capability of the toxin to induce lectin-mediated cytolytic activity, as shown in this study. Since the in vivo role of lectin-mediated cytotoxicity is not at all clear,
future studies will concentrate on the effect of PA on antibody-dependent cell cytotoxicity. Furthermore, in a preliminary experiment, it was observed by the author that PA augments to a low but significant extent the antibody-dependent cell cytotoxicity against anti-erythrocyte antibody-coated chicken erythrocytes (results not shown), results that may be relevant to PA cytolytic cell induction during Pseudomonas infections.

LITERATURE CITED