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Inhibition of Human Lymphocyte Proliferation and Cleavage of Interleukin-2 by *Pseudomonas aeruginosa* Proteases

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This study was undertaken to determine the effect of *Pseudomonas aeruginosa* alkaline protease (AP) and elastase (ELA) on human lymphocyte function. AP at 50 μg/ml and ELA at 12 μg/ml caused a 50% inhibition of phytohemagglutinin-induced proliferation. There was no difference in the effect of proteases on CD4- and CD8-positive cells. To determine the effect of proteases on interleukin-2 (IL-2)-induced cell proliferation, the proteases and IL-2 were added to the IL-2-dependent CTLL-2 cell line. AP and ELA inhibited the proliferation of these cells. When IL-2 was added in excess, the inhibition was partly reversed. ELA at 10 μg/ml cleaved IL-2, as judged by size chromatography of a reaction mixture containing ^{125}I-labeled IL-2 and the proteases. The ELA-digested IL-2 exhibited a reduced binding capacity to IL-2 receptors on the lymphocytes. Furthermore, treatment of phytohemagglutinin-stimulated lymphocytes with AP and ELA resulted in inhibition of binding of intact IL-2 to IL-2 receptors on the stimulated lymphocytes. These results indicated that *P. aeruginosa*-derived enzymes are able to interfere with human lymphocyte function in vitro and that this effect might be due to cleavage of IL-2.

Pseudomonas aeruginosa is an important cause of infection in compromised hosts such as patients with neoplastic disease, cystic fibrosis, or severe burns (4, 7, 14). *P. aeruginosa* and its products have been shown to modulate cellular immune reactions (1, 2, 11, 32), and immunosuppression may contribute to the virulence of the infections. Several mechanisms seem to be operating. The bacteria have been shown to activate suppressor cells (5, 17, 30), and *P. aeruginosa*-derived exotoxin A (15, 16) and phenazine pigments (35) have been shown to inhibit the function of immunocompetent cells. Alkaline proteases (APs) and elastase (ELA) produced by the bacteria seem to play an important role in the pathogenesis of the infections (3, 8, 13, 27, 34, 36). Studies of the interactions between different parts of the host defense system and the proteases have shown that the enzymes interfere with the function of human neutrophil granulocytes (18–20) and natural killer (NK) cells (28) and that they cleave complement components (33) and immunoglobulin A (10). We have recently reported that the enzymes cleave CD4 receptors on human lymphocytes without affecting the CD3, CD5, CD8, HLA-ABC, HLA-DR, HLA-DQ, and HLA-DP/DR receptors on these cells (29). The present study was undertaken to examine the effect of *P. aeruginosa* AP and ELA on human lymphocyte function and on interleukin-2 (IL-2) and its receptor.

MATERIALS AND METHODS

AP and ELA. AP and ELA were purchased from Nagase Chemical Sangwo (Osaka, Japan). The purity of each protease was confirmed by the demonstration of single bands in sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Laemmli (21). The specific activities of AP and ELA were 5.39 and 56.5 proteolytic units per milligram of protein, respectively. Other characteristics are given elsewhere (20). In some experiments, the proteases were inactivated by incubation at 65°C for 60 min. The enzymes were diluted in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) before addition to the cell cultures.

BMNC preparation and separation. Heparinized blood was collected from healthy donors without medication. Blood mononuclear cells (BMNC) were isolated by metrizoate sodium-Ficoll (Lymphoprep; Nyegaard, Oslo, Norway) density gradient centrifugation and washed three times in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS). The cells were then frozen (controlled gradient freezing machine) in RPMI 1640 containing 10% FCS and 10% dimethyl sulfoxide and stored in liquid nitrogen until use. On the day of use, the cells were thawed, washed two times in RPMI 1640 supplemented with 10% pooled human serum, and counted. The BMNC were either used directly in proliferation assays or separated into subsets. The cells for subset determination were washed twice in phosphate-buffered saline with 3% FCS. Monoclonal antibodies against the CD4 and CD8 receptor (fluorescein isothiocyanate-conjugated leu 3 and phytoerythrin-conjugated leu 2; Becton Dickinson and Co., Mountain View, Calif.) were incubated with the cells for 45 min at 4°C in the dark. Before use, the cells were washed twice and resuspended in phosphate-buffered saline. CD4- and CD8-positive lymphocytes were separated by positive selection with a fluorescence-activated cell sorter (FACStar; Becton Dickinson). Only cells strongly positive for CD4 or CD8 were sorted as phenotypic T-helper/inducer (T_h) and T-suppressor/cytotoxic (T_s) cells, respectively. After sorting, the suspensions of $CD4^+$ and $CD8^+$ cells were analyzed. The purity of the sorted cells was 90 to 98%. The amount of $CD4^+$ cells in $CD8^+$ suspensions and of $CD8^+$ cells in $CD4^+$ suspensions was always less than 3%.

Proliferation assays. RPMI 1640 supplemented with 15% pooled heat-inactivated human serum, L-glutamine (58.4 μg/ml), penicillin (10 IU/ml), and streptomycin (20 μg/ml) was used as the culture medium. Cell cultures were performed in triplicate in round-bottom microtiter plates (Nunc, Roskilde,

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Denmark) in a final volume of 170 μl per well. BMNC, T_h, and T_s cultures contained 50,000 cells per well. The T_h and T_s cultures received an additional 10,000 irradiated blood monocytes. Mitogen-stimulated cultures contained phytohemagglutinin (PHA; Difco Laboratories, Detroit, Mich.) in a final concentration of 47 μg/ml. The cells were incubated for 3 days. [^{3}H]thymidine (1 μCi per well; New England Nuclear Corp., Boston, Mass.) was added 24 h before the cells were harvested on glass fiber filters with a harvesting machine (Skatron, Lierbyen, Norway) and [^{3}H]thymidine incorporation was measured in a liquid scintillation counter (Tricarb; Packard Instrument Co., Inc., Rockville, Md.). For each set of triplicate values, the median was recorded. Unstimulated cultures were always included as controls.

IL-2 assay. IL-2-dependent CTLL-2 cells (12) were grown at a concentration of 2×10^3/200 μl of RPMI 1640 supplemented with 10% heat-inactivated FCS. A laboratory standard preparation of IL-2 was used. This standard had previously been compared with an international reference preparation of Jurkat-derived IL-2 (Biological Response Modifiers Program, Frederick, Md.). The cultures were incubated in triplicate for 18 h at 37°C and pulsed with [^{3}H]thymidine for the last 3 h before measurement of thymidine incorporation.

Radiolabeled IL-2. Homogeneous recombinant IL-2 was provided by Takeda Clinical Industries, Ltd. (Osaka, Japan) as a 1.0-mg/ml solution in ammonium acetate buffer (pH 5.0). Human recombinant IL-2 (0.4 mg/ml; ala-125) was purchased from Amersham International plc (Amersham, England; manufactured by AMGEN Biologicals, Thousand, Oaks, Calif.). Both IL-2 preparations were radioiodinated with lactoperoxidase-glucose oxidase (Enzymobeads; Bio-Rad Laboratories, Richmond, Calif.) according to the instructions of the manufacturer. The specific activity of both IL-2 preparations was adjusted to 5×10^4 cpm/pm.

IL-2 binding assay. Binding of ^{125}I-labeled IL-2 to PHA-stimulated BMNC (10 μg/ml, 3 days cultured) from healthy donors was performed as described previously (31), except that the radiolabeled ligands in a final concentration of 2.9 ng/ml were incubated together with the cell suspension (3×10^6 to 4×10^6 cells in 0.2 ml of RPMI 1640 supplemented with 10% FCS). The cell suspension was placed on 0.2 ml of silicone oil suspension (silicone AR 200, $d = 1.05$; Wacker Chemie, Munich, Federal Republic of Germany). After 20 min at 37°C, the tubes were centrifugated at 8,500 × g for 90 s. The top layer containing the supernatant and the bottom layer containing cell-bound IL-2 were collected, and their radioactivities were counted in a gamma counter. The cell-bound IL-2 was expressed as a percentage of the total amount of added IL-2.

In some experiments (see Table 2), radiolabeled IL-2 (143 ng/ml) was incubated in CTTL-2 medium with ELA (10 μg/ml) or AP (50 μg/ml) at 37°C for 1 h. The mixture of enzyme and IL-2 was then diluted 50 times and used in the binding assay. The enzymes did not interfere with the binding of IL-2 to the PHA-induced blasts after dilution. In other experiments (see Table 3), PHA-induced blasts were incubated in CTLL-2 medium with ELA (10 μg/ml) and AP (50 μg/ml) at 37°C for 1 h. Then the blasts were washed free of enzymes and used in the binding assay.

IL-2 size chromatography. A Sephadex G-50 (Pharmacia, Uppsala, Sweden) gel filtration column was prepared according to the instructions of the manufacturer. Phosphate-buffered saline supplemented with polyethylene glycol (0.5 mg/ml) plus NaN_3 (0.04%) was used as the eluent. The column was calibrated with blue dextran, cytochrome c, RNase, and cyanocobalamin (all obtained from Pharmacia). A 2-ml sample of ^{125}I-labeled IL-2 (8.6 ng/ml) was applied to the column, and fractions of 2.7 ml were collected and counted in a gamma counter. Enzyme-treated ^{125}I-labeled IL-2 (8.6 ng/ml) was incubated in eluent with ELA (10 μg/ml) or AP (100 μg/ml) at 37°C for 2 h.

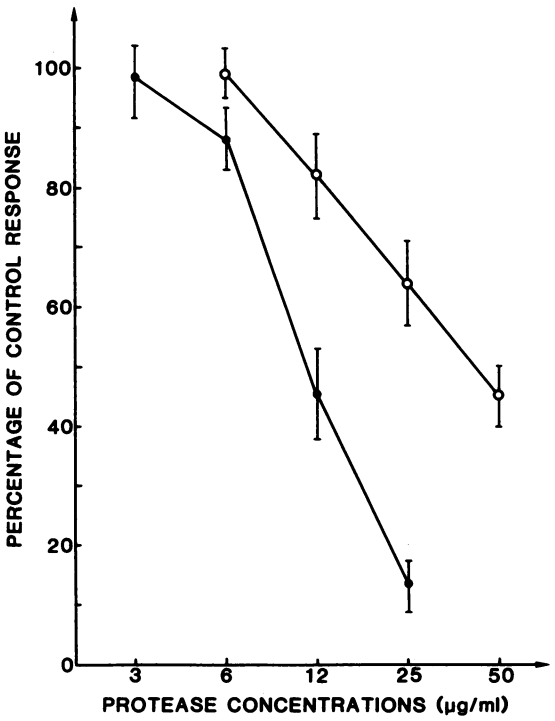

FIG. 1. Effect of AP (○) and ELA (●) on PHA-induced proliferation of BMNC. Mean ± standard error of the mean (SEM) (bars) of 7 to 10 experiments. The incorporation in the control cultures was 71.5 ± 15.9 kcpm (mean ± SEM of 10 experiments).

Statistics. Wilcoxon's rank sum test for paired differences was employed. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Effect of AP and ELA on PHA-induced cell proliferation. Both AP and ELA inhibited the response of PHA-stimulated cultures in a dose-dependent manner when the proteases and PHA were added simultaneously and were present in the BMNC medium during the incubation period (Fig. 1). The inhibition was approximately 50% when AP and ELA were used at concentrations of 50 and 12 μg/ml, respectively. The inhibition was almost abolished if the proteases were heat inactivated at 65°C for 45 min (Fig. 2). The inhibitory effect of the proteases was not due to inactivation of PHA by AP or ELA. Thus, preincubation of PHA with either of the enzymes and subsequent heat inactivation of the enzymes failed to reduce the stimulating effect of PHA (data not shown). To determine whether the inhibitory effect of the proteases was dependent on their presence in the BMNC cultures during cultivation, the cells were preincubated with AP (50 μg/ml) or ELA (25 μg/ml) for 1 h at 37°C and then washed. The cells which had been preincubated with the proteases had the same ability to respond to PHA as untreated cells (data not shown). To investigate the effect of the proteases on different subpopulations of lymphocytes,

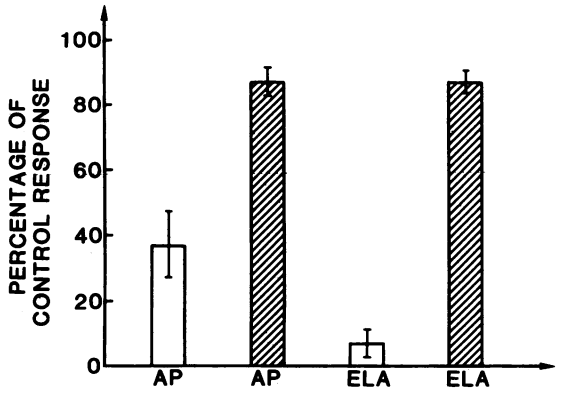

FIG. 2. Effect of untreated (▭) and heat-inactivated (65°C, 45 min) (▨) AP (50 μg/ml) and ELA (25 μg/ml) on PHA-induced proliferation of BMNC. The incorporation in the control cultures was 103.4 ± 26.0 kcpm. Mean ± SEM (bars) of six experiments.

we tested the response of CD4- and CD8-positive cells to protease treatment. AP and ELA inhibited the PHA-induced proliferation of both CD4- and CD8-positive cells (Table 1).

Effect of AP and ELA on IL-2 induced lymphocyte proliferation. IL-2-induced cell proliferation was tested by coincubating IL-2-dependent mouse CTLL-2 cells with IL-2 and AP or ELA. The enzymes and IL-2 were added simultaneously and were present in the CTLL-2 cell medium during incubation. Initial experiments showed that AP at a concentration of 16 μg/ml and ELA at a concentration of 8 μg/ml markedly reduced the proliferation of CTLL-2 driven by IL-2 (1 U/ml). This inhibition, however, decreased when the IL-2 concentrations were increased (Fig. 3). That the enzymes did not mediate their inhibition through a toxic mechanism was also supported by the fact that CTLL-2 cells preincubated with AP or ELA and washed showed the same ability to respond to IL-2 as untreated cells (data not shown).

Effect of AP and ELA on IL-2 binding to human lymphocytes. The data from the CTLL-2 studies indicated that the proteases were able to interfere with the function of IL-2. Therefore, we next tested the ability of AP- or ELA-treated IL-2 to bind to IL-2 receptors on human lymphoblasts. IL-2 treated with ELA showed a statistically significant reduced capacity to bind compared with untreated IL-2 ($P < 0.05$)

TABLE 1. PHA-induced proliferation in cultures of BMNC and BMNC subsets treated with AP and ELA[a]

Treatment (μg/ml) and cell type	% [^{3}H]thymidine uptake in expt:				
	1	2	3	4	Mean
AP (50)					
BMNC	36	7	95	58	49
CD4$^+$	80	3	68	84	59
CD8$^+$	59	2	40	42	36
ELA (25)					
BMNC	5	1	9	ND[b]	5
CD4$^+$	6	2	10	ND	6
CD8$^+$	15	3	3	ND	7

[a] All results are [^{3}H]thymidine uptake as percentage of uptake in cultures that did not receive AP or ELA. The uptakes in the BMNC, CD4$^+$, and CD8$^+$ control cultures were 54.3 ± 7.3, 59.1 ± 23.5, and 43.8 ± 12.9 kcmp (mean ± SEM of four determinations), respectively.

[b] ND, Not determined.

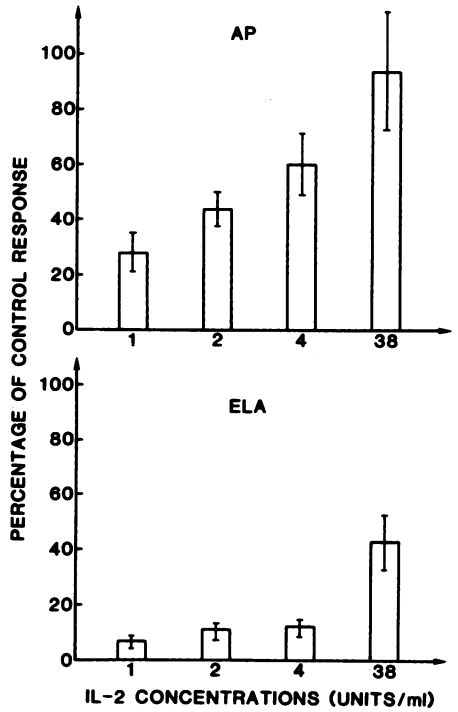

FIG. 3. Effect of AP (16 μg/ml) and ELA (8 μg/ml) on the proliferation of CTLL-2 cells. The proliferation was driven by IL-2 at concentrations indicated on the abscissa. The incorporation in the control cultures was 12.3 ± 2.0, 12.6 ± 2.4, 16.0 ± 2.0, and 8.4 ± 2.0 kcpm in cultures driven by IL-2 concentrations of 1, 2, 4, and 38 U/ml, respectively. Mean ± SEM (bars) of four experiments.

(Table 2). The binding capacity of IL-2 treated with AP was reduced in five of six experiments.

To test whether the enzymes interfered with the IL-2 receptor on the lymphoblasts, we incubated the cells with AP or ELA before the binding assay. The protease-treated cells from six different donors exhibited large variations in their capacity to bind IL-2 (Table 3).

Effect of ELA and AP on IL-2. The result of the binding experiments suggested that ELA and AP had a direct enzymatic effect on IL-2. To test this hypothesis, ^{125}I-labeled IL-2 was incubated with ELA (10 μg/ml) and run through a gel filtration column. The elution profiles of untreated IL-2 and IL-2 preincubated with ELA indicated that some of the IL-2 molecules that had been preincubated with ELA were degraded into smaller fragments (Fig. 4). The elution profile of IL-2 treated with AP (100 μg/ml) indicated that approximately 50% of the IL-2 was degraded (data not shown).

TABLE 2. Binding of IL-2 and IL-2 treated with AP and ELA to IL-2 receptors on human lymphoblasts[a]

Treatment	% Binding of ^{125}I-IL-2 in expt:						
	1	2	3	4	5	6	Mean ± SEM
IL-2 + medium	7	6	12	26	21	20	15.3 ± 3.3
IL-2 + AP (50 μg/ml)	3	4	14	6	10	8	9.2 ± 2.1
IL-2 + ELA (10 μg/ml)	4	3	7	10	10	6	6.7 ± 1.2[b]

[a] All results are percent binding of ^{125}I-labeled IL-2 to PHA-stimulated lymphocytes.

[b] Binding significantly lower than that of IL-2 plus medium ($P < 0.05$).

TABLE 3. Binding of IL-2 to IL-2 receptors on untreated lymphocytes and lymphocytes treated with AP (50 μg/ml) or ELA (10 μg/ml) for 1 h[a]

Treatment	% Binding of ^{125}I-IL-2 in expt:						
	1	2	3	4	5	6	Mean ± SEM
PHA-induced blasts	12	9	12	13	22	22	15.0 ± 2.3
AP-treated blasts	10	6	15	15	1	3	8.3 ± 2.5
ELA-treated blasts	8	9	16	16	8	5	10.3 ± 1.9

[a] All results are percent binding of ^{125}I-labeled IL-2 to PHA-stimulated lymphocytes.

DISCUSSION

It is well established that *P. aeruginosa* is able to induce immunosuppression (1, 2, 5, 11, 16, 17, 30, 32). We have previously shown that *P. aeruginosa* proteases inhibit the function of human polymorphonuclear leukocytes (18–20) and NK cells (28). We have also shown that these proteases cleave the CD4 receptor on human lymphocytes, but fail to interfere with the CD3, CD5, and CD8 receptors or major histocompatibility complex class I and II antigens on these cells (29).

In this study, we examined the effect of *P. aeruginosa* proteases on the in vitro function of human lymphocytes. The proteases inhibited PHA-induced proliferation of human lymphocytes in a dose-dependent manner. This finding seems to disagree with the findings of Sorensen et al. (35), who reported that ELA in concentrations up to 40 μg/ml had little effect on human lymphocyte response to concanavalin A. We found that BMNC preincubated with the enzymes and washed before assay showed the same response as untreated BMNC. This indicates that the inhibitory effect of the enzymes was not due to a toxic mechanism but rather was the result of interference with processes involved in the activation of the cells. The finding that heat-inactivated AP and ELA had no effect on the proliferation response indicated that the inhibition induced by untreated proteases involved enzymatic activity. In a recent study, we reported that AP and ELA selectively cleaved CD4 receptors on T-helper cells, apparently without modulating a number of other receptors on T lymphocytes (29). Cleavage of CD4 receptors alone does not appear to be responsible for the protease inhibition of the proliferative response observed in this study as the effect of enzymes on CD4-positive and CD4-negative lymphocytes were comparable. This was not unexpected since PHA can activate lymphocytes via CD3 and CD2 receptors (22).

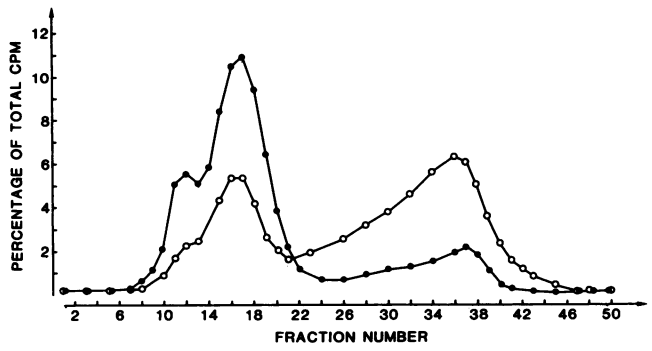

FIG. 4. Size column chromatography (Sephadex G-50) of ^{125}I-labeled IL-2 before (●) and after (○) treatment with ELA (10 μg/ml).

Since IL-2 is important for lymphocyte proliferation, the effect of the proteases on an IL-2-dependent mouse cell line was tested. IL-2-driven proliferation was inhibited when the proteases were present during the incubation period, but this effect could be partly reversed with the addition of large quantities of IL-2. This indicated that the proteases interfered with the binding of IL-2 to its receptor on the cell surface. The inhibition of binding could be either by degradation of IL-2 itself or by modulation of the IL-2 receptor. Binding studies showed that IL-2 treated with ELA had less ability to bind to IL-2 receptors on human lymphoblasts than untreated IL-2, indicating that ELA interfered with the function of IL-2. Gel filtration studies with protease-treated IL-2 demonstrated that ELA degraded IL-2.

It has been suggested that T-cell-mediated responses to *P. aeruginosa* proteases are important for initiating host defenses against the bacterium (25, 26). Human T cells from immune individuals respond to these proteases, and localized infections can develop and progress despite circulating antibody (6). Furthermore, children with cystic fibrosis develop chronic, localized *P. aeruginosa* pulmonary infections despite their extraordinarily high levels of antibody to the various antigens of the infecting organism. Although these high antibody levels prevent bacteremia, they do not prevent the recurrent exacerbation of lung disease associated with *P. aeruginosa* in cystic fibrosis (6). AP is inactivated by α1-proteinase inhibitor (23), and ELA is inhibited by α2-macroglobulin (24). Therefore, the role of these products becomes more important in avascular areas such as in skin burns and the cornea and initially in chronic lung colonization in persons with cystic fibrosis.

A longitudinal study of the immune response to AP and ELA in patients with cystic fibrosis has shown that the antibody response to the proteases developed only after the onset of chronic *P. aeruginosa* lung infection (9). Inhibition of neutrophil (18–20) and NK cell (28) function and cleavage of CD4 receptors on T lymphocytes (29) by *P. aeruginosa* proteases combined with the inhibiting effect of these proteases on T-lymphocyte function described in the present study may provide a mechanism by which *P. aeruginosa* evades the local defense system in the initial stage of the infection. Such an evasion mechanism will enable the bacteria to colonize local tissues such as the lungs of cystic fibrosis patients or the skin of burn patients.

In conclusion, this study demonstrates that the *P. aeruginosa* proteases AP and ELA inhibit the in vitro proliferative response of human lymphocytes, probably by proteolytic attack on IL-2, which inhibits the binding of the growth factor to its receptor on the lymphocytes.

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