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In Vitro Inhibition of Lymphocyte Proliferation by *Pseudomonas aeruginosa* Phenazine Pigments

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Human lymphocyte proliferation is inhibited in vitro in the presence of killed *Pseudomonas aeruginosa* or cell-free *P. aeruginosa* culture supernatants. A comparison of culture supernatants obtained under similar conditions from *Staphylococcus aureus*, *Escherichia coli*, *P. aeruginosa*, and *Pseudomonas cepacia* strains demonstrated that all *P. aeruginosa* supernatants were strongly inhibitory, whereas supernatants from other bacteria were mildly inhibitory or not inhibitory at all. These *P. aeruginosa* inhibitors prevent proliferative responses of resting cells upon mitogen activation and decrease [^{3}H]thymidine uptake when added to human lymphocytes undergoing active proliferation in culture. The inhibitory effect is reversible and not due to cytotoxicity. Most of the inhibitory activity present in crude supernatants was detected in ultrafiltrates of molecular weights below 2,000. Purified *P. aeruginosa* pyocyanine, a low-molecular-weight phenazine pigment present in culture supernatant, was strongly inhibitory for lymphocyte proliferation. Extraction of pyocyanine and phenazine pigments from inhibitory *P. aeruginosa* supernatants eliminated their inhibitory activity. Inhibitors were recovered from reverse-phase chromatographic cartridges by both chloroform and methanol elution, indicating that pyocyanine and other phenazine pigments present in *P. aeruginosa* supernatants are responsible for the inhibition of lymphocyte proliferation. In addition to the identification of phenazine pigments as lymphocyte proliferation inhibitors, several criteria ruled out major contributions of *P. aeruginosa* polysaccharide, exotoxin A, and proteases to this phenomenon. *P. aeruginosa* strains selected for very low protease production or for very low exotoxin A production produced supernatants as inhibitory for lymphocyte proliferation as supernatants obtained from clinical *P. aeruginosa* isolates. Purified *P. aeruginosa* lipopolysaccharide and protease preparations failed to induce reversible lymphocyte proliferation inhibition. Finally, heat inactivation of *P. aeruginosa* supernatants at 100°C for 60 min inactivates exotoxin A and proteases but produced only a moderate decrease of the inhibitory activity for lymphocyte proliferation.

It has been reported previously that high doses of killed *Pseudomonas aeruginosa* inhibit human lymphocyte proliferation and [*methyl*-^{3}H]thymidine ([^{3}H]TdR) uptake in vitro. In lymphocyte cultures exposed simultaneously to optimal doses of several plant lectins and high doses of *P. aeruginosa*, it was shown that lymphocyte proliferation was nonspecifically suppressed. Removal of *P. aeruginosa* from the culture system restored the ability of lymphocytes to proliferate upon activation with plant lectins or bacteria. The inhibition of lymphocyte proliferation did not require the generation of adherent suppressor cells or steroid-sensitive or radiation-sensitive suppressor mechanisms. We further ruled out that the inhibition of lymphocyte proliferation and [^{3}H]TdR uptake was due to a crowding effect, to consumption of nutrients or interleukin 2, or to the presence of cold [^{3}H]TdR competitors (26). Here we report that cell-free *P. aeruginosa* supernatants also cause reversible inhibition of human lymphocyte proliferation. This potent inhibitory activity is found only in *P. aeruginosa* supernatants. Over 75% of the inhibition is caused by heat-resistant small phenazine pigments present in *P. aeruginosa* culture supernatants.

MATERIALS AND METHODS

Lymphocyte preparations. Human peripheral blood mononuclear cell preparations (PBMC) containing approximately 90% lymphocytes were isolated from hep-

arinized blood by Ficoll-Hypaque centrifugation (31). Blood samples were obtained from healthy young adults. Permission to obtain these blood samples was approved by the Institutional Review Board of University Hospitals of Cleveland.

A continuous human lymphocyte line (Daudi) was obtained from the American Type Culture Collection, Rockville, Md. The Daudi cell line is a B-lymphocyte line derived from a Burkitt lymphoma in 1967 (11, 14).

Cell-free bacterial supernatants. Culture supernatants were prepared from *Staphylococcus aureus* Cowan I, *Escherichia coli* ATCC 25922, *Pseudomonas cepacia* 715j (clinical isolate from a cystic fibrosis patient), and several *P. aeruginosa* strains: 2830 (a classic morphology strain), a clinical isolate from a cystic fibrosis patient; PA-103 (very high exotoxin A, low protease production) (17); and WR-5 (high protease, low or negative exotoxin A) (3). In addition, culture supernatants were prepared from five other *P. aeruginosa* strains isolated from cystic fibrosis patients.

In three separate experiments, bacterial culture supernatants were prepared simultaneously from cultures of equivalent cell density of *S. aureus*, *E. coli*, *P. cepacia*, and *P. aeruginosa* strains 2830, PA-103, and WR5. All bacterial strains were initially incubated in rich undefined growth medium (tryptic soy broth [TSB]; Difco Laboratories, Detroit, Mich.) for 18 to 24 h at 37°C with vigorous shaking (180 to 220 rpm) to achieve maximum aeration. Bacteria were then washed in saline and suspended in RPMI 1640 culture medium. Supernatants were collected after an additional overnight incubation at 37°C followed by centrifugation at approximately 17,000 × *g* for 30 min. The supernatants were filtered through 0.22-μm membrane filters, portioned, and stored at −70°C until use. Collection of bacterial culture supernatants in RPMI 1640 culture medium (without phenol red) was used after initial experiments indicated that sterile TSB sometimes became mitogenic for human lymphocyte preparations.

The transfer of rapidly growing bacteria from TSB medium into RPMI 1640 culture medium and the extended total incubation time might conceivably have caused liberation of factors not present in supernatants from organisms grown in either medium alone. As a control for this possibility, *P. aeruginosa* strains were inoculated directly into 50 ml of RPMI 1640 medium and incubated as described above for 18 to 24 h. Collection and processing of supernatants were otherwise performed as in experiments utilizing sequential TSB/RPMI 1640 growth conditions.

For some experiments, *P. aeruginosa* supernatants were collected from antibiotic-killed bacterial preparations. A clinical *P. aeruginosa* isolate with classic morphology (strain P3, international serotype 6) was prepared for lymphocyte stimulation studies as previously described (27). Ten percent (vol/vol) stock preparations of killed bacteria were centrifuged, and the supernatant was collected and frozen at −70°C until use.

Supernatant ultrafiltration. Bacterial culture supernatants of *P. aeruginosa* were fractionated by ultrafiltration (24). By sequential use of membranes with decreasing pore diameters (XM50, PM10, and UM5; Amicon Corp., Lexington, Mass.), a filtrate containing molecules of less than 2,000 daltons was obtained. Each filtration step was taken to dryness. Over 80% of the starting supernatant fraction was recovered in the filtrate.

Extraction of phenazine pigments. Extraction of nonpolar compounds from crude *P. aeruginosa* culture supernatants was performed by adsorption to an octadecylsilane bonded-phase chromatographic material (Sep-Pak C_{18} cartridge; Waters Associates, Inc., Milford, Mass.). After thorough washing of Sep-Pak cartridges with Sorensen buffer, pH 7.6, 20 ml of bacterial culture supernatants diluted 1:1 in buffer were passed through the cartridges. After additional washing with buffer, sequential elutions with 5 ml of chloroform and 5 ml of methanol were performed. After evaporation of these solvents under nitrogen, the eluted components were solubilized in sterile distilled water and tested for inhibition of lymphocyte proliferation.

Purified *P. aeruginosa* components. *P. aeruginosa* lipopolysaccharide (LPS) extracted by the trichloroacetic acid technique from the seven Fisher immunotypes was provided by Parke, Davis & Co., Detroit, Mich. LPS activity in this preparation was verified by the *Limulus* assay. Exotoxin A was prepared as described by Liu (17) in TSB dialysate medium from strain PA-103. Exotoxin A yields were increased by the addition of low concentrations (0.13 μg/ml) of mitomycin C to the bacterial cultures (K. Wood-Klinger and C. W. Shuster, Fed. Proc. **38**:823, 1979). Under similar conditions, strain WR5 did not produce detectable exotoxin A. Final exotoxin A preparations were purified by the use of an anti-exotoxin A antibody affinity column (30). Purified exotoxin A was shown to be toxic in vitro for mouse L-929 cells. Activated toxin was examined for ADP-ribosylation activity in rabbit reticulocyte lysate by a standard method with a lower limit of detection of approximately 3 ng (13). Specific antigenicity was tested by using reference anti-exotoxin A antisera for immunodiffusion reactions in agarose which could detect levels of exotoxin A ≥1.0 μg/ml. Bacterial supernatants were also screened for exotoxin A by the above methods. Purified *P. aeruginosa* proteases were obtained from Nagase Biochemical Ltd., Tokyo, Japan. The protease activities of *P. aeruginosa* culture supernatants and purified protease preparations were assayed by using dye-release methods (hide powder azure and orcein-elastin) (25). Protease activity was expressed as absorbance at 590 or 595 nm (A_{590} or A_{595}), indicating relative dye release by using equal amounts of supernatant protein from test supernatants. Pyocyanine was prepared by chloroform extraction of *P. aeruginosa* culture supernatants (6) or by photochemical reaction of phenazine methosulfate followed by sequential chloroform and aqueous extractions and crystallization from water (15). The dark-blue crystals gave a melting point of 133°C as expected (21, 29), and acid and alkaline solutions gave the same UV/visible spectra as that reported for pyocyanine (21, 33).

Heat inactivation of *P. aeruginosa* culture supernatants was performed under reflux conditions at 100°C for 60 min.

In vitro lymphocyte proliferation assay. PBMC preparations were suspended in RPMI 1640 medium supplemented with 20% heat-inactivated normal plasma pool obtained from 20 donors and with HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (Microbiological Associates, Bethesda, Md.),

penicillin, and streptomycin. Lymphocyte cell lines were suspended in RPMI 1640 medium containing 20% heat-inactivated fetal calf serum. All cell preparations were adjusted to a concentration of 10^6 cells per ml. Cell preparations (0.1 ml, 10^5 cells) were dispensed into Falcon 96-well flat-bottomed microassay plates (Falcon Plastics, Oxnard, Calif.).

The ability of *P. aeruginosa* culture supernatants or purified products to activate lymphocyte proliferations was tested by adding 10 μl of each of several dilutions of these preparations to PBMC cultures. An equal volume of saline was added to control cultures. Cultures were incubated at 37°C in a CO_2 incubator for 5 or 6 days. Five hours before the end of the incubation period, 0.5 μCi of [^{3}H]TdR (specific activity, 24 Ci/mmol) was added to each culture. The cells were harvested with a lymphocyte microharvester (Otto Hiller Co., Madison, Wis.). Incorporation of [^{3}H]TdR was measured with a Searle Isocap 300 liquid scintillation counter. Proliferative responses were expressed as net counts per minute.

The inhibitory activity of *P. aeruginosa* supernatants or purified products was assessed routinely by adding optimal doses of concanavalin A (ConA; usually 25 to 50 μg/ml final concentration) to PBMC cultures 1 h after the addition of bacterial products. Cell cultures were pulsed with [^{3}H]TdR and harvested after 3 days of incubation as above. Occasionally, other plant lectins (pokeweed mitogen or phytohemagglutinin) were used instead of ConA to test the specificity of the inhibition of lymphocyte proliferation by *P. aeruginosa* supernatants. The timing of the addition of *P. aeruginosa* supernatants and of ConA was varied in specific experiments to study the mechanisms of lymphocyte inhibition. The inhibitory activity of *P. aeruginosa* products on the [^{3}H]TdR uptake by Daudi cells was tested by simultaneous addition of *P. aeruginosa* supernatants or of purified *P. aeruginosa* exoproducts and [^{3}H]TdR. Cells were harvested after 60 min of incubation. Inhibition of [^{3}H]TdR uptake was expressed as cpm in inhibited cell culture/cpm in control cell culture × 100.

The viability of cell preparations exposed to *P. aeruginosa* supernatants was assessed by cell counts and by direct fluorescence with ethidium bromide-acridine orange at final concentrations of 1 μg/ml.

RESULTS

Supernatants obtained from antibiotic-killed *P. aeruginosa* preparations inhibited ConA-induced PBMC [^{3}H]TdR uptake by 98%. The concentration of inhibitory factors in all these supernatants was low, i.e., inhibitory activity could no longer be detected in dilutions above 1:2.

The biochemical properties of the first supernatants prepared simultaneously from six bacterial strains are summarized in Table 1. The effect of these supernatants on the responses of six human PBMC preparations to ConA are shown in Fig. 1A and B. All *P. aeruginosa* strains produced strongly inhibitory supernatants, whereas the other bacterial supernatants tested produced no inhibition or only mild inhibition. [^{3}H]TdR incorporation in cultures with the highest degree of inhibition (99%) was similar to the background incorporation by control lymphocyte cultures not exposed to ConA or to *P. aeruginosa* supernatants. At supernatant dilutions of 1:4 and 1:8, the *P. aeruginosa* WR5 supernatant was found to be less inhibitory than the *P. aeruginosa* 2830 and PA-103 supernatants. Two subsequent batches of bacterial culture supernatants confirmed the difference between *P. aeruginosa* and other bacteria, but in both cases the WR5 supernatants were as inhibitory as the other *P. aeruginosa* supernatants (also see Tables 4 and 6, where supernatants from the third batch were used). Supernatants from five additional clinical isolates of *P. aeru-*

TABLE 1. Biochemical analyses of bacterial supernatants[a]

Supernatant from:	Wet wt of cells (g)[b]	pH	Osmolarity (mosM)	Protein (μg/ml)[c]	Lipid[d]	Protease[e] (A_{595})	Hexose (μg/ml)[f]	Elastase[e] (A_{590})	LPS (ng/ml)[g]
P. aeruginosa strains									
2830	0.66	8.8	358	3.6	0	0.645	92	0.85	0.1
PA-103	0.70	8.7	215	7.6	0	0.035	42	ND	0.1
WR5	0.58	8.9	248	4.5	0	0.76	67.5	ND	5
P. cepacia 715j	0.64	9.0	257	8.0	0	0.03	141.0	ND	100.000
S. aureus Cowan I	0.56	8.8	273	7.3	0	0	0.21	ND	ND
E. coli ATCC 25922	0.6	9.0	298	5.3	0	0.01	61	ND	10

[a] Each strain was inoculated in 50 ml of TSB in 250-ml flasks and incubated for 24 h at 37°C with vibrating aeration. After thorough washing, supernatants were collected after incubating the bacteria for 18 h in RPMI 1640 at 37°C.

[b] Weight of bacterial cell pellet after supernatant collection.

[c] Protein determined by the $A_{260/280}$ and the Bradford Coomassie blue assay (5).

[d] Lipid phosphorus determination by ascorbic acid-phosphomolybdate assay (7).

[e] Protease was assayed by dye-release methods (hide powder azure [A_{595}] and orcein-elastin [A_{590}]). Values are absorbance units (A_{595}), indicating relative release with equal amounts of supernatant protein.

[f] Hexose was determined by the anthrone method, using a glucose standard (18).

[g] LPS was determined by *Limulus* assay on supernatant dilutions.

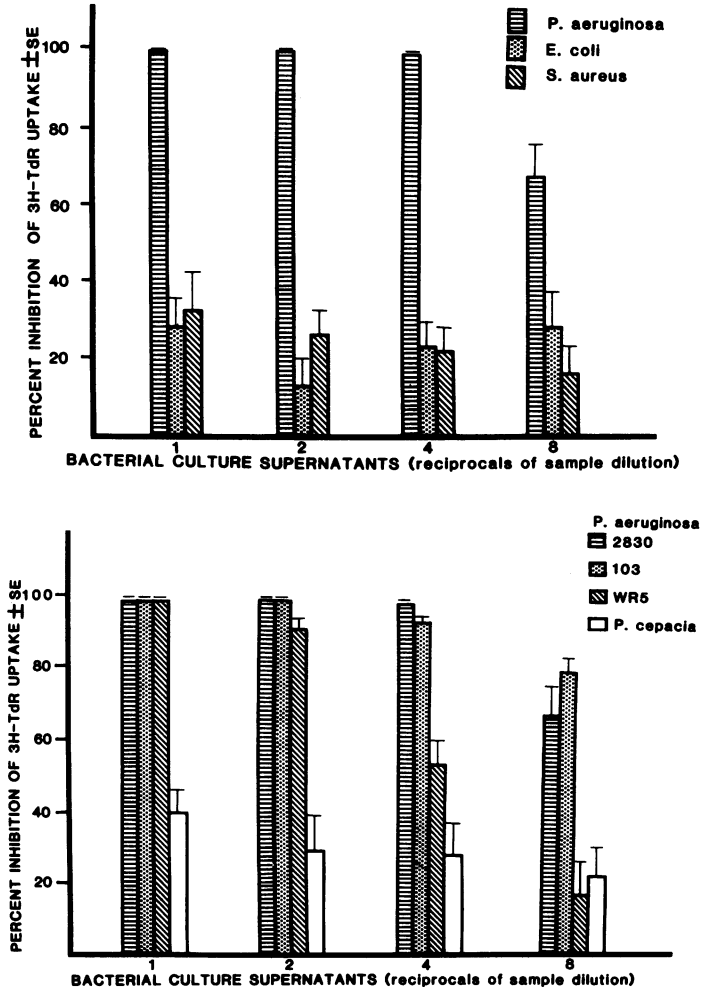

FIG. 1. Inhibition of ConA-induced lymphocyte proliferation by bacterial culture supernatants. Triplicate samples (0.1 ml) of a 10^6 lymphocyte per ml cell suspension were exposed to 0.01 ml of bacterial culture supernatants for 1 h before the addition of ConA (50 μg/ml final concentration). Cultures were pulsed with [^{3}H]TdR after 3 days, 5 h before harvest. Responses to ConA ranged from 71,000 to 147,000 cpm. Counts in control, nonstimulated cultures ranged from 500 to 1,300. Counts in cultures inhibited by the addition of undiluted *P. aeruginosa* supernatants ranged from 500 to 1,700. At sample dilutions of 1:1, 1:2, and 1:4, *P* values for *P. aeruginosa* versus *E. coli*, *S. aureus*, and *P. cepacia* were 0.0005. At a 1:8 dilution, *P* was 0.0075 for *E. coli* and 0.005 for *S. aureus* and *P. cepacia*. *P. aeruginosa* 2830 versus WR5 at a 1:4 dilution, $P = 0.01$; at a 1:8 dilution, $P = 0.005$. All other differences were not significant.

ginosa and two supernatants from *P. aeruginosa* strains grown in RPMI 1640 also were strongly inhibitory for lymphocyte proliferation. The inhibitory effect was not specific for ConA-stimulated PBMC cultures. In two experiments, pokeweed mitogen or phytohemagglutinin were used instead of ConA to activate lymphocytes. *P. aeruginosa* 2830 culture supernatants inhibited proliferative responses in a manner comparable to the inhibition of ConA responses (data not shown).

Culture supernatants from *P. aeruginosa* strains 2830, WR5, and PA-103 were shown to be negative for ADP-ribosylation activity and negative for material reactive with anti-exotoxin A antiserum. These results were expected since growth conditions were not suitable for exotoxin A production (17). These culture supernatants and the *E. coli* supernatant were heat inactivated for 60 min at 100°C. Consistent with the known heat lability of *P. aeruginosa* proteases and exotoxin A (12) and the lack of demonstrable exotoxin A in the supernatants, no protease or exotoxin A activity could be detected in these

TABLE 2. Effect of heat inactivation on the inhibitory properties of bacterial culture supernatants for ConA-induced lymphocyte proliferation[a]

Bacterial supernatant	[^{3}H]TdR incorporation (mean cpm I SD) at reciprocals of sample dilution of:[b]			
	1	2	5	10
P. aeruginosa 2830				
Native	1,276 ± 228	2,252 ± 787	62,082 ± 10,710	51,261 ± 1,1291
Heat inactivated	10,334 ± 2168	21,014 ± 2,935	62,814 ± 8,941	56,249 ± 12,119
P. aeruginosa WR5				
Native	390 ± 113	1,166 ± 826	3,148 ± 1,079	41,368 ± 6,676
Heat inactivated	819 ± 374	991 ± 488	9,365 ± 991	56,175 ± 4,568
P. aeruginosa PA-103				
Native	663 ± 120	2,291 ± 593	19,474 ± 4,960	56,685 ± 17,685
Heat inactivated	2,572 ± 298	5,646 ± 255	48,670 ± 2,846	63,464 ± 3,534
E. coli ATCC 25922				
Native	66,096 ± 14,488	62,353 ± 6,876	79,636 ± 10,272	82,419 ± 5,561
Heat inactivated	58,012 ± 535	59,906 ± 4,217	86,521 ± 3,137	90,602 ± 8,439

[a] Heat inactivation of culture supernatants was performed under reflux conditions at 100°C for 60 min. ConA (50 μg/ml final concentration) was added to cultures 1 h after the addition of 0.01 ml of bacterial culture supernatants.

[b] [^{3}H]Tdr uptake was measured after 3 days of incubation. ConA-stimulated cultures in absence of bacterial supernatants, 86,392 ± 9,972 cpm; unstimulated cultures, 1,031 ± 320 cpm.

boiled supernatants. Table 2 shows that although up to 10-fold increases in counts per minute occurred with strains PA-103 and 2830 after heat inactivation, more than 75% of the inhibitory activity was still present in all *P. aeruginosa* supernatants after boiling for 60 min.

The inhibitory effect of the ultrafiltrate from *P. aeruginosa* 2830 culture supernatants is shown in Table 3. Molecules less than about 2,000 daltons were highly inhibitory for lymphocyte proliferation.

The effects of purified *P. aeruginosa* LPS, protease, elastase, and pyocyanine are summarized in Table 4. Only *P. aeruginosa* pyocyanine was strongly inhibitory for [^{3}H]TdR uptake by ConA-stimulated lymphocytes. A similar inhibition was produced by pyocyanine prepared from phenazine methosulfate (data not shown). Lower concentrations of pyocyanine produced an augmentation of ConA-stimulated proliferation.

The experiments with supernatants from strain WR5 (low or negative for exotoxin A) and with heat-inactivated supernatant established that lymphocyte inhibition can occur in absence of exotoxin A. Additional evidence for an independent inhibitory activity due to phenazine pigments was obtained in experiments with the Daudi lymphocyte line. The use of a cell line was preferred in short-term experiments, since prolonged exposure of lymphocytes to exotoxin A, as in ConA-stimulated cultures incubated for 3 days, results in inhibition of protein synthesis, cell death, and concomitant arrest of [^{3}H]TdR incorporation (12, 20). No inhibition of [^{3}H]TdR uptake was observed after 1 h of incubation with several concentrations of *P. aeruginosa* exotox-

TABLE 3. Ultrafiltration of *P. aeruginosa* 2830 culture supernatant inhibitory activity for ConA-induced lymphocyte proliferation in the supernatant filtrate containing molecules less than 2,000 daltons[a]

Fraction	[^{3}H]TdR incorporation (mean cpm ± SD) at reciprocals of sample dilution of:[b]			
	1	2	5	10
Native supernatant	496 ± 87	527 ± 281	56,988 ± 11,734	85,593 ± 4,393
Filtrate[c]	262 ± 97	4,268 ± 1,807	83,802 ± 5,908	100,224 ± 7,461

[a] ConA (50 μg/ml final concentration) was added 1 h after the addition of 0.01 ml of bacterial culture supernatant or its filtrate.

[b] [^{3}H]TdR uptake measured after 3 days of incubation. ConA-stimulated culture in absence of bacterial supernatants, 84,813 ± 9,358 cpm; unstimulated cultures, 496 ± 87 cpm.

[c] Filtrate was prepared by sequential use of membranes of decreasing molecular sieve size. The last filter used was UM2 (Amicon). Each filtration step was taken to dryness. Starting supernatant volume, 75 ml; UM2 filtrate, 62 ml.

TABLE 4. Effect of purified *P. aeruginosa* exoproducts on lymphocyte proliferative responses to ConA[a]

P. aeruginosa exoproduct concn[b]	Response[c]
LPS (μg/ml)	
0	107,521 ± 10,054
0.1	85,183 ± 7,790
1	105,077 ± 3,877
10	115,056 ± 1,318
100	117,922 ± 8,400
350	102,341 ± 5,732
Protease (μg/ml)	
0	68,602 ± 9,844
0.1	65,047 ± 9,451
1	60,998 ± 123
10	70,774 ± 10,391
40	63,203 ± 6,335
Elastase (μg/ml)	
0	68,602 ± 9,844
0.1	83,080 ± 4,453
1	72,154 ± 8,005
10	71,022 ± 9,047
40	57,374 ± 5,543
Pyocyanine (μM)	
0	49,206 ± 9,103
0.1	75,118 ± 10,364
0.5	43,332 ± 11,827
1	8,912 ± 1,184
5	172 ± 57
10	178 ± 71

[a] ConA (final concentrations, 50 μg/ml) was added 1 h after *P. aeruginosa* products.
[b] Final concentration in culture medium.
[c] Responses expressed as mean ± standard deviation of the counts per minute of [^{3}H]TdR uptake after 3 days of incubation.

in A. In the same experiments, a crude *P. aeruginosa* 2830 supernatant and purified pyocyanine were strongly inhibitory (Table 5).

The extraction of phenazine pigments from *P. aeruginosa* culture supernatants by the use of octadecylsilane bonded-phase chromatographic materials eliminated the inhibitory activity in these supernatants. These results are shown in Table 6. Subsequent elution of pyocyanine with chloroform and then elution of other phenazine pigments with methanol recovered strong inhibitory activity in both eluates. Prior washing of the chromatographic material with distilled water did not recover detectable amounts of inhibitory components.

Several experiments were performed to determine whether prior exposure of lymphocytes to *P. aeruginosa* culture supernatants was required to prevent proliferation and [^{3}H]TdR incorporation in response to ConA stimulation. The addition of culture supernatants either 1 h before or 1 h after ConA addition resulted in inhibition of [^{3}H]TdR uptake by over 95%. The addition of undiluted *P. aeruginosa* 2830 supernatants 3 days after ConA activation, at the time of addition of [^{3}H]TdR, produced a small inhibition of [^{3}H]TdR uptake. In these experiments, ConA-stimulated cells with *P. aeruginosa* supernatants added at day 3 incorporated 55,861 ± 2,886 cpm; with supernatants added at day 1, 2,252 ± 787 cpm; and for cells stimulated with ConA in the absence of *P. aeruginosa* supernatants, 86,392 ± 9,972 cpm. In other experiments, lymphocyte cultures started in the presence of both ConA and inhibitory *P. aeruginosa* supernatants were washed thoroughly to eliminate *P. aeruginosa* supernatants before [^{3}H]TdR addition after 3 days of incubation. No increased [^{3}H]TdR uptake was observed in this situation, indicating that lymphocytes were not proliferating. In these experiments, cells kept in the presence of *P. aeruginosa* supernatants incorporated 429 ± 251 cpm; cells washed before [^{3}H]TdR addition incorporated 495 ± 388 cpm. Subsequent incubation of these cells for 24 h without additional stimulation increased [^{3}H]TdR uptake to 5,718 ± 645 cpm; after 72 h uptake was 29,169 ± 10,173 cpm.

The lack of lymphocyte proliferation suggested by decreased [^{3}H]TdR uptake was confirmed by morphological studies showing the absence of lymphoblasts in PBMC cultures incubated with *P. aeruginosa* 2830 culture supernatants.

At lower concentrations, *P. aeruginosa* culture supernatants no longer caused inhibition of lymphocyte proliferation. *P. aeruginosa* antigens present in these preparations are able to trigger lymphocyte responses in most normal

TABLE 5. Effect of *P. aeruginosa* 2830 culture supernatants and purified exotoxin A and pyocyanine on [^{3}H]Tdr uptake by Daudi cells[a]

Supernatant	Uptake (cpm ± SD)
Background uptake	56,234 ± 15,202
P. aeruginosa crude supernatant, undiluted	1,924 ± 100
Pyocyanine (10 μM)	903 ± 206
Exotoxin A (ng/ml)	
4	78,013 ± 18,722
8	72,440 ± 10,523
12	60,513 ± 5,365
16	62,008 ± 4,651
20	69,552 ± 5,345

[a] Daudi cells were suspended in fresh medium to a 10^6 lymphocyte per ml cell concentration and placed in 0.1-ml portions in culture cells. Cultures were exposed at time 0 to 0.01 ml of bacterial culture supernatant or purified products and pulsed immediately with [^{3}H]TdR. All cells were harvested at 60 min. Cell viability was 95% in cell cultures.

TABLE 6. Effect of Sep-Pak extraction on the inhibitory properties of bacterial culture supernatants for ConA-induced lymphocyte proliferation[a]

Bacterial supernatant	[^{3}H]TdR incorporation (mean cpm ± SD) at reciprocals of sample dilutions of:[b]		
	2	4	10
P. aeruginosa 2830			
Native	2,468 ± 1,254	13,747 ± 1,042	64,365 ± 5,307
Sep-Pak filtrate	57,865 ± 3,880	58,750 ± 10,095	70,594 ± 6,198
P. aeruginosa WR5			
Native	845 ± 505	812 ± 204	47,017 ± 919
Sep-Pak filtrate	69,879 ± 7,833	70,896 ± 13,407	64,906 ± 11,949
P. aeruginosa PA-103			
Native	2,291 ± 593	19,474 ± 4,960	56,685 ± 17,693
Sep-Pak filtrate	59,500 ± 2,535	73,050 ± 7,649	79,609 ± 6,879

[a] ConA (50 μg/ml final concentration) was added 1 h after 0.01 ml of bacterial culture supernatants or filtrates.
[b] Bacterial culture supernatants were diluted in equal volumes of buffer (pH 7.6) before Sep-Pak extraction. [^{3}H]TdR uptake was measured after 3 days of incubation. ConA-stimulated cultures in absence of bacterial supernatants, 64,866 ± 7,316 cpm; unstimulated cultures, 649 ± 535 cpm.

individuals. Proliferation of PBMC can be detected after 5 days of incubation with dilutions of *P. aeruginosa* 2830 supernatant above 1:100 (data not shown).

DISCUSSION

The results presented here indicate that all strains of *P. aeruginosa* tested produce heat-resistant inhibitor(s) of lymphocyte proliferation and [^{3}H]TdR uptake. The culture supernatants obtained from other bacterial strains under the same conditions did not show similar inhibitory activities. The differences between the inhibitory activities of *P. aeruginosa* culture supernatants and supernatants from other bacteria are not likely to be due to differences in bacterial growth, since nearly identical cell yields (CFU per milliliter) were obtained from all strains. There was no significant difference in the wet weight of cells of the various species after the collection of RPMI 1640 culture supernatants. Supernatants from *P. aeruginosa* strains grown in RPMI 1640 medium alone (i.e., no transfer from TSB to RPMI 1640 medium) were equally inhibitory (data not shown). Therefore, increased inhibitory activity was unlikely to be due to higher rates of cell death or lysis with release of intracellular factors due to change of growing bacteria from TSB medium into RPMI 1640 culture medium.

Several *P. aeruginosa* products have to be considered as possible lymphocyte inhibitors. LPS, exotoxin A, and proteases are common factors for most *P. aeruginosa* strains investigated. Yet, our results indicate that these substances are not likely to be reponsible for the observed inhibitory activity in *P. aeruginosa* supernatants. Purified *P. aeruginosa* LPS tested over a large concentration range failed to inhibit [^{3}H]TdR uptake by lymphocytes. In addition, another gram-negative bacterium, *E. coli*, did not show similar inhibitory activity. Arguments against *P. aeruginosa* exotoxin A or proteases being a major cause for lymphocyte inhibition come from three types of experiments. First, *P. aeruginosa* strains selected for very low production of either exotoxin A or proteases showed a similar degree of inhibitory activity. Second, heat inactivation of *P. aeruginosa* supernatants, which completely destroys the activity of these heat-labile protein exoproducts (12), produced only a moderate reduction in the inhibitory activity for lymphocyte proliferation in some supernatants. Third, purified protease or elastase did not inhibit ConA-induced lymphocyte proliferation. Purified exotoxin A failed to inhibit [^{3}H]TdR uptake by Daudi cells after short-term (1 h) exposure. Over that same time span, crude *P. aeruginosa* supernatants completely prevented [^{3}H]TdR uptake by these cells. It is likely that exotoxin A may contribute to a decrease in [^{3}H]TdR uptake by causing lymphocyte death in ConA-stimulated cultures (12, 20), which are incubated for 3 days. The extent of this contribution has not been determined. Cell viability studies failed to reveal a significant decrease in viable cells in cultures inhibited by *P. aeruginosa* supernatants. Moreover, further incubation of cells after removal of inhibitory *P. aeruginosa* supernatants by washing revealed a capacity for significant cell proliferation after three days; this would not be expected with exotoxin A cytotoxicity.

The only purified *P. aeruginosa* factor which consistently showed inhibition of [^{3}H]TdR uptake was the phenazine pigment, pyocyanine. The role of very small molecules in the inhibition of lymphocyte proliferation was further supported by ultrafiltration of inhibitory *P. aeruginosa*

supernatants. The extraction of phenazine pigments from culture supernatants by reverse-phase chromatography confirmed that lymphocyte inhibition is caused by these pigments. The recovery of inhibitory activity in chloroform and methanol eluates suggests that other pigments, in addition to pyocyanine, may be responsible for the observed inhibition. The relative contribution of different phenazine pigments to this inhibition is currently under active investigation in our laboratory.

The inhibition caused by cell-free supernatants is not due to interference with ConA binding to cells. Cell lines are already proliferating when the *P. aeruginosa* supernatants inhibit [^{3}H]TdR uptake. The responses of PBMC to ConA can be suppressed by *P. aeruginosa* supernatants added before or after ConA. The binding of fluorescein-conjugated ConA to lymphocytes is not prevented by the presence of *P. aeruginosa* supernatants (unpublished observations). In addition, the response to other plant lectins is also inhibited by *P. aeruginosa* supernatants.

Pyocyanine and 1-hydroxyphenazine have been shown to inhibit both cytoplasmic and mitochondrial respiration in mammalian cells. They may manifest their inhibitory effect via inhibition of electron transport. This is suggested by their known ability to undergo oxidation and reduction by members of the electron transport chain, particularly ubiquinone (1, 2, 28). Both pyocyanine and 1-hydroxyphenazine have been shown to act at the site of ubiquinone-cytochrome *b* in studies utilizing isolated mouse liver mitochondria (1). In intact guinea pig peritoneal macrophages, 1-hydroxyphenazine was shown to be significantly more effective than pyocyanine in inhibiting oxygen uptake (28). Similarly, in mitochondria isolated from livers of mice injected intravenously with both of these compounds, those derived from 1-hydroxyphenazine-treated animals displayed a more severe inhibition of respiration than mitochondria from pyocyanine-treated animals. (28). These differences might be due to differential ability to interact with or to penetrate the plasma membrane, or to differences in their action on the electron transport chain once taken up. Cytolysis can also occur depending on the amount of pigment absorbed by the cell and on the duration of exposure (4, 16, 28).

Although the effects of phenazine pigments on cell membranes have not been studied extensively in mammalian systems, they have been reported to have direct effects on glucose transport in bacterial membrane preparations (8). In that study (8), two phenazines were examined. Phenazine methosulfate-ascorbate inhibited active transport of glucose in *P. aeruginosa*. Significantly, active transport in *P. aeruginosa* was also inhibited by a second phenazine, 5-*N*-methyl-phenazonium-3-sulfonate, which is membrane impermeable. The effectiveness of the impermeable phenazine implies that the transport inhibition seen may be a direct effect on the membrane of the cells rather than an indirect effect on intermediary or energy metabolism. Although bacteria, unlike mammalian cells, take up glucose via the phosphotransferase system (32), these studies still suggest the possibility that the phenazine pigments may produce direct membrane perturbations resulting in inhibition of metabolite uptake in PBMCs. Whether the effect of purified pyocyanine on human lymphocytes is entirely due to its effect on cell respiration or whether this pigment has an additional inhibitory effect on cell membrane transport needs to be determined. Recent experiments in our laboratory indicating that *P. aeruginosa* supernatants produce a drastic inhibition of an early event of lymphocyte activation, aminosobutyric acid uptake (10), support the possibility of direct interference with membrane metabolite uptake.

The effect of crude *P. aeruginosa* supernatants on 3-day lymphocyte cultures is likely to be multifactorial. A contribution of heat-labile components to a decreased [^{3}H]TdR uptake is expected to occur after prolonged exposure. Consistent with this possibility, a decrease in inhibitory activity was observed in some *P. aeruginosa* supernatants after heat inactivation. This decrease in inhibitory activity never surpassed 25% of the total, indicating that in all supernatants, most of the inhibitory activity was caused by heat-resistant components. That phenazine pigments exert effects which can be clearly separated from the effects of heat-labile components like exotoxin A is further supported by our experiments with Daudi cells. In these experiments, the incubation time precludes an effect of exotoxin A (12, 20), but a significant inhibition of [^{3}H]TdR was observed.

The biological importance of lymphocyte inhibitors produced by *P. aeruginosa* needs to be established. Although lymphocyte proliferation is not a necessary requirement for lymphokine production, lymphocyte proliferation in response to antigens usually is indicative of other manifestations of cellular immunity like lymphokine production and delayed hypersensitivity. A protective role of cellular immunity against *P. aeruginosa* infection has been recently documented in mice (23). Therefore, inhibition of lymphocyte proliferation and of cell-mediated immunity may contribute to the establishment and progression of *P. aeruginosa* infection. The direct inhibition of phagocytic cells represents an alternative mechanism of activity for phen-

azine pigments. Such a mechanism has been postulated to explain the persistence of *P. aeruginosa* in burn wounds (28). We have identified heat-resistant lymphocyte inhibitors in the sputum of patients with chronic *P. aeruginosa* pulmonary infection. No inhibitors were found in sputa from similar patients without *P. aeruginosa* infection. The inhibitory activity of *P. aeruginosa* supernatants is not diminished or neutralized in the presence of plasma containing high titers of agglutinating anti-*Pseudomonas* antibodies, suggesting that phenazine pigments may not be antigenic and thus escape control by specific humoral immunity (R. U. Sorensen, *in* E. Shapira and G. B. Wilson (ed.), *Immunological Aspects of Cystic Fibrosis*, in press).

In systemic infections, it is unlikely that *P. aeruginosa* inhibitory factor(s) can reach the necessary concentration to exert an inhibitory activity. However, it (they) may modify lymphocyte subpopulations and produce a secondary suppression of cellular immunity as described in systemic inoculation of killed *P. aeruginosa* into experimental animals (9, 19, 22).

P. aeruginosa supernatants also contain antigens capable of inducing lymphocyte proliferation. When the *P. aeruginosa* culture supernatants are diluted, the inhibitory effect for lymphocytes disappears and lymphocyte proliferation is induced. This proliferation can be detected after 5 or 6 days in culture. Further studies of *P. aeruginosa* culture supernatant fractions will be necessary to clarify whether the same factors, at different concentrations, are responsible for lymphocyte inhibition and lymphocyte activation or whether once the inhibitory factors are beyond their effective range, different activating substances take over and induce lymphocyte proliferation.

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