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In Vitro Response of Human T Cells to *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is a gram-negative bacillus that is a major cause of morbidity and mortality in immunosuppressed patients, burn patients, and patients with cystic fibrosis. Although immunity to these bacteria has been associated with serum antibody, more recent evidence suggests that T-cell-mediated immunity may also be important. To evaluate human T-cell responsiveness to these bacteria, the optimal conditions were determined for in vitro proliferation of human peripheral blood lymphocytes and T-lymphocytes to Fisher-Devlin immunotype 1 *P. aeruginosa*. The proliferative response of normal adult peripheral blood lymphocytes to heat-killed *P. aeruginosa* was studied in 34 subjects (range, 7,600 to 111,500 net cpm). Analysis of cell subpopulations indicated that T-lymphocytes are the major proliferating cells and that this response is enhanced by the presence of adherent cells. Data from fetal cord lymphocyte responses suggest that the proliferation seen in normal adult lymphocytes is induced by antigenic and not mitogenic stimulation.

Pseudomonas aeruginosa is an extracellular gram-negative bacterium which is a major cause of morbidity and mortality in burn victims, immunocompromised patients, and children with cystic fibrosis (CF). Studies of the basis for immunity to this organism have focused primarily on antibody-mediated protection (1, 8). Although passively transferred antibody is protective against *P. aeruginosa* infection in certain model systems (11), high anti-*P. aeruginosa* antibody titers do not appear to protect burn patients and CF patients from localized skin or pulmonary infections (4, 5). Furthermore, increased mortality attributed to *P. aeruginosa* in burn patients has been correlated with the loss of T-cell-mediated immune functions such as skin test responsiveness to streptokinase-streptodornase, to mumps, and to tuberculin, and responsiveness in mixed lymphocyte culture (9). More recent observations in a murine model suggest a direct role for T-cell-mediated immunity to *P. aeruginosa* (13).

Sorensen et al. studied the in vitro proliferative response of peripheral blood lymphocytes (PBL) to gentamicin-killed *P. aeruginosa* (16–19). Although they did examine proliferation of normal adult lymphocytes in response to *P. aeruginosa* challenge, their main objective was correlating the clinical status of CF patients with the proliferative responsiveness of their PBL. Thus, the range of normal human PBL proliferative responses to *P. aeruginosa* has not been clearly defined, nor has the subpopulation of responding cells been determined. It is also unknown whether the in vitro proliferation occurred in response to an antigenic or to a mitogenic stimulus.

To expand the basis for investigating the role of cellular immunity to *P. aeruginosa* in human infections, we defined more extensively the proliferative response of normal human PBL to a single immunotype of *P. aeruginosa*. Our data demonstrate that the lymphocytes of normal adults have a wide range of proliferative responses to this bacterium, that these responses reflect T-cell stimulation, and that the responses are enhanced by the presence of adherent cells. Since fetal cord blood lymphocytes (FCB) gave a statistically significant lower response, it appears that the proliferation in adult lymphocytes can be attributed to antigenic and not mitogenic stimulation of these cells.

MATERIALS AND METHODS

Subjects. Thirty-four normal, adult Caucasian subjects ranging in age from 19 to 47 years were studied. There were 19 males and 15 females in the study. In addition, cord blood was obtained from seven full-term neonates immediately after delivery.

P. aeruginosa. Fisher-Devlin immunotype 1 *P. aeruginosa* (kindly provided by M. Fisher, Parke-Davis Co., Detroit, Mich.) was grown on Trypticase soy agar plates. The bacteria were harvested and diluted in phosphate-buffered saline to a concentration of 4×10^9 colony-forming units per ml and heated to 56°C for 45

min. No viable bacteria were recovered after this treatment. Bacteria were washed twice and suspended in tissue culture medium at the appropriate concentrations.

PHA. Phytohemagglutinin (PHA) was obtained from Difco Laboratories (Detroit, Mich.). The PHA vial was reconstituted in 5 ml of distilled water and then diluted to a final concentration of 1:200 with culture medium.

Culture medium. RPMI-1640 medium (GIBCO Laboratories, Grand Island, N.Y.) was supplemented with 2.0 mM L-glutamine, 25 mM HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 50 μg of gentamicin per ml, and 10% heat-inactivated, pooled human serum obtained from nontransfused male donors.

Cell separation. PBL from normal adults and FCB were purified over Ficoll-Hypaque by the method of Boyum (2). Cells were fractionated into T-cell-enriched and T-cell-depleted populations by rosetting with sheep erythrocytes and layering this mixture over a second Ficoll-Hypaque gradient. The non-T fraction recovered at the interface was washed twice in phosphate-buffered saline and suspended in culture medium at 10^6 cells per ml. Labeling of this non-T fraction with fluorescein isothiocyanate-conjugated goat anti-human immunoglobulin (Tago Inc., Burlingame, Calif.) revealed 51 to 56% immunoglobulin-positive cells. Rerosetting of this non-T fraction revealed 2 to 3% E rosette-positive cells.

The T-cell fraction was enriched further by passing it through a third Ficoll-Hypaque gradient. The T-cell fraction was >98% E rosette positive. The erythrocytes were lysed with fresh normal human serum (6). Fluorescein isothiocyanate labeling of this doubly enriched T-cell population revealed <1.5% immunoglobulin-positive cells.

Lymphocyte cultures. PBL or subpopulations of these cells were cultured in microtiter wells at 10^5 cells per 0.2 ml of culture medium. Adherent cells were added by one or both of two methods, as follows. (i) A sample of 10^5 PBL in 0.1 ml of culture medium was placed in each microtiter well, and the plates were incubated for 4 h at 37°C. Wells were aspirated and washed twice with culture medium to remove nonadherent cells. For proliferation studies, T cells were then added to the wells coated with adherent cells. (ii) A portion of the non-T-cell fraction was irradiated with 3,100 rads (Gamma Cell 1000, Atomic Energy of Canada, Ltd., Ottowa, Canada). Approximately 20,000 irradiated non-T cells were added to each microtiter well containing T cells. Both methods gave equivalent results. Wells were run in triplicate. Microtiter plates were incubated at 37°C in a 5% CO_2-air mixture. Heat-killed bacteria were added at the start of culture. Wells were pulsed with 1 μCi of tritiated thymidine (2 Ci/mmol; New England Nuclear Corp., Boston, Mass.) 18 h before harvesting. Wells were harvested with a Titer-Tech harvesting device (Flow Laboratories, McLean, Va.).

Statistics. The results from individuals were obtained by averaging the results of triplicate wells and subtracting the appropriate background. The data shown on graphs and tables are the mean of groups of individual samples (i.e., 9 normal adults, 7 neonates, 34 normal adults, 6 normal adults). The bracketed numbers are the standard errors of the mean. A two-tailed Student *t* test was used to determine the significance of the differences observed.

RESULTS

Dose effect and kinetics of in vitro response to *P. aeruginosa*. To determine the optimal culture conditions for studying the in vitro responsiveness of lymphocytes to *P. aeruginosa*, the dose effect and kinetics of the response were examined. The solid line in Fig. 1 depicts the response of normal adult PBL to varying concentrations of heat-killed *P. aeruginosa*. Experiments with PBL from nine normal adults were performed with triplicate wells of each concentration (10^3, 10^4, 10^5, 10^6, 10^7, or 10^8 *P. aeruginosa* per microtiter well). The dose that most often gave the maximum response was 10^7 organisms per well.

The solid line in Fig. 2 demonstrates the proliferative response of normal adult PBL after 4, 6, 7, 8, 9, and 10 days of incubation. Values depicted are the mean of the nine individual responses to the optimal dose of *P. aeruginosa* (10^7) for each day. The mean responses peaked on day 8 (37,035 cpm). There was, however,

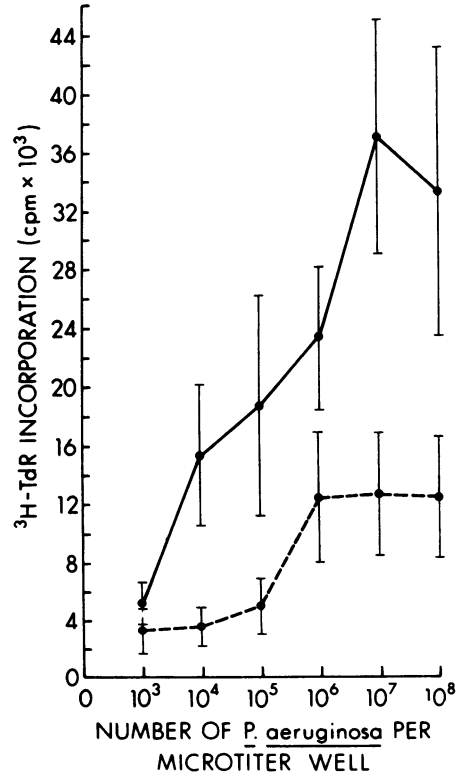

FIG. 1. In vitro peak responses of peripheral blood lymphocytes from nine normal adults (●——●) and seven FCB samples (●------●) to varying doses of *P. aeruginosa*. Values given represent the mean of the individual peak cpm minus background cpm.

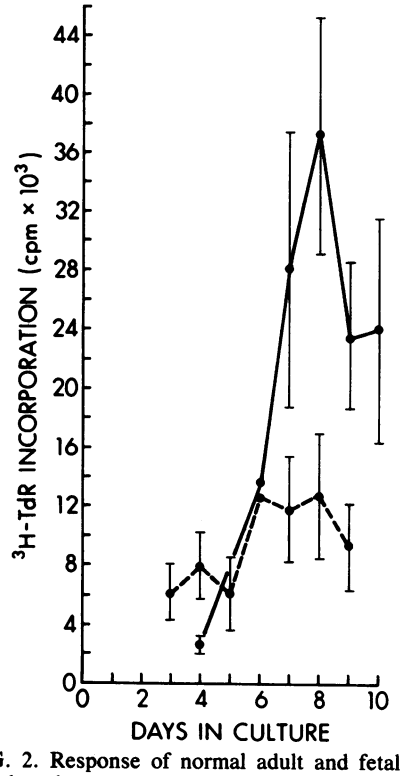

FIG. 2. Response of normal adult and fetal cord blood lymphocytes to the optimal concentration of heat-killed *P. aeruginosa* (10^7) as a function of days in culture. Values depicted represent the mean lymphocyte responses of nine normal adults (●——●) and seven FCB samples (●-------●) to 10^7 *P. aeruginosa* organisms per well. The differences are statistically significant on days 8 and 9 with two-tail *P* values of <0.001 and <0.02, respectively.

some individual variation, with two subjects having peak responses on day 7 and two other subjects having peak responses on day 9. Individual peak responses among these nine subjects ranged from 21,750 to 72,200 cpm.

Response of normal adult PBL to *P. aeruginosa*. PBL from a total of 34 normal individuals without known previous *P. aeruginosa* infection were tested for their ability to respond to these bacteria. Line A in Table 1 depicts the mean of the maximal response of the PBL samples of these 34 individuals (day 7, 8, or 9) to *P. aeruginosa*. Individual peak responses varied from 7,500 to 111,500 cpm.

Table 2 shows proliferation responses from specific individuals determined on different occasions. The results demonstrate the reproducibility of the responses in assays run at different times and indicate that T-cell proliferation reflects a stable form of immune responsiveness to *P. aeruginosa*.

Response of FCB lymphocytes to *P. aeruginosa*. In an effort to determine whether the response to *P. aeruginosa* was due to antigen-specific stimulation or nonspecific mitogen stimulation, the response of FCB was examined. Both dose-response and kinetic studies were performed on seven FCB samples. The dotted line in Fig. 1 depicts the response of seven FCB specimens to concentrations of *P. aeruginosa* from 10^3 to 10^8 per microtiter well. Concentrations of 10^6, 10^7, and 10^8 organisms per well gave essentially identical maximum responses. The dotted line in Fig. 2 demonstrates the kinetics of the seven FCB samples studied on days 3, 4, 5, 6, 7, 8, and 9. Values depicted are the mean of the seven FCB responses to 10^7 *P. aeruginosa* organisms for each day. The mean peak response of FCB (12,000 cpm) was observed after 6 days in culture and was maintained through day 8 in culture. Line B in Table 1 demonstrates the mean of the maximal responses of seven FCB samples (day 6, 7, or 8) to *P. aeruginosa*. Peak responses ranged from 4,000 to 27,600. All seven samples responded vigorously to PHA despite their poor response to *P. aeruginosa*. The difference between the maximal responses to *P. aeruginosa* of normal adult lymphocytes and FCB was statistically significant ($P < 0.01$). The responses of adult PBL and FCB to PHA were essentially identical.

Response of mononuclear cell subpopulations to *P. aeruginosa*. Experiments were performed comparing the response of purified T-cell populations (with and without adherent cells added back), non-T-cell populations, and peripheral blood mononuclear cells. Blood from six normal adults was separated into these three cell populations. Cell populations from each individual were plated the same day. Optimal culture conditions using 10^7 and 10^8 *P. aeruginosa* were used as in the kinetic studies. The enriched T-

TABLE 1. Response of PBL to *P. aeruginosa*

Subjects (no.)	Unstimulated response (cpm)	Stimulated response (cpm)	PHA (day 4)
A. Normal adults (34)	2,420 (±430)	41,985[a] (±4,299)	82,029 (±5,767)
B. FCB samples (7)	5,845 (±2,209)	15,086 (±3,012)[b]	87,005 (±9,835)

[a] The mean (± standard error) of individual's average net cpm.
[b] $P < 0.01$, comparing the stimulated responses of normal adult and FCB samples.

TABLE 2. Reproducibility of proliferative responses to *P. aeruginosa* in individual subjects

Subject	Exp. no.	Proliferative response[a] (cpm)
1	1	59,476
	2	42,818
	3	47,916
2	1	32,178
	2	22,288
	3	30,568
3	1	49,676
	2	63,368
4	1	7,137
	2	7,869
5	1	111,500
	2	95,952

[a] Results for each experiment represent the mean of three microwells. Most of the experiments for individual subjects were performed at intervals of approximately 2 weeks.

cell population always showed a greater response than did the non-T-cell population. Adding adherent cells or radioresistant non-T cells to the T-cell populations produced responses that mimicked those shown by the unseparated PBL (Table 3). No proliferative response occurred when irradiated T cells (3,100 rads) were added to the non-T-cell fraction (data not shown).

DISCUSSION

The present study utilizes in vitro lymphocyte proliferative responses to study cell-mediated immunity to *P. aeruginosa*. Our results indicate that: (i) normal adults' lymphocytes proliferate in the presence of *P. aeruginosa*; (ii) this proliferation most likely represents antigen-specific stimulation; (iii) the response is due to proliferating T cells; and (iv) this T-cell response is enhanced by the presence of adherent cells.

The observation that normal adults' lymphocytes respond in vitro to *P. aeruginosa*, plus the fact that normal adults have detectable low anti-*P. aeruginosa* antibody titers (12), indicates that most normal adults have had previous exposure either to *P. aeruginosa* or to a related antigen. Although *P. aeruginosa* is not ordinarily considered part of the normal bowel flora, it is a common soil and water organism and can be cultured from the gastrointestinal tract of 5 to 15% of burn patients on admission (15). It seems likely that humans are at least transient carriers of this organism.

To answer the question of whether this proliferation represented antigen-specific stimulation or whether *P. aeruginosa* was simply acting as a nonspecific mitogen, we assayed the responses of FCB lymphocytes, which are presumably immunologically naive. The responses of adult and FCB lymphocytes were clearly different, not only in magnitude ($P < 0.01$) but also in kinetics. FCB generated a peak response on day 6, whereas the response of adult lymphocytes peaked on day 8. Because the low response seen in FCB peaked early, this stimulation probably reflects a nonspecific mitogenic effect. There are numerous polysaccharides and glycoproteins in the *P. aeruginosa* cell wall which could act as mitogens. Papamichail et al. demonstrated low-level mitogenic responses of human lymphocytes to *P. aeruginosa* extracellular slime glycoprotein (10). Chen et al. demonstrated three outer membrane proteins which were mitogenic for murine PBL and T-cell-depleted populations (3). Regardless of what substance(s) is acting as a mitogen(s) for FCB, adult PBL generate a response that is clearly different. These data indicate an antigenic stimulation of adult PBL beyond weak mitogenic stimulation and suggest the previous exposure of adults to *P. aeruginosa* or a related antigen(s).

Of particular interest is the observation that the proliferative responses observed in adult PBL were essentially identical to the proliferative responses of T-cells plus adherent cells. This observation is consistent with other studies which suggest that T-cell-mediated immunity may be elicited in response to *P. aeruginosa* antigens. Gibbons et al. have demonstrated leukocyte migration inhibition by *P. aeruginosa* in

TABLE 3. Response of mononuclear cell subpopulations from normal adults to *P. aeruginosa*

Cell population	No. of expts	Nonstimulated[a] (cpm)	Stimulated[a] (cpm)
Unfractionated PBL	6	3,337 (±1,381)	30,950 (±8,088)
Non-T cells[b]	5	2,091 (±519)	1,151 (±566)
T cells	6	494 (±200)	10,567 (±2,269)
T cells + macrophages[c]	7	4,312 (±1,611)	34,129 (±8,767)

[a] The mean (± standard error) of the average net cpm of the individual samples.

[b] Containing 51 to 56% surface immunoglobulin-positive cells.

[c] Adherent cells were used as a macrophage source in this experiment. Equivalent results were obtained when irradiated non-T cells were used as a macrophage source in other experiments.

CF patients (7). Reynolds demonstrated the same phenomenon in rabbits (14). Furthermore, a *P. aeruginosa* polysaccharide vaccine conferred protection to live bacterial challenge in mice that did not produce antibody after vaccination (13). This protection was adoptively transferred to unimmunized mice with T cells from the immune mice (13).

Although the present study does not define the particular immune function of the proliferating T cells, T-cell proliferative responses are a prerequisite for maximal expression of cell-mediated immune responses. Interestingly, preliminary data we have obtained with PBL from six patients with documented *P. aeruginosa* infections but without CF, burns, or active malignancy show no in vitro proliferation to *P. aeruginosa*. Further studies are needed to verify these results and to explore the functional significance of in vitro T-cell proliferation in host defenses against this ubiquitous bacterial pathogen.

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