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Cellular Immunity to Bacteria: Impairment of In Vitro Lymphocyte Responses to *Pseudomonas aeruginosa* in Cystic Fibrosis Patients

RICARDO U. SORENSEN,* ROBERT C. STERN, AND STEPHEN H. POLMAR

Department of Pediatrics, Case Western Reserve University School of Medicine, and Rainbow Babies and Children's Hospital, Cleveland, Ohio 44106

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Lymphocyte responses to the mitogens phytohemagglutinin and concanavalin A and to *Streptococcus pyogenes*, *Staphylococcus aureus*, *Haemophilus influenzae*, and *Pseudomonas aeruginosa* were evaluated in patients with cystic fibrosis and in normal individuals. Lymphocyte proliferation in vitro was stimulated by gentamicin-killed whole bacteria, and the proliferative response was measured by [^{3}H]thymidine incorporation. The in vitro lymphocyte responses to antibiotic-killed bacteria reached maximum thymidine incorporation after 5 days in culture and followed a unimodal dose-response curve for each of the bacteria studied. A significant specific incapacity to respond to *P. aeruginosa* was detected in cystic fibrosis patients with advanced clinical disease.

Progressive pulmonary infection is the major cause of morbidity and mortality in patients with cystic fibrosis (CF). Although respiratory infection with *Staphylococcus aureus* and *Haemophilus influenzae* occurs in CF patients, chronic *Pseudomonas aeruginosa* bronchiolitis and bronchitis ultimately occurs in virtually all patients. *P. aeruginosa* usually becomes the dominant, and is often the only, pathogen recovered from cultures of sputum or of lung tissue obtained at autopsy or at thoracotomy.

The basic abnormality that results in the association of *P. aeruginosa* infection with CF remains unknown. The mucociliary clearance mechanisms of the pulmonary tract are probably compromised in this disease, but this nonspecific alteration does not in itself explain the prevalence of pseudomonas infection (20, 21). The ability to synthesize immunoglobulins and to produce specific antibodies to pseudomonas is not impaired in CF (2, 7, 8, 15, 18). Infections with candida, fungi, mycobacteria, viruses, and other pathogens usually associated with cell-mediated immunodeficiencies are not frequent. The finding suggests that, if a defect in cellular immunity exists, it should be relatively specific for *P. aeruginosa*.

The sterility of the lower respiratory tract is largely maintained by pulmonary phagocytic cells, the pulmonary macrophages, and by neutrophils. Besides opsonizing antibodies, the most important specific and nonspecific activating factors for phagocytic cells are lymphokines secreted by stimulated lymphocytes (5, 6, 10-12, 16). In addition to lymphokine secretion, antigen-stimulated lymphocytes usually undergo a proliferative response, which can be measured in vitro by incorporation of tritiated thymidine into the deoxyribonucleic acid of the proliferating lymphocyte.

In this study, we examined the in vitro proliferative responses of peripheral blood lymphocytes to killed bacteria. After establishing the major characteristics of these responses, 29 CF patients in different stages of the disease and 14 normal individuals were tested with the mitogens phytohemagglutinin (PHA) and concanavalin A (ConA) and with several pseudomonas strains as well as *Streptococcus pyogenes*, *Staphylococcus aureus*, and *Haemophilus influenzae*. A specific incapacity to respond to *P. aeruginosa* was detected in CF patients with advanced disease.

MATERIALS AND METHODS

In vitro lymphocyte proliferation assay. Mononuclear cells, consisting of approximately 90% lymphocytes, were isolated from heparinized peripheral blood by Ficoll-Hypaque centrifugation (19). Approximately 70 to 80% of peripheral blood lymphocytes and monocytes were recovered with this procedure. Lymphocyte proliferation studies were performed in microcultures containing 10^5 cells per 0.1-ml culture in RPMI 1640 medium supplemented with 20% heat-inactivated autologous or homologous plasma, HEPES buffer (N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid) (Microbiological Associates, Bethesda, Md.), penicillin, and streptomycin. Cultures were performed in 96-well flat-bottom microculture plates (no. 3040, Falcon Plastics, Oxnard, Calif.). Ten microliters of mitogen solution or of antigen (bacteria) suspension was added

to each culture. Cultures were incubated at 37°C for 72 h for mitogen-stimulated cultures and for 5 days for bacteria-stimulated cultures. Five hours before the end of the incubation period, 0.5 μCi of [^{3}H]thymidine (specific activity, 5 Ci/mmol) was added to each culture, and the cells were harvested by using a lymphocyte microharvester (Otto Hiller Co). Tritiated thymidine incorporation was measured with a Searle Isocap 300 liquid scintillation counter. Proliferative responses were expressed as net counts per minute (total − background).

Mitogen and bacterial antigen preparations. PHA and ConA were added to cultures in the following final concentrations: PHA, 1.5 μg/ml, and ConA, 25 μg/ml. Pseudomonas lipopolysaccharide (LPS) (0.01 to 100 μg/ml; pseudomonas vaccine, heptavalent, Parke-Davis) and heat-killed bacteria (60 min at 70°C) were used to stimulate lymphocyte cultures in preliminary tests. For all patient-related studies, bacterial antigens were prepared as follows: recently isolated pseudomonas strains from CF patients were inoculated into Trypticase soy broth and incubated for 48 h at 37°C. The cultures were then centrifuged, and the bacterial pellet was made to a 5% (vol/vol) suspension in sterile saline containing 80 μg of gentamicin per ml; then the culture was incubated again for 24 h at 37°C. In some instances, gentamicin was added directly to the broth after 24 h of incubation. Killed bacteria were washed by centrifugation with sterile saline and resuspended to 5% (vol/vol) in sterile saline containing 10 μg of gentamicin per ml. This suspension was stored in equal portions at 4°C as stock suspension. Before use, the supernatant was discarded and the bacterial pellet was resuspended to a 5% (vol/vol) concentration in fresh saline without gentamicin, and serial dilutions, usually 1/10, 1/50, and 1/100, were prepared. Gentamicin-killed bacterial antigen preparations were also prepared from strains of *Staphylococcus aureus* coagulase positive, strains of *Streptococcus pyogenes*, and strains of *H. influenzae*. The same antigen preparation of each of these bacteria was used through all experiments involving CF patients. *P. aeruginosa* strains used included three mucoid strains isolated from three different CF patients in poor clinical condition. Two of these mucoid strains were classified serologically as Homma type 8 (9).

All bacterial antigens were shown to be sterile before use. Agglutination tests were performed before and after killing with sera containing specific antibodies to the bacteria used. Bacterial agglutination tests were performed on glass slides at room temperature, mixing 1 drop of undiluted serum with 1 drop of a homogeneous, dense bacteria suspension, either killed or collected fresh from Trypticase soy broth after 18 to 24 h cultured at 37°C. Stability of the antigen was tested by analyzing the responses of the same individuals against the same antigens at different intervals of time. Dose-response curves were obtained for all antigens. In subsequent experiments, at least two antigen concentrations eliciting the strongest responses were used. Identical lymphocyte cultures were prepared for kinetic studies and then harvested after 2, 3, 4, 5, and 6 days.

Patients and normal controls. Healthy adult hospital workers were used as normal controls. CF was diagnosed by a positive sweat test and either typical pulmonary or gastrointestinal manifestations or a family history of CF. Clinical and roentgenological scoring was performed by a modification (3) of the system of Shwachman and Kulczycki (14). Studies were carried out according to a protocol approved by the Human Experimentation Committee of University Hospitals, and informed consent was obtained from each participant. The effect of normal heat-inactivated plasma was always tested in duplicated assays on the same day the CF patient was studied. Patients' plasma was stored at −70°C before use. For each patient or control, the responses to all mitogens and bacteria used were tested stimultaneously in the same experiment. Most patients were receiving antibiotic treatment with carbenicillin and either colistin or an aminoglycoside at the time of the study. There was no difference in the treatment schedules for patients with high and low scores. None had symptoms of active viral infection.

Statistical methods. All results are indicated as mean counts per minute over background for triplicate cultures. For the normal control groups and each patient group, the mean and standard deviation of the highest response to each antigen was used in calculating means and standard deviations. The differences between the means of groups were statistically analyzed by Student's t test for unpaired variables. When appropriate, Student's t test for paired variables was also employed.

RESULTS

Lymphocyte responses to bacterial antigens. No proliferative responses to pseudomonas LPS or heat-killed pseudomonas were observed in studies of normal individuals or of CF patients. All gentamicin-killed strains were able to induce lymphocyte proliferation. These antibiotic-killed bacterial antigens were also agglutinated by the same sera that agglutinated them before antibiotic treatment. Gentamicin alone in concentrations up to 20 μg/ml did not affect proliferative responses to mitogens or antigens. Unimodal dose-response curves were obtained for all antigens, with absence of proliferation at both high and low bacteria concentrations. The dose-response curves differed for each antigen. For all four bacteria used, there was some response after 3 days, but the peak was reached only after 5 days. Occasionally a further increase was observed upon the 6th day. Background [^{3}H]thymidine incorporation at 5 days had a mean of 416 ± 493 (range, 58 to 1,990) cpm for all experiments performed with cells from CF patients and a mean of 523 ± 417 (range, 71 to 1,894) for all experiments performed with cells from normal individuals. Individuals whose responses to the same bacterial antigens were tested repeatedly over periods of several months in general showed consistent patterns of response.

Patient studies. A summary of the in vitro

responses to all mitogens and bacterial antigens is shown in Table 1. The most remarkable findings are the significantly higher responses of CF patients to both gram-positive bacteria used, staphylococcus and streptococcus, and the very low responses to three mucoid pseudomonas strains in CF patients with low case history scores. In several instances, patients with very low responses were tested two or more times during a 3-month period with identical results. CF patients in good condition did not differ from the noninfected normal controls.

The effects of homologous (normal) and autologous plasma upon CF and normal lymphocyte responses to mitogens and bacterial antigens are shown in Table 2. The responses of CF patients against streptococcus and staphylococcus were significantly reduced in normal plasma. No reduction was observed when normal lymphocytes were incubated in the presence of nor-

TABLE 1. *In vitro responses to PHA, ConA, Streptococcus pyogenes, Staphylococcus aureus, Haemophilus influenzae, and three isolates of mucoid Pseudomonas aeruginosa in CF patients and normal controls*

Antigen	Response[a]					
	CF patients				Normals	
	Poor scores (1-14)		Good scores (15-25)			
	No.	Mean ± SD[b]	No.	Mean ± SD	No.	Mean ± SD
PHA	10	56,798 ± 15,155	11	64,227 ± 13,918 G vs N: $P < 0.025$	12	46,886 ± 23,042
ConA	8	13,978 ± 9,075	11	14,082 ± 6,757	12	15,922 ± 6,959
Streptococcus pyogenes	13	24,416 ± 15,662 P vs N: $P < 0.01$	19	23,804 ± 11,731 G vs N: $P < 0.005$	14	11,393 ± 8,170
Staphylococcus aureus	10	16,816 ± 14,665 P vs G: $P < 0.05$ P vs N: $P < 0.01$	19	9,327 ± 8,540	11	5,009 ± 2,423
Haemophilus influenzae	8	5,692 ± 4,287	15	9,130 ± 5,841	10	6,092 ± 2,388
Pseudomonas aeruginosa (mean response to 3 mucoid strains)	13	753 ± 861 P vs G: $P < 0.0005$ P vs N: $P < 0.005$	19	3,234 ± 3,041	13	2,781 ± 1,719

[a] Results in counts per minute over background. P value indicated only when <0.05.
[b] SD, Standard deviation; P, poor; G, good; N, normal.

TABLE 2. *Influence of normal sera on in vitro responses to PHA, ConA, Streptococcus pyogenes, Staphylococcus aureus, Haemophilus influenzae, and three isolates of mucoid Pseudomonas aeruginosa in CF patients and normal individuals*

Antigen	Plasma	Response[a]					
		CF patients				Normals	
		Poor scores (1-14)		Good scores (15-25)			
		No.	Mean ± SD[b]	No.	Mean ± SD	No.	Mean ± SD
PHA	Own	10	56,798 ± 15,155	10	64,227 ± 13,918		ND[c]
	Normal	10	57,273 ± 15,739	10	63,254 ± 17,183		ND
ConA	Own	8	13,987 ± 9,075	11	14,082 ± 6,757		ND
	Normal	8	16,612 ± 10,164	11	18,126 ± 9,132		ND
Streptococcus	Own	13	24,416 ± 15,662	16	23,804 ± 11,731	14	11,393 ± 8,170
	Normal	13	10,075 ± 6,659 $P < 0.005$	16	12,837 ± 7,768 $P < 0.005$	14	9,479 ± 5,098
Staphylococcus	Own	10	16,816 ± 14,651	16	9,321 ± 8,540	5	5,009 ± 2,423
	Normal	10	4,198 ± 2,658 $P < 0.025$	16	4,131 ± 2,889 $P < 0.025$	5	3,240 ± 2,330
Haemophilus	Own	8	5,692 ± 4,287	15	9,130 ± 5,841	5	6,092 ± 2,388
	Normal	8	6,275 ± 2,942	15	7,831 ± 3,223	5	7,410 ± 5,015
Pseudomonas aeruginose strains	Own	13	753 ± 861	16	3,234 ± 2,041	8	2,572 ± 1,783
	Normal	13	2,652 ± 1,995 $P < 0.005$	16	4,478 ± 2,275 $P < 0.005$	8	2,781 ± 1,719

[a] Results in counts per minute over background. P value indicated only when <0.05.
[b] SD, Standard deviation.
[c] ND, Not done.

mal homologous plasma. In contrast, normal plasma significantly enhanced the lymphocyte responses to pseudomonas in CF patients. This increase in response did not occur in all patients with very low scores: in three cases the response was not enhanced in normal sera (two of them were analyzed on two occasions). The increase in the group in good condition was variable: patients with higher responses in their own plasma often did not increase the response in normal plasma.

The effect of CF plasma on the in vitro response of normal lymphocytes is summarized in Table 3. Plasma from CF patients significantly increased the responses of normal lymphocytes to streptococcus and staphylococcus. However, CF plasma neither enhanced nor inhibited normal lymphocyte responses to pseudomonas.

Two of the three mucoid pseudomonas strains used throughout all the experiments were clinical isolates from two severely ill CF patients. The responses of both these patients against their own pseudomonas strains were considerably lower than the mean response of all CF patients to those same strains (Table 4).

Agglutinating antibodies were present in all CF patients against their own pseudomonas strains isolated at the time of the tests. Only two patients did not have detectable antibodies to one of their own pseudomonas strains. In both cases, no agglutination of the mucoid pseudomonas was found, whereas strong antibodies were present for the other morphological forms of pseudomonas. Similarly, with very few exceptions, all CF plasmas agglutinated all the *P. aeruginosa* strains used as antigens. No consistent differences in proliferative responses were noted between lymphocytes from patients with antibodies and lymphocytes from patients without detectable antibodies.

DISCUSSION

A variety of soluble extracts have been used to evaluate lymphocyte proliferative responses in vitro to bacterial mitogens or antigens. In this work we used gentamicin-killed bacteria (whole cells). This method proved to be useful and reproducible in obtaining bacterial preparations that stimulated lymphocyte proliferation in vitro. Unimodal dose-response curves for each antigen preparation permitted selection of antigen concentrations that induced the highest proliferative responses in lymphocytes. The fact that the peak of the response was invariably obtained after 5 or 6 days in culture suggests that the whole-cell bacterial antigen induced predominantly a clonal-type proliferation, similar to that observed in the mixed-leukocyte culture, in which killed allogeneic leukocytes are used as stimulating antigen (4). The continuous increase in proliferation may also reflect the release of mitogenic lymphokines and recruitment of additional cell populations for this response (4). Bekierkunst (1), using deep-frozen and then heat-killed bacteria, found high responses of mice spleen cells after 3 days in culture, suggesting a mitogenic-type response. Although his system has important differences from ours, we cannot exclude a mitogen-type stimulation in our culture system. The coexistence of mitogenic substances like LPS and antigenic structures on the cell wall makes it likely that a whole-cell bacterium represents, in fact, a combination of mitogens and antigens that may contribute to the response.

The data obtained evaluating the responses

TABLE 3. *In vitro cellular responses to bacterial antigens: influence of CF sera on the response of lymphocytes from normal individuals and CF patients*

Antigen	Plasma	Normal lymphocytes and CF plasma		CF lymphocytes and homologous CF plasma	
		No. of pairs	Mean ± SD[a]	No. of pairs	Mean ± SD
Streptococcus pyogenes	Own	17	8,083 ± 2,303	(2)	33,416 ± 9,598
	CF		26,473 ± 9,521 $P < 0.0005$[b]	6	28,677 ± 9,163
Staphylococcus aureus	Own	5	4,185 ± 1,122		ND[c]
	CF		10,376 ± 5,146 $P < 0.05$		ND
Haemophilus influenzae	Own		13,334 ± 357		ND
	CF	6	9,831 ± 6,874		ND
Pseudomonas aeruginosa (mean response to 3 mucoid strains)	Own		2,284 ± 611	(2)	1,477 ± 105
	CF	15	2,896 ± 1,070 $P < 0.05$	6	1,696 ± 552

[a] SD, Standard deviation.
[b] P value of paired t test indicated when $P < 0.05$.
[c] ND, Not done.

TABLE 4. *Responses of two CF patients in poor condition (scores 6 and 12, respectively) against their own P. aeruginosa isolates as compared with the responses of all CF patients to the same antigens*

Mucoid *P. aeruginosa* from:	Patient responses		All CF patients	
	Score	Best response obtained	No.	Mean ± SD[a]
Patient 1	6	422	35	2,687 ± 2,489
Patient 2	12	709	35	4,303 ± 3,607

[a] SD, Standard deviation.

of normal individuals and CF patients reveal that all have the ability to respond to bacterial antigens, but with a great degree of individual variation. The single most important finding is that lymphocytes from patients with CF who have advanced pulmonary disease have low responses to *P. aeruginosa* antigen when cultured in their own heat-inactivated plasma. Most of these patients improve their responses if their lymphocytes are cultured in normal plasma, but the cells of some patients in poor condition do not respond even in normal plasma. Newborns' lymphocytes do not respond to *P. aeruginosa*, whereas newly diagnosed CF patients do respond even before a pseudomonas organism has been recovered from cultures of respiratory tract secretions (Sorensen, Stern, and Polmar, unpublished data). CF patients infected with pseudomonas who are still in good clinical condition have a higher proliferative response to pseudomonas than normal, noninfected individuals. These findings suggest the following chain of events for the in vitro lymphocyte responses to *P. aeruginosa* in patients with CF: during early life, cellular immune response to pseudomonas is acquired, and this capacity persists for a long period of time, before and during detectable pseudomonas infection. At some point this ability to proliferate in response to pseudomonas antigen is inhibited specifically by autologous plasma, but cells cultured in normal plasma are able to recover some degree of responsiveness. The end phase seems to be a complete lymphocyte unresponsiveness to pseudomonas, either in autologous or normal homologous plasma (longitudinal studies are under way, but will take a long time to be completed). All these events are highly specific, since responses to the mitogens PHA and ConA, and to streptococcus, staphylococcus, and haemophilus, are normal or elevated. This appears to represent an acquired specific unresponsiveness to *P. aeruginosa* in CF patients. We cannot say whether non-CF patients with chronic pseudomonas bronchitis may eventually develop the same unresponsiveness, or whether this is a peculiar characteristic of CF patients.

Other chronic infections, in which the impairment of a specific immune mechanism is involved, are not comparable to the chronic pseudomonas infection in CF patients. Patients with chronic mucocutaneous candidiasis have a variety of underlying immune defects, but even in the cases in which only a very specific unresponsiveness to candida is detected, the defect is probably not acquired. In chronic staphylococcal infection due to neutrophil dysfunction, lymphocyte responses to whole-cell staphylococcus are normal (Sorensen and Polmar, unpublished data).

The mechanism of the acquired unresponsiveness to pseudomonas cannot be defined with the present data. One possible explanation for an inhibitory effect present in the plasma of CF patients is the presence of specific antibodies to pseudomonas, which CF patients have consistently in high titers. Immunoglobulin M antibodies are known to be able to suppress immune responsiveness specifically (13, 17). Our data do not support this possibility. First, CF plasma fails to inhibit the proliferative responses of normal lymphocytes to pseudomonas, and second, in the few cases in which we did not detect specific antibodies to the pseudomonas used as antigens, responses did not differ from the majority, in which strong antibodies to those antigens were easily detectable. Further studies are required to clarify this point.

The increased responses of CF lymphocytes in autologous plasma to streptococcus and staphylococcus remain unexplained. This appears to be primarily a plasma effect, since the response of CF cells decreases in normal plasma and that of normal cells increases in CF plasma.

Immunity to pseudomonas involves specific antibodies of all immunoglobulin classes, as well as complement and properdin (7), which activate phagocytic cells. The role of specific lymphocyte responses has not been previously investigated, but it is conceivable that lymphocyte proliferation and the release of lymphokines also play an important role.

In CF patients, in whom high levels of antibodies to pseudomonas are present, an acquired specific incapacity of their lymphocytes to react to pseudomonas antigens may play an important role in the irreversibility and increasing destructiveness of their chronic pulmonary pseudomonas infection.

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