

Defective Cellular Immunity to Gram-Negative Bacteria in Cystic Fibrosis Patients

RICARDO U. SORENSEN,^{1*} ROBERT C. STERN,¹ PATRICIA CHASE,¹ AND
STEPHEN H. POLMAR^{1,2}

Departments of Pediatrics¹ and Pathology,² Case Western Reserve University School of Medicine and Rainbow Babies and Childrens Hospital, Cleveland, Ohio 44106

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In vitro lymphocyte responses to *Pseudomonas aeruginosa* have been found to be impaired in cystic fibrosis patients with advanced clinical disease. The responses to *Klebsiella pneumoniae*, *Serratia marcescens*, and *Proteus mirabilis* were studied in a similar group of cystic fibrosis patients and normal individuals. Cystic fibrosis patients found to be unresponsive to pseudomonas were also unresponsive to klebsiella, serratia, and proteus. Responsiveness to *Staphylococcus aureus* was not impaired in cystic fibrosis patients. We postulate that *in vitro* lymphocyte responses to several gram-negative bacteria require the function of a lymphocyte subpopulation which may be impaired in some cystic fibrosis patients.

The major cause of morbidity and mortality in cystic fibrosis (CF) patients is progressive pulmonary infection with bacterial pathogens, particularly *Pseudomonas aeruginosa*. Normally, alveolar macrophages and polymorphonuclear cells play an important role in clearing bacteria from the lungs. The phagocytic activity of these cells is enhanced by opsonic humoral factors (immunoglobulins and complement) and by lymphokines released by lymphocytes stimulated by specific antigens (4, 5, 7, 9, 18). Because of the role played by lymphocytes in the activation of pulmonary macrophages, we have explored the possibility of a specific lymphocyte dysfunction as a mechanism for increased susceptibility to bacterial infection in CF patients. In previous studies, we found that lymphocytes from CF patients with advanced disease were unresponsive *in vitro* to antibiotic-killed *P. aeruginosa* used as antigens. Lymphocytes from these same patients had normal proliferative responses to the mitogens phytohemagglutinin and concanavalin A and to antibiotic-killed *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Haemophilus influenzae* (14). The unresponsiveness to pseudomonas was not dependent on the morphological or serological type of pseudomonas used as antigen and did not vary with the source of the pseudomonas. We now present data indicating that this severe lymphocyte dysfunction also involves other gram-negative bacteria.

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 (16). Lymphocyte proliferation studies were performed in microcultures containing 10^5 cells per 0.1 ml of culture in RPMI 1640 medium supplemented with 20% heat-inactivated autologous plasma, HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid) buffer (Microbiological Associates, Bethesda, Md.), penicillin, and streptomycin. Cultures were performed in 96-well flat-bottom microculture plates (catalog no. 3040; Falcon Plastics, Oxnard, Calif.). A 10- μ l amount of antigen (bacterial suspension) was added to each culture. Cultures were incubated at 37°C for 5 days since previous experiments had indicated a maximal proliferative response after 5 days. At 5 h before the end of the incubation period, 0.5 μ Ci of [³H]thymidine (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 tritiated thymidine was measured with a Searle Isocap 300 liquid scintillation counter. Proliferative responses were expressed as net counts per minute (total counts per minute minus background counts per minute).

Bacterial antigen preparations. Bacterial antigens were prepared by using a modification of a previously described method (14). Well-identified and pure bacterial strains were grown under optimal conditions in Trypticase soy broth without dextrose. Gentamicin was then added to the cultures in a final concentration of 200 μ g/ml. The bacteria remaining viable after 2 h were killed by addition of carbenicillin to a final concentration of 4 mg/ml of culture fluid and incubation for 30 min. The antibiotic-killed bacteria were washed by repeated centrifugation with sterile isotonic saline, diluted to a 10% (vol/vol) stock suspension in saline, and stored at 4°C. Before use, the supernatant was discarded, and the bacterial pellet was resuspended to the original concentration in fresh saline; 1, 0.1, 0.01, and 0.001% dilutions were used in lymphocyte culture assays.

Clinical isolates of *P. aeruginosa* (one classic strain and one mucoid strain), *Klebsiella pneumoniae*, *Ser-*

ratia marcescens, and *Proteus mirabilis*, as well as a Cowan 1 strain of *S. aureus* (American Type Culture Collection), were prepared as antigens. Fresh antigens were prepared every 2 months from the original strains and tested before use with normal lymphocytes of known reactivity. All bacterial preparations were shown to be sterile before use.

Patients and normal controls. Healthy young adults served as controls. CF was diagnosed by a positive sweat test and either typical pulmonary or gastrointestinal manifestations or a family history of CF. Case history and roentgenological scoring was performed by a modification of the system of Shwachman and Kulczycki (2, 13). CF patients were divided into groups of high (15 to 25) or low (1 to 14) case history scores. Patients with high scores are in good clinical condition, and those with low scores have advanced disease. Patients with net in vitro lymphocyte responses below 1,000 cpm to a given antigen were classified as nonresponders. Lymphocyte cultures were performed during the first few days after admission in hospitalized patients. Most patients were receiving similar antibiotic treatment with carbenicillin and either colistin or an aminoglycoside. Treatment with these antibiotics or addition of the antibiotic to lymphocyte cultures has been shown not to influence lymphocyte response to bacteria (14). Patients with symptoms of active viral infections or who also had other diseases not related to CF and chronic pulmonary infection were omitted from the study. No patient was receiving corticosteroids when studied. For each patient or control, the responses to all bacteria studied were tested simultaneously on the same blood sample. At least one normal control was included in each experiment. These studies were approved by the Clinical Research Review Committee of University Hospitals of Cleveland, and written informed consent was obtained.

Statistical methods. All results are given as mean counts per minute over background for triplicate cultures. For each series of antigen dilutions, the highest mean response was used for statistical analysis. For pseudomonas antigens, the mean of the optimal responses to both strains tested was used. The differences between the means of groups were analyzed by Student's *t* test for unpaired variables. Correlation coefficients were calculated by using standard statistical methods.

RESULTS

All bacterial strains prepared as stimulating agents were able to induce increased [³H]thymidine uptake in lymphocytes from normal individuals and some CF patients. Unimodal dose-response curves were obtained with all bacterial preparations. Typical examples are shown in Fig. 1. Optimal responses were usually obtained with a 0.01 to 0.001% final concentration of bacterial antigen in the culture medium.

The in vitro lymphocyte responses of controls and CF patients to all bacteria studied are summarized in Table 1. Background thymidine incorporation and responses to staphylococcus were similar in both groups. Responses to all gram-negative bacteria were lower in CF patients, and these differences were statistically significant for pseudomonas, klebsiella, and *Serratia*. In addition, 40 to 53% of CF patients were nonresponsive (<1,000 net cpm) to pseudomonas, *Serratia*, or klebsiella, whereas only 4 to 17% of the normal controls were nonresponsive to these bacteria (Table 2). Although CF lymphocyte responses to proteus were lower than

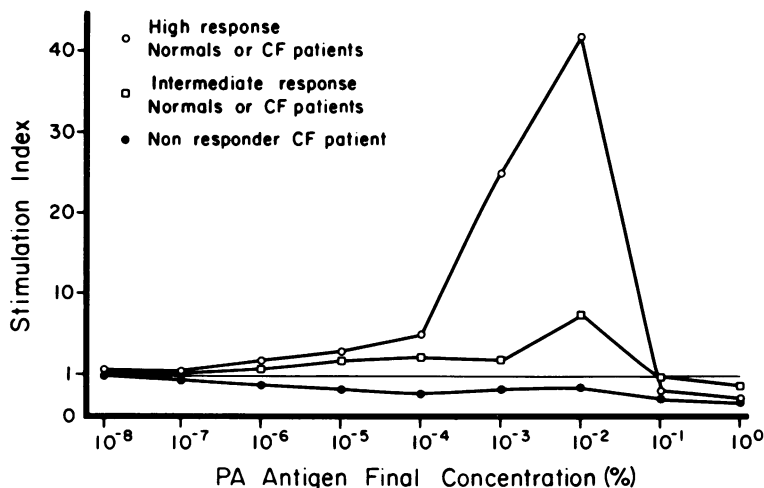


FIG. 1. Typical unimodal dose-response curves of normal and CF lymphocytes to pseudomonas antigens. Lymphocytes from nonresponder CF patients did not increase [³H]thymidine uptake over background at any pseudomonas concentration tested. Frequently the [³H]thymidine uptake of nonresponders was below background at pseudomonas concentrations which stimulated normal lymphocytes (see text). The stimulation index is the [³H]thymidine uptake of stimulated lymphocytes divided by that of unstimulated lymphocytes (background). PA, *P. aeruginosa*.

TABLE 1. *In vitro* lymphocyte responses to *S. aureus*, *P. aeruginosa*, *S. marcescens*, and *P. mirabilis* in normals and CF patients grouped according to case history scores and responsiveness to pseudomonas

Group	No.	Response (cpm over background)					
		Unstimulated	<i>Staphylococcus</i>	<i>Pseudomonas</i>	<i>Klebsiella</i>	<i>Serratia</i>	<i>Proteus</i>
Normals	23	702 ± 78 ^a	44,811 ± 4,695	8,510 ± 1,323	5,156 ± 1,071	6,758 ± 1,249	3,826 ± 886
CF patients	43	756 ± 99	50,402 ± 4,300	3,774 ± 617 (CF vs N, <i>P</i> < 0.0005) ^b	2,836 ± 605 (CF vs N, <i>P</i> < 0.025)	2,011 ± 413 (CF vs N, <i>P</i> < 0.0005)	2,462 ± 727
CF patients High case history scores (15-25)	19	538 ± 58	49,886 ± 6,650	5,973 ± 1,055	4,027 ± 968	3,297 ± 803 (H vs N, <i>P</i> < 0.025)	3,766 ± 1,413
CF patients Low case history scores (1-14)	24	928 ± 163 (L vs H, <i>P</i> < 0.025)	50,810 ± 5,153	1,960 ± 516 (L vs N, <i>P</i> < 0.0005; L vs H, <i>P</i> < 0.0005)	1,893 ± 731 (L vs N, <i>P</i> < 0.01; L vs H, <i>P</i> < 0.05)	1,039 ± 278 (L vs N, <i>P</i> < 0.0005; L vs H, <i>P</i> < 0.005)	1,451 ± 644 (L vs N, <i>P</i> < 0.025)
CF patients Responders to <i>Pseudomonas</i> (>1,000 net cpm)	26	687 ± 96	56,642 ± 5,756	5,921 ± 758	3,888 ± 694	3,365 ± 625	3,893 ± 1,117
CF patients Nonresponders to <i>Pseudomonas</i> (<1,000 net cpm)	17	814 ± 212	40,975 ± 5,830 (NR vs R, <i>P</i> < 0.05)	386 ± 76 (NR vs N, <i>P</i> < 0.0005; NR vs H, <i>P</i> < 0.0005; NR vs L, <i>P</i> < 0.01; NR vs R, <i>P</i> < 0.0005)	309 ± 137 (NR vs N, <i>P</i> < 0.0005; NR vs H, <i>P</i> < 0.0005; NR vs L, <i>P</i> < 0.05; NR vs R, <i>P</i> < 0.0005)	235 ± 83 (NR vs N, <i>P</i> < 0.0005; NR vs H, <i>P</i> < 0.0005; NR vs L, <i>P</i> < 0.025; NR vs R, <i>P</i> < 0.0005)	274 ± 71 (NR vs N, <i>P</i> < 0.005; NR vs H, <i>P</i> < 0.025; NR vs R, <i>P</i> < 0.01)

^a Values are mean ± standard error of the mean.^b Abbreviations: CF, CF patients; N, normals; H, CF patients with high case history scores; L, CF patients with low case history scores; R, responders to *Pseudomonas*; NR, nonresponders to *Pseudomonas*.

TABLE 2. Frequency of responders and nonresponders to different bacterial antigens among normal individuals and CF patients

Group	Total no.	No. of responders and nonresponders to the following antigens:									
		<i>Staphylococcus</i>		<i>Pseudomonas</i>		<i>Serratia</i>		<i>Klebsiella</i>		<i>Proteus</i>	
		R ^a	NR	R	NR	R	NR	R	NR	R	NR
Normals	23	23	0	22	1	19	4	19	4	13	10
CF patients	43	42	1	26	17	20	23	22	21	17	26
CF patients High case history scores (15-25)	19	19	0	16	3	12	7	13	6	11	8
CF patients Low case history scores (1-14)	24	23	1	10	14	8	16	9	15	6	18
CF patients Responders to <i>Pseudomonas</i>	26	26	0	26	0	20	6	21	5	17	9
CF patients Nonresponders to <i>Pseudomonas</i>	17	16	1	0	17	0	17	1	16	0	17

^a Abbreviations: NR, nonresponder (<10,000 net cpm for staphylococcus and <1,000 net cpm for all other bacteria); R, responder.

the control and 60% of CF lymphocytes (compared with 43% of controls) were unresponsive to this bacterium, these differences were not statistically significant.

When CF patients are grouped according to case history scores (Table 1), it becomes apparent that low responsiveness to pseudomonas was associated with low case history scores. In ad-

dition, the majority of patients unresponsive to pseudomonas, serratia, klebsiella, and proteus were in the group with low case history scores (Table 2). Only responses to serratia were significantly lower ($P < 0.025$) than normal in patients with high case history scores.

In Tables 1 and 2 CF patients are also grouped according to the mean responses of their lymphocytes to two pseudomonas antigens, i.e., responders ($>1,000$ net cpm) and nonresponders ($<1,000$ net cpm). Nonresponders to pseudomonas also had lower responses to klebsiella, serratia, and proteus than did responders to pseudomonas (Table I). As shown in Table 2, CF patients unresponsive to pseudomonas generally did not respond to serratia, klebsiella, or proteus, but did respond to staphylococcus. In contrast, the frequency of unresponsiveness to serratia, klebsiella, and proteus was the same in CF patients responsive to pseudomonas and in normal controls (Table 2).

To determine whether there was a quantitative correlation between responses of lymphocytes to any two of the various gram-negative bacteria studied, correlation coefficients were calculated. No significant correlations were found between these responses in normal individuals and in those CF patients capable of responding to pseudomonas.

Lymphocytes from several CF patients failed to incorporate [3 H]thymidine above the unstimulated background rate for all bacterial antigen concentrations tested. In these cases, [3 H]thymidine incorporation was even depressed below background. This phenomenon was not seen in 92 tests with normal lymphocytes, but was present in 21 of 96 responses to gram-negative bacteria in CF patients with low case history scores. Viability assessment with trypan blue at the end of the culture period showed no differences between normal cultures with positive stimulation and cultures with thymidine incorporation below background. A typical example is shown in Fig. 1.

DISCUSSION

In previous studies, lymphocytes from CF patients with low case history scores were found to have decreased or absent tritiated thymidine incorporation when cultured with antibiotic-killed *P. aeruginosa* (14). In these patients, responses to *S. aureus*, *H. influenzae*, and *S. pyogenes* were the same as for CF patients with high case history scores and normal controls. The present studies extend these observations to include other gram-negative bacteria. CF patients with low case history scores were found to have significantly decreased responses to *K.*

pneumoniae, *S. marcescens*, and *P. mirabilis*. In contrast, the frequency of unresponsiveness to pseudomonas, klebsiella, serratia, and proteus was similar in CF patients with high case history scores and normal controls. Patients found to be unresponsive to pseudomonas were also unresponsive to these other gram-negative bacteria. Therefore, defective lymphocyte responses in CF patients with low case history scores are not restricted to pseudomonas but extend to certain other gram-negative bacteria.

The cellular bases of unresponsiveness to pseudomonas and certain other gram-negative bacteria in CF patients with low case history scores are poorly understood at this time. One may think of bacterial cell surfaces as hapten-carrier complexes and that lymphocyte responses to bacteria may be analogous to lymphocyte reactions involving hapten-carrier complexes (11). One lymphocyte subpopulation may be carrier specific, while the other subpopulation is hapten specific; response to the hapten requires normal functioning of both subpopulations. Inability to respond or tolerance to the carrier would also ablate responses to the hapten (19-21). In the present system, a common gram-negative antigen may function as the carrier, while each bacterial species has certain unique antigens similar to the haptens. Inability to respond to, or tolerance to, the common gram-negative antigen would be expected to result in loss of responses to species-specific hapten-like antigenic determinants. This may be the situation which exists in CF patients with low case history scores who are unresponsive to pseudomonas, klebsiella, serratia, and proteus. CF patients who have not lost the ability to respond to pseudomonas (or the common gram-negative antigen) would be expected to show the same frequency of responses to other gram-negative bacteria as normal individuals, and this is precisely what we have observed. This is not the only hypothesis, but it is testable by identifying the subpopulation requirements for these responses and performing coculture experiments with isolated subpopulations from responding and nonresponding individuals.

Recently, Lieberman and Kaneshiro found that the lymphocytes from CF patients and CF carriers had decreased total proteins and β -glucuronidase synthesis 2 days after exposure to phytohemagglutinin (8). We (14) and others (17) have not found decreased deoxyribonucleic acid synthesis in CF patients studied by [3 H]thymidine incorporation when their cells were stimulated with phytohemagglutinin or concanavalin A. However, we have found that there is a depression of [3 H]thymidine incorporation below background levels in nonresponding CF patients

when their cells are exposed to pseudomonas and other gram-negative bacterial antigens. Whether this phenomenon represents an active suppression of cellular function is not clear and requires further investigation.

Defective lymphocyte responses to certain gram-negative bacteria appear to be specific for chronically infected CF patients. Non-CF patients with chronic pseudomonas infections have not been found to have a similar defect (15). However, none of these patients was as seriously ill at the time of study as the CF patients with low case history scores. Immunodeficient patients who became infected with pseudomonas usually also have low responsiveness to staphylococcus and mitogens, reflecting their underlying immune defect (Sorensen and Polmar, unpublished data). Malnutrition, present in some CF patients (nonresponders and responders as well), is frequently associated with T-cell deficiency (1, 3, 10, 12). CF patients, however, are not lymphopenic and have normal numbers of T-cells (6) and normal lymphocyte responses to phytohemagglutinin and concanavalin A (14, 17).

CF patients in good clinical condition but with pseudomonas present in their respiratory tract secretions have responses to pseudomonas comparable to noninfected normal individuals (14). This fact does not necessarily preclude an underlying intrinsic lymphocyte defect since it is difficult to establish what an adequate lymphocyte response should be for these patients. Why patients with a generalized defect in lymphocyte responses to many gram-negative organisms should have an almost specific susceptibility to pseudomonas infection is not clear. It can be postulated that CF patients have a progressive lymphocyte dysfunction which becomes increasingly apparent with advancing clinical disease and that, when this defect is fully expressed, the pseudomonas infection is already so well established that other infections become unlikely.

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