Influence of Cystic Fibrosis Plasma on Lymphocyte Responses to *Pseudomonas Aeruginosa* In Vitro

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Summary

Peripheral blood lymphocytes from cystic fibrosis (CF) patients with advanced diseases do not proliferate following exposure to *Pseudomonas aeruginosa* (PA) antigens in *in vitro*. In this study, we sought to determine if CF lymphocyte unresponsiveness to PA is due to inhibitory factors present in CF plasma. Nineteen low-responder CF (LRCF) patients increased their mean lymphocyte proliferative response (^3H]thymidine incorporation) from 703 ± 133 to 3178 ± 811 net cpm when incubated in normal plasma. These increases do not reach the level of response seen in normal individuals (8510 ± 1323 net cpm). Ten of 19 patients did not increase their responses over 2000 net cpm. Plasma from LRCF patients does not inhibit responses of normal or homologous CF lymphocytes. Responses of normal individuals in autologous plasma were 7807 ± 1164 net cpm. The same lymphocytes incubated in 16 plasmas from LRCF patients gave responses of 7146 ± 1317 net cpm. Preincubation of the PA antigen in LRCF plasma increases rather than inhibits normal lymphocyte responses. LRCF plasma absorbed with PA no longer supports normal lymphocyte responses to PA. LRCF and normal plasma mixtures increase responses of normal lymphocytes to PA over responses in autologous plasma. Extensive preincubation and washing of LRCF lymphocytes to eliminate blocking immune complexes failed to restore the ability to respond to PA. These data suggest that the unresponsiveness to PA of lymphocytes from CF patients with advanced disease is due to alterations occurring at a cellular level *in vivo*. This lymphocyte dysfunction cannot be reversed by normal plasma *in vitro*, nor can it be induced in normal lymphocytes by the use of CF plasma.

Speculation

Lymphocytes from cystic fibrosis (CF) patients with advanced disease fail to proliferate in response to *Pseudomonas aeruginosa* antigens. This abnormality cannot be reversed by the use of normal plasma or by preincubation and washing of CF lymphocytes *in vitro*. Heat-inactivated CF plasma does not inhibit proliferation of normal lymphocytes in response to *Pseudomonas*. These results indicate that changes in lymphocyte reactivity occur *in vivo* and are unresponsive to plasma substitution *in vitro*. Further investigation of quantitative and functional aspects of lymphocyte and macrophage subpopulations may elucidate the nature and significance of the unresponsiveness of CF lymphocytes to *Pseudomonas aeruginosa*.

Patients with cystic fibrosis (CF) are unusually susceptible to chronic pulmonary infection with *Pseudomonas aeruginosa* (PA), a ubiquitous organism which rarely infects healthy individuals (5, 14). The cause of persistent PA infection in the bronchopulmonary tract of CF patients remains unexplained. Defective mucociliary transport has been detected in some CF patients, but this does not

explain the predilection for PA infection over other common pulmonary pathogens (32, 34). Pulmonary alveolar macrophages and neutrophils play an important role in clearing bacteria from the lung. The phagocytic function of these cells is nonspecific, but can be enhanced by antibodies and by lymphokines produced by lymphocytes specifically stimulated by antigens (8, 11, 12, 15, 28). Therefore, if a specific immune defect is responsible for PA infections in CF, it is likely to occur at the lymphocyte level. We have reported a CF-specific lymphocyte unresponsiveness to PA and other gram-negative bacteria *in vitro* (22, 23). In some patients, this defect was less pronounced when the lymphocytes were incubated in normal plasma. Conversely, in some instances, plasma from an unresponsive CF sibling reduced the lymphocyte response to PA of the responding CF sibling (24). These findings suggested the presence of heat-stable lymphocyte-inhibiting factors in the plasma of CF patients. A heat-stable serum factor has been shown to cause specific inhibition of PA phagocytosis by rabbit and human alveolar macrophages (26). In this study, we explored the possibility that plasma factors similar or identical to those reported to inhibit macrophages could also cause the lymphocyte unresponsiveness to PA in CF patients.

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 (27). Lymphocyte proliferation studies were performed as previously described (23, 24). Mononuclear cells at a concentration of $10^5/0.1$ ml in Roswell Park Memorial Institute (RPMI) 1640 culture medium supplemented with 20% heat-inactivated plasma. 4-(2-hydroxyethyl)-piperazineethanesulfonic acid buffer, and antibiotics were incubated in flat-bottom microculture plates. Ten μl of antigen (bacterial suspension) were added to each culture. Cultures were incubated at 37°C for 5 days and then pulsed with $0.5 \mu\text{Ci}$ of ^3H]thymidine (specific activity, 24 Ci/mmol) 5 hr before the end of the incubation period. The cells were harvested with a lymphocyte microharvester (Otto Hiller Co., Madison, WI). Incorporation of tritiated thymidine was measured with a Searle Isocap 300 liquid scintillation counter. Proliferative responses were expressed as cpm.

BACTERIAL ANTIGEN PREPARATION

Bacterial antigens were prepared by using a modification of a previously described method (22). Well-identified and pure bacterial strains were grown under optimal conditions in trypticase soy broth without dextrose and then killed by the addition of gentamicin. The antibiotic-killed bacteria were washed by repeated centrifugation with sterile isotonic saline, diluted to a 10% (v/v) stock suspension in saline, and stored at 4°C . Before use, the supernatant was discarded, and the bacterial pellet was resus-

pended 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), and Cowan 1 strain of *Staphylococcus 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. We have previously shown that there is a significant correlation between unresponsiveness to PA and low case history scores in CF patients (22-24). To analyze the effect of plasma factors, both normal controls and CF patients were grouped into three categories according to their responses to PA in autologous plasma: low responders <2000; medium responders, 2000 to 8000; and high responders >8000 net cpm. In experiments involving CF lymphocytes, at least one normal control was always included. These studies were approved by the Clinical Research Review Committee of University Hospitals of Cleveland, and written informed consent was obtained.

PLASMA PREPARATION

Plasma was obtained from heparinized peripheral blood by 20 min centrifugation for 20 min at $800 \times g$. Plasma samples were then heat inactivated for 30 min at 56°C and spun again for 30 min at $1600 \times g$. In most instances, the plasma substitution experiments were performed on the same day, and the remaining plasma was stored in aliquots under sterile conditions without preservatives at -70°C . When used after storage, the plasma samples were centrifuged before use. Plasma pools were prepared by mixing equal amounts of stored plasma from low responder cystic fibrosis (LRCF) patients or from normal individuals. Mixtures of normal and LRCF plasma were prepared by adding 5, 10, and 15% of CF heat-inactivated plasma to autologous normal plasma to a total plasma concentration of 20%. PA-absorbed plasma was prepared by mixing plasma thoroughly with 0.05 ml of packed killed PA. After 30 min incubation at 37°C , the mixture was spun 30 min at $1600 \times g$. The supernatant plasma, shown to

be devoid of agglutinating activity for PA, was used as "absorbed plasma."

STATISTICAL METHODS

Results are given as net cpm (mean cpm over background for triplicate cultures). For each series of antigen dilutions, the highest mean of the optimal response to both strains tested was used. For experiments in which specific variables of the culture conditions were tested, the percentage response compared to control response was calculated (experimental cpm: control cpm $\times 100$). In addition, stimulation indices (SI = experimental cpm: background cpm) were calculated when modifications of the culture system resulted in background [^3H]thymidine incorporation changes. Statistical analyses were performed using the Student *t* test for paired variables. *P*'s are indicated only when <0.05 .

RESULTS

PLASMA SUBSTITUTION EXPERIMENTS

The effect of substitution of homologous for autologous plasma on the lymphocyte responses to PA is shown in Table 1. Both CF and normal low responders increased their responses significantly when cultured in homologous normal plasma. There were only three normal individuals with low responses that could be tested. The effect of different plasmas on the same normal lymphocytes showed variable individual increases in responsiveness. Only in one of 13 instances did the response not increase over 2000 net cpm. This is in contrast with the results obtained with 19 LRCF lymphocytes. Although there was a significant increase in the responsiveness to PA in the group as a whole, 10 of the 19 patients did not increase their response to greater than 2000 net cpm. Only two of the nine patients increased their responses about 8000 net cpm. The mean response for normal individuals in autologous plasma is 8510 ± 1323 net cpm. A similar increase in response was not observed when individuals with low response to *Staphylococcus* in autologous plasma were cultured in homologous plasma.

The effect of CF plasma on the responses of normal and homologous CF lymphocytes is shown in Table 2. Plasma obtained from low responder CF patients did not inhibit the responses of normal lymphocytes to PA nor did it further decrease the responses to CF lymphocytes. Background values of normal

Table 1. Effect of homologous normal plasma on lymphocyte responses to *Pseudomonas* I influence of degree of responsiveness in autologous plasma

Source of lymphocytes	Source of plasma	Degree of response in autologous plasma (net cpm)		
		Low (<2000 cpm)	Medium (2000-8000 cpm)	High (>8000 cpm)
Normal donors	Autologous Plasma	(3-13) ¹ $1,304 \pm 40^2$	(9-12) $4,108 \pm 429$	(10-26) $14,195 \pm 1,082$
	Homologous Normal plasma	$4,513 \pm 890$	$5,114 \pm 1,122$	$14,808 \pm 1,529$
		$P < 0.0025^3$		
CF Patients	Autologous Plasma	(19) 703 ± 133	(27) $4,111 \pm 239$	(16) $10,824 \pm 657$
	Homologous Normal plasma	$3,178 \pm 811^4$	$5,932 \pm 675$	$10,132 \pm 1,129$
		$P < 0.005$ $P < 0.01$		

¹ Numbers in parentheses, number of pairs. When two numbers are given, the first number indicates individual lymphocyte preparations, and second number indicates number of individual plasmas tested.

² Mean $\pm$ S.E.

³ *P* is indicated only when <0.05 .

⁴ Although this is a significant increase, 10 of 19 patients did not increase their responses over 2000 cpm in normal plasma.

Table 2. Effect of CF plasma on the responses of normal and CF lymphocytes to PA

Source of lymphocytes	Source of plasma	Degree of responsiveness of CF plasma donors ¹ (net cpm)		
		Low ² (<2000 cpm)	Medium (2000–8000 cpm)	High (>8000 cpm)
Normal donors	Autologous Plasma	(16) ³ 7,807 ± 1,164 ⁴	(13) 8,241 ± 1,660	(9) 11,031 ± 2,330
	Homologous CF plasma	7,146 ± 1,317	8,078 ± 1,493	12,221 ± 2,848
CF patients	Autologous Plasma	(6) 3,400 ± 1,129	(7) 4,921 ± 1,846	(5) 2,967 ± 802
	Homologous CF plasma	3,442 ± 936	4,939 ± 1,162	5,720 ± 3,489

¹ For this study, CF plasmas were grouped according to the degree of responsiveness to PA of the CF patient from whom the plasma was obtained.

² Effect of LRCF plasma on normals or unrelated CF patient responses.

³ Numbers in parentheses, number of pairs.

⁴ Mean ± S.E.

lymphocytes incubated with CF plasma did not show a significant variation in comparison to background values in autologous plasma. Table 3 summarizes the results obtained with plasma substitution experiments on LRCF patients and normal controls. Although CF plasma from the three groups of patients increase the responses of normal lymphocytes to *Staphylococcus*, the differences are not significant (Table 4). The changes of responses to *Staphylococcus* and to PA induced by individual plasmas were not correlated.

The use of pooled normal or low responder CF plasma did not give different results from those obtained with large numbers of individual plasma-lymphocyte combinations. Both normal and CF lymphocytes with low responses in autologous plasma increased their responses to PA when homologous plasma pools were used.

ABSORPTION EXPERIMENTS

These experiments were performed to determine if inhibitors in CF plasma could be removed by preincubation with PA. Three LRCF plasma samples and three normal control plasma samples were absorbed with antibiotic killed PA and then used in experiments with autologous lymphocytes. When absorbed, normal plasma was used, previously responding lymphocytes became unresponsive to PA. Absorption of LRCF plasma did not increase the autologous CF lymphocyte responses. The SI for both normal and LRCF individuals in PA absorbed plasma were ≤2, indicating that no lymphocyte activation by PA occurs under these conditions. By contrast, in both normal individuals and patients, the SI of the responses to *Staphylococcus* in PA absorbed plasma ranged from 12 to 63.

The high concentration of specific PA antibodies in CF plasma is likely to change the antigenicity of PA. To test this possibility, the PA used to absorb LRCF was resuspended to original v/v concentrations and used as antigen. The effect of the preincubation of killed PA in LRCF plasma on its antigenicity is shown in Table 5. PA incubated in LRCF plasma increased the responses of normal lymphocytes. Lymphocytes from two of three LRCF patients showed a moderate increase in their responses. Similar increases were observed with PA preincubated with plasma from high responder CF patients or normal individuals.

Normal and LRCF plasma mixtures were used to test whether an inhibitory factor in CF plasma could lose activity when diluted in normal plasma. An increase in activity in plasma mixtures indicates complementary function of factors present in both types of plasma. Experiments were performed by incubating lymphocytes from five high-responding normal individuals in 20% autologous plasma, LRCF plasma, or plasma mixtures. In all instances, an increased responsiveness to PA was observed with plasma mixtures compared to autologous or LRCF plasma alone. The

Table 3. Summary of plasma effects on lymphocyte proliferative responses to PA in LRCF patients and normals

	LRCF plasma	Normal plasma
LRCF lymphocyte	Low or absent	Moderate increase in only 50% of cases
Normal lymphocyte	Normal	Normal

increases ranged from 8 to 23% over the responses in autologous plasma and from 29 to 211% over the responses in 20% homologous LRCF plasma alone. The peak increase with plasma mixtures was usually found with either 5 or 10% LRCF plasma.

To determine if CF plasma factors interfered with early events in the triggering of lymphocyte proliferation by PA antigens, lymphocyte cultures were started in the absence of plasma. Plasma was then added 60 min after antigen addition to the cell suspension. Some control cultures had plasma included before addition of antigen. Others were kept in plasma-free conditions throughout the experiment. Cultures to which no plasma was added over the 5 days incubation period showed proliferative responses to mitogens (phytohemagglutinin, concanavalin A, and pokeweed mitogen) and *Staphylococcus*. No response to PA was found under plasma-free culture conditions, in normal individuals, or in CF patients. The delayed addition to plasma after incubation of lymphocytes and bacterial antigens for 60 min allowed responses of normal individuals to PA antigens, but did not increase the responses of three LRCF patients.

Preincubation of lymphocytes was performed to allow shedding or pinocytosis of blocking complexes on the lymphocyte surface. Two types of experiments were performed. In short-term preincubation studies, lymphocytes were kept in plasma-free culture medium at 37°C for 30 min before cultures were set up in normal heat-inactivated plasma. This preincubation was shown to reduce the amount of immunoglobulin-bearing lymphocytes by 20 to 80%. None of the four LRCF patients increased their responses to PA under these conditions. In a second group of experiments, the preincubation was performed in plasma-poor medium for prolonged periods of time. Lymphocytes were kept at 37°C in 1-ml aliquots in RPMI medium and 2% heat-inactivated plasma for 24 and 48 hr and for 5 days. The cells were resuspended daily in fresh medium. At the completion of the preincubation period, lymphocytes were resuspended in fresh medium. Viable cells were counted, readjusted to 1×10^6 cells/ml, and cultured for additional 5 days in the presence of antigens. Cells from normal individuals and from responding CF patients maintained their capacity to respond to *Staphylococcus* and PA antigens after incubation for up to 5 days, with daily changes of culture medium. None of the

Table 4. Influence of CF plasma on the response of normal lymphocytes to *Staphylococcus*

Source of lymphocytes	Source of plasma	Degree of responsiveness to <i>Pseudomonas</i> of CF plasma donors		
		Low (<2000 cpm)	Medium (2000–8000 cpm)	High (>8000 cpm)
Normal donors	+Autologous	(12) 40,984 ± 7,587 ¹	(13) 37,271 ± 4,926	(9) 52,254 ± 8,487
	+Homologous CF plasma	65,729 ± 6,225	44,050 ± 4,990	62,855 ± 6,897

¹ Mean ± S.E.

Table 5. Effect of preincubation of PA in low responder of plasma on lymphocyte responses to PA

	Untreated PA (net cpm)	PA preincubated with LRCF plasma (net cpm)
Normal Lymphocytes		
Control 1	4,763	9,336
Control 2	20,416	24,592
Control 3	8,881	12,158
LRCF Lymphocytes		
Patient 1	359	374
Patient 2	146	1,150
Patient 3	163	2,326
	<i>P</i> < 0.01	

eight LRCF patients studied increased their responses to PA after 1, 2, or 5 days preincubation. Control responses to staphylococci were present in all instances under these conditions. In two additional experiments, CF lymphocytes were preincubated for 24 hr in RPMI medium and 1% human albumin only, with similar results.

DISCUSSION

In previous studies, we have shown that *in vitro* lymphocyte responses to PA are low or absent in CF patients with advanced disease. In some instances, these responses could be increased by substituting normal for CF plasma (24). Here we show that this increase of low responses, rather than being specific for CF lymphocytes, is also observed in low responder normal lymphocytes. Thus, plasma appears to exert a regulatory effect on lymphocyte responses to PA both in CF patients and normal individuals. Experiments performed under plasma-free conditions show that the response to PA is strictly plasma dependent. Unlike responses to PA, lymphocytes do respond to *Staphylococcus*, and to the mitogens phytohemagglutinin, concanavalin A, and pokeweed mitogen in plasma-free conditions. Further evidence for the plasma dependence of the lymphocyte response to PA was gained by absorbing plasma with killed PA. This absorption rendered plasma completely unresponsive of lymphocyte reactivity to PA. The specificity of this effect was shown by the fact that responses to staphylococci occur in the presence of PA absorbed plasma. In accordance with the lack of response to PA in absorbed plasma, PA preincubated with unabsorbed plasma becomes a stronger antigen than the untreated PA control.

Several CF factors have been found in serum from CF patients. One group of these factors appears to be genetically determined and has been found in homozygous as well as heterozygous individuals. These factors are inactivated by heat and by heparin (4, 6) and are not present in the heat-inactivated heparinized plasma used in our assay. A heat-stable factor has been shown to inhibit PA uptake by alveolar macrophages (26). A partially heat-stable CF plasma factor was shown to inhibit the increase of total protein and β -glucuronidase in CF lymphocytes stimulated with phytohemagglutinin (13). We failed to see a similar effect of CF plasma on PHA-induced [³H]thymidine incorporation (24). Here we show that heat-inactivated CF plasma does not inhibit [³H]thymidine incorporation of either normal or homologous CF lymphocytes in response to bacterial stimulation. Although reduc-

tions of responsiveness are seen in some combinations of CF plasma with normal or CF lymphocytes, increased responses are observed as well. Under certain circumstances, LRCF plasma consistently increases responses to lymphocytes to PA. When bacteria are preincubated with this plasma, they elicit higher responses in normals and also increase responses to PA of LRCF lymphocytes. The increased responses in the presence of CF and normal plasma mixtures indicates that, rather than inhibitors, CF plasma contributes factors which collaborate with normal plasma factors in eliciting a lymphocyte response to PA. These results are in keeping with our earlier observation that the presence of PA-agglutinating antibodies did not correlate with lymphocyte unresponsiveness (23). Hoiby *et al.* (10) had shown that CF plasma with multiple precipitins to PA antigens did not interfere with the responses of normal lymphocytes to heat-killed PA, soluble PA antigens, and heat killed *Escherichia coli* (10). In this paper, we present additional evidence against an inhibiting effect of antibodies by showing that their absorption out of CF plasma does not restore lymphocyte responsiveness to PA.

Several findings in this paper support the concept that the defective responses to PA *in vitro* are due to cellular factors rather than heat-stable plasma inhibitors present in CF patients. Only 50% of low responder CF patients increase their lymphocyte responses to PA in normal plasma. Moreover, the observed increases do not reach to the level of responses seen in normal individuals. To rule out the inhibition of lymphocytes by CF factors or immune complexes adhered to their surface, cells were incubated and washed for up to 5 days before stimulation with PA (7, 17, 30, 31). No recovery or increase of responsiveness was seen under these conditions. A similar finding had been reported earlier by Hoiby *et al.* (10). Repeated washing of CF lymphocytes failed to increase their responses to microbial antigens.

The presented evidence does not rule out the possibility that immune complexes play a role in creating a state of unresponsiveness of CF lymphocytes *in vivo*. Immune complexes are likely to occur in CF patients who have high specific antibody titers for PA (11, 20) together with heavy airway infection with these bacteria (5, 14). Several reports confirm their presence in CF serum (18, 19, 25). Experimentally induced tolerance to orally administered antigens is based on the presence of such complexes (1). Similar mechanisms could operate for antigens presented to the bronchopulmonary lymphoreticular system. Moreover, immune complexes have been shown to be required for the expression of suppressor function by suppressor T-cells (16). Our work has been carried out with heat-inactivated plasma as conventionally used for lymphocyte culture assays. Heat inactivation is known to alter immune complexes and destroy them if they are heat labile (21, 34). Therefore, it is conceivable that immune complexes *in vivo* alter monocyte and lymphocyte subpopulations in such a way that the phenomena become irreversible to incubation, washing, or plasma substitutions *in vitro*. The *in vivo* reversibility of the lymphocyte unresponsiveness observed in some CF patients after hospitalization and intensive antibiotic therapy is compatible with this hypothesis.

Although further experiments using fresh CF serum or purified heat labile CF-factors are required for a definitive answer, it is unlikely that they cause the lymphocyte unresponsiveness to PA. Their presence is genetically determined and independent of progression of disease or severity of PA infection (3, 29). In contrast, lymphocyte unresponsiveness is associated with advanced disease and PA infection (22–24). Experiments in which

CF lymphocytes were incubated in a low concentration of heat-inactivated plasma or in complete absence of CF plasma provide further evidence against *in vivo* blocking of lymphocyte receptors by heat-labile CF factors.

A strong macrophage inhibiting factor has been described in the serum of CF patients (21). Although we have not observed a similar plasma effect on the *in vitro* lymphocyte responses to PA, both mechanisms could play a role in the pathogenesis of lymphocyte unresponsiveness to PA. Benacerraf and Germain (2) have shown that in mice, the response to the synthetic copolymer L-glutamic acid⁶⁰-L-alanine³⁰-L-tyrosine¹⁰ depends on the presentation of the antigen (2). The presenting cells are most likely macrophages. In L-glutamic acid⁶⁰-L-alanine³⁰-L-tyrosine¹⁰ responder strains of animals, T-helper cells are generated by this antigen. In nonresponder animals or in responding animals in which there is interference with the presentation of the antigen by the macrophage, T-suppressor cells develop instead of T-helper cells. It is, therefore, conceivable that in CF patients, abnormal macrophage function results in lymphocyte subpopulation changes in such ways that proliferative responses to PA are abrogated. This is a testable hypothesis which is currently being investigated in our laboratory.

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- Signed informed consent was obtained from patients and normal controls. The research protocol was approved by the Institutional Review Board for Human Investigation.
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