IDENTIFICATION OF THE PRINCIPAL T LYMPHOCYTE-STIMULATING ANTIGENS OF *PSEUDOMONAS AERUGINOSA*

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Pseudomonas aeruginosa is an opportunistic pathogen responsible for chronic infections in patients with underlying injury and disease, such as those with burns and cystic fibrosis (CF).[1] Antibodies to a variety of *Pseudomonas* antigens are produced in these patients (1–4), but the persistent nature of their infections suggests that immune responses are frequently ineffective. The potential role of cell-mediated immunity in limiting *Pseudomonas*[2] infections has been suggested by the work of Pier and Markham (5) and Sorensen et al. (6, 7). In the former study, protection of mice against bacterial challenge was afforded by the adoptive transfer of Thy-1$^+$ spleen cells from *Pseudomonas*-immune donors. In the latter series of reports, T lymphocytes from CF patients in advanced stages of infection were shown to be hyporesponsive to *Pseudomonas* bacterial challenge in vitro. Using crude soluble antigens prepared from several mucoid *Pseudomonas* strains, we too have observed unresponsiveness on the part of CF T cells in vitro.[2] For this reason we have been particularly interested in identifying the antigenic components of the microorganism that are recognized by T cells of normal, healthy individuals.

A number of exoproducts from *P. aeruginosa* have been well characterized with regard to their pathogenic properties (4, 8–12). We were interested, therefore, in the potential antigenicity of these components for human T cells, since this information might be useful in identifying the nature of immune defects in CF. Our experimental approach has been to clone human T lymphocytes responding to the antigens in crude bacterial culture fluids and to test these clones for reactivity to known *Pseudomonas* antigens. Clones of *Pseudomonas*-immune T cells were selected by limiting dilution and expanded with the aid of T cell growth factor (TCGF). They were then analyzed with antigen panels that permitted enumeration of the most frequent clones and identification of their specificities. This approach not only has identified the relevant bacterial antigens,

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[1] *Abbreviations used in this paper:* AP, alkaline protease; CF, cystic fibrosis; E, elastase, LDA, limiting dilution analysis; MC, mononuclear cell; Mϕ, monocyte; PA, *Pseudomonas* aeruginosa; SI, stimulation index; TCGF, T cell growth factor.

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but has yielded information on the immune repertoire diversities of several donors. The results indicate that immune human donors develop predominant repertoire patterns to select *Pseudomonas* antigens, including two proteases secreted by the organism.

Materials and Methods

Cell Preparations. Mononuclear cells (MC) were isolated from the blood of healthy adult immune donors by centrifugation through Hypaque-Ficoll. Adherent monocytes (Mϕ) were prepared as previously described (13), except that Mϕs were irradiated (2,500 rad; ^{137}Cs) rather than being treated with mitomycin.

Antigens. Crude *Pseudomonas* antigens consisted of sterile (0.22 μm Millipore filtered) culture supernatant fluids collected after 48 h of bacterial growth in trypticase soy broth. The bacterial growth medium was not antigenic for human T cells. Crude antigens were titrated against at least five immune donors' MCs for optimum tissue culture doses, (generally found to be 0.1–1 μl/well). *Pseudomonas* alkaline protease (AP) and elastase (E) were purchased from Nagase Co, Ltd. (Tokyo) and *Pseudomonas* exotoxin A was prepared as previously described (14). Inactivation of the enzyme activity of AP involved heating at 65°C for 30 min (15, 16). Total protease activity was measured by the assay described by Rinderknecht et al. (17) using hide azure blue powder substrate, and elastolytic activity was determined as previously described (18). Mumps, tuberculin PPD, and *Candida albicans* were purchased from commercial sources and used at doses reported earlier (13).

Cloning of Pseudomonas-specific Human T Cells. Screening MC donors for immunity to *Pseudomonas* involved culturing MC with various *Pseudomonas* antigens (Table I) under conditions previously described (13) and assessing tritiated thymidine ([^{3}H]TdR) uptake 7 d later. Donors were designated highly immune if the stimulation index, *SI* (cpm, antigen-stimulated cultures/cpm, control cultures) was >10.

Monoclonal T cell populations were isolated and propagated according to Lamb et al. (19) with the following modifications. All cloning and assay medium contained 10% human AB$^+$ serum. After initial stimulation with crude *Pseudomonas* antigens for 6 d, lymphoblasts were purified by a two-step (35%, 45%) discontinuous Percoll gradient and plated under limiting dilution conditions (30–40 wells/dose) in Teresaki Microtest II plates (Falcon Labware, Oxnard, CA). Input cell doses ranged from 27 to 0.11 lymphoblasts/well. After 7 d, the contents of each well were transferred to a 200 μl microculture and expanded for 7 additional days in the presence of 10–20% TCGF, antigen, and 10^5

TABLE I

Characteristics of Pseudomonas Strains Used in this Study

Strain	Properties	Ref.
GoM	Clinical isolate from cystic fibrosis patient	
JoS	Clinical isolate from cystic fibrosis patient (elastase deficient)	
Ka	Clinical isolate from cystic fibrosis patient	
PAO-1	Parental strain	20
PAO-1-T1	Exotoxin A-deficient mutant of PAO-1	13
PAO-1-E64	Altered elastase mutant of PAO-1	18
PAO-1-1641	Protease and elastase deficient mutant of PAO-1	*
Pa-103‡	Protease-deficient parental strain	21
Pa-103-29‡	Exotoxin A-deficient mutant of Pa-103	22

* Howe and Iglewski, unpublished observation.

‡ While Pa-103 and Pa-103-29 produce low levels of AP when grown under certain conditions (22), they failed to produce detectable amounts of AP when grown in TSB under the conditions used for this study.

irradiated, autologous MC. Wells then were scored for growth and Poisson analysis was used to identify those populations arising from single precursors (i.e., input cell doses giving >37% negative wells). Clones were expanded in 15-mm wells containing antigen, 10% TCGF, and 10^6 autologous irradiated (2,500 rad, ^{137}Cs) MC as feeder cells. Subcloning was performed in an analogous fashion on clones aa6, aa10, ff1, and dd3 (see Fig. 1) using an input cell dose of 0.33 cells/well and in all cases verified monoclonality. Typically, two passages (3–4 d each) in 15-mm wells were adequate to expand clones and subclones to populations of $>2 \times 10^7$ cells, at which time they were frozen (−80°C) in cloning medium containing 7.5% dimethyl sulfoxide. Subsequent expansion, when necessary, was always performed with autologous irradiated MC, antigen, and 10–20% TCGF.

TCGF was prepared by stimulating tonsillar lymphocytes (10^6/ml) in 12-mm polystyrene tubes with 0.1% PHA-P (Difco Laboratories Inc., Detroit, MI); the reagent was diluted to a concentration of 10 mg/ml according to the manufacturer's instructions and further diluted 1:100 in culture medium). Culture supernatant fluids were recovered 48 h later, filter sterilized, and used without further purification.

In Vitro Antigen-induced T Cell Proliferation. Specificity assays with uncloned MC (10^5/well) were performed in 96-well flat-bottom plates as previously described (13). To determine the specificity of cloned T cell populations, the cells were thawed, washed three times, and cultured ($1–2 \times 10^4$/well) with irradiated (2,500 rad), autologous adherent Mϕs (10^4/well) and various antigens in 200-μl flat-bottom wells. Generally, better responses were obtained when the Mϕs were precultured with antigen for 16–18 h before addition of the T cell clones to the wells. All specificity assays were performed with cloning medium (19) containing 10% human AB+ serum. Cultures were labeled with 1 μCi [^{3}H]TdR per well for 7 h after 3 d incubation at 37°C. The results are expressed as mean cpm ± 1 SD for quadruplicate cultures.

Limiting Dilution Analysis (LDA) of Clonal T Cell Frequencies. A two-stage LDA was used to maximize detection of proliferative activity. Immune MC were cultured in 96-well round-bottom plates under limiting dilution conditions ($3.1–50 \times 10^3$ MC/well) in the presence of excess (5×10^4) irradiated (2,500 rad), autologous MC and optimal concentrations of antigen. 7 d later, each well was washed three times and the cells were

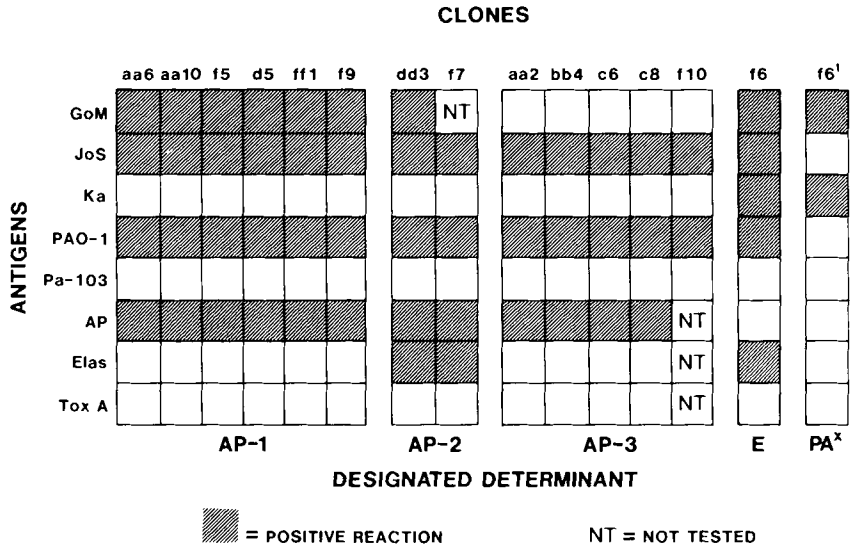

FIGURE 1. Reaction patterns of 15 human T cell clones from two donors selected and expanded with either GoM or JoS antigens (see Table I). The patterns are clustered so as to achieve maximum homology and are assigned specificities that reflect, where possible, reactivity with purified *Pseudomonas* exoproducts. A positive reaction is one in which [^{3}H]TdR uptake exceeded control levels by a factor of 10.

transferred to flat-bottom wells containing 25×10^3 irradiated autologous MC. After an additional 5 d rest, antigen was added and cultures were labeled 2 d later with 1 μCi [^{3}H]TdR for 7–8 h. Controls consisted of MC cultured at the same cell doses in the presence of feeder cells, but stimulated with antigen only during the second in vitro challenge. For all data points, 24–36 replicate wells were assayed. Wells were scored positive if the uptake of [^{3}H]TdR exceeded that of control cultures by 3 SD. Cell doses in which all wells were positive were not included in the frequency calculations. The data were then plotted as the fraction of negative wells as a function of input cell dose by the least squares method. Frequencies were extrapolated for $F_0 = 0.37$ and correlation coefficients and y-intercepts were calculated. The final statistic served as a measure of the number of interacting cell types being titrated.

Results

To assure that a wide range of potential *Pseudomonas* antigens could be examined, we prepared crude culture supernatant fluids from nine mucoid strains, three recovered from cystic fibrosis patients and six that previously had been characterized for their pathogenicity and secreted exoproducts (13, 18, 20–22). Some of the known attributes of these bacterial strains are shown in Table I. Of particular interest were those strains that failed to secrete proteases or toxins, because these would be useful in implicating specific products as T cell antigens.

The antigen preparations were then used to stimulate blood MC from a large number of immune donors. Results with six representative donors (Table II) revealed that most of the preparations were highly mitogenic. Additional experiments not reported here showed that proliferative responses were adherent cell dependent, reached maximum levels on days 6–7 of culture, and were mediated by E rosette-forming lymphocytes. ~20% of the normal donors tested showed reactivity to one or more of the antigens listed in Table II. Only an occasional donor was immune to the protease-deficient Pa-103 preparation, whereas the majority of immune donors responded to PAO-1 (a normal protease-producing strain). This implied that one or more of the *Pseudomonas* proteases was a common antigen.

In an attempt to identify the bacterial antigens responsible for this activity, T

TABLE II

T Cell Proliferative Responses of Six Immune Donors to a Panel of Pseudomonas* Antigens

Antigen‡	Mononuclear cell donors					
	1	2	3	4	5	6
—	59	506	172	219	141	2,618
GoM	8,555	48,812	33,308	50,525	44,098	48,869
JoS	48,974	74,000	11,924	14,724	11,310	31,725
Ka	544§	70,458	13,408	57,911	33,516	64,038
PAO-1	37,500	79,159	8,489	21,005	36,971	71,981
Pa-103	69§	1,266§	265§	1,038§	2,890	13,473§

* Mean cpm ^{3}H-TdR uptake assessed 7-d after initiation of MC cultures. Data represents the optimum responses observed when several doses of the antigens were tested.

‡ All antigens were crude 48-h sterile culture supernatant fluids from growing bacteria. The bacteriological medium (trypticase soy broth) was nonantigenic.

§ Responses differing from controls (without antigen) by a factor less than 10.

cells from donors 1 and 2 (Table II) were selected with the GoM or JoS antigens and cloned by limiting dilution. Limiting dilution cloning after only 6 d of in vitro stimulation assured recovery of a large number of clones with diverse antigen specificities.

Monoclonal populations were then expanded with a minimum number of passages (usually only 3) to large numbers ($\sim 2 \times 10^7$) and frozen in dimethyl sulfoxide-containing medium. Aliquots were later thawed, washed, and assayed for specificity in the presence of freshly prepared autologous antigen-presenting Mϕs. 29 clones were initially isolated from these two donors and tested for specificity. Approximately half (i.e., 14) were unreactive or very weakly reactive to *Pseudomonas* antigens in the absence of TCGF. 15 *Pseudomonas*-specific clones were identified that reacted ($SI > 10$) with the antigens listed in Table I. None of these T cells responded to antigens in the absence of Mϕs.

Fig. 1 shows those reactions that were useful in distinguishing between different clones. The figure also groups the clones into five subsets, presumably representing distinct antigenic determinants (here designated AP-1, AP-2, AP-3, E, and PA^x). 12 clones reacted with purified Pseudomonas alkaline protease (AP) and two of these showed a weak cross-reactivity with elastase (E). One clone (f6) reacted selectively with elastase (E). Three distinct AP determinants were implied by the data and these together constituted the most frequent specificities. For example, determinant AP-1 was present in both GoM and JoS crude antigens and on the purified AP molecule. By contrast, AP-3 was present on pure AP and in the crude JoS preparation, but was lacking in the GoM crude antigen.

A titration of AP against clone GoMaa6 as well as the donor's uncloned MCs revealed that AP was antigenic at very low concentrations (~ 1 ng/ml) and stimulated vigorous proliferative responses (Fig. 2). These responses were adherent cell dependent and could be elicited with AP-pulsed Mϕs (data not shown).

Since the antigen AP was proteolytic, we wanted to exclude the possibility that its mitogenicity was dependent simply on its enzymatic activity. Heat treatment of the enzyme (65°C for 30 min) reduced caseinolytic activity 1,600-fold, but only partially affected antigenicity (Fig. 3). Proliferative responses to AP were suboptimal at doses $>10^{-1}$ μg/well and declined to background levels when doses were increased above 1 μg/well. This inhibition was apparently attributable to enzyme-dependent toxicity, because heat inactivation of the protein reversed the effect. Moreover, addition of high doses of AP to mumps-stimulated cultures inhibited anti-mumps responses as well (Fig. 4). Here too, the immunosuppressive effect was reversed when the AP was first heat treated.

The finding that so many clones from these two donors were AP specific implied that immunity to the protease might be common among *Pseudomonas*-immune donors. Indeed, four of the six donors included in this study were highly immune to the antigen (Table III). To test the hypothesis that AP-specific T cells are frequent among T cells committed to *Pseudomonas* antigens, we measured clonal frequencies by LDA. Dilutions of MC from known immune donors were cultured either with AP, PAO-1, JoS, or mumps antigens for 7 d. Then the cells were washed, rested on fresh irradiated MC, and restimulated with the

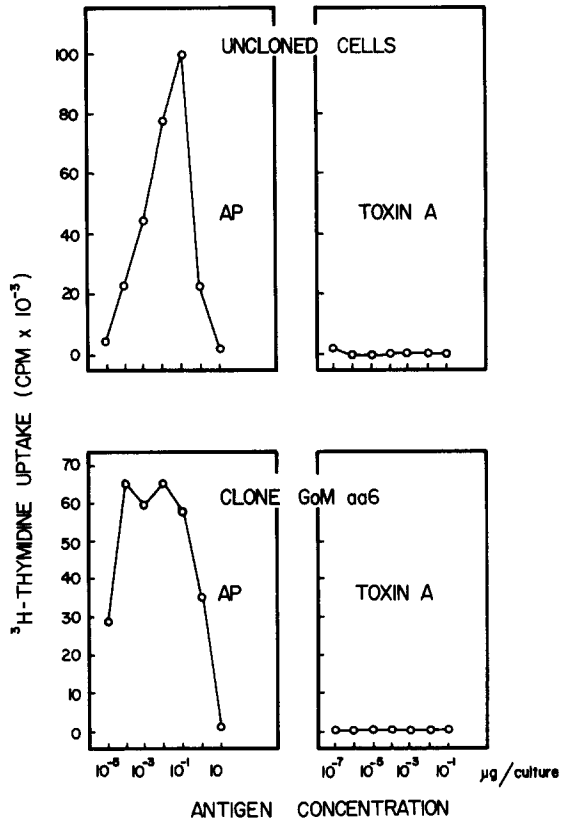

FIGURE 2. Responses of uncloned cells and clone GoMaa6 (both from donor number 1, Table II) to AP and Exotoxin A.

homologous antigen. Proliferation was assessed 2 d later. A typical experiment (Fig. 5) revealed that the frequencies of AP-specific proliferating T cells were comparable to the frequencies of T cells committed to all secreted *Pseudomonas* antigens in the crude PAO-1 preparation. This was a consistent finding (Table IV). The frequencies obtained were also comparable to those calculated for T cells reacting to non-*Pseudomonas* antigens (e.g., mumps antigen, exp. 5; references 23, 24). Values for y-intercepts (y = 0.84–1.06) indicated that a single precursor cell type was being titrated.

As a final test of the hypothesis that most of the T cells of these donors were reacting to AP secreted by *Pseudomonas* organisms, we cloned cells from donor 5 (Table III), using AP as the selecting antigen. We predicted that clones obtained in this manner should show specificity for AP and express the same specificity for the crude antigen panel (Fig. 1) as did the AP-specific clones initially selected with crude antigens. This was indeed the case. Table V summarizes data on six such clones. Five showed reactivity to the GoM, JoS, PAO-1, and AP antigens, while failing to respond to the Ka, Pa-103, or E antigens (i.e., they were AP-1 specific; see Fig. 1). Therefore, by selecting clones with either pure AP or crude *Pseudomonas* antigens, we obtained T cells with the same apparent specificities.

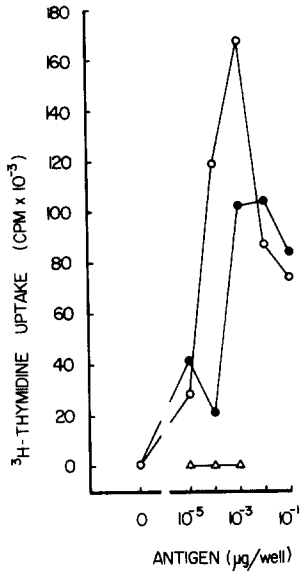

FIGURE 3. Effect of heat inactivation of AP on its mitogenicity. O, alkaline protease; ●, heat treated (65 C/30 min) AP; Δ, E. Reported are proliferative responses of clone GoMaa6.

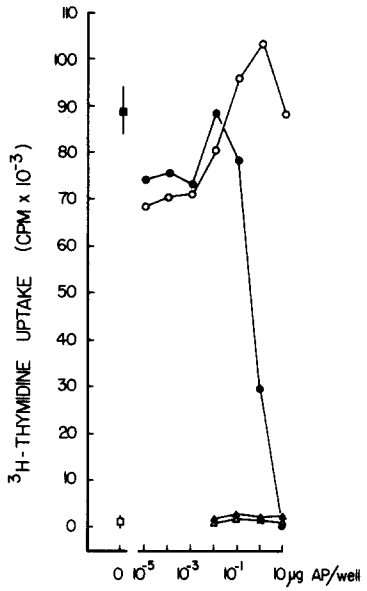

FIGURE 4. Heat inactivation of AP destroys its high dose immunosuppressive activity. Cells from a donor immune to mumps antigens, but showing no response to AP, were cultured with mumps antigens and various doses of AP (●) or heat-inactivated AP (O). [^{3}H]TdR uptake was assessed 7 d later. ▲ and Δ, cultures with AP or heat-treated AP, respectively, but no mumps antigens. □ and ■, control response with and without mumps.

Discussion

Many human donors recognize AP as the predominant T cell–stimulating immunogen secreted by *Pseudomonas* organisms. Several lines of evidence sup-

TABLE III

Uncloned Cells from Pseudomonas-immune Donors Respond to Alkaline Protease

MC donors*	[^{3}H]Thymidine uptake (cpm × 10^3)	
	No antigen	AP (10^{-1} μg per well)
1	1.9 ± 0.4	110.9 ± 10.2
2	0.3 ± 0.1	135.1 ± 7.1
3	0.2 ± 0.1	41.1 ± 14.3
4	0.2 ± 0.2	1.9 ± 3.3
5	0.2 ± 0.2	29.2 ± 8.2
6	2.6 ± 1.2	1.5 ± 0.7

* Same as in Table II.

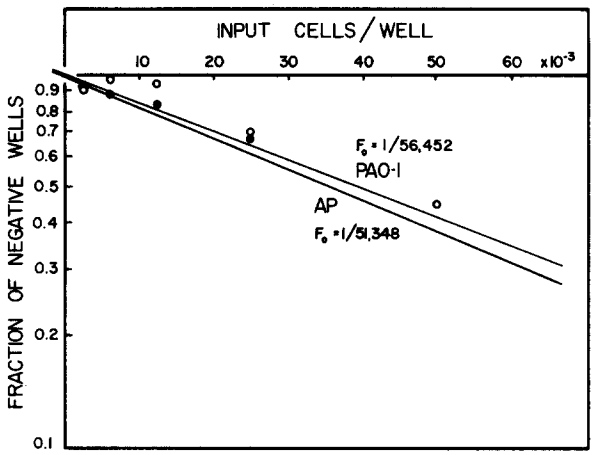

FIGURE 5. Limiting dilution measurement of the frequencies (F_0) of proliferating T cells specific for AP (●) or PAO-1 (○) antigens.

TABLE IV

Limiting Dilution Measurement of the Frequency of T Cells Specific for AP and Other Pseudomonas Antigens in Peripheral Blood

Exp.	Antigen	Clonal frequency	r*	y-intercept‡
1	PAO-1	1.8×10^{-5} (1/56,453)	0.97	1.02
	AP	1.9×10^{-5} (1/51,348)	0.99	0.96
2	JoS	2.4×10^{-5} (1/42,511)	0.94	1.01
	AP	2.6×10^{-5} (1/37,890)	0.99	0.96
3	PAO-1	6.0×10^{-5} (1/16,703)	0.94	0.84
	AP	2.7×10^{-5} (1/37,090)	0.99	1.03
4	JoS	1.3×10^{-5} (1/75,801)	0.99	1.05
	AP	2.5×10^{-5} (1/39,795)	0.94	1.00
5	AP	1.5×10^{-5} (1/67,454)	0.99	1.06
6	Mumps	3.3×10^{-5} (1/30,135)	0.98	0.94

* r, correlation coefficient. At least 4 points were used to define each line.
‡ y-intercept values approaching 1.0 indicate titration of a single cell type.

TABLE V
Fine Specificity of AP-Specific T Cell Clones

Antigen*	Lymphoproliferative response‡					
Clone:	ee2	ee10	ff9	bb2	ee5	ee6
—	8	21	31	13	19	67
GoM (0.4)	31,673	17,572	1,522	63	339	231
JoS (2.0)	7,884	5,100	497	329	253	84
Ka (0.4)	35	141	54	18	9	103
PAO-1 (0.4)	40,018	25,669	2,343	3,618	200	308
Pa103 (2.0)	21	81	3	9	14	17
AP (10^{-2} μg)	121,419	46,948	46,948	30,070	59,200	26,902
E (10^{-2} μg)	52	22	8	22	117	12
TCGF (20%)	80,053	70,184	18,726	38,584	63,580	43,340

* Doses in parentheses are μl or μg/well.
‡ Assessed by ^{3}H-TdR uptake on d3.

port this conclusion. First, soluble crude antigens prepared from *Pseudomonas* strains that were known to produce little or no protease (e.g. Pa-103, Pa-103-29, and PAO-1-1641; reference 22 and Howe and Iglewski, unpublished observations) have consistently failed to stimulate MCs from most donors tested to date. This did not appear to reflect nonspecific toxicity of the preparations, since they did not inhibit MC responses to non-*Pseudomonas* antigens. Second, monoclonal T cells selected for reactivity to soluble crude *Pseudomonas* antigens reacted specifically with purified AP at fairly low doses (10^{-1}–10^{-5} μg/ml). Pure AP also stimulated uncloned MC from four of the six donors included in this report. Third, when absolute measurements of the frequencies of AP-specific blood T cells were made, we found that these were not trivial clones that had simply survived the pressures of in vitro selection. Rather, their numbers among uncloned cell populations were comparable to the frequencies of T cells committed to all released *Pseudomonas* antigens of the JoS and PAO-1 strains. These values (1/67,454–1/37,090 MC) are also comparable to published data on the frequencies of human blood T cells specific for streptokinase-steptodornase, tetanus toxoid, tuberculin PPD, and keyhole limpet hemocyanin (23–26). It should be noted, however, that frequencies calculated in this manner represent minimum values, particularly for the crude antigens that contain inhibitory substances, such as endotoxin, that might interfere with the detection of antigen-reactive T cells (13). Finally, we selected clones from *Pseudomonas*-immune donors with AP and re-tested these for reactivity against the panel of crude antigens. Clones with the same specificity patterns were obtained regardless of whether they were selected with crude or purified AP antigens.

We have considered the possibility that the purified AP used in this study was contaminated with another antigen, particularly *Pseudomonas* E. However, direct measurements of the two enzyme activities have detected <0.1% E contamination of the AP preparation. Three additional observations argue against E contaminating the AP. First, very small quantities of purified AP were antigenic for many of the clones (e.g., 10^{-2} ng/ml), whereas elastase-specific clones, like f6, typically showed optimal responses at doses of 10^{-2}–10^{-1} μg E/ml. Therefore, a minor E contaminant in the AP probably would have been insufficient to activate

the cells. Second, at least one strain of bacteria included in this study (Ka) produced very high levels of E but was nonantigenic for AP-specific clones. Third, although two AP-2 specific clones cross-reacted with E, responses to E were modest (i.e., mean cpm [^{3}H]TdR uptake for AP and E were approximately 42,000 and 6,000, respectively). This is not to say that *Pseudomonas* E is not an important antigen. In fact, clone f6 (Fig. 1), which did not react with AP, reacted vigorously with crude Ka antigen and pure E. Recent analysis of cells from donor 6 (Table II) selected with the PAO-1 antigens has revealed a preponderance of E-specific clones (data not shown).

A number of observations reported here can be explained by postulating that allotypic variations exist between the proteases produced by different *Pseudomonas* strains. For example, clone aa6 recognized a determinant (designated AP-1) present on AP molecules secreted by GoM and JoS strains. By contrast, the AP produced by the GoM strain apparently lacked the AP-3 determinant (Fig. 1). The presence of allotypic markers capable of distinguishing between different *Pseudomonas* strains suggests that T cell clones may prove useful as monoclonal typing reagents in defining antigens associated with pathogenicity traits. Alkaline protease has been implicated in animal models as a virulence factor in *Pseudomonas* infections (12, 27–31), although to our knowledge allotypic variants of the molecule have not heretofore been described. Such information may prove important in identifying bacterial properties and altered host immune responses that permit chronic *Pseudomonas* infections like those in CF. In this regard it is significant that CF patients' blood MC failed to mount proliferative response to Ka and JoS crude antigens despite ongoing pulmonary *Pseudomonas* infections.[2] Because CF patients are known to produce antibodies to AP and E (3), these findings suggest that protease-specific T cells may be involved in limiting such infections.

Summary

To aid in understanding the role of cellular immunity in limiting *Pseudomonas* aeruginosa infections, we have identified some of the principal antigens of the organism that are recognized by human T cells. Clones of T cells were selected in such a manner that they would provide information not only about the identity of *Pseudomonas* antigens, but also the T cell repertoires of immune donors. Most clones were found to be specific for *Pseudomonas* alkaline protease (AP). Such clones could be physically isolated by selecting with crude *Pseudomonas* antigens or purified AP. In either case, their fine specificities were the same when tested against a panel of *Pseudomonas* antigens. The conclusion that AP is the principal immunogen for many donors was confirmed by measuring the absolute frequencies of proliferating T cells committed to AP and all other *Pseudomonas* antigens. Frequencies of AP-specific clones ($1.5–2.7 \times 10^{-5}$) were comparable to those from the same donors that were specific for all secreted *Pseudomonas* antigens ($1.3–6.0 \times 10^{-5}$). These results provide a model system for studying human T cell–mediated immunity to bacteria by identifying discrete antigens and measuring the repertoire diversities of cells responding to them.

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