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Human Polymorphonuclear Leukocyte Interaction with Cyclosporine A

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The effects of cyclosporin A (cyA) on human polymorphonuclear leukocyte function, including phagocytosis, its associated metabolic burst, bacterial killing, and chemotaxis, were evaluated. Both *Pseudomonas aeruginosa* and *Staphylococcus aureus* were used as test particles. Polymorphonuclear leukocytes incubated in 10 and 50 μg of cyA per ml behaved normally with respect to phagocytosis and hexose monophosphate shunt activity at both high (10:1) and low (2:1) *S. aureus*/leukocyte ratios. With a small bacterial inoculum, killing of *S. aureus* was slightly impaired at early times only in the presence of 50 μg of cyA per ml. Phagocytosis and killing of *P. aeruginosa* with both large and small bacterial inocula were unaffected by cyA. Chemotaxis was within normal limits under all conditions. In addition, polymorphonuclear leukocytes from four renal transplant recipients receiving both cyA and prednisone demonstrated normal metabolic bursts and bacterial killing with both small and large inocula of *S. aureus*.

Cyclosporine A (cyA) is a fungal metabolite that is undergoing extensive evaluation as an immunosuppressive agent in organ transplantation (9, 11, 14). Previous immunosuppressive regimens required cytotoxic chemotherapy combined with high doses of corticosteroids. These drugs have many effects (5), with the most significant complication being infection (15). cyA does not cause neutropenia (1), and concomitant steroid dosage is usually much smaller (9). At the University of Pittsburgh School of Medicine, cyA and prednisone are being used as immunosuppressive therapy for liver, heart, and kidney allografts. One case of deep-seated *Staphylococcus aureus* infection and four cases of aspergillosis have occurred in patients in the posttransplant period (4a). As these infections are reminiscent of those seen in chronic granulomatous disease (3, 7), a condition in which human polymorphonuclear neutrophil (PMN) killing is markedly abnormal, a study of the effect of cyA on PMN function seemed to be in order.

MATERIALS AND METHODS

Human PMNs and monocytes were obtained from normal donors by layering heparinized whole blood over a Ficoll-sodium diatrizoate gradient (Sigma Chemical Co., St. Louis, Mo.) (2). The erythrocytes-PMN-rich fraction was harvested, purified by sedimentation through dextran followed by hypotonic lysis of residual erythrocytes (4), and suspended in Hanks balanced salt solution (HBSS M. A. Bioproducts, Walkersville, Md.) at a concentration of 10^6 to 10^7 PMNs per ml. Monocytes were harvested from the gradient interface and used at a concentration of 1×10^6 to 2×10^6/ml. Patient PMN were obtained from consenting donors at various intervals after renal transplantation and processed in the same manner. These individuals were receiving both cyA and prednisone. Autologous serum or plasma was used in all experiments.

S. aureus 502A was a kind gift from Gerald Mandell, University of Virginia, Charlottesville. A serum-resistant strain of *Pseudomonas aeruginosa* was a kind gift from Bruce Farber, University of Pittsburgh, Pittsburgh, PA. Both organisms were grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) for 18 h before experimentation. When ready for use, the bacteria were washed three times in normal saline and adjusted spectrophotometrically to a concentration of 10^9 CFU/ml. In some experiments *S. aureus* D2C was used. Bacteria used in hexose monophosphate shunt activation studies were heat killed.

cyA was a kind of gift from Monto Ho, University of Pittsburgh. Various concentrations of cyA were dissolved in 0.1% dimethyl sulfoxide (DMSO) in HBSS (HBSS-cyA) immediately before use.

The chemoattractant *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) was dissolved in DMSO at a concentration of 10^{-3} M and further diluted with medium 199 to a concentration of 2×10^{-8} M. Zymosan-activated plasma (ZAP) was prepared by incubating autologous plasma with 10 μg of zymosan (Sigma Chemical Co.) per ml for 30 min at 37°C. This suspension was centrifuged at 10,000 rpm for 5 min in a Beckman Microfuge at 4°C.

PMNs or monocytes were incubated in HBSS-cyA at cyA concentrations of 10 and 50 μg/ml at 37°C for 45 min. This time and temperature were chosen because of previous work which showed that these conditions are optimum for other phagocyte inhibitors (17). After being washed, PMNs or monocytes were resuspended in HBSS-cyA and ready for use in the phagocytosis, killing, metabolic, and chemotactic assays. Cell viability remained greater than 95%, as determined by trypan blue exclusion.

Phagocytosis and killing by PMNs and monocytes were evaluated by using differential centrifugation assays (4). Preparations with high (10:1) or low (1:1 or 2:1) bacterium/PMN ratios were added to HBSS-cyA and fresh serum to yield a final volume of 1 ml containing 10% serum. PMNs in polypropylene tubes (Falcon Plastics, Oxnard, Calif.) were tumbled on a rotator at 12 rpm for 120 min at 37°C. At various intervals, samples were removed, and the PMNs were lysed by sonication. Bacterial counts were performed

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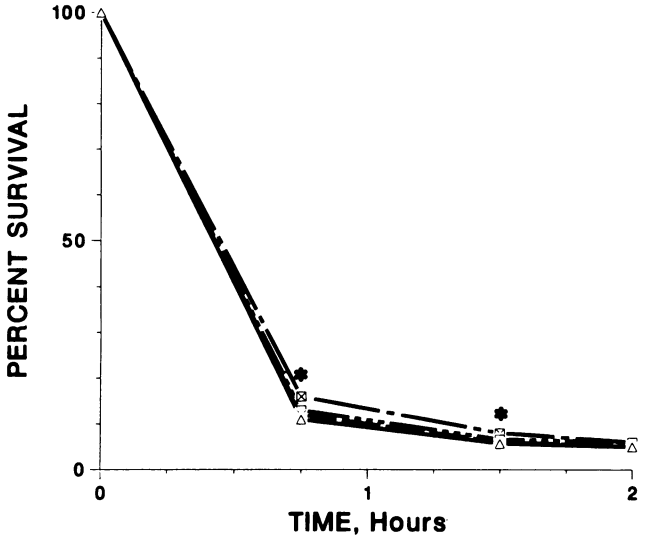

FIG. 1. PMN killing of *S. aureus* at low (2:1) bacterium/cell ratios. DMSO is the diluent for cyA. Symbols: *, $P < 0.05$; △, control; ×, DMSO; □, cyA, 10 μg/ml; ⊠, cyA, 50 μgl/ml.

by plating serial dilutions on Trypticase soy agar. These counts represented total bacteria present at each time period. At the same times, a second sample was removed and centrifuged at 150 × *g* for 15 min at 4°C to sediment the PMNs and PMN-associated bacteria. Plate counts of the supernatant, lysed sediment, and total were determined by manual counting after a 48-h incubation. Phagocytosis was calculated from changes in CFU of the supernatant over time, and bactericidal activity was determined from changes in the number of total CFU over time. All of the above experiments were run in duplicate and repeated a minimum of three times.

In some experiments with *S. aureus*, a modification of the above assay was employed (8). Bacteria and PMNs or monocytes at a 5:1 bacterium/leukocyte ratio were placed in 25-ml Erlenmeyer flasks containing Krebs ringer phosphate,

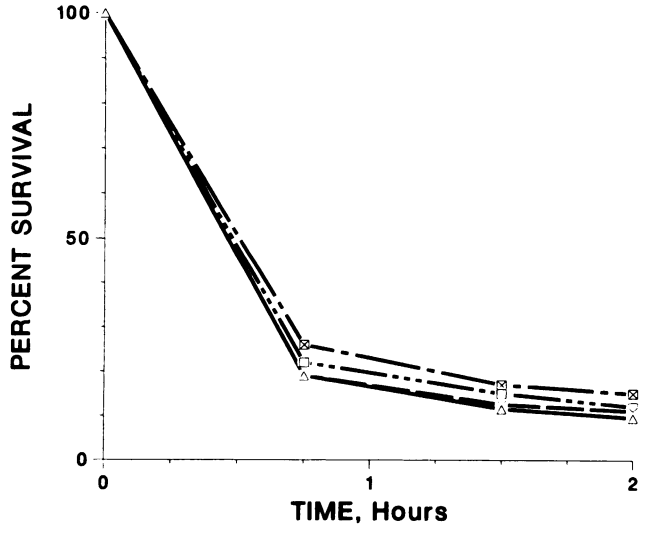

FIG. 2. PMN killing of *S. aureus* at high (10:1) bacterium/cell ratios. DMSO is the diluent for cyA. Symbols same as in Fig. 1.

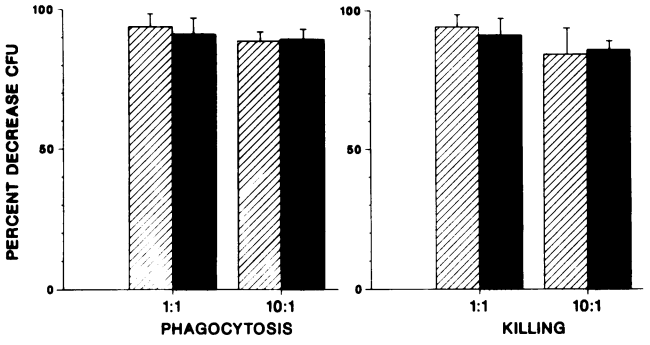

FIG. 3. Phagocytosis and killing of *P. aeruginosa* at low (1:1) and high (10:1) bacterium/cell ratios. At low ratios, phagocytosis and killing were measured after a 2-h bacterium-PMN incubation. At high ratios, results were obtained after a 30-min bacterium-PMN incubation. Control represents DMSO-treated PMNs. Symbols: ▨, cyA; ■, control.

glucose-cyA, and 10% serum. Samples were removed at 0, 45, 90 and 120 min, and the phagocytes were lysed by distilled water. After serial dilutions, 0.01-ml samples were dropped on Trypticase soy agar, and sterile 18-mm-diameter glass cover slips were placed on the drops. Bacterial killing was determined as above. Controls included bacteria, serum, and HBSS-cyA without phagocytes; bacteria and serum plus the diluent for cyA; and phagocytes, bacteria, and serum.

Metabolic activation was determined by incubating leukocytes with [1-^{14}C]glucose in the presence of *S. aureus*/leukocyte ratios of 2:1 and 10:1. The evolved $^{14}CO_2$ was quantitated by established methods (8).

Chemotaxis was measured in a modified 48-well micro-Boyden chamber (6) by placing 25 μl of FMLP or ZAP in the lower wells of the chamber and 40 μl of leukocytes consisting of 2.5×10^6 PMNs per ml or 1×10^6 monocytes per ml in the upper wells. A polycarbonate filter with a pore size of 5 μm separated the upper and lower chambers. The assembled apparatus was placed in a humidified incubator at 37°C for 13 min for PMNs and 90 min in 5% CO_2 in room air for monocytes. The filters then were removed and stained with hematoxylin and eosin. The number of cells migrating to the bottom of the filter was counted, and the number of cells reaching the bottom of the filter in the absence of a chemoattractant was subtracted from this value.

Statistical evaluation. All results are expressed as the mean ± standard error of the mean. The Student *t* test was used to evaluate significance in all experiments.

RESULTS

cyA had no effect on phagocytosis or bacterial killing of *S. aureus* 502A at a bacterial/PMN ratio of 1:1. Phagocytosis and killing of *S. aureus* by PMNs in the presence of 50 μg of cyA per ml (99.3 ± 2% and 94.2 ± 1.9%) were identical to those in 0.1% DMSO (99.2 ± 0.3 and 92.3 ± 2.2%) and to PMNs in HBSS only (99.4 ± 0.1 and 94.7 ± 1.8%). Results when 10 μg of cyA per ml with *S. aureus* 502A was used were similar.

The effect of cyA on bacterial killing of *S. aureus* D2C at inocula of 2:1 and 10:1 at 45, 90, and 120 min is shown in Fig. 1 and 2. cyA at 10 μg/ml had no significant effect on bacterial killing with a small (2:1) or a large (10:1) inoculum at any incubation time. cyA at 50 μg/ml had a small, but significant,

TABLE 1. Effect of cyA on hexose monophosphate shunt activity

Bacterium/ PMN ratio	Hexose monophosphate shunt activity[a]			
	Control	DMSO	CyA, 10 μg/ml	CyA, 50 μg/ml
Resting	5.2 ± 1.3	8.2 ± 2.0	7.0 ± 2.4	8.0 ± 2.0
2:1	41.4 ± 8.6	40.8 ± 6.9	38.8 ± 4.8	37.8 ± 8.6
10:1	139.4 ± 12	141.6 ± 15	134 ± 11.5	121 ± 7.6

[a] Nanomoles of CO_2 per 10^7 PMNs per 30 min.

effect on bacterial killing when the bacterium/PMN ratio was 2:1 at the earlier times. After 45 min of incubation, the percentage of the inoculum killed was reduced from 89 ± 1.6 to 83 ± 2.8, and at 90 min, it was reduced from 94.2 ± 1.1 to 91.8 ± 1.8. By 120 min there was no difference between the groups. When the effect of cyA at 50 μg/ml on monocyte killing of *S. aureus* was evaluated, no difference was seen between control and cyA-treated cells, with 58 and 65% of the inoculum killed, respectively, after 90 min of incubation.

Figure 3 shows the effect of cyA on PMN phagocytosis and killing of *P. aeruginosa*. When a small bacterial inoculum and 50 μg of cyA per ml were used, phagocytosis and killing of the cyA-treated cells (94.4 ± 3.71 and 94.3 ± 3.6%) were equal to those of controls (91.3 ± 5.3 and 91.6 ± 5.4%). Examination at 30 and 60 min likewise revealed no differences. When a large bacterial inoculum and 50 μg of cyA per ml were used, phagocytosis and killing were evaluated at 30 min. cyA-treated PMNs ingested and killed (89.2 ± 2.5 and 84.4 ± 9.0%) equal numbers of bacteria to those of controls (89.6 ± 3.4 and 86 ± 3.2%). cyA at 10 μg/ml likewise had no effect on PMN phagocytosis or killing of *P. aeruginosa* with a large or small bacterial inoculum.

The effect of cyA on hexose monophosphate shunt activity is shown in Table 1. There was no significant reduction in activity at either concentration of cyA with a small (2:1) or large (10:1) inoculum of *S. aureus*.

The effect of cyA on PMN and monocyte chemotaxis is shown in Table 2. Neither PMN nor monocyte chemotaxis to FMLP or ZAP was altered by cyA.

Four patients who had received renal transplants were treated with cyA for 8 to 11 days when blood was obtained for leukocyte function studies. Evaluations of bacterial killing and hexose monophosphate shunt activity are shown in Table 3. Although there was considerable patient-to-patient variability, the mean activity was not significantly impaired for bacterial killing or metabolic activation at low or high bacterium/PMN ratios. One of the patients, however, appeared to show markedly reduced bacterial killing at an *S. aureus*/PMN ratio of 2:1 but not at 10:1. Hexose monophosphate shunt activity was not evaluated in this patient.

TABLE 3. Bacterial killing and hexose monophosphate shunt (HMPS) activity in patients receiving cyA

Bacterium/ PMN ratio	Bacterial killing[a]			HMPS activity[b]
	45 min	90 min	120 min	
2:1				
Controls	89 ± 1.6	94.2 ± 1.1	94.8 ± 1.1	41.4 ± 8.6
Patients[c]	70.5 ± 7.7	74 ± 7.9	82 ± 7	28.7 ± 6.4
10:1				
Controls	80.4 ± 2.2	88.2 ± 2.4	90.2 ± 1.6	139.4 ± 12
Patients	73.8 ± 7.9	82.6 ± 4.7	83 ± 1.8	139.7 ± 15.9

[a] Percentage of inoculum killed at each time.
[b] Nanomoles of CO_2 produced per 10^7 leukocytes per 30-min incubation.
[c] Patients receiving cyA for 8 to 11 days.

DISCUSSION

Phagocyte response in host defense involves chemotaxis, phagocytosis, and intracellular killing (16). The act of phagocytosis is accompanied by a respiratory and metabolic burst in which cellular oxygen consumption is markedly increased and glucose is channelled through the hexose monophosphate shunt pathway (12). We therefore examined the effect of cyA on different facets of this defense system. The 50-μg/ml concentration of cyA represents an amount ca. 45 times the peak blood level and 100 times the steady-state level attained after the usual maintenance dose of 10 mg/kg per day in humans (13). Neither chemotaxis, phagocytosis of *S. aureus*, nor hexose monophosphate shunt activity was impaired at these high concentrations. Interestingly, intracellular killing of *S. aureus* was diminished at early times with low but not with high bacterial ratios.

We also examined the effect of cyA on PMN phagocytosis and killing of *P. aeruginosa*. The reasons for choosing this organism were twofold. First, gram-negative rods are a major cause of morbidity and mortality in the posttransplant period (10, 15). Second, PMN killing of *P. aeruginosa* is not dependent on oxidative killing mechanisms, as the bacteria are killed effectively by PMNs in an anaerobic environment (12). We found no significant effect on phagocytosis or killing with a large or small bacterial inoculum at doses of 10 and 50 μg of cyA per ml. In contrast to the data from when *S. aureus* was used, no differences were found in any parameters studied at early times.

Our in vitro data were further corroborated by examining PMNs and serum from patients maintained on both cyA and prednisone. Again, no effect on bacterial killing or hexose monophosphate shunt activity was found. These studies are encouraging and lend additional support for the optimism surrounding cyA.

TABLE 2. Effect of cyA on chemotaxis

Condition	Chemotaxis[a]			
	PMNs		Monocytes	
	ZAP	FMLP	ZAP	FMLP
Control	18,900 ± 2,400	7,680 ± 770	2,800 ± 425	3,150 ± 1,000
1% DMSO	18,450 ± 2,100	7,970 ± 1,050	2,550 ± 550	3,200 ± 940
cyA, 10 μg/ml	18,400 ± 1,750	7,970 ± 1,250	3,700 ± 1,100	4,000 ± 840
cyA, 50 μg/ml	16,250 ± 1,800	8,900 ± 1,800	3,150 ± 680	3,900 ± 730

[a] Mean number of cells migrating to the lower surface of the filter ± standard error of the mean.

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