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Concanavalin A-Induced Transformation of Mononuclear Cells: Influence of Plasma from Patients Receiving Anti-inflammatory Agents (40846)

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The response of peripheral blood mononuclear cells (PBMC) to mitogen stimulation has been studied in patients with a variety of rheumatic diseases in attempts to illuminate the pathogenesis of disease (1–8). More recently, the response of PBMC from normal individuals to lectin stimulation has been studied after *in vitro* addition of nonsteroidal anti-inflammatory drugs (NSAID) in attempts to explain anti-inflammatory drug activity (9–12). There is evidence suggesting that PBMC responsiveness to lectins may be affected by these drugs (9–15). It is now recognized that some studies investigating immune function of lymphocytes from patients with rheumatic diseases may have been performed at a time when significant blood concentrations of NSAID might have been present, perhaps explaining why conclusions have at times been conflicting and inconclusive (7, 9).

It has been claimed that decreased responsiveness to phytohemagglutinin (PHA) occurred when lymphocytes of patients receiving phenylbutazone were cultured in media containing autologous plasma, while no such effect was observed in patients receiving ibuprofen or indomethacin (9). To confirm and extend this finding, we have examined the effect of plasma from patients with spondyloarthropathies on concanavalin A (Con A)-induced transformation of PBMC from normal individuals; these patients were receiving either NSAID or no therapy at the time of study.

Materials and methods. We compared the effect of plasma from 12 patients with spondyloarthropathies with that of plasma from seven age-matched normal individuals on Con A-induced transformation of PBMC obtained from the latter group. A total of 15 experiments were performed employing varying combinations of patients' plasma with different normal PBMC. Ten of the patients had ankylosing spondylitis and two had Reiter's disease; 11 were HLA-B27 positive. At the time of the study, five patients were receiving phenylbutazone, two ibuprofen and two indomethacin. Phenylbutazone was administered in doses of 100 mg one to three times daily, ibuprofen 400 mg three times daily and indomethacin 250 mg three times daily. All patients had been on this therapy for at least 4 months. Two of the three untreated patients had not received drugs for many months, the other had been off therapy for 1 month. All of the drug-treated patients and one of the untreated patients were judged to have active disease on clinical grounds.

Peripheral blood was collected into syringes containing sodium heparin (The Upjohn Co., Kalamazoo, Mich.) and centrifuged for 15 min at 800*g*. The plasma was decanted, heated for 30 min at 56°C, and centrifuged twice at 800*g* for 10 min, after which the clear supernatant was removed. The buffy coat and red cells were resuspended in medium consisting of RPMI 1640 (GIBCO, Grand Island, N.Y.), 100 units/ml penicillin G, 100 μg/ml streptomycin sulfate and 2.5 ml of a 1 *M* Hepes buffer (GIBCO, Grand Island, N.Y.) per 100 ml. The PBMC from normal individuals were then separated by Ficoll/Hypaque (Sigma Chemical Co., St. Louis, Mo./Winthrop Laboratories, New York, N.Y.) gradient centrifugation. The interface layer of cells was removed and washed three times in medium, after which suspensions of 1×10^6 PBMC/ml containing 15% of either autologous normal plasma or plasma from patients were prepared.

Cell cultures were prepared as follows: 0.2 ml of cell suspension was placed in flat-bottom wells of Micro-Test II Falcon tissue culture plates. Ten microliters of Con A solutions ranging in amounts from 3.75 or 7.5 to 30 μg was added to each well in tripli-

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cate or quadruplicate, after which the plate was sealed with pressure-sensitive film and incubated at 37°C for 64 hr. Five hours prior to termination of culture, 10 μl of methyl-[^{3}H]thymidine (Schwarz-Mann, Orangeburg, N.Y.), specific activity 1.9 Ci/mmole, was added to each well.

A solution of Con A (Pharmacia, Uppsala, Sweden) was prepared and sterilized by passage through 0.45-μm Millipore filters. A stock solution of 10 mg/ml was prepared and stored at -20°C until required.

Cells were aspirated onto glass-fiber filter paper by means of a semiautomated multiple sample harvester (Otto Hiller Co., Madison, Wis.). The papers were dried for 30 min in a warming oven, after which the disks arising from the O ring impression were punched into scintillation vials containing 5 ml Scinti Verse (Fisher Scientific Co., Fairlawn, N.J.) and counted for 10 min in a Packard Model 3320 Tri-Carb scintillation spectrometer.

The arithmetic mean of the counts per minute for each set of triplicate or quadruplicate cultures was determined and used to plot the dose–response curve. Lymphocyte responsiveness was assessed by two means: (a) direct measurement of the area described by the dose–response curves and (b) response to a single suboptimal dose of Con A (7.5 μg/well), based on the findings of Goodwin *et al.* (15). The difference between the responsiveness of normal PBMC cultured in autologous plasma (N/A) and in plasma from patients (N/P) was determined and expressed as ΔR. Statistical significance was determined by paired sample analysis and the t test (16).

All plasma samples were tested for the concentration of C-reactive protein (CRP) by radial immunodiffusion (17). Glycoprotein levels in the plasma of nine patients were determined through the kind assistance of Dr. C. Willis of the Cleveland Clinic Research Foundation. Total plasma phenylbutazone concentrations were determined by Dr. G. Lukas of the Ciba–Geigy Corporation according to the method of Burns *et al.* (18).

Results. Table I shows the response to Con A of normal PBMC cultured in autologous plasma compared with that of cells cultured in plasma from untreated patients or from patients treated with phenylbutazone. By paired sample analysis a significant difference in ΔR was found for the phenylbutazone-treated group ($P < 0.001$). Similarly significant differences were found whether the area under the curve was determined from point to point drawings between the mean counts per minute for each Con A concentration or from computer-drawn curves which followed best-fit analysis. Comparison of the responses to 7.5 μg of Con A, a suboptimal dose, similarly showed a significant decrease in PBMC response for the phenylbutazone-treated group ($P < 0.05$).

In three experiments using plasma from ibuprofen-treated patients, decreased responsiveness was observed. In contrast, increased responsiveness was noted in the two experiments in which PBMC were cultured in plasma from patients receiving indomethacin. In these experiments employing plasma from ibuprofen or indomethacin-treated patients, statistical analysis was not performed due to insufficient numbers of samples.

Diminished responsiveness to Con A in plasma from phenylbutazone-treated patients might be attributed to binding of the lectin by plasma glycoproteins, whose concentrations may be increased in individuals with severe inflammation. Evidence that this was not the case was provided by the results of dose–response curves. If plasma from the patients bound to Con A more than normal plasma did, then the area under the curve would have been the same but shifted to the right. This was not found, indicating that the decrease in Con A responsiveness was not due to binding to plasma proteins. Two additional methods were employed to rule out this possibility. Glycoprotein concentrations were directly measured in nine patients. In addition, as an indirect measure of the acute phase response, serum CRP concentrations were determined in all plasma samples. No correlation between altered mitogen responsiveness and either CRP or glycoprotein concentrations was found.

In four of the five experiments employing

TABLE I. RESPONSIVENESS TO CON A OF NORMAL PBMC CULTURED IN AUTOLOGOUS PLASMA OR PLASMA FROM UNTREATED, OR PHENYLBUTAZONE-TREATED PATIENTS

Plasma source	Expt No.	Responsiveness (area under dose–response curve, cm^2)			Total phenylbutazone concentration (μg/ml)
		N/A[a]	N/P[b]	ΔR[c]	
Treated patients	1	6.25	5.39	0.87	73.5
	2	3.46	2.73	0.73	45.5
	3	4.40	3.77	0.63	23.5
	4	4.40	3.86	0.54	14.5
	5	4.33	3.86	0.47	88.0
			($P < 0.001$)		
Untreated patients	6	3.38	2.76	0.62	
	7	3.38	4.22	−0.84	
	8	4.33	4.46	−0.13	
	9	4.33	4.10	0.23	
	10	4.33	3.60	0.73	
			N.S.[d]		

[a] Normal PBMC cultured in 15% autologous plasma.
[b] Normal PBMC cultured in 15% patient plasma.
[c] Response in autologous plasma minus response in patient plasma.
[d] Not significant.

plasma from patients receiving phenylbutazone, a rough correlation between plasma phenylbutazone concentration (Table I) and degree of altered responsiveness appeared to exist.

Discussion. The question addressed in this study was whether the plasma of patients receiving phenylbutazone, a potent NSAID, would suppress PBMC responsiveness to Con A, compared to plasma from normal individuals. As controls, plasma from untreated patients and from patients receiving other NSAID were studied. We found that plasma from phenylbutazone-treated patients caused decreased PBMC responsiveness compared to plasma from normals, while plasma from untreated patients did not significantly alter responsiveness to Con A. Our finding that normal PBMC achieved the same response to Con A whether cultured in autologous normal plasma or in the plasma of untreated patients with spondyloarthropathies suggests that plasma factors related to disease in these patients do not influence the PBMC response to mitogenic stimulation. This finding serves as a control for studies of patients receiving medication.

Most previous studies of drug effect on PBMC responsiveness to mitogens have employed *in vitro* addition of NSAID. In our experiments, the effect of whole human plasma from drug-treated patients was studied. While such a system permits evaluation of the effects of drug concentrations ordinarily present *in vivo* in plasma of treated patients, it is possible that substances may be present in plasma, unrelated to drug administration, which might effect Con A responsiveness of PBMC. This difficulty is compounded by uncertainties as to the mechanism of action of NSAID.

It is uncertain whether the effect of plasma from phenylbutazone-treated patients is mediated through phenylbutazone, its metabolites, or through nondrug-related constituents of plasma from these patients. Our finding that phenylbutazone concentration may correlate with decreased responsiveness suggests, but does not prove, that phenylbutazone itself may be the effective agent. Alternatively, the effects of patients' plasma upon the Con A response need not necessarily be attributed to drug or drug metabolite effects. Patients receiving phenylbutazone generally tend to have more symptomatic disease than untreated patients or those receiving other, potentially less toxic drugs. It may be that factors

are present in these patients' plasmas related to severity of disease, which affect the responsiveness to Con A.

The major known physiologic effect of phenylbutazone and other NSAID which might be expected to influence cellular responsiveness is inhibition of cyclooxygenase, a critical enzyme for the synthesis of prostaglandins (PGs) (19). While there is abundant evidence suggesting that PGs of the E type decrease the mitogenic response of PBMC (14, 15, 20–23), the effects of inhibition of PG synthesis on PBMC response to various lectins have not been entirely elucidated. Several workers have shown that *in vitro* addition of inhibitors of PG synthesis, such as phenylbutazone and aspirin, produced a dose-dependent inhibition of PBMC responsiveness. Our findings are consistent with data reported in such studies (9–13). However, it is far from certain that the effects of phenylbutazone necessarily involve effects on PG synthesis; there is evidence of enhanced responsiveness when the cyclooxygenase inhibitor indomethacin was added *in vitro* to PBMC suboptimally stimulated with PHA (14, 15). This finding is consistent with our observation that sera from two indomethacin-treated patients appeared to enhance responsiveness.

Our studies suggest that plasma from patients receiving phenylbutazone diminish PBMC responsiveness to the mitogenic effects of Con A. They suggest caution in presuming that effects on lymphocyte transformation are mediated through an effect on PG metabolism alone, in experiments involving *in vitro* addition of NSAID.

Summary. The effects of plasma from patients with spondyloarthropathies on Con A-induced transformation of PBMC from normal individuals were evaluated. Plasma from untreated patients did not produce a significant difference in responsiveness when compared with normal autologous plasma, while plasma from patients treated with phenylbutazone caused a significant decrease in PBMC responsiveness. It is not clear whether this phenomenon is mediated by effects on prostaglandin metabolism or through other mechanisms.

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