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Evaluation of the *in Vitro* Production of Interferon γ and Other Lymphokines in Uremic Patients (42464)

S. YOUSEFI, N. D. VAZIRI, G. CARANDANG, AND T. CESARIO

Department of Medicine, University of California, Irvine, California 92717

Abstract. It has been suggested that a deficient immune response can be responsible at least partially for the high risk of infections and neoplasia in uremic patients. Since interferon (IFN) is critical to the immune response, we have evaluated the *in vitro* production of IFN-γ and other lymphokines by peripheral blood mononuclear cells (PBMC) drawn from patients with end-stage renal disease and appropriate controls. We have correlated production of lymphokines by these cells with proliferative response to different mitogens. It was found that the secretion of IFN-γ in response to all three mitogens was elevated in these patients compared with the control group. This elevation was significant with both phytohemagglutin and staphylococcal enterotoxin A, but not with Con A. No significant difference was observed in production of lymphotoxins, IL-2, and leukocyte migration inhibition responses. In contrast the proliferative response appeared diminished in the PBMC of uremic patients. We concluded that defective lymphokine generation is not a major immunological problem in patients with end-stage renal disease. Indeed, they appear to release excess amount of IFN-γ which is known to be a macrophage-activating factor. It is suggested that high IFN-γ activity could enhance the secretion of IL-1 or endogenous pyrogen and result in development of febrile reactions in dialysis patients.

Despite many recent advances, infection (1) and neoplasia (2) continue to be the major threats to patients with end-stage renal disease (ESRD) undergoing maintenance hemodialysis. Previous studies have suggested that impaired cellular immune response, nonspecific uremic toxins and malnutrition may be at least partially responsible for the increased risks of both infection and malignancy. Most of the immunologic studies performed to date have relied on measurements of lymphocyte proliferative responses (3–7), mixed-lymphocyte reactions (8, 9), delayed-type hypersensitivity to common antigens (10), skin transplantation (11, 12) and phagocytic activity (13, 14). A few studies on antibody-dependent lymphocytotoxicity (ADCC) and spontaneous lymphocytotoxic (NK cells) activities also have been reported. (15). The results of these studies are not conclusive and often contradictory. Therefore, the need for a better understanding of the immunological defenses in ESRD patients is evident.

The fact that lymphokines released by stimulated T lymphocytes play a major role in coordinating the immune system prompted us to measure the release of several lymphokines, including immune interferon (IFN-γ), lymphotoxins (LT), lymphocyte migration inhibition factor (LMIF), and interleukin 2 (IL-2) from the peripheral blood mononuclear cells (PBMC) of patients with ESRD. PBMC of these patients were stimulated with three different mitogens, phytohemagglutinin (PHA-P), concanavalin A (Con A), and staphylococcal enterotoxin A (SEA) in order to evaluate lymphokine production in ESRD patients. We believe that assessment of these parameters of cellular immunity may aid in understanding the pathogenesis of infections and neoplasms in ESRD patients.

Materials and Methods. *Patients.* Twelve patients with ESRD (seven men, five women) aged 18 to 68 years (average 41.8 years) were included in the study. The etiology of ESRD included chronic glomerulonephritis in five patients, diabetic nephropathy in three patients, hypertensive nephrosclerosis in two, chronic interstitial nephropathy in one, and Henoch-Schonlein purpura in one patient. Duration of dialysis ranged from 6 months to 14 years and dialysis schedule consisted of 3- to 4-hr treatments three times weekly. Hemodialysis was performed using hollow fiber cuprophane dialyzers and single-pass dialysate delivery systems. Dialysate contained Na, 140 mEq/liter; Ca, 3.5 mEq/liter; K, 0–2 mEq/liter; Mg, 1.5 mEq/liter; Cl, 107 mEq/liter;

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Acetate, 38 mEq/liter; glucose, 200 mg/dl. Anticoagulation was achieved using systemic heparinization during hemodialysis. Dialysate flow rate was 500 ml/min while blood flow rates ranged from 200 to 300 ml/min. Sodium, potassium, and water restrictions were prescribed to control volume overload and hyperkalemia. No caloric or protein restrictions were imposed. Multivitamins and folate supplements were given to prevent deficiency states and aluminum carbonate was used to control hyperphosphatemia. Hypertension was controlled by salt and fluid restriction and ultrafiltration. In addition antihypertensive agents were prescribed when indicated. Oral or parenteral iron compounds were administered in patients with iron deficiency. Blood transfusions were given sparingly in those with severe symptomatic anemia. Unstable patients, those with discernible infections and autoimmune disorders, were excluded from the study. None of the patients were receiving corticosteroids or immunosuppressive medications at the time of the study and for several months previously. Predialysis BUN and creatinine values ranged between 70 and 80 mg/dl and 9.3 and 13 mg/dl, respectively.

Control group. Eleven age- and sex-matched healthy individuals were studied simultaneously for comparison. Blood was collected from controls by aspiration into heparinized tubes and immediately returned to the laboratory. Control specimens were handled identically to those of the dialysis patients described below.

PBMC stimulation and lymphokine inductions. Thirty-milliliter arterial blood samples were obtained from the arteriovenous fistula in heparinized tubes (preservative free) shortly before dialysis. PBMC were separated from the blood samples by centrifugation on Ficoll–Hypaque gradients as described by Boyum (16). The mononuclear cell layer was aspirated and washed three times with PBS solution and resuspended to RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 200 m*M* L-glutamine (complete medium). Finally, cell counts were adjusted to a concentration of 5×10^6/ml in complete medium. The viability of the cells was 98% as determined by trypan blue dye. PBMC preparation (2 ml) was stimulated independently with 8 μg/ml PHA-P, 8 μg/ml Con A, and 0.8 μg/ml of SEA. These concentrations were utilized because preliminary studies (Table I) found them to be optimal for proliferative responses and lymphokine induction. Cells were incubated for 3 days at 37°C as previously described (17). The viability of the cells was rechecked after incubation was terminated. Portions of mitogen-stimulated and unstimulated cell suspensions were used to assess the proliferative response as described below. Supernatants of stimulated and unstimulated cells were centrifuged and harvested for subsequent lymphokine assays.

Proliferative response. The proliferative responses were determined by uptake of [H^3]thymidine. At the end of 3 days incubation, 180 μl stimulated and unstimulated PBMC was pulsed 4 hr with 0.5 μCi/well of [H^3]thymidine having a specific activity of 6.7 Ci/mmole (ICN, Irvine, CA). Cells then were harvested onto glass filter paper using a multisample automatic cell harvester and counted in a liquid scintillation counter (Beckman LS 100C). All experiments were run in duplicate.

IFN-γ assay. IFN-γ levels were titered by a microtiter method described previously (17). Briefly, the assay is based on the inhibition of viral cytopathic effects (CPE) on WISH cells

TABLE I. RELATIONSHIP BETWEEN THE CONCENTRATION OF MITOGEN AND IMMUNOLOGICAL RESPONSE[a]

Concentration (μg/ml)	PHA	Con A	SEA[b]
	Proliferative responses (cpm × 10^3)		
0	.9 ± .7	.3 ± 0	.3 ± 0
2	1.9 ± .7	1.5 ± .0	13.1 ± 1
4	5.0 ± .3	8.4 ± 1	16.4 ± 3
8	26.3 ± 5	8.5 ± 1	16.9 ± 3
16	20.3 ± 4	2.6 ± .3	5.2 ± 1
32	21.3 ± 4	3.4 ± .3	9.3 ± 2
	Interferon titers (units/ml)		
2	160 ± 10	80 ± 10	160 ± 10
4	320 ± 0	80 ± 0	160 ± 0
8	320 ± 10	160 ± 0	640 ± 0
16	320 ± 10	80 ± 10	160 ± 10
32	160 ± 10	40 ± 10	320 ± 0

[a] Summary of three experiments.

[b] For SEA, the concentrations used were those given in far left-hand column divided by a factor of 10. Hence rather than a concentration of 2 μg/ml, the concentration was 0.2 μg/ml.

(human amnion cell line). Confluent monolayers of WISH cells were exposed overnight to serial twofold dilutions of IFN-γ in 6-mm wells of a 96-well culture plate, challenged with vesicular stomatitis virus (VSV), and incubated for 24 hr at 37°C. The plates then were read by light microscopy and the titer of the IFN was considered to be the last dilution of IFN which inhibited cell destruction by 50% as compared to the control cells. This crude titer was corrected to an internal reference standard prepared in our laboratory by inoculating eggs with Newcastle disease virus (NDV). This internal standard was titrated against international standard from the National Institute of Allergy and Infectious Disease (NIAID), NIH (Bethesda, MD). Each unit of IFN in our system was equal to 1.5 units of international standard. Our results are expressed in international reference units (IU/ml).

Lymphotoxins assay. Lymphotoxin levels were determined by methods previously reported by others (18) with a minor modification. Monolayers of mitomycin C-treated mouse L 929 cells were exposed to serial twofold dilutions of samples on the bottom of 96-well microtiter plates. After overnight incubation, plates were stained with gentian violet, washed thoroughly with tap water, and dried completely. The bound dye was eluted by ethanol-HCl mixture and measured colorimetrically in a Titertek Multiscan plate reader. The lymphotoxin titers were estimated by plotting the absorbance as a function of the lymphotoxin dilution and measuring the endpoint graphically as that dilution of lymphotoxin which inhibited cell lysis by 50% (or 50% reduction in dye uptake). A standard lymphotoxin preparation generously provided by Dr. G. Granger (University of California, Irvine) was employed in every assay. The results are expressed in units per milliliter (U/ml).

LMIF assay. LMIF levels were measured by methods previously described by Clausen (19). The LMIF effect of supernatant preparations was tested on leukocytes obtained from buffy coat fractions of normal human blood donors (American Red Cross). Buffy coats were diluted 1:2 with phosphate-buffered saline (PBS) and 8 ml of diluted buffy coat was mixed with 3 ml of 5% Dextran 250 (in saline) in 115 × 13-mm polystyrene tubes. Tubes were incubated at 37°C for an hour at which time leukocyte-rich supernatants were collected and washed twice with PBS and once with TC 199 medium supplemented with 10% horse serum, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 200 m*M* L-glutamine (complete TC 199 medium). The cells were resuspended in the different culture supernatant fluids at a leukocyte concentration of 2.5×10^8 cells/ml and incubated at 37°C for 1.5 hr. After incubation, a volume of 10 μl from each of the cell suspensions was placed in individual wells cut into agarose plates and having a diameter of 2 mm. The migration area around the wells was measured under an inverted microscope. The degree of migration inhibition was expressed as the migration index which indicates the ratio between the mean squared radius calculated from four wells of mitogen-stimulated cultures and that calculated from the same number of unstimulated control wells. For calculating this mean radius, the distance between the furthest point of leukocyte migration and the edge of the cut well was measured at two horizontal and two vertical directions for each of the four replicate wells containing the individual samples. Thus the mean radius was calculated from a total of 16 different measurements.

IL-2 assay. IL-2 levels were titrated by the microtiter method of Gillis (20). This method is based on the [H^3]thymidine incorporation of a cytotoxic T-cell line (CTLL). Serial twofold dilutions of the samples were made in 96-well microtiter plates using complete RPMI 1640 medium. This medium was supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 200 m*M* L-glutamine. Complete RPMI 1640 medium was enriched with 10% IL-2 for maintenance of CTLL cells. CTLL cells were washed free of maintenance medium and resuspended into complete RPMI 1640 medium at concentration of 1×10^5 cells/ml. A volume of 100 μl of the cell suspension was added into each well of microtiter plate containing diluted samples. Plates were incubated overnight at 37°C. Then cells were pulsed with [^{3}H]thymidine as described above and harvested onto glass filter paper using a multiple automated cell harvester. Subsequently filter papers were counted in a liquid scintillation counter. The IL-2 titer

was determined by plotting cpm as a function of the IL-2 dilution and estimated the endpoint graphically as that dilution of IL-2 which enhances CTLL cell growth by 50% (50% increase in uptake of the [^{3}H]thymidine). A standard IL-2 preparation purchased from Amgen, Inc. (Los Angeles, CA) was included in every assay. The results are expressed in units per milliliter (U/ml).

Statistical analysis. Data in this study were analyzed by the unpaired Student *t* test working at 95% confidence interval ($P < 0.05$).

Results. Table II demonstrates the results of these studies. It can be seen that the proliferative responses in PBMC from patients tended to be lower than those from controls, although only with PHA-P was the difference statistically significant. Conversely, titers for IFN-γ tended to be higher when PBMC from patients with ESRD were induced as compared to controls. This difference was significant when both PHA and SEA were used as inducers. Lymphotoxin, LMIF, and IL-2 reponses did not differ significantly between PBMC from patients and controls; however, limited volumes of supernatants precluded IL-2 and LMIF analysis from being performed on samples from tests utilizing each of the three mitogens. It thus was necessary to perform IL-2 and LMIF determinations on samples induced using only one of these mitogens. The magnitude of the proliferative response and lymphokine production showed no significant correlation with predialysis BUN and serum creatinine values. Likewise the nature of the underlying disease did not seem to influence significantly the parameters studies.

Comments. The high incidence of infection and cancers in patients with ESRD may be related to a depressed immune system. Since in recent years lymphokines have received considerable attention as immunoregulatory agents for both T-cell and B-cell responses, we felt it would be appropriate to assess simultaneously mitogens in patients with ESRD. In this study, we have demonstrated a significant depression of the lymphocyte proliferation in response to PHA-P compared with normal controls. These results contrast with those of Byron *et al.* (6) who found normal proliferative

TABLE II. DIAGNOSES OF PATIENTS WITH END-STAGE RENAL DISEASE INCLUDED IN THIS STUDY

Patient	Sex	Age (years)	Duration of dialysis (monthly)	Diagnoses	Medication
CS	F	68	3	Nephrosclerosis	Digoxin, allopurinol
GC	F	64	4	Chronic interstitial nephritis	—
TC	M	63	2	Diabetes	Insulin
SA	M	50	96	Malignant nephrosclerosis	Minoxidil, propranolol
VV	M	53	8	Nephrosclerosis	Nitroglycerin, hydroxyzine
RD	F	51	9	Diabetes	Insulin lente, propranolol
PR	M	34	122	Chronic glomerulonephritis	Minoxidil, propranolol
NM	M	22	6	Thrombotic microangiopathy	Clonidine, Fe So4
LL	F	18	24	Chronic glomerulonephritis	Propranolol
VH	M	24	3	Chronic glomerulonephritis	Atenolol, clonidine
LD	F	25	168	Chronic glomerulonephritis	Clonidine
VR	F	30	3	Diabetes	Clonidine, amitriptyline, furosemide

Note. Patients with discernible infections and autoimmune disorders were excluded from the study. None of the patients were receiving immunosuppressive medications at the time of the study and for several months prior to the study.

TABLE III. PROLIFERATIVE RESPONSE AND LYMPHOKINE PRODUCTION IN PERIPHERAL BLOOD MONONUCLEAR CELLS FROM UREMIC PATIENTS AND NORMAL CONTROLS

Assay		PHA (8.0 μg/ml)	Con A (8.0 μg/ml)	SEA (0.80 μg/ml)
Proliferative response (cpm × 10^3)	Patients	11.2 ± 8.6	6.5 ± 5.8	8.5 ± 9.9
	Controls	30.6 ± 18.4	10.8 ± 7.1	14.8 ± 7.4
		($P < 0.01$)	($P > 0.05$)	($P > 0.05$)
IFN-γ (U/ml)	Patients	206 ± 142	719 ± 79	3,661 ± 2135
	Controls	95 ± 23	484 ± 192	1,792 ± 896
		($P < 0.05$)	($P > 0.05$)	($P < 0.025$)
Lymphotoxins (U/ml)	Patients	3.4 ± 2.7	8.4 ± 7.3	38.4 ± 27.5
	Controls	11.4 ± 1.5	20.0 ± 22.6	70.0 ± 55.9
		($P > 0.05$)	($P > 0.05$)	($P > 0.05$)
LMIF (mitogens/PBS)	Patients	Not tested	0.50 ± 0.48	Not tested
	Controls		0.41 ± 0.26	
			($P > 0.05$)	
IL-2 (U/ml)	Patients	Not tested	Not tested	19,268 ± 11.454
	Controls			14,899 ± 12,342

responses to mitogens in patients with ESRD and those of Daniels *et al.* (21) who reported an increase in this response. However, two groups (10, 22) have observed significant reductions in the PBMC proliferation response to mitogens in these patients in accord with our own data. Since we have tested patients and controls simultaneously and have used the same fetal calf serum for the entire experiments, the changes in the mitogen response must be due to changes in the lymphocytes rather than in the autologous plasma as reported by Nelson *et al.* (23). In this regard, they have reported hemodialysis removed molecules (e.g., IL-2) necessary for lymphocyte responsiveness and mitogenic proliferation. Undoubtedly, the use of different assay systems for the lymphocyte proliferation response can contribute to the disparate results in the proliferation response of patients with ESRD.

Since we did not find significant differences in the release of this lymphokine by prehemodialysis PBMC in response to Con A and pokeweed mitogen in our previous measurement of IL-2 levels in patients with ESRD (24) compared with normal controls, we thought it would be important to measure other lymphokines such as IFN-γ, lymphotoxins, and LMIF as well as IL-2. Such assays were performed simultaneously using various mitogens for a better evaluation of immune mechanisms in patients with ESRD. Of interest was the fact that IFN-γ was the only lymphokine whose mean titer produced in response to PHA and SEA was elevated in PBMC from ESRD patients as compared with the mean titer of PBMC from the control group. While the titer of IFN-γ induced with Con A was higher in patients with ESRD, the difference was not statistically significant. This elevation was statistically significant when PHA-P or SEA was used as the inducing agent.

Previous reports have not examined the production of IFN-γ by PBMC from uremic patients; however, it has been reported that the uremic state results in a consistent suppression of IFN-α production. These earlier authors have suggested that either a factor in uremic serum depressed the production of IFN-α or a defect existed in the cells of uremic patients which precluded the normal production of IFN-α (25).

In examining our results, we cannot find a direct correlation between the proliferative response and IFN-γ secretion in the patient population. Indeed, the correlation is almost inverse. These findings are not unexpected since it has been shown that the proliferation response is disassociated from the production of IFN-γ (17, 26–29).

In contrast to the IFN-γ titer, the mean titer of lymphotoxin in response to all three mitogens and the mean titer of LMIF in response to Con A (the only mitogen tested) were slightly lower in patients compared with normal controls, but this reduction was not sta-

tistically significant. Our findings may suggest some disassociation in IFN-γ production and production of other lymphokines, particularly lymphotoxins. The PBMC producing maximal amounts of IFN-γ may not be identical with those PBMC releasing lymphotoxins of LMIF. It is also possible that the different PBMC populations are differentially affected by the uremic state. It could also be suggested that genes coding for lymphocyte proliferation and production of lymphotoxins and possibly LMIF are under the control of the same common regulatory gene(s) which is different from the genes regulating IFN-γ production, or that dialysis removes a factor(s) which is inhibitory to the production of IFN-γ. While limited volumes of samples precluded the evaluation of IL-2 production in response to PHA-P and Con A, the mean titer of IL-2 in response to SEA was slightly higher in patients compared with normal controls. This difference was, however, not statistically significant.

The proliferative responses and lymphokine production did not show a significant correlation with BUN or serum creatinine levels. It should be emphasized, however, that all participants had end-stage renal disease and dialytic and dietary prescriptions had been tailored to provide an adequate uremia control for each patient. Accordingly, despite mild predialysis variations of BUN levels, the degree of uremia control was approximately similar in all cases studied. This may account for the failure to show significant correlation between BUN and creatinine levels with the lymphocytes responses studied. The nature of the underlying disease responsible for ESRD did not seem to have a significant effect on the lymphocyte responses evaluated in this study. This may be due to the small number of cases in each subgroup which may have masked additional changes associated with the underlying process. Further studies of large groups of diabetic and nondiabetic patients with ESRD are required to provide definitive information concerning the possible effect of the underlying etiology of ESRD.

Taken together, the data in our experiments demonstrate significantly lower PBMC proliferation in response to at least certain mitogens (PHA-P) in patients with ESRD compared with normal controls, while the secretion of IFN-γ in these same patients is elevated in response to at least PHA-P and SEA. These findings may very well be the result of the suppression of certain T-cell subsets or the stimulation of other T-cell subpopulations in ESRD patients resulting in the high level of IFN-γ production.

The implications of this work are several. First it is apparent that defective lymphokine generation does not appear to be an immunological problem for uremic patients on dialysis. Second, it is of interest that these individuals appear to have an adequate IFN-γ response. Since IFN-γ is known to be an important macrophage activating factor (35), it is likely that this initial step in macrophage function is not impaired in uremic patients on dialysis. Finally, IFN-γ is known to enhance the secretion of IL-1 (36) or endogenous pyrogen, and the enhanced production of IFN-γ observed conceivably could influence the propensity of uremic patients on dialysis to develop febrile reactions.

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