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Cytokine Production in Cell Culture by Peripheral Blood Mononuclear Cells from Immunocompetent Hosts

ROHIT K. KATIAL,[1]* DORIS SACHANANDANI,[2] CAROLYN PINNEY,[2] AND MICHAEL M. LIEBERMAN[2]†

Department of Allergy and Immunology, Walter Reed Army Medical Center, Washington, D.C.,[1] and Department of Clinical Investigation, Fitzsimons Army Medical Center, Aurora, Colorado[2]

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The production of interleukin 2 (IL-2) gamma interferon, IL-4, tumor necrosis factor alpha (TNF-α), TNF-β, IL-5, and IL-10 in vitro by peripheral blood mononuclear cells cultured from healthy immunocompetent subjects after mitogen stimulation was determined. The mitogens used were concanavalin A, phytohemagglutinin, pokeweed mitogen, and *Staphylococcus aureus* Cowen. The results obtained provide a normal range for the production of these cytokines under specified conditions in vitro.

Cytokines are proteins which play an integral role in the human immune response. The functions of these proteins are diverse and include roles in normal T-cell-mediated immunity, the inflammatory response, cancer, autoimmunity, and allergy (2). Therefore, various pathologic conditions will be accompanied by changes in cytokine levels. In order to develop a better understanding of immune-mediated disorders, it becomes imperative to measure cytokine production. Only recently has refined laboratory technology provided the ability to measure serum cytokine levels. However, these serum measurements are plagued with difficulties due to soluble receptors, anticytokine antibodies, and receptor antagonists (6, 15). The emphasis has since shifted to measuring in vitro cytokine production in cell culture supernatants. This has been accomplished by the use of various enzyme-linked immunosorbent assay (ELISA) kits. Although the number of cytokines described is increasing, the normal cytokine production profile has not been established for many of the cytokines. The purpose of this study was to evaluate cytokine production in vitro by peripheral blood mononuclear cells (PBMC) from healthy immunocompetent individuals after mitogen stimulation and to determine normal ranges. The cytokines measured include Th1-cell cytokines (gamma interferon [IFN-γ] and tumor necrosis factor beta [TNF-β]), Th2-cell cytokines (interleukin 4 [IL-4] and IL-5), macrophage/lymphocyte cytokines (TNF-α and IL-10), and a cytokine produced by both Th1 and Th2 cells (IL-2) (2).

MATERIALS AND METHODS

Blood donors. Blood samples were obtained from healthy donors from the Allergy/Immunology Clinic (clinic personnel) and from laboratory personnel at Fitzsimons Army Medical Center. Subjects aged 30 to 50 years were enrolled for the study. All subjects except three were active-duty military personnel and had had routine physical examinations that included yearly human immunodeficiency virus (HIV) testing. None of the participants reported any history of acute or chronic medical problems. These subjects were all in good health, and any individuals with recent upper respiratory tract infections were excluded. Males and females were represented in equal numbers. Heparinized blood was obtained, and PBMC were isolated according to the techniques described below. Informed consent was obtained from all subjects.

* Corresponding author. Mailing address: Department of Allergy and Immunology, Walter Reed Army Medical Center, Washington, DC 20307. Phone: (202) 782-8085. Fax: (202) 782-7093. E-mail: RKatial@POL.NET.

† Present address: Department of Chemistry, University of Hawaii at Manoa, Honolulu, HI 96822.

Media and reagents. All chemical reagents and media components were obtained from Sigma Chemical Co., St. Louis, Mo., unless otherwise noted. The cell culture medium consisted of RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 10 mM HEPES buffer, 2 mM L-glutamine, 50 μg of gentamicin/ml, 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 0.25 μg of amphotericin B/ml. PBMC were isolated from whole blood by centrifugation through Ficoll-Hypaque solution (Histopaque-1077 or Accuspin-1077).

Cell culture. PBMC isolated from whole blood were washed twice in RPMI 1640 and resuspended in culture medium at a concentration of 10^6/ml. Then, 0.5 ml of cell suspension was added to wells of a 24-well tissue culture plate. Next, 0.5 ml of mitogens at a 2× final concentration in culture medium or 0.5 ml of additional medium (for the cell control) was added to the wells, yielding a final concentration of 5×10^5 cells/ml. The final concentrations for concanavalin A (ConA), pokeweed mitogen (PWM), phytohemagglutinin (PHA), and *Staphylococcus aureus* Cowen (SAC) were 5, 5, 10, and 10 μg/ml, respectively. Plates were incubated for 3 days (37°C, 95% air, 5% CO_2, 100% humidity).

Cytokine assays. Cell culture supernatants were harvested and analyzed for cytokines by ELISA techniques with commercially available kits (or were frozen for later analysis). IL-2, IL-4, IL-5, and IL-10 kits were obtained from R & D Systems, Minneapolis, Minn. TNF-α, TNF-β, and IFN-γ kits were obtained from Biosource International, Camarillo, Calif. All cytokine assays were calibrated against the World Health Organization international standards by the kit manufacturer. The lower limits of detection for the individual assays are as follows: IL-2, 20 pg/ml; IL-4, 0.2 pg/ml; IL-5, 5 pg/ml; IL-10, 1 pg/ml; TNF-α, 1 pg/ml; TNF-β, 10 pg/ml; and IFN-γ, 10 pg/ml.

Data analysis. The association between cytokine levels for each mitogen was examined by using Spearman's correlation coefficient. Given the large number of possible pairwise comparisons, only correlations with P values less than 0.01 are presented to reduce the probability of reporting spurious associations.

RESULTS

The range and median values for the production of each cytokine by PBMC from a set of healthy subjects are given in Tables 1 to 7. In general, the data show a wide range of values

TABLE 1. IFN-γ production by stimulated PBMC in culture[a]

Mitogen[b]	IFN-γ production (pg/ml) (n = 22)	
	Range	Median
ConA	<10–82,000	1,300
PHA	450–66,000	11,900
PWM	3,600–98,000	39,000
SAC	100–12,000	4,400
None (cell control)	<10–42	<10

[a] IFN-γ production was determined with supernatants from PBMC cultures as described in Materials and Methods.

[b] ConA and PWM were used at 5 μg/ml; PHA and SAC were used at 10 μg/ml.

TABLE 2. IL-2 production by stimulated PBMC in culture[a]

Mitogen[b]	IL-2 production (pg/ml) (n = 16–18)	
	Range	Median
ConA	<20–40	<20
PHA	<20–135	<20
PWM	1,170–4,000	1,930
SAC	<20–532	<20
None (cell control)	<20–35	<20

[a] IL-2 production was determined with supernatants from PBMC cultures as described in Materials and Methods.
[b] ConA and PWM were used at 5 μg/ml; PHA and SAC were used at 10 μg/ml.

for each of the mitogens used. Because of this wide range, the median rather than the mean values are considered more indicative of the expected normal values. The results for IFN-γ production are presented in Table 1 and show a particularly wide range of values. The median values demonstrate a clear mitogen stimulatory effect in the following order: PWM > PHA > SAC > ConA > cell control.

In contrast, IL-2 levels seen with all four mitogens were within a somewhat narrower range (Table 2). PWM was much more effective in generating IL-2 synthesis than the other mitogens and was the only mitogen which stimulated IL-2 production at a median value above the baseline (cell control) value.

The results for IL-4 production are given in Table 3. The range of values obtained is also narrower than those for some of the other cytokines. However, all of the mitogens stimulated production at median values above the cell control value. The order of preferential stimulation was PWM > PHA > ConA > SAC.

The results of IL-5 production in stimulated cell cultures are shown in Table 4 and again demonstrated a relatively narrow range of values. PWM and PHA were the only mitogens capable of stimulating production at median values above baseline, with PHA > PWM.

IL-10 production (Table 5) showed one of the wider ranges of values. The preferential order of mitogen stimulation based on median values was PWM > PHA > SAC > ConA > cell control.

The two forms of TNF have similar functions, and both showed a broad range with all four mitogens (Tables 6 and 7). However, the preferential order of mitogen stimulation based on median values was different. For TNF-α, this order was SAC > PWM > PHA > ConA > cell control, while for TNF-β the order was PHA > PWM >> (much greater than) SAC ≥ ConA > cell control.

The association between cytokine levels for each mitogen and for all individuals was assessed, and the significant results are shown in Table 8. Stimulation with ConA demonstrated direct correlations between IL-4 and TNF-β and between IL-10 and IFN-γ. A direct correlation was noted for PHA between IL-2 and IFN-γ. For PWM there was a direct correlation between IL-4 and IL-5. SAC stimulation produced direct correlations between IL-2 and TNF-β and between IFN-γ and TNF-β. There were no inverse correlations noted for any of the four mitogens.

TABLE 3. IL-4 production by stimulated PBMC in culture[a]

Mitogen[b]	IL-4 production (pg/ml) (n = 16–20)	
	Range	Median
ConA	<0.2–9	0.5
PHA	1.4–12	4.2
PWM	3.9–19	11
SAC	<0.2–2.25	0.2
None (cell control)	<0.2–1	<0.2

[a] IL-4 production was determined with supernatants from PBMC cultures as described in Materials and Methods.
[b] ConA and PWM were used at 5 μg/ml; PHA and SAC were used at 10 μg/ml.

TABLE 4. IL-5 production by stimulated PBMC in culture[a]

Mitogen[b]	IL-5 production (pg/ml) (n = 18–24)	
	Range	Median
ConA	<5–270	<5
PHA	<5–145	73
PWM	<5–125	35
SAC	<5–113	<5
None (cell control)	<5–13	<5

[a] IL-5 production was determined with supernatants from PBMC cultures as described in Materials and Methods.
[b] ConA and PWM were used at 5 μg/ml; PHA and SAC were used at 10 μg/ml.

DISCUSSION

This study determined normal ranges for IL-2, IL-4, IL-5, IL-10, TNF-α, TNF-β, and IFN-γ levels. The increased levels of IL-2 noted secondary to PWM may have been due to dual stimulation of both T and B lymphocytes. The B cells may have produced an amplification factor which then stimulated the T lymphocytes to increase IL-2 production. The seeming lack of IL-2 production by PBMC from the majority of donors in response to ConA and PHA (Table 2) may have been due to the length of incubation of the cell cultures (3 days). If the culture supernatants had been harvested after 24 h of incubation, some IL-2 production by PBMC from a majority of donors may have been observed. The absence of detectable IL-2 after 3 days may have been due to upregulation of IL-2 receptors on activated lymphocytes, with subsequent binding and uptake of this cytokine. IL-4 is classically associated with allergic disease, as it is the immunoglobulin E switch factor. The relatively low values of IL-4 observed may have been due to the nonallergic state of the study participants or the absence of aeroallergens during the study period (winter months in Denver, Colo.). IL-5 is involved with eosinophil proliferation. Therefore, in a healthy donor one would not expect the lymphocytes to be primed to produce increased IL-5 levels. This would explain the narrow range noted for IL-5 production. The high levels of TNF-α obtained as a result of stimulation with SAC may be due to preferential stimulation of monocytes/

TABLE 5. IL-10 production by stimulated PBMC in culture[a]

Mitogen[b]	IL-10 production (pg/ml) (n = 16–21)	
	Range	Median
ConA	<1–338	40
PHA	20–450	240
PWM	90–950	280
SAC	45–870	175
None (cell control)	<1–85	4

[a] IL-10 production was determined with supernatants from PBMC cultures as described in Materials and Methods.
[b] ConA and PWM were used at 5 μg/ml; PHA and SAC were used at 10 μg/ml.

TABLE 6. TNF-α production by stimulated PBMC in culture[a]

Mitogen[b]	TNF-α production (pg/ml) (n = 14–17)	
	Range	Median
ConA	40–4,840	440
PHA	420–2,960	1,940
PWM	980–4,760	2,210
SAC	1,960–9,600	5,800
None (cell control)	<1–480	46

[a] TNF-α production was determined with supernatants from PBMC cultures as described in Materials and Methods.
[b] ConA and PWM were used at 5 μg/ml; PHA and SAC were used at 10 μg/ml.

macrophages with this mitogen and preferential production of this cytokine by monocytes/macrophages.

This study also assessed whether a pattern existed between cytokine levels for each individual for a given mitogen. The PHA and PWM results were consistent with Th1 and Th2 profiles, respectively. For PHA, as IL-2 levels increased or decreased IFN-γ levels increased or decreased accordingly. For PWM, the same relationship existed between IL-4 and IL-5. For SAC there was an association between TNF-β and IL-2, as well as one between TNF-β and IFN-γ. These correlations are of the Th1 variety. Stimulation with ConA showed that IL-10 levels were directly related to IFN-γ, and this would not corroborate the classic Th1-Th2 paradigm in which these two cytokines would be inversely related. ConA stimulation also demonstrated a positive relationship between TNF-β and IL-4. Again, this may not fit the classic Th1-Th2 differentiation model. However, recently there has been further elucidation of the Th1-Th2 model for humans (2). The majority of the literature has described cytokines such as IL-2 as being produced by Th1 cells. However, this was originally described for the murine model. The review by Borish and Rossenwasser demonstrates that the breakdown of the Th1- and Th2-cell cytokines in humans is not as clearly divided. For example, IL-2 is produced by both Th1 and Th2 lymphocytes. The cytokine associations noted in this study are very interesting, and most appear to fit the Th1-Th2 paradigm. However, this was not the primary objective of this study, and thus further investigation is needed to delineate the direct and inverse relationships between cytokines in humans.

The explanation of why each mitogen stimulates the cells to produce different levels of cytokines is not clear, although the spectrum of target cells for each mitogen is known to be somewhat different. ConA is reported to stimulate cytotoxic T cells (14), suppressor inducer T cells (12), or "virgin" T cells (11). PWM, on the other hand, stimulates helper T cells and, in association, B cells (11). SAC directly stimulates B cells (13);

TABLE 7. TNF-β production by stimulated PBMC in culture[a]

Mitogen[b]	TNF-β production (pg/ml) (n = 14–17)	
	Range	Median
ConA	<10–82	10
PHA	390–1,640	750
PWM	135–>1,000	505
SAC	<10–37	18
None (cell control)	<10	<10

[a] TNF-β production was determined with supernatants from PBMC cultures as described in Materials and Methods.
[b] ConA and PWM were used at 5 μg/ml; PHA and SAC were used at 10 μg/ml.

TABLE 8. Significant associations between cytokine levels for each mitogen

Mitogen	Correlated cytokines	Correlation coefficient	P value
ConA	IL-4 to TNF-β	0.67	0.009
	IL-10 to IFN-γ	0.71	0.003
PHA	IL-2 to IFN-γ	0.63	0.009
PWM	IL-4 to IL-5	0.81	<0.0005
SAC	IL-2 to TNF-β	0.64	0.007
	TNF-β to IFN-γ	0.76	0.001

however, T cells may possibly be stimulated indirectly through cytokines elaborated by the stimulated B cells. As a result this study not only highlights what cytokine levels one should expect in culture but also shows that different levels are produced depending on the mitogen used.

Cytokine levels vary not only in vitro secondary to different mitogens but also in vivo in different clinical diseases. A study by Friberg et al. showed varying cytokine levels in cancer patients who had received biologic response modifiers (5). Al-Janadi et al. (1) demonstrated greatly increased synthesis of IL-6 and IFN-γ by ConA-stimulated PBMC from patients with systemic lupus erythematosus (SLE) with nephrotic syndrome (NS) or SLE with lymphadenopathy (LN), compared to that by ConA-stimulated PBMC from healthy controls. However, ConA-stimulated PBMC from SLE patients with thrombocytopenia (TP) showed significantly smaller increases in IL-6 production and no increase in IFN-γ synthesis relative to those from healthy controls. In contrast, ConA-stimulated PBMC from SLE patients with TP, but not from SLE patients with either NS or LN, produced greatly increased amounts of TNF-α compared to those from healthy controls (1). Kobrynski et al. (8) found that PBMC from chronic mucocutaneous candidiasis patients produced more IL-4, but not IL-10, IL-2R, or IFN-γ, in response to PHA than PBMC from controls. Additionally, there has been increasing use of in vitro cytokine measurements to classify disease states according to the Th1-Th2 cytokine paradigm. In a study of the effects of the immunomodulatory drug thalidomide, McHugh et al. (10) found that the drug effected an early switch from a Th1- to a Th2-type cytokine profile. A switch from a Th1- to a Th2-type cytokine profile has also been associated with disease progression in HIV-infected patients (3, 4). In fact, pediatric HIV patients have been found to produce more IL-4 and less IL-2 than healthy controls (16). Moreover, in a comparative study of rapidly progressing (RP) and seroreverting (SR) vertically infected pediatric HIV patients, Lee et al. (9) found that the RP patients produced less IL-2 and IFN-γ than the SR patients. In contrast, the RP patients produced more IL-4 mRNA than did the SR patients. Finally, in a case study of a patient with a γδ T lymphocytosis (7), it was found that the production of all cytokines tested, including both Th1- and Th2-cell cytokines, was severely depressed.

The results presented above indicate that one needs to be very aware of the culture conditions, the mitogens used, and the clinical state of the patient when performing in vitro cytokine measurements. This is the first study to provide reference ranges for cytokine production in mitogen-stimulated cell cultures, while demonstrating variable cytokine levels in response to different mitogens.

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