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

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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.

Materials and methods. 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, 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 10⁶/ml. Then, 0.5 ml of cell suspension was added to wells of a 24-well tissue culture plate. Next, 0.5 ml of mitogen at a 2:1 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 × 10⁴ 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% CO₂, 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.

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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.

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 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.

**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 2. IL-2 production by stimulated PBMC in culture

| Mitogen | 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   |        |

* IL-2 production was determined with supernatants from PBMC cultures as described in Materials and Methods.

### Table 4. IL-5 production by stimulated PBMC in culture

| Mitogen | 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    |        |

* IL-5 production was determined with supernatants from PBMC cultures as described in Materials and Methods.

### Table 3. IL-4 production by stimulated PBMC in culture

| Mitogen | 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 |        |

* IL-4 production was determined with supernatants from PBMC cultures as described in Materials and Methods.

### Table 5. IL-10 production by stimulated PBMC in culture

| Mitogen | 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    |        |

* IL-10 production was determined with supernatants from PBMC cultures as described in Materials and Methods.

### Table 6. IL-10 production by stimulated PBMC in culture

| Mitogen | 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    |        |

* IL-10 production was determined with supernatants from PBMC cultures as described in Materials and Methods.

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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- 
a and IL-5. For SAC there was an association between TNF- 
a 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- 
and IL-2. Again, this may not fit the classic Th1-Th2 differentiation 
model. However, there is further elucidation of the 
Th1-Th2 model for humans (2). The majority of the liter- 
ature 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 Rosenwasser dem- 
onstrates that the breakdown of the Th1 and Th2-cell cyto-

kines 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 be-
tween 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 some-
what 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);

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 ex-
pect 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 pa-
tients 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 thrombocy-
topenia (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). Kobryn-
ski 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 immu-
nomodulatory 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 cyto-
kine measurements. This is the first study to provide reference 
ranges for cytokine production in mitogen-stimulated cell cul-
tures, while demonstrating variable cytokine levels in response 
to different mitogens.

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### TABLE 6. TNF-α production by stimulated PBMC in culture

| Mitogen | 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    |        |

* TNF-α production was determined with supernatants from PBMC cultures as described in Materials and Methods.

### TABLE 7. TNF-β production by stimulated PBMC in culture

| Mitogen | TNF-β production (pg/ml) (n = 14–17) | Range | Median |
|---------|-------------------------------------|-------|--------|
| ConA    | <10–82                              | 10    |        |
| PHA     | 390–1,640                           | 750   |        |
| PWM     | 125–5,000                           | 505   |        |
| SAC     | <10–37                              | 18    |        |
| None (cell control) | <10                           | <10   |        |

* TNF-β production was determined with supernatants from PBMC cultures as described in Materials and Methods.

* ConA and PWM were used at 5 μg/ml; PHA and SAC were used at 10 μg/ml.
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