Temporal sequence and cellular origin of interleukin-2 stimulated cytokine gene expression

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Summary  A study of activation of the cytokine network by interleukin 2, IL-2, may provide a rationale for devising cytokine combination and cytokine antagonist treatments with increased anti-tumour efficacy and decreased toxicity. We have investigated the expression of mRNA for 13 cytokines and three transcription factors during in vitro culture of peripheral blood mononuclear cells, PBMC, with IL-2. A consistent pattern of induction was seen in nine individuals, with early (2–24 h) induction of IL-1β, IL-6, tumour necrosis factor, TNF, lymphotoxin, LT, and gro. TNF and LT mRNA was expressed continually throughout culture, but levels of mRNA for IL-1β, IL-6, and gro declined by 24–48 h. After 48 h, PBMC began to express mRNA for IFN-γ, IL-5, GM-CSF, and M-CSF. At 15 min to 1 h post IL-2 mRNA for c-fos, c-jun, and c-myc, and TNF was induced in three individuals studied. IL-4, IFN-α, and IL-1a mRNA was not detected. Only a minority of cells expressed mRNA for TNF, IL-1β, IL-6 and IFN-γ, and monocytes were the main source. Levels of cytokine protein in culture supernatants mirrored the pattern of mRNA induction. This in vitro model shows clear parallels with the reported in vivo production of cytokines during IL-2 therapy, and may prove useful in designing new therapeutic strategies.

Therapy with IL-2, with or without ex vivo activated lymphocytes, has resulted in partial, and occasionally complete, tumour regressions in a minority of patients with melanoma and renal cell carcinoma (Dutcher et al., 1989; Negri et al., 1990). The induction of cytolytic activity in peripheral blood mononuclear cells, PBMCs, by IL-2 has been well documented both in vitro and in vivo (Grimm et al., 1982; Rosenberg et al., 1985). The mechanisms of activation of this heterogeneous population of cells are not fully elucidated. Several studies have shown that in vitro activation results in production of several cytokines. Early (24 h) induction of IL-1α and IL-1β mRNA has been reported, as has the induction of TNF, LT and IFN-γ mRNA (Kovac et al., 1989). In other studies mRNA for GM-CSF and IL-6 was detected in PBMC after 3 days of culture with IL-2 (Beldeegrain et al., 1989; Kasid et al., 1989).

The study of cytokines induced by IL-2 in vitro and in vivo is important for several reasons. First, these secretory products may contribute to the antitumour effects of IL-2 in vivo. Cytokines such as TNF and IFN-γ have well documented antitumour activity in animal models (Kelly et al., 1989), and synergy between IL-2 and other cytokines has been reported (Truitt et al., 1989). Second, the toxicity of IL-2 therapy may be related to the cytokine release induced by activated PBMC. For instance, anti-TNF antibody abrogated some of the toxic side effects of IL-2 in tumour bearing mice (Fraker et al., 1989), and antibodies to IL-5 abolished the IL-2 induced eosinophilia in mice (Yamaguchi et al., 1990). Third, studies of cytokine induction in a mixed population of mononuclear cells also gives us indications of the range of cytokines which may be induced after other mitogenic or antigenic stimuli.

In order to understand the potential of other cytokines or their antagonists, for influencing the anti-tumour activity or toxicity of IL-2 in humans, we have studied in detail the range and temporal sequence of cytokine mRNA and proteins induced by IL-2, and the producer cells of those cytokines. In this paper we present data obtained in in vitro cultures of human PBMC with IL-2.

Materials and methods

Reagents

Human rIL-2 was generously supplied by Glaxo IBM (Geneva, Switzerland) (specific activity 3.1 × 10^6 U mg^-1), and Roussel-UCLAF Centre de Recherche (Romaine, France) (specific activity 10^6 U mg^-1). Maximum cytotoxicity was induced when the cells were cultured with 1000 U mg^-1 of IL-2.

Preparation of PBMCs

Peripheral blood mononuclear cells from healthy donors were separated from heparinised venous blood on Ficoll-Hypaque gradient (Lymphoprep TM, Nycomed Pharma AS, Oslo, Norway). The cells were resuspended at 1 × 10^8 ml^-1 in RPMI 1640 containing 10% heat inactivated foetal calf serum with or without 1000 U ml^-1 rhIL-2. Cellular cytotoxicity was assayed after 3 days of culture. For northern blot analysis cells were lysed with guanidine-isothiocyanate solution for RNA extraction. For in situ hybridisation cells were immunostained for FACS sorting.

Cell lines as positive controls for Northern blot analysis

The HL60 human promyelocytic leukaemic cell line was maintained in 5% FCS/RPMI 1640. Total cellular RNA was extracted after a 3 h incubation with 50 ng ml^-1 PMA (Sigma, Dorset, UK). This constituted a source of TNF, IL-1α, IL-1β, M-CSF, and TGF-β mRNA as a positive control for Northern blot analysis. Human T-cell line Jurkat cells were stimulated overnight with 1 ng ml^-1 phytohaemagglutinin (Sigma, Dorset, UK) and 20 ng ml^-1 PMA. Extracted RNA provided a positive control for IFN-γ. Human foreskin fibroblasts were stimulated with 10 ng ml^-1 PMA for 1 h, providing a positive control for IL-6 mRNA. MLA-144 T cell line stimulated with PHA and PMA provided a positive control for GM-CSF mRNA. It was not possible to obtain reliable controls for IFN-α and IL-4 expression using Northern analysis however the IFN-α factor reacted on a Southern blot.

Labelling of target cells

Freshly thawed T 24 cells were washed three times in complete medium. 0.1 mCi of ^51 Cr (Na^51CrO_4) (Amersham International UK) was added to the cell pellet. Cells were incubated at 37°C for 1 h, then washed in complete medium three times to remove unbound ^51 Cr. Cells were resuspended in medium at a final concentration of 5 × 10^6 ml^-1.

Isolation of cellular RNA

Non-adherent cells from PBMC cultures were harvested. The adherent cells were scraped with rubber policeman into
guanidine-isothiocyanate solution which was then used to
lyse the non-adherent fraction. Total cellular RNA
was isolated after centrifugation through cesium chloride followed
by precipitation with 3m sodium acetate and ethanol (Chirg-
win et al., 1979).

Northern blot analysis
Fifteen μg aliquots of total cellular RNA were electro-
phoresed through a 1.4% agarose-formaldehyde denaturing
gel then capillary blotted onto Biodyne A membrane (Pall
Ultranfine Filtration Corp., Glen Cove, NY). Membrane
were hybridised to 32P-labelled inserts of human complemen-
tary DNA probes under standard conditions as outlined by
Church and Gilbert (1984) and labelled with [32P]dCTP
(Amersham International UK) by the random priming
method of Feinberg and Vogelstein (1984). Membranes
were subsequently washed to high stringency and exposed to
Kodak XAR5 film at ~70°C with two intensifying screens
(Dupont, Stevenage, Herts, United Kingdom). Films were
exposed for 7 days.

In situ hybridisation
The technique used was essentially as described in Naylor et
al. (1990) except that no prehybridisation stages were needed
with cytokins. 10 μl of the hybridisation buffer containing
35S-labelled RNA probe at 5 × 106 dpm μl-1, was added to
each slide, which was then covered with a siliconised cover-
slip and left to hybridise at 50°C for 16 h. Slides were washed
to high stringency at 50°C for 30 min, then 65°C for 16 h.
Slides were washed to high stringency at 50°C for 30 min,
then 65°C, followed by RNase treatment to digest the un-
bound RNA followed by autoradiography. Slides were devel-
op after 10 days of exposure and stained with methylene
blue.

Probes
For Northern analysis The following probes were used:
TNF: PstI fragment of p-hTNF 1; IFN-γ: BamHI fragment
of pBR327G-2; IL-1α: HindIII-EcoRI fragment of pSPHI
-1α-2; IL-1β: HindIII fragment of pSPHl-1β-2; IL-6: BglII
fragment of pSP63T-IL-6; TGF-β EcoRI EcoRI fragment phas
TGF-β1 (Genentech, California); β-actin: EcoRI-HindIII
fragment of M13 β-actin; IL-5: Xbal-BamHI fragment of
pGEM4 IL-5; M-CSF: PstI-SmaI fragment of pUC18 M-
CSF; GM-CSF: HXh-Smal fragment of pXm GM-CSF; Gro-
genecolone fragment of pGEM3 Gro; IFN-α: EcoRI fragment
of MIP 211-hIFN-α (Wellcome, Beckenham, Kent, UK);
LT- HindIII-PVUII fragment of pOKBLT-6-HT; IL-10:
cDNA clone (Vieira et al., 1991). c-myc: BamHI-Sall fragment
of DOR h-c-myc; c-jun: EcoRI-BamHI fragment of M13 c-jun; fos:
BglII-PvuII fragment of pHBH-1-fos; ras: EcoRI fragment of
pBR322 ras-Ha (Dhar et al., 1982).

For in situ hybridisation An antisense β-actin riboprobe
was generated from HindIII cleaved Bluescript M13 β-actin
using T7 RNA polymerase. This was used as a positive
controls in all runs. Antisense TNF was generated from the Apal
cleaved pGEM1-hTNF using T7 RNA polymerase (Promega
Biotech, Madison WI). The negative control was sense TNF
generated from BamHI cleaved pGEM-hTNF using Sp6
RNA polymerase (Promega Biotech). The antisense IL-1β
riboprobe was generated from Apal cleaved pGEM-3 using
T7 polymerase, the sense control was generated from EcoRI
cleaved pGEM-3 using Sp6 polymerase. Antisense IL-18
riboprobe was generated from EcoRI linearised pSP h IL-1-
β.2 Antisense IL-6 riboprobe was generated from AccI
linearised pGEM-3 using T7 polymerase, sense control from
XbaI linearised pGEM-3 using Sp6 polymerase. In all
transcriptions were performed using Promega Biotech transcrip-
tion kits to incorporate 35S-UTP (Amersham Interna-
tional plc, UK). Restriction enzymes were obtained from
Pharmacia.

Flow cytometry and cell sorting
On days 1 and 4 of culture in the presence or absence of
IL-2, aliquots of PMBC were stained by direct immuno-
fluorescence following the standard procedure recommended
by the supplier of the fluorescent conjugates (Becton Dickinson
Immunochemistry Systems, San Jose, Ca, USA). Non
adherent and adherent cell populations were pooled for the
analysis, adherent cells being removed from the culture by
rubber policeman after a period of incubation on ice for 1 h.
Cells were labelled with either (a) anti-Leu-3I/phycocerythrin (PE) (CD-
14), (b) Simultest anti-Leu-4/FITC (CD3) + anti-Leu-11C/PE
(CD16) + anti-Leu-19/PE (CD56) or (c) Simultest anti-Leu-
3a/FITC (CD4) + anti-Leu-2a/PE (CD8). All samples were
examined using a FACStarPLUS (Becton Dickinson
Immunochemistry Systems) and relevant populations sorted.
The sorted populations were then recycled through the FAC-
StarPLUS to ensure a high degree of purity. The flow cyto-
meter was flushed with 0.1% diethylcarbamate in
phosphate buffered saline for 2h prior to sorting. The col-
lected cells were centrifuged at 1000 r.p.m. for 5 min at 4°C
resuspended at a concentration of 1 × 106 cells ml-1.

Preparation of slides
Sorted cells were then spun onto slides using a Shandon
Cytospin centrifuge (Shandon Scientific Ltd., Astmoor,
Runcorn, UK) at 500 r.p.m. × 5 min. Slides were then fixed in
4% paraformaldehyde (Sigma, Dorset, UK) in phosphate
buffered saline, alcohol dehydrated and stored at ~70°C.

Cytokine protein assays
IL-1β was measured using EASIA kit from Medgenix
( Brussels, Belgium) with a range of 33–1400 pg ml-1. It was
standardised against an international reference prepara-
tion (86/680) from the National Institute of Biological
Standards and Controls, NIBSC, and was used at detection
limits of 33 pg ml-1. TNF-α and IFN-γ were measured using IRMA
kits from Medgenix (Brussels, Belgium). The TNF-α IRMA
range was from 15–5000 pg ml-1. It was standardised against
an international reference preparation (87/650) from the
National Institute of Biological Standards and Controls,
NIBSC, and was used at detection limits of 30 pg ml-1. The
IFN-γ/IRMA range was from 0–90 U ml-1. It was
standardised against an international reference preparation
(88/606) from NIBSC and was used at detection limits of 2.5 U ml-1.
The IFN-α was measured using RIA kits from Medgenix
( Brussels, Belgium). The range was from 0–200 U ml-1.
It was standardised against an international reference prepara-
tion (82/576) from NIBSC and was used at detection limits
of 8.0 U ml-1. IL-6 was an ‘in house’ RIA (anti sera G150
BM from Dr S. Poole, NIBSC). The assay was used at a
final dilution of 1:1,750,000 (equivalent to a theoretical
dilution of 1:350,000). The assay was standardised against an
international reference preparation (88/514) from NIBSC and
was used at detection limits of 70 pg ml-1.

Results
Induction of cytokine and proto-oncogene mRNA in IL-2
stimulated cultures-Northern analysis
In the first series of experiments we studied the induction of
mRNA for 13 different cytokines in PBMC stimulated with
10,000 U ml-1 IL-2, a concentration of IL-2 that generated
maximal in vitro cytotoxicity against T24 cells. Table 1 and
Figure 1 show the results obtained in cultures from one
normal individual. In the interests of clarity, Table 1 only
shows the mRNA induction in IL-2 stimulated cultures. The
figure, however, clearly shows that there was no appreciable
induction of cytokine mRNA in the cultures without IL-2. In
this first individual, there were three different patterns of
cytokine induction: TNF and LT were detected as early as
2h after IL-2 addition and the mRNA was then present


Table I: Summary of Northern blot analysis of cytokine mRNA induction in PBMC cultured with or without IL-2.

| Time | C | 2 h | 1 d | 2 d | 3 d | 4 d | 5 d | 6 d | 7 d |
|------|---|-----|-----|-----|-----|-----|-----|-----|-----|
| mRNA |   |     |     |     |     |     |     |     |     |
| TGF-β | + | + | + | + | + | + | + | + | + |
| TNF | − | + | + | + | + | + | + | + | + |
| LT | − | + | + | + | + | + | + | + | + |
| Gro | − | + | + | − | − | − | − | − | − |
| IL-1β | − | + | + | + | + | − | − | − | − |
| IL-6 | − | + | + | + | − | − | − | − | − |
| IL-5 | − | − | + | + | + | + | + | + | + |
| IFN-γ | − | − | + | + | + | + | + | + | + |
| GM-CSF | − | − | − | + | + | + | + | + | + |
| M-CSF | − | − | − | + | + | − | − | − | − |
| IFN-α | − | − | − | − | − | − | − | − | − |
| IL-1α | − | − | − | − | − | − | − | − | − |
| IL-4 | − | − | − | − | − | − | − | − | − |
| β-actin | + | + | + | + | + | + | + | + | + |

Exp. no. 1 (h = hour, d = day) (+) indicates the intensity of mRNA induction within each individual case. In this experiment, all time points were studied without IL-2 but only one representative time point is shown as (C).

Throughout the 7 days of culture; IL-1β, IL-6 and the gro gene (a member of the interleukin family, related to IL-8) were also induced 2 h after stimulation, but their induction was only transient, and none of these were detected after 3 days of culture; IL-5, IFN-γ, GM-CSF, and M-CSF were induced at a later time, being first present at 2–3 days. Expression was then sustained throughout the culture period in all of these except M-CSF. mRNA for IFN-α, IL-1α, and IL-4 were not detected during this time. TGF-β mRNA was present in all cultures, stimulated or unstimulated (data not shown).

This pattern of cytokine gene expression during culture of PBMC with IL-2 was found to be reasonably consistent in PBMC from another eight individuals who were studied (Table II). TNF and IL-1β were induced in PBMC from 8/8 individuals, LT, gro, IL-6, GM-CSF, IFN-γ and M-CSF were induced in 4/4 studied. IL-5 mRNA was only detectable in PBMC from 1/4 individuals studied but was also expressed in the unstimulated culture from that individual, and IFN-α and IL-4 were again not found. A probe for IL-10 was also included in this series. In one individual IL-10 mRNA was expressed in unstimulated cells after 24 h of culture. The intensity of the message started to decline during the first 2 days.

![Figure 1](image)

Figure 1: Northern blot analysis of cytokine mRNA in PBMC. Total cellular RNA isolated from unstimulated PBMC (−), or IL-2 stimulated PBMC (+) at the given time points (expt. 1) (h = hours, d = days).
of culture with IL-2 and was not detected at later time points (Figure 2).

In a third series of experiments, the induction of nuclear proto-oncogenes and two of the cytokines, TNF and gro, was studied during the first hour after IL-2 stimulation. There was evidence of a transient increase in c-myc levels at 15 min in 2/3 individuals, for was induced in all three individuals at 15 min, gro gene was induced at 30-60 min, and TNF mRNA induction was seen as early as 30 or 60 min. There was no detectable mRNA for Ha-ras (Table III).

Cell populations in IL-2 stimulated cultures

The proportion of monocytes (CD14), T cells (CD3), NK (CD16/CD56), and cells coexpressing NK/CD3 markers, were studied in IL-2 stimulated PBMC cultures. Significant changes were seen in the relative proportions of these populations after 4 days culture with or without IL-2, in PBMC from five out of six individuals. In the unstimulated cultures the mean % T cells was 70 (± 11) of total cell count, NK cells 4 (±4%) and cells coexpressing NK and CD3 markers 2 (±3%). After IL-2 stimulation the mean percentage of T cells was 74 (±7), NK cells 5 (±4%) and cells expressing NK and T cell markers 3 (±3%). In one case the number of cells coexpressing T and NK cell markers rose from 8% of the total population in unstimulated cultures to 76% of the IL-2 stimulated cultures. The percentage of CD14 +ve monocytes after 4 days of culture was 10% (±1) in unstimulated cultures and 9% (±1) in IL-2 stimulated cultures.

Identification of cells producing the cytokine mRNA

Using FACS sorting and in situ hybridisation with cytokine riboprobes, we identified the producer cells of four different cytokines, TNF, IL-1β, IFN-γ, and IL-6 in the total population and the sorted cells. These cytokines were studied at the time of peak message induction in the Northern blots, in three individuals. The proportion of cells in the total population with detectable cytokine mRNA ranged from 2–16% after 1 day of culture for IL-6 and IL-1β, and 4 days of culture for IFN-γ and TNF. As a control for cytokine mRNA stability during FACS sorting, the total PBMC population was passed through the sorting process and the percentage of cells expressing cytokine mRNA compared to that in cells cyto spun at the beginning of this process. No differences were seen. Because of the small numbers of cells involved in these experiments, the gated populations were resorted to ensure high degrees of purity. Resorting of cells likewise did not affect mRNA stability.

IL-1β In two of the three individuals, 2–3% cells in the total population expressed IL-1β mRNA. These cells were monocytes, and in sorted populations 8–35% of monocytes expressed IL-1β mRNA. In cells from a third individual, IL-1β mRNA was induced in 19% of monocytes, 6.6% of NK cells, 3% CD4 + and 8% CD8 + cells on IL-2 stimulation, making a total of 16% expressing cells in the total population. In the unstimulated control cultures a small proportion of monocytes and CD8 + cells expressed IL-1β mRNA (0.1–0.5% and 0.2% of the total population respectively).

IL-6 In the three individuals 0.4–6% of the total cells expressed IL-6 mRNA after 24 h stimulation with IL-2. In two out of three cases IL-6 mRNA was detected in the monocyte (2–6% monocytes positive) and NK (1–4% cells positive) populations. In the third case IL-6 mRNA was detected only in monocytes (22% positive).

IFN-γ Between 2 and 5% of the total cell population expressed IFN-γ mRNA. IFN-γ mRNA was detected in all sorted populations except the CD8 cells. NK (6–13% cells positive) and CD4 cells (2–3% positive) made a significant contribution to levels of mRNA in the total population, but,
Figure 2 Northern blot analysis of IL-10 mRNA expression in PBMC cultured with or without IL-2. Cells from exp. 6 were lysed at the indicated time points (h = hours, d = days) for RNA isolation. (-) without IL-2 (+) with IL-2.

Table III Induction of transcription factors and cytokine mRNA in PBMC during the first hour of culture with or without IL-2

| C | Exp. 10 | Exp. 11 | Exp. 12 |
|---|---------|---------|---------|
|   | 5' 15' 30' 60' | 5' 15' 30' 60' | 5' 15' 30' 60' |
| C-myc | + + + + + + + + + + + + + + + + + | + + + + + + + + + + + + + + + + + |
| C-jun | + + + + + + + + + + + + + + + + + | + + + + + + + + + + + + + + + + + |
| fos | + + + + + + + + + + + + + + + + + | + + + + + + + + + + + + + + + + + |
| gro | + + + + + + + + + + + + + + + + + | + + + + + + + + + + + + + + + + + |
| ras | + + + + + + + + + + + + + + + + + | + + + + + + + + + + + + + + + + + |
| TNF | + + + + + + + + + + + + + + + + + | + + + + + + + + + + + + + + + + + |

(+) indicates the intensity of mRNA induction within each individual case. RNA from cells cultured without IL-2 (C) was analysed at 30 and 60 min with identical results.

Interestingly, in all three individuals 3–4% of monocytes expressed IFN-γ mRNA on IL-2 stimulation. No IFN-γ production was detected in unstimulated control cultures. Figure 3 shows results from in situ hybridisation of the sense and anti-sense IFN-γ riboprobes in total population and NK/CD3 populations in one individual.

**TNF** Between 1 and 3% of cells in the total population expressed TNF mRNA. These were 4–5% of the monocytes population and 1–3% of the CD3 cells. TNF mRNA was also detected in a small minority (0.1%) of monocytes from unstimulated cultures.

**Production of cytokine protein**

Cytokine protein levels were measured in the culture fluids from eight different experiments by immunoassay. The results are shown in Figure 4.

**IL-1β** The production of IL-1β in the IL-2 stimulated cultures was maximal in the first 2 days of culture. The IL-1β concentration varied from 90 pg ml⁻¹ to 1300 pg ml⁻¹. In unstimulated cultures it peaked at 100 pg ml⁻¹.

**IL-6** IL-6 protein concentration peaked maximally after 2–4 days of culture with IL-2, occurring after the first

Figure 3 In situ hybridisation of an 35S labelled IFN-γ riboprobe to IL-2 stimulated PBMC after 4 days of culture. a, Antisense IFN-γ riboprobe hybridised to total population of cultured cells. b, Sense IFN-γ riboprobe hybridised to the same population. c, Antisense IFN-γ riboprobe hybridised to FACS sorted cells co-expressing NK and CD3 marker. d, Sense IFN-γ riboprobe hybridised to the same population.
evidence of IL-6 mRNA induction. In one of seven unstimulated cultures, the spontaneous production of IL-6 protein was at a level (3029 pg ml\(^{-1}\)) comparable to the IL-2 stimulated culture. In the other unstimulated cultures IL-6 concentration ranged between 0–1206. After IL-2 stimulation, the maximal concentration ranged from 2903–3512 pg ml\(^{-1}\).

**IFN-γ** Maximal production of IFN-γ protein by IL-2 stimulated PBMC was invariably found at 4 days of culture. The maximum IFN-γ concentration varied from 28–90 IU ml\(^{-1}\). No spontaneous production was detected in the unstimulated culture supernatants. The pattern of protein production matched exactly IFN-γ mRNA induction.

**TNF** Maximum levels of TNF were detected at 4 days of culture, in seven experiments and at 7 days in one experiment. Spontaneous production occurred one out of six cultures (1100 pg ml\(^{-1}\)). The TNF concentration in control cultures was 30–1100 pg ml\(^{-1}\), while maximal production on IL-2 stimulation ranged between 5,300–10,000 pg ml\(^{-1}\).

**Discussion**

Previous *in vitro* and *in vivo* human studies have reported the induction of IL-1, TNF, LT, IFN-γ, IL-6 and GM-CSF mRNA in IL-2 stimulated PBMC but there has been little information on the time course of induction or cellular origin of these mRNA. In this paper we show that PBMC incubated *in vitro* with IL-2 produced a wide range of cytokine mRNA. IL-1β, TNF, LT, gro gene, and IL-6 mRNA were induced early in the culture (30 min–2 days). Later in culture IFN-γ, IL-5, M-CSF and GM-CSF mRNAs were induced. The induction was transient in some of the cases and persistent in others. The temporal pattern of cytokine mRNA induction was consistent among individuals. Within limits of detection of the assay mRNA for IL-1α, IL-4 and IFN-α were not found. This does not preclude their production as indicated by the results of the assay for TNF protein. TNF mRNA in cultures without IL-2 was generally undetectable, but in some cases the protein was found in the cultures.

In at least four of the cytokines, TNF, IL-1β, IFN-γ and IL-6, the production of the protein mirrored the expression of mRNA. Previous *in vitro* studies reported the production of IL-1α and β proteins in culture supernatants of IL-2 stimulated PBMC (Numerof *et al.*, 1989).

*In situ* hybridisation studies on FACS sorted PBMC revealed that the monocytes were the main producers of the above mentioned cytokines, but the NK cells shared in the production of IL-6 and IFN-γ, while the CD4+ cells produced IFN-γ as well. At each given time point only a small percentage of cells in the total or in the sorted population was positive for each cytokine mRNA. This low figure may influence any firm conclusions as to the cellular source of the cytokines. However, each gated population was resorted in each experiment, and this resulted in high (<95%) purity. We do not think the low level of positivity is due to limits of detection of this technique because a high percentage of positive cells was seen in controls incorporated in each experiment. PMA induced HL60 cells were used as positive controls for TNF, IL-β and IFN-γ mRNA, PMA induced HFF cells were used for IL-6 mRNA induction. More than 90% of cells counted were positive for the relevant mRNA. In addition, control experiments proved that the low percentage was not due to instability of RNA during the sorting process, as described in the Results section.

The production of cytokines by the monocytes is unlikely to be due to a direct effect of IL-2 on the monocytes. These cells express the IL-2Rα subunit of the IL-2 receptor only when activated (Waldmann, 1991). It is more likely that an intermediary molecule, possibly one of the other cytokines induced early on, is responsible for the monocyte cytokine production. A more detailed study using *in situ* hybridisation at earlier time points would be of interest, as would study of *in situ* production of some of the other cytokine mRNAs.
identified in the Northern blots. The expression by monocytes of mRNA for one of the cytokines, IFN-γ, is not, to our knowledge a common finding, although murine macrophages have been reported to produce IFN-γ after stimulation with polyinosinic-polycytidylic acid (Dijou et al., 1979). The beta chain of the IL-2 receptor is expressed on 90% freshly isolated monocytes (Espinoza-Delgado et al., 1990), but the alpha chain only on IFN-γ activation (Waldmann, 1991). This again suggests that intermediary cytokines, or other factors, produced by lymphocytes, act as a stimulus for IFN-γ mRNA expression in these cultures. It is not however certain whether monocytes in the cultures can produce IFN-γ protein. We are currently investigating this.

Early induction of the cytokines by IL-2 suggests that they might share in the generation of cytolytic activity of PBMC, particularly as certain cytokines when added exogenously to culture, have been found to synergise with IL-2. TNF enhanced the cytolytic function in PBMC by IL-2, possibly through the induction of high affinity IL-2 receptor complex (Blay et al., 1989), and high serum levels of TNF have been correlated with response to therapy in one clinical study (Blay et al., 1990).

The production of IFN-γ by IL-2 stimulated PBMC in vivo might induce the expression of class I MHC antigen on tumours thus assisting the CTL cytolytic function (Dustin et al., 1986). This function might be helped by TNF which induces the expression of the same antigen (Weber & Rosenberg, 1990). IFN-γ might also induce tumour antigen on tumour cells thus helping the non-MHC restricted cytolytic activity (Mai et al., 1981).

IL-1β was detected in supernatants of IL-2 stimulated PBMC, and its exogenous addition to the culture synergised with IL-2 (Crump et al., 1989). IL-1β exerts a cytotoxic effect on its own (Gaffney & Tsai, 1986). It was found to induce 

The cause of IL-2 induced toxicity remains unknown. The most significant problems are cardiovascular with hypotension, renal failure, fluid retention and a capillary leak syndrome. It has been suggested that TNF and IFN-γ contribute to the toxic effects (Kohler & Sørdal, 1989). Passive immunisation against TNF by antibodies increased the number of doses of IL-2 that could be given to mice before fatal toxicity (Fraeker et al., 1989).

C-fos and c-myc genes and their relevant proteins, exert a role in cellular activation (Kaczmarek & Kaminska, 1989) and together with c-jun are part of the regulatory network of gene expression (Berti et al., 1989). The induction of cytokine mRNA may be dependent on the induction of these genes and their relevant proteins. We are currently studying the role of protein synthesis in these effects.

The detection of IL-10 (cytokine synthesis inhibitory factor) mRNA in one of the cases suggests an autoregulatory function. IL-10 mRNA was expressed in unstimulated PBMC and declined in IL-2 stimulated PBMC. IL-10 downregulates the induction of other cytokines (IL-1, IL-6, IL-8, TNF, GM-CSF, G-CSF) and exerts an autoregulatory function on its mRNA induction in LPS stimulated monocytes (De Waal-Malefyt, 1991).

Recent clinical studies have suggested that this simple in vitro model may help in understanding the activation of the cytokine network in patients treated with IL-2, and in designing trials with cytokine combinations. For instance, List et al. (1992) found transient induction of TNF, IL-1β, IL-6, and IFN-γ in the cerebrospinal fluid of patients receiving IL-2 by the intraventricular route. In other clinical studies, blood from IL-2 treated patients has been reported to contain IL-6, TNF and IFN-γ (Schaafsma et al., 1991; Boccoli et al., 1990) and using PCR mRNA for M-CSF, GM-CSF, IL-3, and IL-5 was detected in PBMC form IL-2 treated patients (Schaafsma et al., 1991). The sequence of release of these cytokines resembled that seen in cultured PBMC, with the exception of IFN-γ which was released earlier in vitro compared with in vivo (e.g. List et al.). However, it is probable that other cell populations may be able to respond to IL-2 or cytokines induced during its use in vivo. Although IL-2 receptor expression is thought to be restricted primarily to haemopoietic cells, there are reports that some tumour cells will respond directly to IL-2 (Saachi et al., 1990). Moreover, tumour cells may well respond to cytokines induced by IL-2 tumour infiltrating lymphocytes.

In summary, IL-2 induced the mRNA of ten cytokines in PBMC in vitro which might reflect the in vivo release of cytokines during IL-2 therapy and this is likely to contribute to the therapeutic and the toxic effects of IL-2 in patients. Studies such as these may help to rationalise the use of submaximal doses of cytokines or specific cytokine inhibitors/binding proteins to obtain a final combination with greater or equivalent therapeutic efficacy and less toxicity.

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