J. Biosci., Vol. 21, Number 4, June 1996, pp 455-469. © Printed in India.

Identification and partial purification of a human natural killer cell proliferation-inducing factor

QUEEN B SAXENA, RAJIV K SAXENA*†, RONALD H GOLDFARB, RONALD B HERBERMAN and THERESA L WHITESIDE

Pittsburgh Cancer Institute, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA

*School of Life Sciences, Jawaharlal Nehru University, New Delhi 110067, India

MS received 2 September 1995; revised 29 February 1996

Abstract. Culture supernatants of Concanavalin A activated human peripheral blood mononuclear cells were found to contain a factor which induced proliferative response in normal peripheral blood mononuclear cells. This proliferation-inducing factor specifically induced and sustained proliferation of purified human NK cells but not of T or B cells. Although interleukin 2 (IL12) also has proliferation-inducing effects on NK cells, the partially purified proliferation-inducing factor preparations contained no measurable IL2 contamination. Moreover, neutralizing anti-IL2 antibodies did not block the growth effect of proliferation-inducing factor on purified human NK cells. Other cytokines which were tested, including IL4, IL6, IL7, IL12, TNF and IFN, were all found to be inactive in the proliferation-inducing factor assay. While proliferation-inducing factor by itself had no effect on T-cell proliferation, IL2-induced proliferation of T cells was significantly enhanced in the presence of proliferation-inducing factor, as was IL2-induced NK-cell proliferation. NK cells could be maintained in culture for at least a month in the presence of proliferation-inducing factor alone, but the cells lost their cytolytic activity after 3-4 weeks in culture. Addition of IL2, to NK cells which had been cultured in the presence of proliferation-inducing factor, restored their cytotoxicity. Proliferation-inducing factor activity was partially purified on an anion exchange HPLC column. The molecular weight of proliferation-inducing factor appeared to be about 10 kDa, based on its elution profile on a sizing HPLC column. Our results indicate that proliferation-inducing factor is a novel NK-cell proliferation-inducing factor.

Keywords. NK cells; mitogen; cytokines; interleukin; MHC-AF; protein purification.

1. Introduction

Clonal expansion of activated T cells is mediated by the release of IL2 by T-helper cells. While T cells need to be activated to express receptors for and to become sensitive to IL2, natural killer (NK) cells are immediately responsive to IL2, apparently due to their constitutive expression of intermediate affinity receptors for IL2 (Caligiuri *et al* 1990). In addition to IL2, a variety of other cell proliferation-stimulating lymphokines are known, which induce B-cell proliferation (Paul and Ohara 1987; Alderson et al 1987; Namen *et al* 1988) or proliferation of mast cells and granulocytes (Metcalf 1985; Ihle and Weinstein 1986). Such proliferation-inducing factors may play a crucial role in generating and sustaining the normal immune response. These factors may also be important as immuno-therapeutic agents and, when used singly or in combinations, for correcting defective immune functions. In addition, attempts are being made to use

†Corresponding author (Fax, 091-11-6865886; Email, R K Saxena@jnuniv.ernet.in)

lymphocytes or their subsets for adoptive immunotherapy in various human diseases and sufficient *in vitro* expansion of these cells depends on growth factors able to support their proliferation. IL2 has been extensively used for expansion of LAK cells or tumour-infiltrating lymphocytes (TIL5) *in vitro* (Rosenberg and Lotze 1986; Heo *et al* 1987; Whiteside *et al* 1987; Wibke *et al* 1987). However, although IL2 is a growth factor for NK cells, it has been unable to support NK-cell growth for more than 15-21 days in culture (Rabinowich *et al* 1991). IL2, a recently discovered lymphokine (Kobayashi *et al* 1989; Stern *et al* 1990) appears to stimulate NK activity but does not support long-term culture of NK cells (Kobayashi *et al* 1989).

In the present communication, we describe an additional cytokine which may have a role in regulating human NK and T cell proliferation. Evidence for this factor, named proliferation-inducing factor (P1F), was first obtained from our studies of a different factor, the MHC-I expression-augmenting factor or MHC-AF (Saxena *et al* 1992, 1996). While studying MHC-AF, we found that partially-purified MHC-AF preparations induced proliferation in normal human peripheral blood mononuclear cells (PBMC). This observation has suggested that either the MHC-AF preparations were contaminated with IL2, a known T-cell or T-cell growth factor, or that they contained a distinct growth-promoting cytokine. Since the MHC-AF preparations had no detectable IL2, we considered the possibility that a new PIF, different from IL2, was present in MHC-AF. Evidence is presented here for the presence of PIF, distinct from IL2 or other known cytokines, in the Con A supernatants of human PBMCs, and the characteristics of this factor are described.

2. Materials and methods

2.1 *Reagents*

Various cytokines used in this study were obtained as gifts from the following sources: IL2 was kindly provided by Cetus Corp. (Emeryville, CA, USA); IL2, Hoffmann LaRoche (Nutley, NJ, USA); IL4 and IL7 by Sterling Drug Inc. (Malvern, PA, USA); IL6 by Sandoz (Vienna, Austria); IFN-α by Roussel Uclaf (Romainville, France); and TNF-α by Knoll Pharmaceuticals (Whippany, NJ, USA). The hybridoma cell line, HB95 (secreting anti-MHC class I mAb), was obtained from American Type Culture Collection (ATCC; Bethesda, MD, USA). Neutralizing monoclonal antibodies to IL2 were obtained from Genzyme (Cambridge, MA, USA). Reagents for gel filtration and isoelectric focusing were obtained from Sigma (St. Louis, MO, USA).

2.2 *Initial fractionation of PIF*

Preparations enriched in PIF activity were derived from culture supernatants of Con-A-activated peripheral blood derived mononuclear cells (PBMC). PBMC were isolated by Ficoll Hypaque density gradient centrifugation (Boyum 1974). Cells obtained by leukapheresis (Central Blood Bank of Pittsburgh) were diluted 20-fold in cold RPM1 1640 medium (Gibco), and a 20 ml aliquot was layered on top of 10 ml of Lymphoprep solution (density 1·077 g/ml; Pharmacia) and centrifuged at 600*g* for 15 min. Mononuclear cells were harvested from the interface and washed three times with RPMI. PBMC (5×10^6 cells/ml) were cultured in RPMI 1640 medium supplemented with 5% fetal calf

serum (FCS; Gibco), glutamine (2 mM), penicillin (50U/ml), streptomycin (50 μg/ml) and 25 mM HEPES (complete medium). Con-A was added to give a final concentration of 5 μg/ml and, after 3 days, culture supernatants were collected by centrifugation (2000 *g* for 15 min). Con-A supernatants were processed in batches of 1 to 4 liters. For each liter of a supernatant, 599 g of ammonium sulphate were added gradually at 4°C, and the supernatant allowed to remain overnight at 4°C. Precipitated proteins were pelleted by centrifugation at 10,000 g for 20 min and resuspended in 0.1 M glycine-HCl buffer at pH 2.0. The solution was dialyzed against the pH 2.0 buffer for 2 days, and then neutralized back to pH 7.0, using 10 N NaOH. Any insoluble material present at this stage was removed by centrifugation, and the solution concentrated to about 30 ml for each 1 liter of the starting material. The concentrate was fractionated on a 5 × 115 cm Sephadex G 100 column. For each Sephadex column separation, 30 ml of the concentrated supernatant were used. The column was run at 4°C, at a flow rate of 1 ml/min, using phosphate-buffered saline (PBS).

2.3 *Preparative isoelectric focusing* (*IEF*)

The fractions with PIF activity separated on the Sephadex G 100 column were pooled, concentrated and dialyzed against 1% glycine. This pool was fractionated further by preparative IEF, as described elsewhere (Saxena 1987). The IEF fractions were dialyzed against PBS and assayed for PIF activity. Active fractions were pooled and concentrated using a YM-5 Amicon ultrafiltration membrane.

2.4 *HPLC fractionation*

PIF preparations obtained after the Sephadex G 100 or IEF step, were further fractionated on a HR8 (anion-exchange HPLC column, Waters) or a PP125 (sizing HPLC column, Waters) column. PIF preparation in 10 mM Tris-HCl buffer pH 8·2 was applied on HR8 column and eluted using a gradient of 20 to 100% v/v of 0·5 M NaCl in 10mM Tris-HC1 buffer pH 8·2. Column fractions were filter-sterilized and assayed for PIF activity. For PP125 column fractionation, 0·2 ml aliquots of PIF-containing material in PBS were fractionated on a 10 ml column.

2.5 *Bioassay for PIF*

PBMC were adjusted to a concentration of 5×10^5 cells/ml and cultured (0·2 ml/well in 96-well plates) with or without the test PIF preparations for 5 days. A pulse of [^{3}H] thymidine (0·5 μCi/well, specific activity, 6·7 Ci/mrnol) was given during last 12 h of culture and cells harvested for [^{3}H] thymidine incorporation. In some experiments, purified NK or T cells were used in proliferation assays.

2.6 *MHC-AF bioassay*

Human tumour cells (HR, a gastric carcinoma cell line) were cultured at $0{\cdot}1 \times 10^6$ cells/ml in MEM complete medium, in the presence of the desired concentrations of the test fractions, for 2 to 3 days. At the end of culture, cells were harvested and stained for

class I MHC antigens as described before (Saxena *et al* 1988). Culture supernatant of HB95 hybridorna was used as a source of a mouse monoclonal antibody reactive to a non-polymorphic region of MHC class I antigens. Stained cells were fixed with 1% paraformaldehyde and analysed in a FACStar flowcytometer.

2.7 *Determinations of IL2 by ELISA or a bioassay*

ELISA for IL2 was performed using reagents from Collaborative Research, Bedford, MA, USA. The assay detects > 3IU/ml of 1L2 (WHO standard). An IL2 bioassay, using a CTLL line obtained from ATCC was performed as previously described (Gillis *et al* 1978).

2.8 *Isolation of T, B and NK cells*

PBMC preparations, isolated by Ficoll-Hypaque density-gradient centrifugation, were depleted of macrophages and B cells by passage through a nylon wool column, as described before (Saxena *et al* 1980). Cells were washed and adjusted to 4×10^6/ml and mixed with fetal calf serum and a 0·5% v/v suspension of sheep erythrocytes, in a 1:2:2 ratio. Mixtures were centrifuged at 400 *g* for 5 min and incubated for 1 h at 29°C. The cell pellet was gently resuspended, and rosetted T-cells and non-rosetted cells were separated by Ficoll density gradient centrifugation. Erythrocytes in the rosette population were lysed by a brief hypotonic shock (water for 30 s), washed and T cells were purified further by panning, using flasks coated with anti-CD3 mAb (AIS, Menlo Park, CA, USA), as described before (Letessier *et al* 1991; Pricop *et al* 1991). Non-rosetted cells were treated with an anti-CD3 monoclonal antibody (1:50 diluted of an anti-CD3 from DAKO Corp., Carpinteria, CA, USA). Cells were washed and anti-body-coated cells (T cells) removed by using magnetic beads coated with a goat anti-mouse Ig antibody (30 beads/cell, 30 min at 4°C, Advance Magnetics; Cambridge, MA, USA). T cells bound to the beads were removed by using a powerful magnet. The process was repeated twice to ensure a complete removal of T cells. Residual cells (NK cells) were washed and suspended in RPMI 1640 medium containing 10% v/v of human AB serum. In our experiments, this process enriched NK cells (identified by flowcytometric analysis as CD3– , CD16+ , CD56+ population) to levels ranging from 92-95%, (Pricop *et al* 1991).

For B cell isolation, the nylon wool step was replaced with a plastic adherence step to deplete macrophages. T cells were removed from this preparation by E-rosetting as described above. B cells were isolated by positive selection on plastic flasks pre-coated with anti-CD19 mAb (Wysocki and Sato 1978).

3. Results

3.1 *Proliferation inducing activity in MHC-AF preparations*

We have recently described a novel factor in the culture supernatants of Con A-activated human PBMC which induces marked augmentation of the MHC class I antigen expression on a variety of tumour cell lines (*Saxena et al* 1992, 1996). This factor

named MHC-AF (MHC I expression-augmenting factor) was also found to induce proliferation in PBMC preparations. Figure 1 shows a dose-response curve of this effect. In this representative experiment, maximum incorporation was 10,000 cpm/well, which is significantly lower than a typical mitogen induced proliferative response (results not shown). To determine if proliferation-inducing activity could be separated from MHC-AF, fractions obtained by preparative IEF of the MHC-AF preparation recovered from a Sephadex G 100 column were tested for MHC augmenting as well as proliferation-inducing activity. Figure 2 shows the major peak of MHC-AF activity in fractions 9-11 (pI 5·7-6·2) and the same fractions also contained the proliferation-inducing activity. Fractions 1 to 6 however had significant MHC-AF activity but no proliferation-inducing activity (henceforth called proliferation-inducing factor or PIF).

3.2 *Further separation of PIF and MHC-AF*

Preparations obtained by preparative IEF were further fractionated on HR8 anion exchange HPLC column (figure 3). PIF and MHC-AF elution profiles in figure 3A and

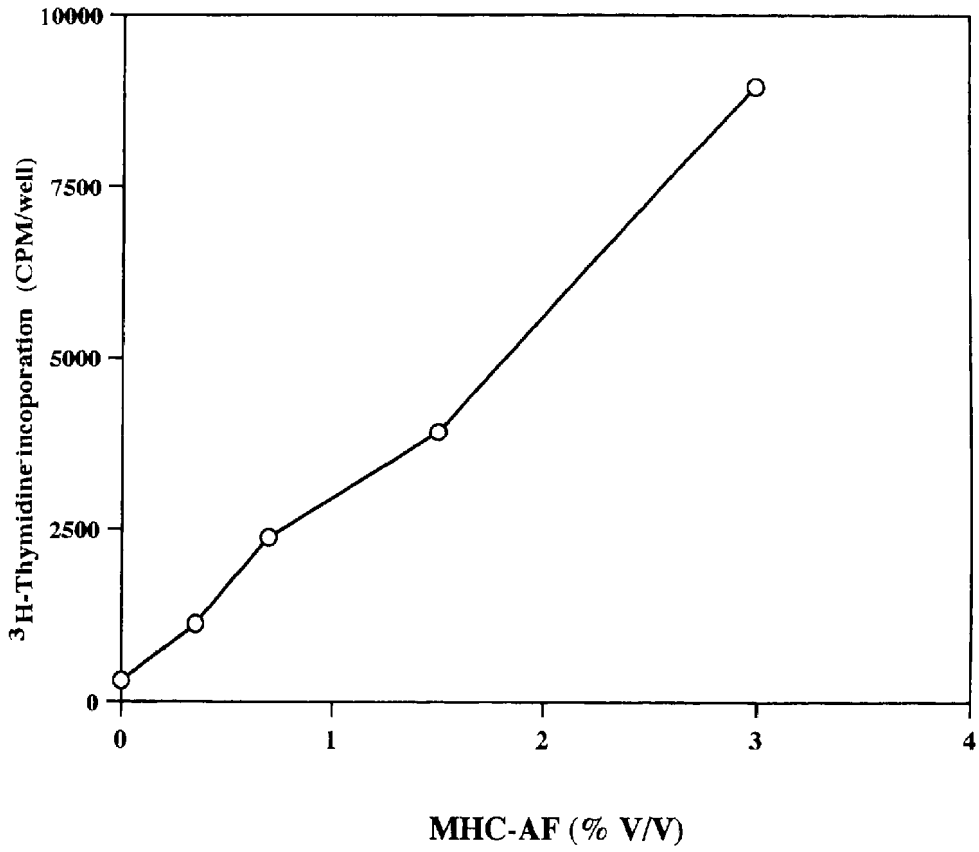

Figure 1. Proliferation of human PBMC in response to partially purified MHC-AF. Human PBMC were cultured (5×10^5/ml, 0·2 ml/well) with various concentrations of a Sephadex G100 purified preparation of MHC-AF (Saxena *et al* 1995) for 5 days. 12 h prior to harvest, cells were pulsed with 0·5 μCi of [^{3}H] thymidine/well. Each point in the graph represents a mean of three replicate assay wells.

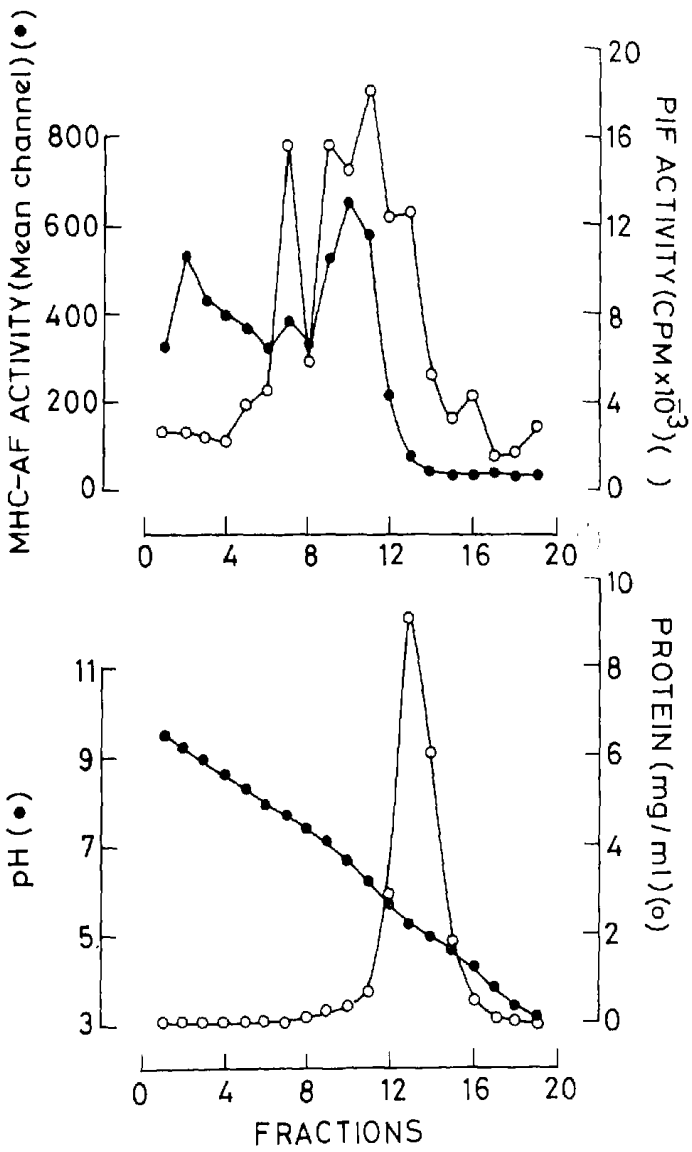

Figure 2. Isoelectric focusing of PIF and MHC-AF activities. Preparation obtained after Sephadex G100 column fractionation (160 mg of protein) was fractionated by preparative isoelectric focusing as described before (Saxena 1987). Fractions were assayed for MHh-AF activity at 10% (v/v), using HR cells as indicator cells and for PIF activity at 5% (v/v), using purified human PBLs as described in § 2.

B respectively, indicate that the two activities could be resolved as two different peaks on HR8 columns. Fractions positive for PIF activity did not have IL2 activity, as determined by CTLL assay. The IL2-containing peak eluted earlier than PIF (figure 3A) MHC-AF and PIF activities could also be resolved on HPLC sizing column (figure 4) The PP125 column was calibrated with protein markers of known molecular weight and using the calibration curve (not shown), the molecular weight of PIF was estimated to be 10 kDa, as compared to 35 kDa for MHC-AF.

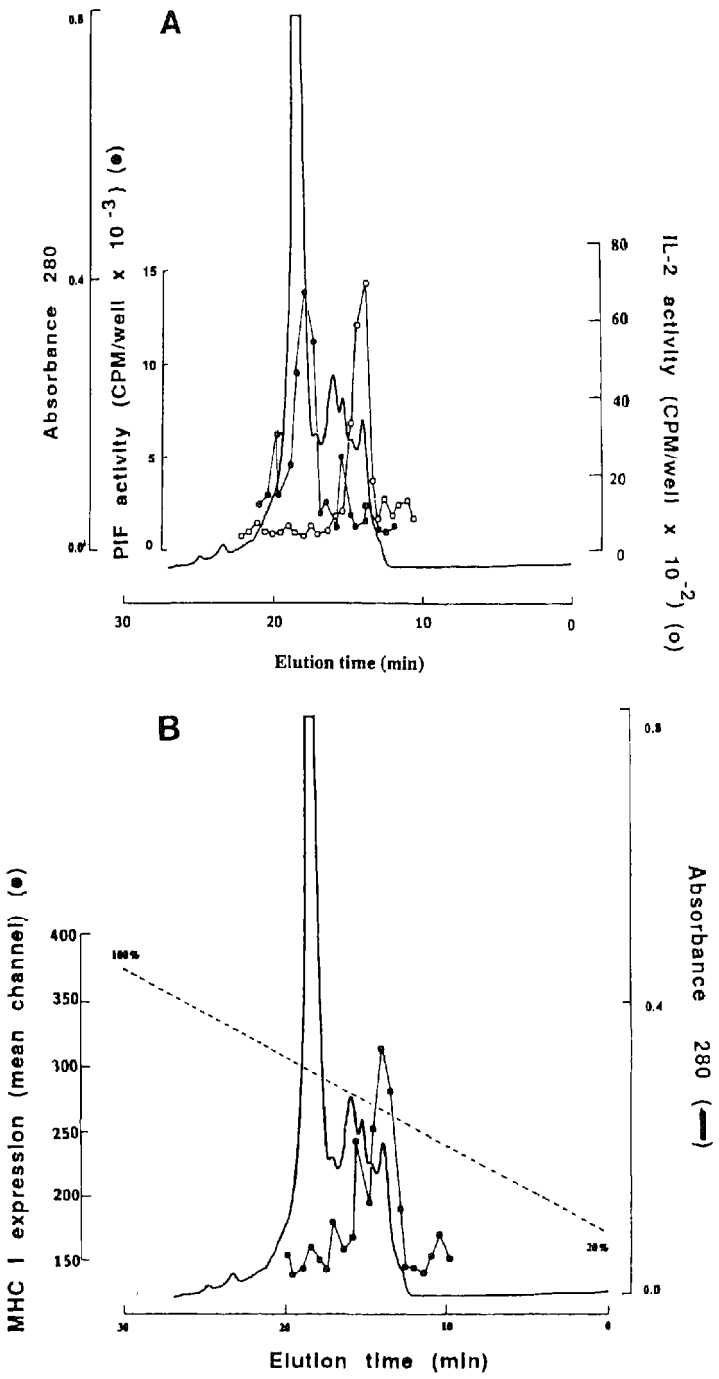

Figure 3. Fractionation of PIF, on an HR8 ion-exchange HPL column. A pooled Sephadex G-100 purified MHC-AF preparation was fractionated on HR8 ion-exchange column. The NaCI gradient used for elution was from 20% of 0.5 M NaCI in 0·01 M Tris-HCI buffer at pH 8·2 to 100% of 0·5 M NaCI in the same buffer. The fractions obtained were assessed for PIF ( ) and IL2 (O) (**A**) and MHC-AF (**B**) activities as described in §2.

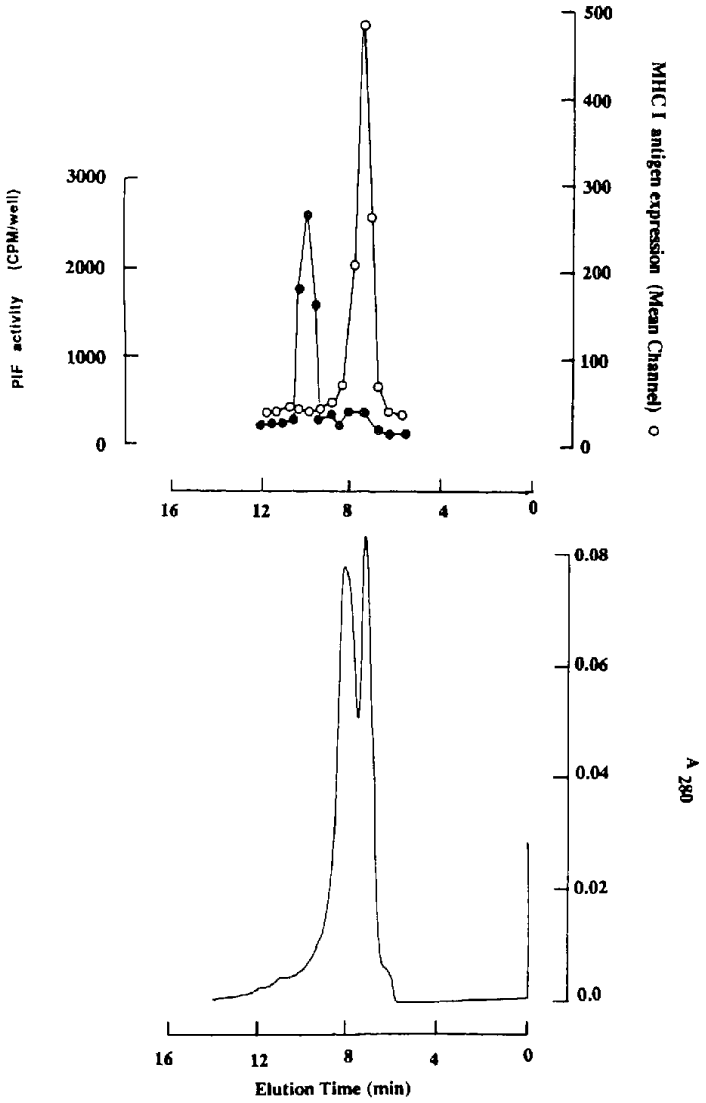

Figure 4. Fractionation of PIF and MHC-AF activities on a PP125 HPLC column. 100 μl of a PIF preparation pooled after the IEF step were fractionated on a PP125 sizing HPLC column. Fractions of 250 μl were collected and assayed for PIF and MHC-AF activities at 10% (v/v) by the procedure described in §2. The lower panel shows the protein profile, and the top panel shows the separate peaks with PIF (●) or MHC-AF ( ) activities.

3.3 *Determination of cells responsive to PIF*

In order to determine the cell population (s) which proliferated in response to PIF, highly enriched populations of T cells, B cells and NK cells were obtained from PBMC and cultured in the presence of PIF-containing preparations. Figure 5 shows that human NK cells proliferated in response to PIF, while B and T cells did not. Proliferation of NK cells was dose-dependent on P1F with about 20-fold increase in

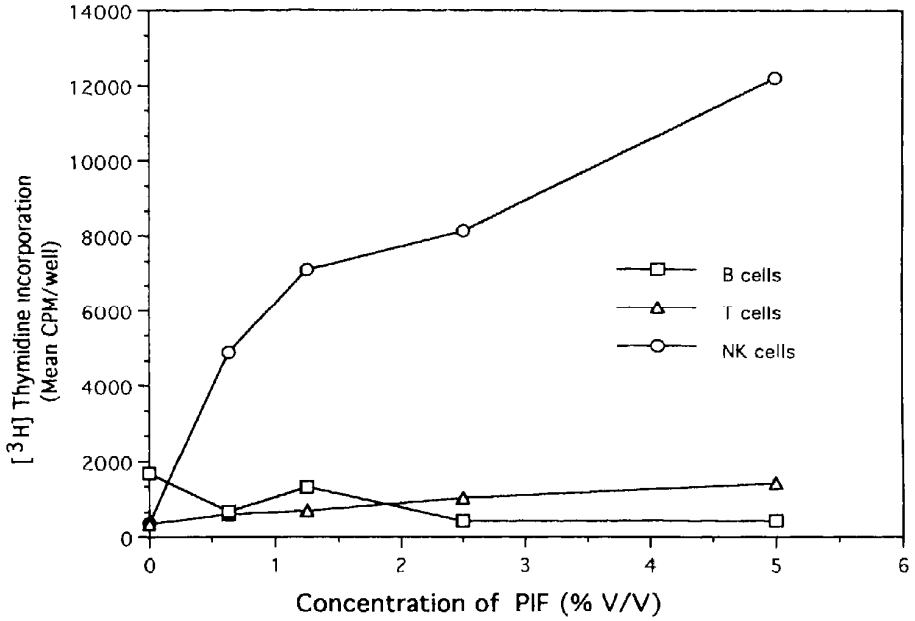

Figure 5. Effect of PIF preparations on proliferation of T, B or NK cells. The purified cell preparations were cultured (5×10^5 cells/ml, 02 ml/well) with various concentrations of PIF purified by IEF for 5 days. 5% V/V of PIF preparation corresponded to a final protein concentration of 10 μg/m1 in the assay medium. Cells were pulsed with [^{3}H] thymidine for 12 h before harvest. Each point represents a mean of three replicate assay wells. Results of one representative experiment of the three done, have been shown.

[^{3}H] thymidine incorporation at the highest concentration tested, and was consistently observed with HPLC column-purified as well as partially-purified PIF. These observations indicated that PIF induced selective growth of human NK cells.

3.4 *Relationship of PIF to other known cytokines*

Since IL2 is known to induce proliferation of NK cells and IL12 is also known to have NK-cell stimulating effects (Kobayashi *et al* 1989; Stern *et al* 1990), we next compared the proliferative response of human purified NK cells to IL2, IL12 and PIF. Both IL2 and PIF induced significant proliferative responses in NK cells (table 1). IL12 was, however, not active in our assay. In other similar experiments, recombinant IL12, did occasionally induce a low level proliferative response in NK cells. However partially purified PIF preparations had no activity in IL12 bioassay (Maurice Gately, personal communication).

In order to further clarify a possible relationship between PIF and IL2, we attempted to neutralize P1F activity by antibodies against human IL2. Results of a typical experiment, shown in table 2, indicate that monoclonal anti-human IL2 antibodies completely neutralized the proliferation-inducing abilities of IL2 but had no effect on PIF-induced NK-cell proliferation. Taken together, our results indicate that PIF and

Table 1. Proliferative response of human NK cells to IL2, IL12 and PIF.

Agent	Concentration	[^{3}H] thymidine incorporation (cpm/well)
None	—	102 ± 43
IL2	10·0 U/ml	6,657 ± 526
	5·0 U/ml	4,755 ± 76
	2·5 U/ml	2,602 ± 665
	1·25 U/ml	1,178 ± 243
IL12	100·0 U/ml	196 ± 84
	50·0 U/ml	86 ± 20
	25·0 U/ml	113 ± 45
	12·5 U/ml	75 ± 3
PIF	30 μg/ml	7,697 ± 397
	15 μg/ml	5,147 ± 243
	7·5 μg/ml	2,539 ± 185
	3·7 μg/ml	1,357 ± 176

Purified human NK cells were cultured (5 × 10^5/ml, 0.2 ml/well) in the presence or absence of various concentrations of I L2, IL12 or PIF (a post-IEF preparation) for five days. hells were pulsed with [^{3}H] thymidine for 12 h prior to harvest. The data are mean cpm ± SD of Four replicate assay wells.

Table 2. Effect of anti-IL2 Mab on the activity of PIF on NK cells.

Proliferation inducing agent	Addition of anti-IL2 Mab	Thymidine incorporation (cpm/well)
—	No	47 ± 14
IL2	No	4,465 ± 446
IL2	Yes	387 ± 178
PIF	No	4,206 ± 971
PIF	Yes	4,285 ± 446

NK cells purified from PBMC were cultured (5 × 10^5/ml, 0·2 ml/well) with IL2 (5 U/ml) or PIF purified by IEF (30 kig/m1) in the presence or absence of an anti-IL2 mAb capable of neutralizing 5 U/ml of IL2. After five days of culture, cells were pulsed with 0·5 μCi/ml of [^{3}H] thymidine for 12 h before harvest. The PIF preparation used had no detectable IL2 levels. The data are mean cpm/well ± SD from one representative experiment of three performed.

IL2 are distinct factors capable of inducing proliferation of human NK cells. A variety of other known cytokines, including IL4, IL6, IL7, IFN-α and TNF-α were also examined in proliferation assays with human NK cells and found to be unable to induce proliferation of these cells (results not shown).

3.5 *Potentiation of IL2 activity by PIF*

Effects of a combination of PIF and IL2 on different subsets of human lymphocytes were also examined. When freshly isolated, purified T cell populations were used, IL2 (up to a dose of 10 U/ml) had only a marginal proliferative effect (figure 6). PIF preparation by itself had no effect on T cell proliferation, but when added to the cultures together with IL2, a marked boosting of T cell proliferation was observed (figure 6).

The effects of various combinations of IL2 and PIF on NK cell proliferation are shown in table 3. Human NK cells cultured in medium alone showed little [^{3}H] thymidine incorporation, and addition of IL2 at 20-100 U/ml had a dose-dependent proliferation-inducing effect on these cells. Increasing the dose to 1,000 U/ml IL2 did not further augment proliferation of these cells. At all IL2 dose levels, addition of PIF resulted in an additive proliferative effect on NK cells.

Induction of NK cell proliferation by PIF preparations was investigated further to determine if purified human NK cells could be cultured in the presence of PIF for prolonged time periods. We found that NK cells could be cultured for up to 1 month in the presence of PIF alone. Although NK cells proliferated in these cultures, they did not retain cytotoxic activity. Addition of IL2 to these proliferating yet non-cytolytic cultures resulted in up-regulation of cytolytic activity. Results in table 4 show that although NK cells cultured with PIF alone for 7 days had considerable cytotoxicity, by day 15, they were no longer cytotoxic. In all experiments, when IL2 was added as the second treatment to these cultures, the cytolytic activity of NK cells was restored. In

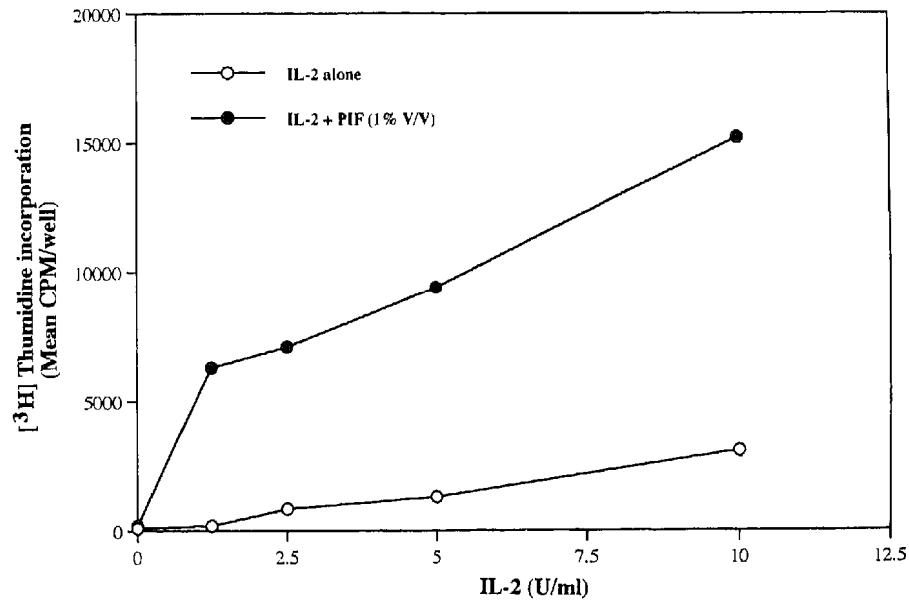

Figure 6. Combined effects of IL2 and a PIF preparation on proliferation of T cells. Purified T cells were cultured (5×10^5/ml, 0·2 ml/well) with different concentrations of IL2 in the presence or absence of post-IEF PIF (1%, v/v). After 5 days of culture, cells were pulsed with [^{3}H] thymidine (0·5 μCi/well) for 12 h prior to harvest. Each point in the graph is a mean of three replicate assay wells. A representative experiment of three performed is shown.

Table 3. Effects of combination of PIF and IL2 on proliferation of human NK cells.

PIF concentration (μg/ml)	IL2 (U/ml)	Thymidine incorporation (cpm/well)
0	—	262 ± 4
10	—	6,240 ± 1429
20	—	10,375 ± 3277
0	10	6,432 ± 577
10	10	20,877 ± 1241
20	10	21,565 ± 1598
0	100	18,476 ± 1211
10	100	32,162 ± 811
20	100	34,098 ± 1000
0	1000	19,510 ± 378
10	1000	33,142 ± 776
20	1000	36,056 ± 440

NK cells purified from peripheral blood were cultured (5×10^5/ml, 0·2 ml/well) with various concentrations of human recombinant IL2 and an IEF-purified PIF preparation for 5 days.Cells were harvested following a 12h pulse with [^{3}H] thymidine (0·5 μCi/well). The data are mean cam ± SD obtained from four replicate assay welis. One representative experiment of six performed is shown.

contrast, when NK cells were cultured in PIF alone, they had no cytolytic activity at the end of 15 or 25 day cultures, although they mediated NK activity on day 7 of culture (table 4). These results show that PIF can support long-term proliferation of NK cells in culture but not their cytotoxicity beyond day 7 of culture.

4. Discussion

In this manuscript, we have provided evidence for the presence of a factor in the culture supernatants of Con A-activated PBMC, which induces and supports proliferation of human purified NK cells. No prior activation of NK cells was required, and addition of PIF to resting NK cells allowed these cells to be cultured and expand for up to 25 days.

During our purification of a different factor, named MHC class I expression-augmenting factor (MHC-AF), it became apparent that some of the fractions of the culture supernatants of Con-A-activated PBMC contained a proliferation-inducing factor which was distinct from MHC-AF. Although initial partially-purified MHC-AF preparations were found to induce proliferation of PBMC, further purification allowed us to separate MHC-AF and PIF. While PIF and MHC-AF activity peaks overlapped considerably on preparative IEFs, they could be resolved on either HR8 ion-exchange or PP125 sizing HPLC columns. The two factors not only had distinct biologic effects but were also different in their characteristics. For example, molecular weights of 35 kDa and 10 kDa have been estimated for MHC-AF and PIF, respectively, based on the PP125 column elution profiles. These results clearly show that the MHC-AF and PIF activities reside in distinct molecules.

Table 4. Cytolytic activity of NK cells maintained in long-term culture with PIF and IL2.

NK cell culture				Target lysis (%)	
Phase I duration	Treatment	Phase II duration	Treatment	E/T 0	E/T 5
4 h	PIF	7 d	Nil	1·3	0·5
4 h	IL2	7 d	Nil	10·2	4·2
7 d	PIF	Nil	Nil	34·2	18·1
7 d	IL2	Nil	Nil	41·6	34·1
15 d	PIF	Nil	Nil	3·7	2·3
15 d	IL2	Nil	Nil	17·0	8·1
21 d	PIF	Nil	Nil	1·3	0·3
15 d	PIF	7 d	IL2	68·5	47·3
21 d	IL2	Nil	Nil	55·1	35·1
25 d	PIF	Nil	Nil	1·1	0·8
20 d	PIF	5 d	IL2	57·0	31·0
15 d	PIF	10 d	IL2	61·0	36·0
25 d	IL2	Nil	Nil	76·0	62·0

Purified human NK cells (5×10^5/m1) were cultured with PIF (10 μg/m1 of a post-IEF preparation) or IL2 (10 U/ml for culture durations up to 15 days, 100 U/ml for culture durations more than 15 days) added at various times during culture, as indicated. At the end of the cultures, cells were harvested, washed and tested for cytotoxicity against K562 targets in 4 h ^{51}Cr-release assays.

Although PIF appears to be distinct from MHC-AF, its relationship to or identity with other known cytokines remained unclear. We, therefore, compared the effects of a variety of recombinant cytokines, including ILL IL2, IL4, IL6, IL7, IL12, IFNs and TNF with those of PIF. When isolated and purified subpopulations of lymphocytes were cultured in the presence of PIF, only preparations enriched in NK cells were able to proliferate in short- and long-term cultures. Besides IL2, none of the other cytokines tested were significantly active in inducing NK-cell proliferation. No IL2 could be detected in PIF preparations, either by a sensitive bioassay or by IL2-specific ELISA. Although these results do not rule out the presence of IL2 in levels below the detection limit of the assays utilized, PIF activity could not be neutralized by monoclonal antibodies to human IL2 which were able to neutralize 5 U of IL2 in parallel experiments. The combination of PIF and IL2 also resulted in improved proliferative responses of purified NK and T cell preparations, to IL2 as compared to those in the presence of IL2 alone.

IL12, a disulphide linked heterodirner of 75 kDa is an NK-cell stimulatory lymphokine that has been described and cloned (Kobayashi *et al* 1989; Stern *et al* 1990). In some of our experiments, IL12 induced a weak proliferative response in purified NK cells. However, this effect was not consistently seen and when observed was much lower than the effect of PIF. Moreover, the molecular weight of PIF, estimated at 10 kDa in preliminary experiments, is not comparable with that of IL12 (75 kDa). Another cytokine IL15 has recently been described, which like IL2, induces proliferation in CTLL cell line (Grabstein *et al* 1994). PIF is unlikely to be IL15 since the former has no effect on the proliferative activity of CTLL cell line (figure 3).

Taken together, our results indicate that a novel proliferation-inducing factor is present in supernatants of Con A-activated PBMC. This factor, PIF when used alone, has activity restricted to NK cells, but when used in combination with IL2, it also promotes T cell proliferation. Further purification and characterization of PIF is necessary to gain a better understanding of its nature. An NK cell proliferating factor is of great potential interest, since it has been very difficult to grow human NK cells in long-term cultures, even in the presence of feeder cells and mixtures of various cytokines (Rabinowich *et al* 1991). With the availability of PIF, it may be possible to expand human NK cells *in Vitro* and to obtain numbers of NK cells necessary for immunotherapy in clinical trials (Hercend *et al* 1990; Whiteside *et al* 1990). Studies are in progress in our laboratory to isolate PIF and to demonstrate its applicability for large-scale culture of human NK cells.

References

Alderson M R, Pike B C, Harada K, Tominaga A, Takatsu K and Kossal G V 1987 Recombinant T cell replacing factor (interleukin 5) acts with antigen to promote the growth and differentiation of single hapten specific B lymphocytes*; J. Immunol*. **139** 2656- 2660

Boyum A 1974 Separation of blood leukocytes, granulocytes and lymphocytes; *Tissue Antigens* **4** 269 274

Caligiuri M A, Zmuidzimas A, Manley T S, Levine H, Smith K A and Ritz J 1990 Functional consequences of interleukin 2 receptor expression on resting human lymphocytes; *.J. Exp. Med*. **171** 1509-1526

Gillis S, Fenn M M, 0u W and Smith K A 1978 T cell growth factor: Parameters of production and a quantitative microassay for activity; *J. I minimal*. **120** 2027-2032

Grabstein K H, Rauch C, Srinivasan S, Fung V, Beers C, Richardson J, Schoenborn A, Andieh M, Johnson I Alderson M R, Watson J D, Anderson D M and Girt J G 1994 Cloning of a T celi growth factor that interacts with the interleukin-2 receptor; *Science* **264** 965 968

Heo D S, Whiteside T L, Johnson J T, Chen K, Lawrence E L and Herberman R B 1987 Long-term interleukin 2-dependent growth and cytotoxic activity of tumor-infiltrating lymphocytes from human squamous cell carcinomas of the head and neck; *Cancer Res*. **47** 1- 6

Hercend T, Farace F, Baume D, Charpenter F, Droz J Triebel F and Escudicr B 1990 Immunotherapy with lymphokine-activated natural killer cells and recombinant interleukin 2. A feasibility trial in metastatic renal cell carcinoma; *.J. Biol. Resp. Modif* **9** 546-555

Ihle J N and Weinstein Y 1986 Immunological regulation of hematopoietic/lymphoid stem cell differentiation by interleukin 3; *Adv. Immanol*. **39** 1-50

Kobayashi M, Fitz L, Ryan M, Hevick R M, Clark S C, Chan S, Loudon R, Sherman F, Perussia B and Trinchieri G 1989 Identification and purification of natural killer cell stimulatory factor (NKSF); *J. Exp Med*. **170** 827-845

Letessier E, Heo D S, karma T, Johnson J T, Herberman R B and Whiteside T L 1991 Enrichment tumor-reactive C^{+} T lymphocytes by positive selection from the blood and lymph nodes of patient with head and neck cancer; *Cancer Res*. **51** 3891-3899

Metcalf D 1985 The granulocyte-macrophage colony stimulating factors; *Science* **229** 16-22

Namen A E, Schmierer A, March h J, Overell R W, Park L S, Urdal D L and Mochizuki D Y 1988 B cell precursor growth-promoting activity. Purification and characterization of a growth factor active on lymphocyte precursors; *J. Exp. Med*. **167** 988-1002

Paul W E and Ohara J 1987 B cell stimulatory factor-l/interleukin 4; *Annu. Rev. Immunol*. **5** 429-459

Pricop L, Galatiuc C, Manciulea M, DeLeo A, Sulica A, Whiteside T L and Herberman R B 1991 Expression of Feu receptors on human natural killer cells; *Clin. Immunol. Immunopathol*. **59** 355-367

Rabinowich H, Sedlmayr P, Herberman R B and Whiteside T L 1991 Increased proliferation, lytic activity and purity of natural killer cells co-cultured with mitogen-activated feeder cells; *Cell. Immunol*. **135** 454-4711

Rosenberg S A and Lotze M T 1986 Cancer immunotherapy using interleukin 2 and IL2-activated lymphocytes; *Annu. Rev. Immunol*. **4** 681-709

Saxena R K 1987 A spleen cell derived factor imparts resistance to NK cell mediated lysis in a mouse lymphoma celi line; *Immunol. Lett*. **15** 105-108

Saxena Q B, Mezey E and Adler W A 1980 Regulation of natural killer activity *in vivo*. II. The effect of alcohol consumption on human peripheral blood natural killer activity; *Int. J. Cancer* **26** 413 -417

Saxena R K, Saxena Q B, Sarin A and Herberman R B 1992 Natural killer-resistance inducing factor (NK-RIF): A new immuno-modulatory cytokine; in *Natural killer cells: Receptors, signalling* and *mechanisms* (eds) E Lotzova and R. B Herberman (New York: CRC Press) pp 381-392

Saxena R K, Saxena Q B, Whiteside T L, Goldfarb R H and Herberman R B 1996 Partial purification and characterization of a novel human factor that augments the expression of class I MHh antigens on tumour cells; *J. Biosci*. **21** 13 25

Stern A S, Podlaski F, Hulmes J D, Pan Y E, Ouiner P M, Wolitzky A G, Fanilleti P C, Stremlo D L, Truitt T, Chizzoniti R and Gately H K 1990 Purification to homogeneity and partial characterization of cytotoxic lymphocyte maturation factor from human B lymphoblastoid cells; *Proc. Natl. Acad. Sci. USA* **87** 6808-6812

Whiteside T L, Ernstoff M S, Kair S, Kirkwood J M and Herberman R B 1990 *In vitro* generation and *in vivo* effects of adherent-lymphokinc activated killer (A-LAK) cells and IL2 in patients with solid tumour; in *Natural killer cells: Biology and clinical application*, (ed.) R E Schmidt (6th Int. NK Cell Workshop, Goslar, Hannover Basel, Karger, Switzerland) pp 293- 302

Whiteside T L, Heo D S, Takagi S, Johnson J T, lwatsuki S and Herberman R B 1987 Cytolytic anti-tumour cells in long-term cultures of human tumour-infiltrating lymphocytes in recombinant interleukin 2; *Cancer Immunol. Immunother*. **47** 6353-6357

Wibke E A, Lotze M T and Rosenberg S A 1987 Tumour cell susceptibility to lysis: marked increase in lysis by tumour-infiltrating lymphocytes following target stimulation with interferon-gamma and tumour necrosis factor-alpha; *Implicat. Immunother. Sing. Forum*. **38** 436-441

Wysocki LJ and Sato V L 1978 "Panning" for lymphocytes: A method for cell selection; *Proc. Natl. Acad. Sci. USA* **75** 2844-2849

Corresponding editor: INDIRA NATH