Lymphocyte recovery and clinical response in multiple myeloma patients receiving interferon $\alpha_{2\beta}$ after intensive therapy

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Summary The recovery of immunoregulatory cells in the peripheral blood of patients with multiple myeloma receiving maintenance therapy with interferon $\alpha_{2\beta}$ (IFN-$\alpha_{2\beta}$) after intensive therapy with high-dose melphalan and autologous bone marrow or peripheral blood stem cell rescue was studied. IFN-$\alpha_{2\beta}$ significantly inhibited the recovery of CD3$^+$, CD4$^+$, CD8$^+$, CD56$^+$/CD3$^-$ and CD16$^+$/CD3$^-$ lymphocytes compared with numbers found in patients who had no further post-transplant treatment, but had no effect on the recovery of CD19$^+$ cells. Among patients who did not receive IFN-$\alpha_{2\beta}$, the number of CD8$^+$, CD56$^+$/CD3$^-$ and CD16$^+$CD3$^-$ lymphocytes recovered to values similar to normal volunteers with increasing time after intensive therapy, however the number of CD4$^+$ cells remained significantly below levels found in normal volunteers. Although CD16$^+$/CD3$^-$ and CD56$^+$/CD3$^-$ cell numbers were reduced in patients receiving IFN-$\alpha_{2\beta}$, natural killer (NK) activity was not effected. The levels of soluble interleukin 2 receptor (sIL-2R) were similar in all patients and IL-2 was not detected in any patient. At the time of writing, of the total of 69 patients, seven have relapsed, of whom three were receiving IFN-$\alpha_{2\beta}$, however there was no correlation between the absolute numbers of any lymphocyte subset with imminent relapse. The data suggest that the recovery of a specific lymphocyte subset(s) in peripheral blood is unlikely to be associated with the maintenance of response after intensive therapy.

Keywords: multiple myeloma; interferon $\alpha_{2\beta}$; immune recovery; B cell; NK activity

Multiple myeloma (MM) is a disease of the B-cell lineage and remains incurable. Encouraging results from two clinical studies suggest that maintenance therapy with interferon $\alpha_{2\beta}$ (IFN-a$_{2\beta}$) after intensive therapy with autologous bone marrow rescue (ABMR) (Attal et al., 1992; Cunningham et al., 1993) increases the duration of response and may prolong survival (Cunningham et al., 1993). Since this therapeutic benefit is confined to patients who have had a complete response to treatment, it has been suggested that IFN-$\alpha_{2\beta}$ is only effective in patients who have had a substantial reduction of the tumour burden (Attal et al., 1992). Thus far, there has been no indication that IFN-$\alpha_{2\beta}$ improves the clinical status after intensive therapy, suggesting that the mechanism(s) involved in its therapeutic efficacy is concerned with the maintenance of tumour homeostasis rather than direct cytotoxicity.

The existence of monoclonal gammopathies of undetermined significance (MGUS) and plateau phase MM shows that monoclonal B cells can be kept under homeostatic control in vivo. In the case of plateau phase disease, this has been attributed to complex interactions between the malignant clone and the immune system (Joshua, 1988). Several lines of evidence suggest that the evolution of the disease is influenced by the behaviour of immunoregulatory cells. Idiotype-reactive T cells have been found in MM patients with early stage disease (Dianzani et al., 1988; Osterborg et al., 1991) and high levels of interleukin 2 (IL-2) have been correlated with prolonged survival (Cimino et al., 1990). In contrast, high levels of soluble IL 2 receptor (sIL-2R) in peripheral blood correlated with active disease (Vacca et al., 1991) and low CD4$^+$ cell numbers have been associated with both advanced clinical disease and shorter survival (San Miguel et al., 1992).

Massaia et al. (1993) have shown that activation of CD3$^+$ lymphocytes from MM patients in vitro results in greater production of IFN-γ than that produced from normal donor cells. This may have an association with putative tumour control in vivo since IFN-γ, as well as IL-2, induces the differentiation of allergen-specific T cells in vitro to TH-1 clones and is induced by IFN-α (Parronchi et al., 1992).

As well as T cell-mediated immunoregulation, there is evidence that natural killer (NK) and lymphocyte-activated killer (LAK) cells may play a role in the regulation of MM. Although the number of precursors of cytotoxic cells may be reduced in patients with MM (Massaia et al., 1988) and LAK activity of peripheral blood T- and non-T lymphocytes is reduced compared with normal donors (Massaia et al., 1989), both cellular and humoral effector mechanisms are retained that can regulate the behaviour of MM cells in model systems (Abbas, 1979; Rohrer and Lynch, 1979). Despite a reduction in NK activity in patients with advanced MM (Osterborg et al., 1990) a signifigant increase has been found in patients with early stage disease (Gonzalez et al., 1992) and in patients following response to treatment or during plateau phase (Osterborg et al., 1990). In vitro exposure of peripheral blood mononuclear cells from MM patients to IFN-$\alpha_{2\beta}$ increases NK activity (Uchida et al., 1984) and in vivo administration of IFN-$\alpha_{2\beta}$ increased NK activity in patients given the cytokine as sole treatment (Einhorn et al., 1982).

The aim of this study was to determine whether the recovery of one or more lymphocyte subsets associated with immune regulation is enhanced in patients receiving high-dose melphalan (HDM) in combination with ABMR or peripheral blood stem cell rescue (PBSCR) followed by maintenance therapy with IFN-$\alpha_{2\beta}$ and may serve as a measure of the therapeutic effect(s) of the cytokine. Comparisons have been made between patients who are receiving IFN-$\alpha_{2\beta}$ with those who have had no further treatment after intensive therapy and with normal volunteers. In addition, the clinical response of patients to HDM followed by IFN-$\alpha_{2\beta}$ has been monitored to determine whether the cytokine improves the anti-tumour effect of intensive therapy.

Materials and methods

Clinical samples

Samples (10 ml) of clotted and heparinised peripheral blood were taken at out-patient clinics from patients and normal

volunteers after informed consent and approval by the local Royal Marsden Hospital Ethics Committee. All patients had received treatment with one of two conditioning regimens, either CY-VAMP [i.v. infusion of vincristine (0.4 mg) and doxorubicin (9 mg m^{-2}) over 24 h for 4 days with bolus of methylprednisolone (1.5 g i.v. or orally for 5 days) plus cyclophosphamide (500 mg i.v. bolus on days 1, 8 and 15)] or VERCY-VAMP (vincristine, doxorubicin, methylprednisolone and cyclophosphamide (doses as before) plus verapamil (10 mg i.v. over 24 h for 5 days) followed by HDM either 200 mg m^{-2} with ABMR or PBSCR or 140 mg m^{-2} alone. Patients who had ABMR were given methylprednisolone (1 g m^{-2} daily for 5 days) after HDM. In the total group of 69 patients, two received high-dose busulphan (HDBu) (16 mg kg^{-1} body weight over 4 days) with ABMR, seven were given IFN-$\alpha_{2\beta}$ after CY-VAMP, one received HDM and two received HDM with methylprednisolone without rescue. When their leucocyte count was greater than 3×10^9 l^{-1} and platelets were greater than 100×10^9 l^{-1}, patients were given IFN-$\alpha_{2\beta}$ (Intron-A, Shering-Plough) at 3×10^6 U m^{-2} subcutaneously three times weekly until relapse.

The distribution of treatment protocols of patients who had received IFN-$\alpha_{2\beta}$ for more or less than 12 months was similar to that of patients who had no further treatment in each group.

Clinical status

A complete remission (CR) was defined as the absence of measurable paraprotein and bone marrow infiltration by myeloma cells of <5%. A partial response (PR) was defined as a paraprotein level reduced by 50% and improvement in all other clinical features sustained for greater than 1 month.

Flow cytometric analysis

All blood samples were counted and used for flow cytometric analysis within 2 h of collection. Aliquots of 50 μl were dispensed into tubes with 10 μl of ready-conjugated antibody agitated on a mixer and incubated at 4°C for 20 min. Lysis solution (1 ml) (Ortho-Diagnostics, UK) was added to each tube and incubation continued at room temperature for 10 min. The tubes were transferred to an ice bath and analysed immediately. The antibodies used were mouse anti-human CD45-FITC/CD14-RPE (Sigma, UK), CD4-FITC/CD8-RPE (Sigma, UK), CD3-FITC, CD19-RPE, CD16-RPE and CD56-RPE (Becton Dickinson, UK).

Analysis was done using an Ortho Cytoronabsolute (OrthoDiagnostics, UK) with gating for lymphocytes. The machine was calibrated before use with normal donor blood that had been assessed for the different count. Calibration for the lymphocyte gate ensured that >99% of the gated cells were lymphocytes. Data with experimental samples were collected in list mode file and analysed using the software package supplied by the manufacturer. All subsequent calculations were based on the total white cell count assayed by Coulter Counter to provide absolute counts of individual lymphocyte subsets.

NK activity

Freshly harvested lymphocytes obtained by Ficoll–Hypaque separation of whole blood (Boyum, 1968) followed by removal of phagocytic cells with carbonyl iron were used as effector cells. The percentage of lymphocytes in the resulting suspensions ranged from 85% to 99%. The K562 cell line was used as target cells for NK activity. Cells were labelled with 200 μCi 10^{-6} cells of ^{51}Cr (1 mCi ml^{-1} Amersham International, UK) in a volume of 200 μl. Samples were tested at four effector-target (E/T) cell ratios (60:1, 30:1, 15:1 and 7.5:1) using 5000 K562 cells per well in a total volume of 200 μl in 96-well microtitre plates. Samples were assayed in triplicate. The cell mixtures were incubated for 18 h at 37°C. The radioactivity in the supernatant was analysed in a gamma counter (Hydragamma 16, Innotron,

UK). Spontaneous release was determined by incubation of ^{51}Cr-labelled cells with medium alone. Maximum release was measured by incubating the cells with lysis buffer (20 mM Hepes pH 6.9, 5 mM potassium chloride, 5 mM magnesium acetate, 1 mM DTT and 0.5% Nonidet 140). The percentage of ^{51}Cr released was calculated from the formula;

$$\% \quad \text{release} = \frac{(\text{experimental } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release})}{(\text{total } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}} \times 100$$

Absolute numbers of CD16$^+$/CD3$^-$ and CD56$^+$/CD3$^-$ lymphocytes were calculated after depletion of phagocytic cells using flow cytometry as above. The cells were labelled with CD45-FITC/CD14-RPE, CD3-FITC/CD16-RPE and CD3-FITC/CD56-RPE.

Detection of IL-2 and sIL-2R

Samples of clotted blood were separated and the serum stored at $-20°$ until use. IL-2 and IL-2R were measured using enzyme-linked assays (Quantikine, R & D Systems, USA, and Boehringer Mannheim Biochemica, UK, respectively). Assays were performed in accordance with the manufacturer's instructions.

Statistics

Data were analysed using a non parametric Mann-Whitney U-test for significance. P-values from two-tailed Mann-Whitney U-tests are given in the text.

Results

Lymphocyte analysis

The clinical status of MM patients used in this study at the time of examining the lymphocyte subsets is shown in Table I. Although total white cell counts (WBCs) remained significantly lower in patients who received IFN-$\alpha_{2\beta}$ for prolonged periods after intensive therapy than in patients who had no further treatment ($P<0.02$) or normal volunteers ($P<0.0002$), this was not a reflection of a general inhibition of recovery among each of the lymphocyte subsets (Table II). Despite a significant reduction in B-cell numbers in all patients after HDM, IFN-$\alpha_{2\beta}$ had no effect on their recovery compared with that seen among patients who had no further treatment, and numbers returned to levels similar to those in normal volunteers 12 months after HDM. In contrast, IFN-$\alpha_{2\beta}$ inhibited the recovery of CD3$^+$, CD4$^+$/CD3$^+$, CD8$^+$/CD3$^+$, CD16$^+$/CD3$^-$ and CD56$^+$/CD3$^-$ lymphocytes. Since HDM also inhibited the recovery of CD4$^+$/CD3$^+$ T cells, the poor recovery in this subset was augmented by IFN-$\alpha_{2\beta}$. The data are summarised in Table II.

NK activity

NK activity correlated with absolute numbers of CD16$^+$/CD3$^-$ lymphocytes ($r=0.616$) (Figure 1) but not with CD56$^+$/CD3$^-$ cells (data not shown). Although absolute numbers of both CD16$^+$/CD3$^-$ and CD56$^+$/CD3$^-$ lymphocytes remained low in patients receiving long-term treatment with IFN-$\alpha_{2\beta}$ (Table II), there was no significant difference between NK activity in patients or normal volunteers. The mean lytic activity was 60%±6.2% in normal volunteers, 57.8%±4.2% in patients who had no further treatment and 51.75%±6.0% in patients on IFN-$\alpha_{2\beta}$ (Figure 2).

sIL-2R and IL-2

Analysis of sIL-2R in serum from the patients who were taking IFN-$\alpha_{2\beta}$ compared with those who were not showed that there was no significant difference between the levels compared with that reported for normal volunteers. The

Table I Clinical status of multiple myeloma patients treated with HDM/ABMR or PBSCR with or without maintenance therapy with interferon α_{2b}

	Median time after HDM in months (range)	Median time on interferon $\alpha_{2\beta}$ in months (range)	Clinical status[a] at time of testing				Change in clinical status to present (July 1995)	Time of status change after HDM
			CR	PR	NC	PD		
No interferon $\alpha_{2\beta}$ group < 12 months (n = 10)	3.5 (1–10)	NA†	1	5	3	1	PR→CR	–
							CR→PD	6 months
							PR→PD	11 months
No interferon $\alpha_{2\beta}$ group > 12 months (n = 10)	47 (20–103)	NA	6	2	1	1	CR→PD	61 months
							PR→PD	107 months
Interferon $\alpha_{2\beta}$ group < 12 months (n = 28)	5 (2–13)	3 (0.5–12)	11	11	6	0	PR→CR	–
							PR→CR	–
							NC→CR	–
							CR→PD	6 months
							CR→PD	9 months
							PR→PD	12 months
Interferon $\alpha_{2\beta}$ group > 12 months (n = 21)	46 (21–102)	27 (13–70)	11	6	3	1	No change in status of any patient	–

[a]CR, complete remission; PR, partial response; NC, no change after disease progression; PD, progressive disease (samples taken on day this was identified). NA, not applicable.

Table II Comparison of surface epitopes in peripheral blood from normal donors with multiple myeloma patients treated with HDM/ABMR or PBSCR with or without maintenance therapy with interferon-$\alpha_{2\beta}$ at time intervals of 1–12 months and more than 12 months therapy

	Normal donors (n = 10)	MM patients without IFN therapy		MM patients given IFN therapy	
		1–12 months after HDM (n = 10)	> 12 months after HDM (n = 10)	1–12 months on IFN (n = 28)	> 12 months on IFN (n = 21)
WBC	7.43 ± 0.60	4.47 ± 0.50	6.60 ± 0.87	3.98 ± 0.34	4.51 ± 0.32[a]
CD19$^+$	0.221 ± 0.043	0.056 ± 0.019[b]	0.396 ± 0.092	0.100 ± 0.016[b]	0.275 ± 0.038
CD3$^+$	1.506 ± 0.181	0.959 ± 0.196	1.252 ± 0.232	0.572 ± 0.037[c]	0.612 ± 0.062[c,d]
CD16$^+$/CED3$^-$	0.179 ± 0.034	0.147 ± 0.049	0.193 ± 0.039	0.058 ± 0.010	0.079 ± 0.010[e]
CD56$^+$/CD3$^-$	0.329 ± 0.035	0.243 ± 0.060	0.342 ± 0.068	0.115 ± 0.015	0.204 ± 0.022[f]
CD8$^+$	0.610 ± 0.094	0.741 ± 0.158	0.724 ± 0.156	0.402 ± 0.032	0.305 ± 0.036[g]
CD4$^+$	0.894 ± 0.128	0.218 ± 0.044[h]	0.528 ± 0.093[i]	0.175 ± 0.016[h]	0.306 ± 0.032[j]

Results are given as absolute counts $\times 10^6$ ml^{-1} (mean ± s.e.) [a]$P < 0.0002$ vs normal donors; $P < 0.02$ vs patients who had no further treatment (NFT). [b]$P < 0.005$ vs normal donors. [c]$P < 0.00001$ vs normal donors. [d]$P < 0.01$ vs NFT. [e]$P < 0.02$ vs normal donors. [f]$P < 0.01$ vs normal donors. [g]$P < 0.005$ vs normal donors. $P < 0.01$ vs NFT. [h]$P < 0.00005$ vs normal donors. [i]$P < 0.05$ vs normal donors. [j]$P < 0.02$ vs NFT.
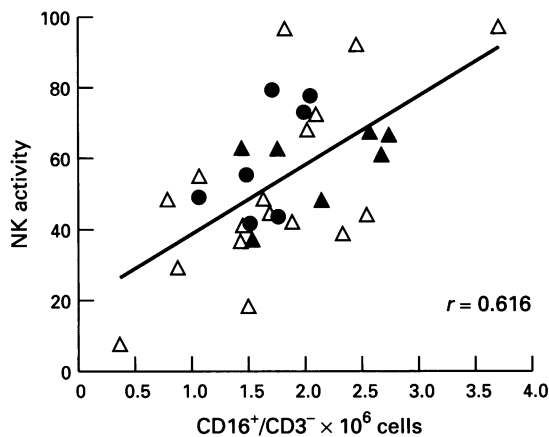

Figure 1 Correlation of NK activity with absolute cell numbers of CD16$^+$/CD3$^-$ cells. ●, Normal volunteers; △, myeloma patients on interferon $\alpha_{2\beta}$; ▲, myeloma patients not on interferon $\alpha_{2\beta}$. NK activity is expressed as per cent lysis of 5000 ^{51}Cr-labelled K562 cells.

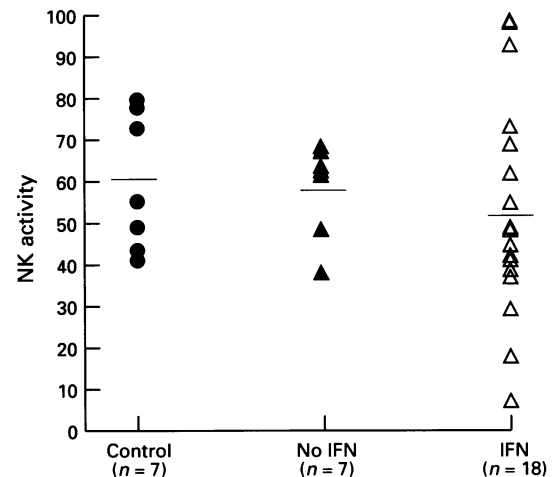**Figure 2** Comparison of NK activity in lymphocytes from normal volunteers (●), myeloma patients on maintenance therapy with interferon $\alpha_{2\beta}$ (△) and myeloma patients not having maintenance therapy (▲). NK activity is expressed as per cent lysis of 5000 ^{51}Cr-labelled K562 cells; bar indicates mean value.

mean level was 116.7 ± 10.1 pmol l^{-1} in patients receiving the cytokine compared with 109.4 ± 15.6 pmol l^{-1} in patients who had no further treatment. IL-2 was not detected in serum from any patient.

Clinical response

At the time of writing, 7/69 patients have relapsed within 6 months after examining their lymphocyte subsets, three of whom were receiving IFN-$\alpha_{2\beta}$. Although CD19$^+$ cell numbers were lower in patients who relapsed subsequently, this was not statistically significant. There was no correlation between absolute numbers of other lymphocyte subsets among patients who relapsed and those in patients who remain in remission, irrespective of treatment with IFN-$\alpha_{2\beta}$.

Discussion

Although IFN-$\alpha_{2\beta}$ has been used as maintenance therapy after HDM for more than 6 years at the Royal Marsden Hospital, the mechanism by which it regulates the proliferation of MM remains elusive. Examination of peripheral blood samples after short (median time, 3 months) and long (>12 months) exposure to IFN-$\alpha_{2\beta}$ failed to identify the enhanced recovery of a specific lymphocyte subset(s) which might be a prognostic indicator for the anti-proliferative effect of IFN-$\alpha_{2\beta}$.

Several authors have reported that continuous treatment with IFN-$\alpha_{2\beta}$ suppresses the peripheral blood WBC but that recovery is rapid following cessation of treatment (Klingemann et al., 1991). Reduced numbers of most lymphocyte subsets contributed significantly to the delayed recovery of the WBC in patients receiving IFN-$\alpha_{2\beta}$ in addition to a long-time inhibitory effect on the recovery of CD4$^+$ cell caused by therapy (Bergmann et al., 1984). However, despite the reduction in B cell numbers in all patients during the first 12 months after intensive therapy, their subsequent recovery was not inhibited by IFN-$\alpha_{2\beta}$ and cell numbers were similar to those in normal volunteers at longer time intervals.

Previous studies have considered the role of immunoregulatory cells as prognostic indicators in untreated patients with MM (Massaia et al., 1988, 1989) and the effects of IFN-$\alpha_{2\beta}$ on these cells in vitro (Uchida et al., 1984) or as a single agent in vivo (Einhorn et al., 1982). The association of low numbers of CD4$^+$ lymphocytes with poor prognosis (Bergmann et al., 1984) is clearly inappropriate as a criterion of imminent relapse after intensive therapy, since their recovery is known to be inhibited by chemotherapy (Bergmann et al., 1984). In our patients, the delayed recovery of CD4$^+$ lymphocytes was increased by IFN-$\alpha_{2\beta}$ whereas the number of CD8$^+$ cells was similar to those of normal volunteers in patients who had no further treatment within 12 months after intensive therapy. These data suggest that despite the differential sensitivity of CD4$^+$ and CD8$^+$ lymphocytes to chemotherapy they are equally sensitive to the anti-proliferative effects of IFN-$\alpha_{2\beta}$.

Although increased numbers of activated NK cells have been found in the peripheral blood of untreated patients with early stage disease (Osterborg et al., 1990; Gonzalez et al., 1992), there was no indication that the magnitude of the IFN-$\alpha_{2\beta}$-induced enhancement of NK activity correlated with clinical response to IFN-$\alpha_{2\beta}$ (Einhorn et al., 1982). While the latter study questions the role of NK cells in vivo in response to IFN-$\alpha_{2\beta}$ it is unlikely to be comparable with mechanisms that are operable after intensive therapy. In a study in which IFN-$\alpha_{2\beta}$ was given to patients early after allogeneic transplantation for advanced haematological diseases, NK function was low before IFN and was not improved with the cytokine (Klingemann et al., 1991), however the report took no account of absolute numbers of NK cells. Although recovery of the two major NK subsets, CD16$^+$/CD3$^-$ and CD56$^+$/CD3$^-$ cells, was delayed in our patients receiving IFN-$\alpha_{2\beta}$ compared with patients who had no further treatment, there was no difference in NK activity measured by the lysis of K562 cells between patients and normal donors. The data suggest that if NK cells have a role in mediating tumour homeostatis in vivo after intensive therapy, the efficacy of this mechanism may be lower in patients receiving IFN-$\alpha_{2\beta}$ because of fewer NK cells.

Given that MM is a disease of the B-cell lineage, the recovery of B-cells in patients receiving IFN-$\alpha_{2\beta}$ raises questions concerning both the putative stem cell in MM as well as the mechanism by which tumour homeostasis is achieved. Montes Borinaga (1993) showed that MM cells can be cultured in vitro from patients receiving IFN-$\alpha_{2\beta}$ as maintenance therapy during remission after HDM/ABMR suggesting that the effect of the cytokine in vivo is likely to be cytostatic rather than cytocydal. Although the stem cell in MM has not been identified several authors agree that it is likely to arise after class-switching immediately before or at the level of the memory B-cell (Ralph et al., 1993; Sahota et al., 1994). The failure of neutralising antibody to IFN-$\alpha_{2\beta}$ to persist in patients receiving the cytokine as maintenance therapy led to the suggestion that memory B-cells may not be involved (Millar and Bell, 1995). An alternative conclusion could be that the proliferation and maturation of memory cells may be inhibited in patients receiving the cytokine. Despite our failure to identify a particular lymphocyte subset in the peripheral blood that may effect tumour homeostasis, we cannot exclude the possibility that changes may have occurred in the milieu of the bone marrow or lymph nodes that might account for inhibition of tumour cell proliferation. Although sequestration of cytotoxic T cells and/or NK cells to those sites may be responsible for localised control, the relapse of three patients while receiving IFN-$\alpha_{2\beta}$ within 6 months of HDM with PBSCR suggets that the efficacy of such a control mechanism would require a fine balance between the residual tumour burden and the anti-tumour effect of the cytokine.

The lack of correlation between absolute numbers of lymphocyte subsets with relapse may be indicative of an alternative mechanism dependent on changes in the cytokine profile at specific sites within the bone marrow or lymph nodes. There is increasing evidence that the cytokine profile of the natural immune response probably determines the phenotype of the specific immune response (Romagini, 1992). For example, Th1 (which produce IL-2 and IFN-γ) and Th2 (which produce IL-4 and IL-5) lymphocytes are associated with markedly different functions whereas Th0 cells show an unrestricted lymphokine pattern (Firestein et al., 1989). Although IFN-$\alpha_{2\beta}$ promotes the differentiation of allergen-specific T cells into Th1 instead of Th2 clones (Parronchi et al., 1992), IL-2 was not detected in the serum from any patient, neither were the levels of sIL-2R different from those in patients who did not receive the cytokine. However, the net effect of INF-$\alpha_{2\beta}$ in vivo may result in the localised release of INF-γ and IL-2 by different T cell subsets depending on their proximity to cells that can respond and may be insufficient to be detected in serum.

In conclusion, there was no enhanced recovery of a particular lymphocyte subset(s) in peripheral blood samples that may account for the anti-tumour effect of IFN-$\alpha_{2\beta}$ as maintenance therapy. It seems likely that the anti-proliferative effect of the cytokine is determined by events that occur at localised sites within the bone marrow or lymph nodes and that such events are dependent critically on the residual tumour mass.

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