Regulation of interleukin-4 production and cytokine-induced growth potential in peripheral T-cell non-Hodgkin’s lymphomas

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Received 9 July 1997; accepted for publication 3 November 1997

Summary. The malignant cells in tumour tissues produce cytokines/growth factors that may influence tumour growth, tumour immunogenicity and host immune response. We demonstrate that lymph node cell (LNC) purified neoplastic T cells from CD4+ peripheral T-cell lymphoma (CD4+ PTCL) and CD8+ PTCL spontaneously, and after stimulation with anti-CD3, secreted high amounts of interleukin-4 (IL-4) as compared to LNC-purified CD4+ and CD8+ non-malignant T cells. Furthermore, IL-4 was observed to be the most potent cytokine that induced in vitro proliferation and growth of the malignant T cells. Moreover, malignant T-cell-derived IL-4 secretion was augmented by exogeneous recombinant human interferon-gamma (IFN-γ) and was profoundly inhibited by IL-2. Because IL-4 was shown to be a locally active cytokine with a wide range of immunoregulatory properties, regulation of IL-4 production by IFN-γ and IL-2 in malignant T cells may be one of the important parameters to be assessed in the design of anticancer-specific immunotherapy. In summary we report that malignant T cells produce IL-4, a type 2 cytokine (Th2 cell response) that acts as a growth factor and which may play a critical role in PTCL disease mechanism.

Keywords: interleukin-4, interferon-gamma, malignant T cells, peripheral T-cell lymphoma.

In various pathological disorders the pathomechanisms of tissue damage are casually associated with the specific pattern of locally produced cytokines (Hsu et al. 1993). Cytokines are soluble mediators that are responsible, at least in part, for orchestrating the immune effector function to achieve a fine balance in the immune response (Cohen & Cohen, 1996). The precise functional significance of unregulated cytokine secretion in various human diseases is an area of intense research. One current area of cytokine research concerns their role in the proliferation and histopathological features of neoplastic cells in lymphoid neoplasm.

Non-Hodgkin’s lymphomas (NHL), the most common malignancy worldwide constitute a heterogenous group of lymphoid tumours, the majority of which are of the B-cell type (Knowles & Halper, 1982; Weiss et al., 1985; Foon & Todd, 1986; Raziuddin et al., 1987, 1991). Of B-cell and T-cell neoplasms (NHL disease), B-cell NHL have been investigated most vigorously. Of the T-cell NHL diseases, peripheral T-cell lymphoma (PTCL) is less commonly found and has distinct clinicopathologic features characterized by the clonal proliferation of neoplastic T cells in lymph nodes (Ohnishi et al. 1990; Merz et al. 1991; Winberg, 1993; Kato et al. 1996). T cells have multiple functional activities and T-cell-derived cytokines, interleukin (IL)-2, IL-3, IL-4 and interferon-gamma (IFN-γ), are essential regulator and effector molecules which serve to ensure that the immune system can respond to distinct immunological challenges (Cohen & Cohen, 1996). T cells often require endogenous or exogenous cytokines for their growth in vitro. We have previously reported various immunologic abnormalities in PTCL (Raziuddin et al., 1987, 1991, 1994b; Sheikha et al., 1993). The identification of specific cytokine production in PTCL may provide a basis for the hypothesis of a continuous neoplastic T-cell growth. Therefore, we determined the cytokine secretion pattern of malignant T cells from well-characterized PTCL disease.

PATIENTS AND METHODS

Patient characteristics. We studied 11 untreated patients

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### Table I. Clinical data, histopathologic features and immunophenotype of PTCL cases.

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(eight men and three women, aged between 54 and 69 years) with PTCL, who were admitted to the Asir Central Hospital, Abha. The patients were diagnosed according to the characteristic clinical, haematological, phenotypic and histological criteria. In all cases there was no serological evidence of infection by human T-cell leukaemia/lymphoma virus (HTLV I/II), Epstein-Barr virus (EBV) or human immunodeficiency virus (HIV).

Fresh cervical lymph node specimens were obtained from the 11 PTCL cases. Non-malignant cervical lymph node specimens (four cases) were selected from those displaying non-specific reactive hyperplasia and were analysed as controls (C0). These non-malignant lymph nodes removed for diagnostic purposes had no signs of malignancy. The only criteria for selection was the recovery of a sufficient number of cells after extraction. At the time that samples were obtained, newly diagnosed patients had not been treated. The characteristics and histopathologic classification of the PTCL patients (case 1–11) are given in Table I. The histologic classification of NHL was based on the International Working Formulation (National Cancer Institute, 1982). The PTCL cases were all diffuse NHL (six cases, diffuse, large cell lymphoma, DLC; two cases, diffuse, mixed small and large cell lymphoma, DMC; and three cases, diffuse, lymphoblastic lymphoma, LBL). T-cell origin of PTCL was established by documentation of >90% of the cells (lymph node cells, LNC) staining for T-cell reagents expressing one or more markers identified by CD2, CD3, CD4, CD5 and CD8, along with the lack of expression of B-cell markers including Sig, anti-kappa/anti-lambda light chains and myeloid markers (Raziuddin et al., 1991b). According to immunophenotypic results, seven cases were characterized as CD4+ PTCL (cases 1–7) and the other four cases as CD8+ PTCL (cases 8–11) (Table I).

Cell separation. Lymph node cell (LNC) suspensions obtained by gently scraping the tissue with a scalpel were purified on Histopaque-1077 (Sigma Chemical Co., St Louis, Mo.) density gradient centrifugation as described previously (Raziuddin et al., 1987, 1994b). LNC were suspended in RPMI-1640 medium containing 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Flow Laboratories, Finland).

T cells were purified from the LNC of controls by the 2-aminoethylisothiouronium-bromide-treated sheep erythrocyte rosetting technique and density gradient centrifugation on Histopaque-1077 (Sigma) (Raziuddin et al., 1991). Purified rosetting cells were referred to as T cells. The resultant T cells were 90–98% CD3- (OKT3 mAb reactive). The CD3+ T cells were further separated into CD4+ and CD8+ T-cell subsets by complement-mediated cell lysis with OKT8 and OKT4 mAbs (Ortho Diagnostics, Raritan, N.J., respectively). The OKT8-treated cells contained 90–96% OKT4+ cells, hereafter referred to as CD4+ T cells. The OKT4-treated cells yielded 89–93% OKT8+ cells, hereafter referred to as CD8+ T cells. The LNC suspensions prepared from the CD4+ PTCL and CD8+ PTCL cases by Histopaque-1077 (Sigma) density gradient centrifugation method stained 90–98% for CD4+ (OKT4 mAb reactive) and CD8+ (OKT8 mAb reactive) antigens, respectively, and were used as such.

**Culture conditions.** CD4+ and CD8+ T cells (LNC, PTCL and CO) (2.5 × 10⁶ cells/ml) were cultured in RPMI-1640 medium containing 10% FCS with or without anti-CD3 (1 μg/ml, Genzyme Corporation, Boston, Mass.) for a total period of 48 h at 37°C, and 5% CO₂ in 24-well tissue culture plates (Linbro no. 76063, Flow Laboratories). The supernatants were collected, centrifuged and frozen at −45°C until assayed for cytokines.

**Proliferation.** Proliferation. [³H]Thymidine incorporation of PTCL T-cell cultures (CD4+ T cells or CD8+ T cells from LNC) was determined as follows: 2.5 × 10⁵ cells/ml were cultured in triplicate in 96-well flat-bottomed microtiter culture plates with or without recombinant cytokines, IL-2 (50 units/ml), IL-3 (10 ng/ml), IL-4 (10 ng/ml), IL-6 (10 ng/ml), IL-10 (10 ng/ml) or IFN-γ (50 units/ml) (all from Genzyme Corporation, Boston, Mass.) in RPMI-1640 medium
supplemented with 10% FCS, 2 mm ω-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco Laboratories, Grand Island, N.Y.) for a total period of 72 h. All cultures were pulsed with 37 kBq [3H]thymidine (Amersham Radiochemicals, U.K.) for 16 h before harvest. At the end of incubation period cells were harvested and incorporation of [3H]thymidine into DNA was measured in a Beckman LS 9800 liquid scintillation counter (Beckman, U.K.). The data are expressed as mean cpm (counts/minute) of triplicate cultures.

Effect of recombinant cytokines on IL-4 production. In another set of experiments, malignant T cells (2.5 × 10⁶ cells/ml in complete RPMI-1640 medium) from CD4⁺ PTCL (case 4) and CD8⁺ PTCL (case 10) were cultured spontaneously or with various human recombinant cytokines IL-2, IL-3, IL-6, IL-10, IFN-γ and TNFα each at 50 ng/ml, and anti-cytokine antibodies (Ab), anti-IL-2 Ab, anti-IL-4 Ab or anti-IFN-γ Ab, each at 2 μg/ml (Genzyme Corporation, Boston, Mass.). All cell cultures were incubated for 48 h at 37°C and 5% CO₂, after which the supernatants were isolated and assayed for IL-4 by ELISA.

Cytokine assays. Detailed methods for determination of IL-2, IL-3, IL-4, IL-6 and IFN-γ from the supernatant fluids were previously described (Raziuddin et al. 1994b). Briefly, IL-2 was measured in a proliferation assay using the murine IL-2-dependent CTLL cell line. IFN-γ was measured in an enzyme-linked immunosorbent assay (ELISA) in which two mAbs recognizing different epitopes of IFN-γ (Genzyme Corporation, Boston, Mass.: Hoffman-La Roche, Basel, Switzerland) were used as catcher and tracer antibodies, respectively. IL-3, IL-4 and IL-6 were each measured with commercially available ELISA kits (Inter-Test, IT-3, IT-4 and IT-6, Genzyme) according to the manufacturer’s protocol. In each cytokine assay the appropriate human recombinant cytokines were used to generate standard curves. Sensitivities of various assays were as follows: IL-2 and IFN-γ (5 units/ml), IL-3, IL-4 and IL-6 (5–10 pg/ml).

Statistical analysis. Statistical significance was determined by the Wilcoxon t-test/Kruskal-Wallis test (Rank Sums).

RESULTS

Cytokine secretion

We measured IL-2, IL-3, IL-4, IL-6 and IFN-γ secretion from culture fluids of CD4⁺ normal, CD8⁺ normal, CD4⁺ PTCL and CD8⁺ PTCL. T cells cultured spontaneously or after stimulation with anti-CD3 for 48 h. IL-3 and IL-6 were not detectable in any of the supernatants of cells cultured either spontaneously or with anti-CD3 (data not shown). The production of IL-2, IL-4 and IFN-γ by these cells are shown in Fig. 1. As shown, LNC purified normal CD4⁺ and CD8⁺ T-cell populations secreted IL-2 both spontaneously and after

![Cytokine secretion graph](https://via.placeholder.com/150)

Fig 1. IL-2, IL-4 and IFN-γ production by normal or malignant T cells (PTCL). LNC-purified CD4⁺ and CD8⁺ normal T cells, and LNC-purified CD4⁺ (CD4⁺ PTCL) and CD8⁺ (CD8⁺ PTCL) malignant T cells were cultured spontaneously (unstimulated) or with anti-CD3 (1 μg/ml) for 48 h, and supernatants were assayed for IL-2, IL-4 and IFN-γ by ELISA. Data shown are mean ± SEM of four normal cases or 11 PTCL cases.
culture with anti-CD3. Spontaneously growing LNC-purified malignant T cells of CD4⁺ PTCL and CD8⁺ PTCL did not secrete any amount of IL-2. However, anti-CD3 stimulated CD4⁺ PTCL and CD8⁺ PTCL secreted IL-2, but these levels were significantly lower as compared to their normal counterparts (P < 0.0001). The results obtained for IFN-γ production demonstrated that anti-CD3 stimulated malignant CD4⁺ PTCL and CD8⁺ PTCL also secreted highly decreased levels of IFN-γ as compared to normal CD4⁺ and CD8⁺ T cells (P < 0.0006). Of interest, however, a highly increased level of IL-4 production was demonstrated by both spontaneously and anti-CD3-stimulated malignant CD4⁺ PTCL and CD8⁺ PTCL as compared to normal controls (P < 0.0006).

**IL-4 stimulated growth of malignant T cells from PTCL**

Purified recombinant cytokines, IL-2, IL-3, IL-4, IL-6, IL-10 and IFN-γ were next compared for their capacity to stimulate DNA synthesis from CD4⁺ PTCL (patients P1–P7), and CD8⁺ PTCL (patients P8–P11). In order to examine the mitogenic or inhibitory effects of recombinant cytokines on these malignant cells, cells were cultured for 72 h and [³H]thymidine uptake was determined. Cytokines were used at optimal concentrations as described (Razinuddin et al. 1994a) according to our own empirical experience with these reagents. As illustrated in Fig 2, although interindividual variations were observed amongst the cytokines tested, IL-4 and IFN-γ were found to enhance [³H]thymidine incorporation in both CD4⁺ PTCL and CD8⁺ PTCL. However, the effect of IL-4 was more pronounced than IFN-γ. We did not test the effects of combinations of cytokines which might be additive or synergistic, and did not evaluate the effects of these cytokines on cell survival, differentiation, activation or other biological processes.

**Effect of cytokines on IL-4 production**

IL-4 and IFN-γ had a wide range of immunoregulatory activities in the immune system. We demonstrated that malignant T cells from both CD4⁺ PTCL and CD8⁺ PTCL cases secreted higher amounts of IL-4 both spontaneously and after stimulation with anti-CD3 as compared to their counterparts from control non-malignant T cells. Because cytokines themselves also have a central role in the regulation of specific cytokines, we further determined the effects of various human recombinant cytokines and neutralizing anti-cytokine antibodies on IL-4 production by malignant T cells (Fig 3). As shown, the spontaneously growing malignant T cells produced IL-4, and the addition of anti-IL-4 Abs neutralized this IL-4 production. Studies on the effect of exogenously added cytokines demonstrated that the additions of IL-3, IL-6, IL-10 and TNFα to CD4⁺ PTCL or CD8⁺ PTCL cells do not have a major influence on IL-4 production (Fig 3). Of interest, however, addition of IFN-γ to such cultures highly enhanced IL-4 production by malignant T cells from both CD4⁺ PTCL and CD8⁺ PTCL cases. The neutralization of IFN-γ by addition of anti-IFN-γ Abs to these cultures, however, suppressed IL-4 production. To the contrary, additions of IL-2 inhibited IL-4 production, and additions of neutralizing IL-2 Abs, anti-IL-2 Ab enhanced IL-4 production. Of interest, malignant T cells from CD4⁺ PTCL

and CD8\(^+\) PTCL cases secreted higher amounts of IL-4 and that exogenously added IFN-\(\gamma\) increased, whereas IL-2 downgraded IL-4 production.

**DISCUSSION**

The concept of malignant cell induced cytokine production and growth potential has attracted much interest recently in attempts to understand the pathogenesis of various neoplastic diseases and to design selective immunotherapy (Hsu et al. 1993; Huang et al. 1995; Behringer et al. 1997).

In this study we have demonstrated that of the various T-cell-derived cytokines, IL-2, IL-3, IL-4, IL-10 and IFN-\(\gamma\), malignant T cells from the CD4\(^+\) PTCL and CD8\(^+\) PTCL cases characteristically secrete higher amounts of IL-4. This was accomplished by comparing the *in vitro* cytokine production in LNC-purified malignant T cells from PTCL to that of LNC purified non-malignant T cells from controls. T cells have been classified according to the cytokine production profile as Th1 phenotype, producing IL-2 and IFN-\(\gamma\), and the Th2 phenotype, producing IL-4. IL-5, IL-6 and IL-10 (Mosmann & Coffman, 1989; Romagnani, 1995). However, IL-10 is a product of monocytes, Th2 cells, and to a lesser extent, Th1 cells (Moore et al. 1993). Based on secretion of IL-4, a prototype Th2 cytokine, malignant T cells from PTCL disease patients appear to be activated T cells of the Th2 phenotype. IL-4, which can direct an immune response towards a Th2 phenotype, might be an important factor for the NHL-specific immune response. Furthermore, we also investigated the influence of different cytokines on proliferation/growth of malignant T cells. The most salient feature of our results was the strong proliferation of malignant T cells from both CD4\(^+\) PTCL and CD8\(^+\) PTCL by IL-4 and to a lesser extent by IFN-\(\gamma\), with very little or no response to various other cytokines, such as IL-2, IL-3, IL-6 and IL-10.

Detailed investigations of malignant T cells from pathologic tissues (lymph nodes) of PTCL and other NHL cancers might promote our understanding of the role of individual cytokines such as IL-2, IL-4 and IFN-\(\gamma\) in malignancy. Control LNC purified T cells secreted all three T-cell cytokines, IL-2, IL-4 and IFN-\(\gamma\). To the contrary, malignant T cells from all PTCL cases irrespective of immunophenotype or histologic types produced very high amounts of IL-4 and little IL-2 and IFN-\(\gamma\). The fact that the spontaneously (unstimulated) growing malignant T cells produced specifically high amounts of IL-4 and that exogenously added IL-4 induced growth of malignant T cells indicated an critical role of IL-4 in the pathogenesis of this malignant disorder. In view of the production and response of the malignant T cells to this unique cytokine (IL-4), it seems possible that the microenvironment structure and tumour growth could be modulated by locally acting IL-4 cytokine in PTCL.

Several recent studies have examined the serum levels of proinflammatory, IL-1\(\beta\), IL-6 and TNF\(\alpha\) and immunoregulatory IL-2, IL-10 and IFN-\(\gamma\) levels in a wide variety of NHL diseases and their associations with various histopathological features as well as with characteristic B symptoms, such as fever, night sweats and/or weight loss (Hsu et al. 1992; Bhuy et al. 1993; Raziuddin et al. 1994b; Kato et al. 1996). Some of the studies have shown that serum IL-6 values were significantly higher in lymphoma patients with B symptoms than in those without B symptoms (Kato et al. 1996; Raziuddin et al. 1994a). In our studies of small numbers of PTCL cases we have not measured the serum levels of the cytokines; however, we have found that malignant T cells, irrespective of histopathologic type, immunophenotype or clinical symptoms, all secreted significantly higher levels of IL-4 and proliferated in response to IL-4 and IFN-\(\gamma\).

Evidence for an important role of T-cell-derived cytokines on IL-4 production in normal tissues and malignant tissues from T-cell NHL disease is scarce. We have shown here that, regardless of the CD4\(^+\) or CD8\(^+\) status of the tumour cells, all cells respond similarly to CD3 stimulation with IL-4 secretion. Moreover, our results have demonstrated, for the

first time, a distinct role of IL-2 and IFN-γ in the regulation of IL-4 production in PTCL. We were able to demonstrate, in one CD4+ PTCL and one CD8+ PTCL patient that exogenously added IL-2 profoundly inhibited IL-4 production whereas IFN-γ up-regulated IL-4 production. The differential modulation of the production of IL-4 by IL-2 and IFN-γ was also apparent from the results obtained upon addition of the respective neutralizing anti-IL-2 and anti-IFN-γ Abs to this system. Our findings that the inhibition of IL-4 production by IL-2 was modified by addition of a neutralizing anti-IL-2 Ab clearly indicated that this effect of IL-2 was related with IL-4 up-regulation. It is known that small amounts of cytokines can act as potential stimulators or inhibitors of cell mass. Thus deficient production of IL-2 by malignant T cells could be due to a loss of the physiological regulation within the cytokine network and thus may be a sign of neoplastic growth. This suggests that, by increasing IL-2 production by biological response modifier, one could restrict the growth of malignant T cells in PTCL disease. However, the significance of these differential effects of IL-2 and IFN-γ on IL-4 production and their role in the pathophysiology of PTCL remains to be defined. As we learn more about the cytokine network pathways in human malignant diseases, it should become possible to design appropriate forms of intervention, including perhaps therapy by implantation of specific cytokine genes. Moreover, our data may also provide a rational basis for the cytokine-mediated therapeutic control of T-cell NHL disease associated with IL-4 hyperproduction.

IL-4 is a pleiotropic type 2 response cytokine that has been shown previously to have both stimulatory and inhibitory effects on antitumour immune responses (Paul, 1991; Kawakami et al. 1992; Huang et al. 1995). Recombinant IL-4 decreases the proliferation of some solid tumour cell lines including lung cancers (Topp et al. 1993; Morisaki et al. 1994). In contrast, IL-4 may be a growth factor for malignant T cells in PTCL. In vitro, IL-4 is also known to inhibit macrophage production of inflammatory cytokines such as IL-1β, IL-6, IL-8 and TNFα (Hart et al. 1989; Standiford et al. 1990), and inhibit lymphokine-activated killer cell function (Swisher et al. 1990). We speculate that IL-4 produced by malignant T cells in PTCL may also cause diminished immune response at the lymph node tumour site and may promote accumulation of neoplastic cells.

Our current protocols of T-cell-derived cytokines in T-cell NHL disease will enable us to further study the immunological reactivities and critical roles of IL-2, IL-4 and IFN-γ in cytokine network regulatory pathways in tumour-draining lymph node tissues. The cytokine-induced growth potential of malignant T cells in cancer is a mechanism that has been recently exploited in the design of cytokine therapies for tumours. Of particular importance in understanding the immune response to cancer is the specific pattern of cytokines produced by neoplastic cells, as immunotherapy is often directed towards altering these patterns. Further detailed immunoregulatory studies, including expressions of IL-4 mRNA and cytokine receptor expressions on malignant T cells, are necessary to determine the biological significance of unfavourable IL-4 production in PTCL disease.

REFERENCES


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