Human tumour-associated NK cells secrete increased amounts of Interferon-γ and Interleukin-4

J. Lorenzen¹, C.E. Lewis¹, D. McCracken¹, E. Horak¹, M. Greenall²
& J.O'D. McGee¹

¹Nuffield Department of Pathology and Bacteriology, University of Oxford and ²Department of Surgery, John Radcliffe Hospital, Oxford OX3 9DU, UK.

Summary Numerous interactions between malignant and stromal/inflammatory cells take place within solid human tumours, which are mediated, in part, by the release of signalling proteins called cytokines. In the present study, we have compared the secretion of two important immunomodulatory cytokines, IFN-γ and IL-4 by individual, immunophenotyped NK cells freshly isolated from either malignant tumour biopsies, or peripheral blood samples from patients with ductal invasive breast cancer. Due to the marked heterogeneity amongst cells isolated from these clinical samples, we have employed a technique called the reverse haemolytic plaque assay to identify and enumerate cytokine-secreting cells at the single cell level. Our data indicate that NK cells isolated directly from the tumour site secrete more IFN-γ and IL-4 than NK cells from the blood of the same patients. However, a greater proportion of CD16+ cells from both sources in breast cancer patients secreted IFN-γ than from those of the blood of healthy donors. We also show that factors secreted by the human breast cancer lines, MCF-7 and MDA-231 PN9, were able to mimic the stimulatory influence of the tumour microenvironment on secretory activity of NK cells.

Natural killer (NK) cells can be defined on the basis of their ability to lyse certain tumour cells in a manner which is not dependent upon the expression of MHC molecules. In man, such 'natural' cytotoxicity is displayed mainly by CD3−/CD16+ cells with the morphology of large granular lymphocytes (LGL) (Timonene et al., 1981; Ortaldo et al., 1986; Lanier et al., 1986). NK cells can also be stimulated by IL-2 or IFN-γ to express a broader range of cytotoxicity, known as lymphokine activated killer (LAK) activity. Their ability to lyse tumour cells in vitro has led to the experimental use of NK and LAK cells in the immunotherapy of some types of human cancer. This has occasionally led to tumour regression and/or improved clinical prognosis in advanced renal cell cancers and melanomas (Lotze et al., 1986; Mule et al., 1986).

However, there is also evidence to suggest that the cytotoxicity of NK cells towards tumour cells may actually be markedly reduced once they infiltrate the microenvironment of a malignant tumour (Moy et al., 1985; Lotzova, 1988; Nakamura et al., 1988). Indeed, malignant tissues release factors which suppress various immune functions in draining lymph nodes (Wen et al., 1989), and cultured tumour cells have been shown to inhibit the generation of LAK cells in vitro (Guillou et al., 1989). That factors produced by the malignant cell population itself may mediate, at least in part, this immunosuppression is given credence by the finding that factors known to be produced by human cancer cells, such as TGF-β or prostaglandin E3, markedly inhibit the cytotoxicity of NK and LAK cells in vitro (Rook et al., 1986; Wahl et al., 1988; Leung; 1989; Lugter et al., 1989).

NK cells secrete a range of cytokines, including IFN-γ, IL-2, (Djeu et al., 1982) and TNF-α (Peters et al., 1986) which are capable of directly lysing neoplastic cells in a time dependent manner (Uchida et al., 1985; Ruggerio et al., 1987). Recently, interleukins 4 and 6, which are also NK cell products (Tamm et al., 1989) have been shown to alter the proliferation and metastatic potential of malignant cells in vitro (Puszta et al., 1991). Taken together, these findings suggest that NK cells may contribute a number of cytokines to the complex cytokine network which exists within the microenvironment of solid human tumours (Lewis, 1991), and which collectively regulates the activity of both resident neoplastic cells and immune cells infiltrating such tissues.

A sensitive immunoassay called the reverse haemolytic plaque assay (RHPA) has recently been introduced for the measurement of cytokine secretion by individual, immunohistologically identified human cells (Lewis et al., 1990). In the present report, the RHPA has been used to investigate the influence of the tumour microenvironment on the release of IFN-γ and IL-4 by individual human CD16+ NK cells. To do this, the secretory activity of NK cells derived from both tumour and the peripheral blood samples of patients with ductal invasive breast carcinoma were compared. In addition, experiments were undertaken to investigate the effects of soluble media tors released by the breast adenocarcinoma cell lines, MCF-7 and MDA-231 PN9, on the secretion of cytokines by NK cells in vitro.

Materials and methods

Isolation of mononuclear cells from the peripheral blood of cancer patients

Peripheral blood samples (10 ml) were obtained from patients undergoing mastectomy (prior to surgery). Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Hypaque density gradients. Briefly, this involved diluting whole blood samples 1:1 (v/v) in calcium and magnesium-free Hank’s buffered salt solution (HBSS). The resultant cell suspensions were then washed three times before being carefully layered onto Ficoll-Hypaque cushions. After centrifugation for 25 min at 450 g, cells in the lymphocyte layer were collected and washed by centrifugation/resuspension three times in HBSS. This procedure routinely yielded 1–5 x 10⁶ cells ml⁻¹.

Enrichment of NK cells from the peripheral blood of healthy donors

Human NK cells were isolated from 1,000 ml buffy coat preparations from five healthy female donors. PBMC were isolated using Ficoll-Hypaque density gradients as outlined above. The resulting cell pellet was resuspended in complete culture medium (see below) and incubated in large plastic culture dishes for 60 min at 37°C to remove plastic-adherent monocytes. Non-adherent cells were harvested and then incubated in nylon wool columns for 30 min at 37°C to
remove B cells (Julius et al., 1973). T cells were removed by the negative panning technique (Genica-Penarrubia & Bankhurst, 1989). Briefly, this involved incubating cells (5 x 10^6 cells ml^{-1}) with monoclonal antibodies (10 µl ml^{-1}) specific for the CD3, CD4 and CD8 surface antigens on T cells (Dako, High Wycombe, UK) for 30 min at 4°C. The cell suspension was then layered onto poly styrene Petri dishes (Sterilin, UK), previously coated with goat anti-mouse IgG, for 60 min at 4°C. Non-adherent lymphoid cells (i.e. mainly NK cells) were carefully aspirated from each dish and used for subsequent experiments.

Unfortunately, experiments using breast cancer patient's PBMC enriched for NK cells could not be performed as the negative panning procedure requires 500–1,000 ml of whole blood. For obvious clinical reasons, the removal of this quantity of blood from each breast cancer patient was not feasible.

**Enzymatic dispersion of cells in malignant breast biopsies**

Mastectomy resections for breast cancer were collected immediately after surgery. Each tumour was dissected out and a portion removed for histopathological diagnosis. At the same time, a small (1 cm^3) piece of tissue containing tumour was also removed and digested with 0.1% collagenase type IV (Sigma, Dorset, UK) in RPMI 1640 medium for 2 h at 22°C. Enzymatically dispersed cells were removed, suspended in 10 ml of HBSS and centrifuged at 444 g for 15 min. The supernatant was discarded and this step repeated four times in order to remove contaminating enzyme. This procedure routinely yielded 1–20 x 10^6 cells ml^{-1}. Monocytes were removed by adherence to plastic culture wells during a 60 min incubation. As a control, peripheral blood mononuclear cells from each of the cancer patients were subjected to similar collagenase treatment for 2 h and their secretory activity assessed in the RHPA.

**Culture conditions and preparation of tumour cell supernatants**

Cultures of MCF-7 or MDA-231-PN-9 cells were maintained in RPMI 1640 medium supplemented with 2 mM of glutamine, 10% foetal calf serum, 100 IU ml^{-1} penicillin G and 100 µg ml^{-1} streptomycin (complete culture medium) at 37°C in air/5% CO₂. All culture reagents used were obtained from Gibco, Paisley, UK. The human breast cancer cell lines, MCF-7 and MDA-231 PN-9, were propagated in complete culture to medium to near (≥90%) confluence. Cells were incubated with fresh medium for 24 h and the medium then removed and centrifuged at 5,000 g for 20 min. The supernatant was harvested (pore size 0.2 µm) and used immediately as conditioned medium in subsequent medium-transfer experiments. Small aliquots of each batch of conditioned medium were transferred to glass slides and stained with haematoxylin/eosin to check for the absence of any contaminating tumour cells.

**Reverse haemolytic plaque assay**

The secretion of IFN-γ and IL-4 was measured using the RHPA as previously described for cytokines (Lewis et al., 1990). Mononuclear human cells were suspended in RPMI 1640 medium supplemented with 0.1% bovine serum albumin, 2 mM glutamine and antibiotics (assay medium) and then mixed with ovine red blood cells (Serotec, Kidlington, UK) to which staphylococcal protein A (Sigma Chemicals, Dorset, UK) had been coupled using the chromium chloride method. This cell suspension was allowed to settle in Cunningham chambers for 45 min at 37°C in air/5% CO₂. Non附 hetached cells were removed with prewarmed assay medium and the chambers filled with a 1:50 dilution of polyclonal rabbit antisera to either native human IFN-γ or recombinant human IL-4 (Genzyme, USA). After incubation for 6 h, plaque formation was induced by injection into the chambers of a 1:50 dilution of guinea pig complement in assay medium. The cell monolayers were then fixed with 2% glutaraldehyde (v/v) in phosphate buffered saline and stored at 4°C for subsequent analysis. All treatments were repeated on 10 separate slides in each assay.

**Immunocytochemistry**

Mononuclear cells were phenotyped by immunocytochemistry with monoclonal antibodies against CD3, CD4, CD8 (Dako, UK) and CD16 antigens (Becton Dickinson, UK). Slides were incubated with the monoclonal antibodies in Tris-buffered saline (TBS) with 5% low fat milk protein ('Marvel', Premier Brands, Birmingham, UK) at optimal dilutions for 4 h at 22°C. After several washes in TBS, bound antibodies were detected by the APAAP technique (Cordell et al., 1984) using fast red as chromogen. Nuclei were lightly counterstained with haematoxylin.

**Morphometry and statistics**

The area of the plaques formed in the RHPA was determined using a WILD M-20 microscope to which a drawing device had been attached. This allowed the superimposition of the microscopic image onto the screen of an Apple Macintosh computer. Plaques were traced manually with a cursor and areas calculated by a program developed by Dr J. Lorenzen in this Department. For IFN-γ secretion, the size of the first 20 randomly selected haemolytic plaques was measured on each of 5–10 replicate slides for each treatment (i.e. minimum of 100 plaques). For IL-4 secretion, all plaques on replicate slides were measured (as there was invariably fewer of the latter). To rule out subjective bias, all plaque sampling was performed 'blind' without prior knowledge of the source of NK cells. Statistical analysis of the data was performed using the Mann Whitney U test. Although data from single, representative experiments have been illustrated in this report, essentially similar results were obtained in five identical assays using blood or tumour samples from at least five separate individuals.

**Results**

**Immunocytochemical labelling of cell preparations used in the RHPA**

Table I indicates the higher proportions of cells expressing T (CD3, CD4 and CD8) and NK cell (CD16) antigens in PBMC isolated from breast cancer patients (after Ficoll-Hypaque purification) than in cell preparations directly isolated from enzymatically-dispersed breast tumours. However, a slightly higher frequency of CD16+ cells were seen in dispersed tumour-associated mononuclear cells. Negative panning of PBMC from healthy donors reproducibly enriched the proportion of CD16+ cells to >65%, and depleted cells bearing the CD3, CD4 and CD8 antigens to <5% (data not shown). Fifteen to twenty per cent of CD16+ NK cells from healthy donors enriched in this way secreted IFN-γ, whereas fewer than 2% of CD3+ or CD4+ cells secreted this cytokine in the RHPA (Table II). By

| Immunophenotype | PMBC | Tumour-associated mononuclear cells |
|-----------------|------|-----------------------------------|
| CD3*            | 64%  | 18%                               |
| CD4*            | 26%  | 16%                               |
| CD8*            | 14%  | 2%                                |
| CD16*           | 10%  | 17%                               |

Peripheral blood mononuclear cells (PBMC) were isolated from the peripheral blood of a breast cancer patient using a Ficoll-Hypaque gradient. Tumour-associated mononuclear cells were isolated by enzymatic dispersion of the malignant breast biopsy from the same patient.
Table II  Proportion of the total number of cells of a given immunophenotype present in the RHPA which secreted IFN-γ

| Immunophenotype | PBMC from: | | |
|-----------------|------------|-----------------|-----------------|
| Healthy donors  | Breast cancer | Tumour-associated |
| (i.e. NK cell enriched) | patients (not enriched) | mononuclear cells |
| CD3+            | 1%         | 4%              | <2%             |
| CD4+            | 2%         | 3%              | 20%             |
| CD16+           | 15–20%     | 30%             | 31%             |

Contrast, only a small subpopulation (i.e. 1–2%) of the total number of enriched CD16+ NK cells present secreted IL-4 (data not shown). However, over 30% of CD16+ cells derived from either the blood or tumours of breast cancer patients secreted IFN-γ (Table II). A higher proportion of CD4+ cells isolated from breast tumours (i.e. 20%) was found to release IFN-γ than those isolated from the peripheral blood (i.e. 3%) of the same patients (Table II).

To determine the identity of the main producer cell type(s) for IFN-γ and IL-4 in the various cell preparations used in these studies, the proportion of the total plaque-forming cells bearing each of the CD antigens was calculated. The majority (i.e. 80–90%) of cells secreting these cytokines in the RHPA were seen to be CD3+ or CD16+, irrespective of whether they were derived from healthy donors or patients with breast malignancies (data not shown).

Comparison of the secretory activity of NK cells derived from the peripheral blood or tumours of breast cancer patients

Figure 1 illustrates the appearance of a haemolytic plaque formed by an NK cell secreting IFN-γ. The secretory cell is surrounded by an area of erythrocyte ghosts (plaque). Tumour-associated NK cells released significantly (P<0.01) more IFN-γ and IL-4 than those from the peripheral blood of the same patients (Figure 2). This difference was not due to the collagenase treatment of tumour-associated NK cells, as incubation of peripheral blood NK cells with collagenase for 2 h did not result in alterations in cytokine release (data not shown).

It should be noted that IFN-γ secretion was consistently detected in every blood and biopsy sample. By contrast, the release of IL-4 was only detected in blood samples from 50% (6/12) of the healthy donors and 10% (2/20) of the cancer patients tested.

Effect of medium conditioned by breast cancer lines on the secretory activity of NK cells

For these experiments, NK cell enriched populations of PBMC were used (see above). Figure 3 illustrates the significant (P<0.01) stimulation of IFN-γ and IL-4 secretion by CD16+ NK cells derived from healthy donors after exposure to medium conditioned by MCF-7 cells. Exposure to conditioned medium did not alter the proportion of CD16+ or CD4+ cells secreting IFN-γ in the RHPA (data not shown). Essentially similar results were obtained using medium conditioned by MDA-231 PN9 cells (data not shown).

Discussion

Any investigation of the activity and regulation of neoplastic cells in solid human tumours is complicated by the presence of considerable numbers of infiltrating stromal/immune cells at the tumour site. Macrophages alone, for example, can comprise over 50% of the total cell number in malignant breast tumours (Kelly et al., 1988).

In the present report we have employed a technique which permits the detection of cytokine secretion at the single cell level to investigate the cytokine communication which occurs in malignant tissues between resident malignant cells and NK cells. Since the area of haemolysis surrounding a secreting cell in the RHPA has been shown to be directly proportional to the amount of product secreted (Neill et al., 1987), this technique allows the quantitation of cytokine secretion by individual cells and its modulation by various stimuli. The coupling of the RHPA with immunocytochemistry to phenotype producer cells allows the contributions made by various cell types to the overall level of secretion of a given cytokine to be assessed.

Cytokine levels in the serum can vary between individuals, but are usually a stable individual property (Moelvig et al., 1988). For this reason, we chose to compare the cytokine-secretting activity of tumour-infiltrating NK cells with those from the blood of the same patients. Our studies indicate that CD16+ NK cells are the main source of IFN-γ spontaneously secreted in short-term cultures of human PBMC or dispersed malignant breast tumours. This correlates well with the finding that it is NK cells, rather than T cells, which are the major producer cells for IFN-γ in vitro in the absence of exogenous stimulation (Young & Ortaldo, 1987).

That NK cells secrete IL-4, as well as IFN-γ and IL-2, was inferred in the early report of Procopio and coworkers (1985). Our studies using the RHPA have both provided unequivocal evidence of IL-4 secretion by CD16+ NK cells, and revealed a marked heterogeneity amongst NK cells in their ability to secrete IFN-γ or IL-4. However, without the application of either a variant of the RHPA called the 'sequential' RHPA, to visualise the secretion of both these cytokines by the same cells (Neill et al., 1987), or their dual labelling by immunofluorescence (Anderson et al., 1990), it is not possible to say whether the 1% of CD16+ cells which were seen to secrete IL-4 also secreted IFN-γ. The possibility

Figure 1  Light micrograph of a haemolytic plaque formed by an NK cell secreting IFN-γ in the RHPA. Human cells were isolated and mixed with SpA-coated ovine red blood cells coated with protein A. These cells were incubated as a monolayer with polyclonal antiserum against human IFN-γ in for 6 h. Plaque formation was induced by the addition of guinea pig complement. (Magnification bar = 50 μm).
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Figure 2 Comparison of the mean (± s.e.m.) size of IFN-γ and IL-4 plaques formed by NK cells derived from either the peripheral blood (open bars) or breast tumour (opaque bars) of the same patient. The identity of plaque-forming cells as predominantly CD16⁺ NK cells was confirmed by immunocytochemistry. *P<0.01 compared to peripheral blood NK cells.

Figure 3 Effect of medium conditioned by MCF-7 cells on the mean (± s.e.m.) size of IFN-γ or IL-4 plaques formed by human CD16⁺ NK cells derived from the peripheral blood of healthy female donors. PBMC which had been enriched for NK cells were incubated for 24 h with incubation medium alone (control group; open bars) or medium conditioned by the breast cancer cell line, MCF-7 (shaded bars), prior to their use in the RHPA. *P<0.01 compared to respective control group.

exists that cells secreting each of these products may have represented functionally distinct subpopulations of NK cells. Although the reason(s) for the greater frequency of NK cells secreting IFN-γ than IL-4 remains unclear, this finding accords well with recent reports showing the differential production of IFN-γ and IL-4 by stimulated human T cells in vitro (Lewis et al., 1988; Anderson et al., 1990).

This study is the first to demonstrate an augmented secretion of IFN-γ and IL-4 by tumour-associated CD3⁻/CD16⁺ NK cells. The finding that NK cells secrete greater quantities of IFN-γ once they reach a malignant tissue is not surprising in view of the finding that LAK cells secrete elevated levels of IFN-γ and TNF-α when cocultured with tumour cells (Chong et al., 1989). The medium transfer experiments conducted in the present study also indicate that factors secreted by tumour cells in culture, may be involved in the activation of NK cell secretory activity by the microenvironment of the tumour.

IL-4 has been shown to inhibit IFN-γ production in mononuclear cells (Peelman et al., 1989). Our finding that both IL-4 and IFN-γ levels were elevated in human breast tumours suggests that, at least for a proportion of the tumour-associated NK cell population, this negative feedback loop may not be operative. The increased secretion of these two cytokines by NK cells within the tumour may have numerous implications for both tumour progression and the regulation of cell-mediated cytotoxicity against tumour cells. It is likely that they are two, amongst many cytokines which have been implicated as mediators of the intercellular communication which takes place within malignant tumours. Indeed, these often act back on the producer cell itself in an autocrine fashion (for review, see Lewis, 1991). Both cytokines stimulate the secretory activity of human NK cells in vitro (Lewis et al., 1991) and can modulate the induction of LAK cell activity (Mule et al., 1987; Widmer et al., 1987; Giovarelli et al., 1988). Within the tumour, IL-4 can act on other non-malignant cells either synergistically or antagonistically with IFN-γ. This is best illustrated in the case of macrophages, where IL-4 can be regarded mainly as the functional antagonist of IFN-γ. Whereas both cytokines increase the expression of MHC class II antigens by macrophages (Crawford et al., 1987), IL-4 also acts to reduce their tumouricidal activity (te Velde et al., 1988; Hart et al., 1989).

It is also known to inhibit macrophage activation by IFN-γ (Lehn et al., 1989). The differential effects of IL-4 on NK cell activity and inhibition of macrophage cytotoxicity make it difficult to predict the overall effect of increased IL-4 levels for the tumour-bearing host. However, in transgenic mice producing high levels of IL-4, there is evidence for reduced tumour growth (Tepper et al., 1989).

The role of IL-4 receptors which have been shown to be expressed on neoplastic epithelial cells and fibroblasts (Al Jabari et al., 1989) has yet to be established, but raises the possibility that IL-4 is able to modulate tumour cell function directly. In the case of IFN-γ, Jabranne-Ferrat and coworkers (1990) have shown that this cytokine protects human breast cancer cells from NK and LAK cell lysis. However, such an effect might be specific to malignant breast cells, as this interferon is reported to increase in susceptibility of a neuroblastoma cell line to cell-mediated cytotoxicity (Handgretinger et al., 1989). In addition, our own recent studies have indicated a role for IFN-γ and IL-4 in regulating the growth of breast cancer cell lines in vitro (Puszta et al., 1991).

The cellular mechanism(s), by which tumour cells might play a part in stimulating the secretion of IL-4 and IFN-γ by NK cells remain(s) to be identified. The possibility exists that the elevated level of cytokine released by tumour-associated NK cells reported here may have resulted, not so much from their exposure to activating factors produced within the tumour, but rather their release from local inhibitory influences. However, this seems unlikely since we have also demonstrated that medium conditioned by breast cancer cell lines stimulates, rather than inhibits, cytokine secretion by human NK cells in vitro. Cytokines may be secreted by neoplastic cells which directly modify the secretory activity of NK cells. Indeed, we have recently shown that one putative tumour cell product, namely basic form of fibroblast growth factor markedly enhances the secretion of IFN-γ by human CD16⁺ NK cells in vitro (Lewis et al., 1991). It is, therefore, possible that this (and/or other cytokines secreted by the neoplastic cell population may play a part in the stimulation of NK cell secretory activity at the tumour site. They may also play a part in the mitogenic stimulation of lymphocytes by tumour cell lines recently reported by Packard (1990).

Taken together, the studies outlined in this report demonstrate that soluble, tumour-specific signals augment the secretion of IFN-γ and IL-4 by human CD16⁺ NK cells. Indeed, the neoplastic cell population is one potential source of such stimulatory factors.
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