AN INVESTIGATION OF THE POTENTIAL ANTI-TUMOUR EFFECTS OF A TUMOUR CELL-LINE ENGINEERED TO SECRETE CYTOKINES

A thesis submitted to the University of Glasgow for the degree of Master of Science in the Faculty of Medicine

by

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DECLARATION

I declare that the work presented in this thesis has been carried out solely by me, except where indicated below. The tissue culture, immunohistochemistry, molecular biology, and tumour harvesting was performed by myself.

Dr. Grant Gallagher performed the tumour inoculations and measurements assisted by myself.

1 - SUMMARY

Systemic administration of cytokines such as IL-2, IL-4 and the interferons have been extensively examined in animal models and clinically to assess their potential as anti-cancer drugs. To date, the promising results from animal studies have not been reproduced in clinical trials. The systemic administration of cytokines in cancer therapy is also complicated by serious side-effects because of the potency of these compunds. New routes of administering cytokines are therefore being investigated in order to prevent these side-effects and thus allow higher doses of drug to be administered. Routes of administration such as peritumoural, isolated limb perfusion, lymphokine activated killer cells and tumour infiltrating lymphocytes have all been tested clinically with various degrees of success.

One of the advantages that tumour cells possess is their lack of immunogenicity. It has been proposed that this lack of immunogenicity could perhaps be overcome by engineering the tumour cells to secrete cytokines thus, rendering them more immunogenic. A variety of tumour cell types have therefore been genetically altered to enable them to secrete one or more different types of cytokines. On injection of the cytokine-secreting tumour cells into the host animal, it has been reported that tumour growth may be slowed or completely prevented. One such model of cytokine-secreting tumour cells is the fibrosarcoma (FS-29) cell line, which has been genetically altered to enable it to secrete IL-2, IL-4 or IFN-γ. It has been reported that these cells exhibit reduced tumourigenicity when injected into mice and in some cases the tumour cell inoculum is completely rejected.

The aim of this project was to further characterize the possible anti-tumour effects of the cytokine-secreting FS-29 cell-lines and to examine the possible effector mechanisms involved in tumour cell rejection. An immunohistochemical technique was developed which allowed the extent of the lymphocytic infiltration into the tumours that formed to be assessed. The cytokine-secreting cells were examined to

check that they contained the appropriate retroviral integrant for one cytokine before they were used in any animal studies. The first study was carried out to assess the ability of animals to reject a two-site injection of cytokine-secreting cells. It was found that the IFN-γ-secreting and the IL-4-secreting cells were significantly less tumourigenic in comparison to the non-secreting cells but only with a larger inoculum of cytokine-secreting cells. However, when IL-2-secreting cells were injected into mice, tumours grew progressively.

It has been reported that when an animal has rejected an inoculum of cytokine-secreting cells, a second inoculum of non-secreting cells may also be rejected. This rejection of tumour cells may correlate with the appearance of tumour-specific cytotoxic lymphocytes in the spleen. The second study examined the ability of these tumour-specific lymphocytes to transfer immunological memory between animals through the transfer of splenocytes from mice that had been previously challenged with tumour cells. Results from this study suggest that there may be a slight transfer of anti-tumour memory between animals, particularly in the group that received splenocytes from an animal that had previously received a small inoculum of IFN-γ-secreting cells and did not develop a tumour.

Previous studies report that cytokine-secreting tumour cells may prevent growth of an equal number of admixed non-secreting tumour cells. However, few studies have been carried out to assess the ability of cytokine-secreting tumour cells to reduce growth of an already established tumour nodule. The final study was carried out to assess the ability of the cytokine-secreting cells alone or in various combinations to reduce growth of an established tumour nodule. Unfortunately, none of the cell lines, either alone or in combination, were able to prevent further growth of an established tumour nodule.

The implications of these results and their relevance to published work is discussed.

2.1 INTRODUCTION: GENERAL

Over the past 50 years there has been a steady rise in the incidence and mortality rates of certain cancers. It has been reported that deaths from prostate cancer have risen 107% between 1970 and 1990, with the overall number of registrations rising by 75% between 1971 and 1986 (Majeed and Burgess, 1994). In females, reports show that the incidence of breast cancer is 40% higher than in 1979 with the total mortality increasing steadily since the 1980's. By the mid 1980's the total mortality for breast cancer in England and Wales was amongst the highest in the world (Quinn and Allen, 1995). This rise in the incidence of cancer is not only confined to adults, as the incidence of childhood cancer, in particular central nervous system tumours, is rising steadily by 1% per year (Bunin et al., 1996).

However, the most alarming cancer statistic lies in the incidence of lung cancer. Lung cancer is the most common cause of cancer death in males and in recent years the incidence of lung cancer in females has risen dramatically to become the biggest cause of cancer death (Travis et al., 1996).

One could argue that the higher incidences of cancer could be attributed to the better detection techniques and the increased number of cancer screening clinics; however with the ever increasing mortality rates, it remains apparent that conventional cancer therapies such as radiotherapy, chemotherapy and surgery are insufficient to control the disease. In the search for more efficient therapies for cancer, the immune system has come under the spotlight as a possible anti-tumour therapy. The presence of an immune infiltration into a tumour is seen to be indicative of a vigorous inflammatory response and indeed, the presence of an immune infiltration has been reported to correlate with a better prognosis in a number of human tumours such as breast (Ioachim, 1976; Lauder et al., 1977). Therefore manipulating the immune system to respond aggressively against tumours could be of benefit therapeutically.

2.2 Immune Surveillance Theory

The idea that the immune system may act as a surveillance mechanism against cancer cells was first suggested by Ehrlich in the early part of this century. It wasn't until the 1950's that Thomas and Burnet independently proposed what later evolved as Burnet's immune surveillance theory. These theories, as reviewed by Kripke (1988), proposed that the immune system was capable of recognising cancer cells through their antigens and then destroying the cancer cells. This lysis of cancer cells depended upon the existence of an effective immune system and therefore, such lysis would not take place if the immune system was suppressed.

Studies on immunity against tumours induced in animals have reported that tumours differ greatly in their immunogenicity. For example, tumours which were induced by oncogenic viruses were generally found to be antigenic (Klein, 1966) whereas tumours which were produced through contact with chemical carcinogens although generally antigenic, varied in their ability to induce protective immunity (Old et al., 1962). In contrast, spontaneous tumours, which arise in aged animals from no apparent cause, have been reported to be poorly or non-immunogenic (Baldwin., 1966). Since immune surveillance as originally defined would only respond against highly antigenic tumours, those tumours that are non-immunogenic will not be recognised and destroyed by the immune system. On this basis, it would be expected that only highly antigenic human cancers would be subject to immune surveillance. However, it has been argued that highly antigenic experimental tumours in animals are not the same as human cancers (Hewitt et al., 1976). It is thought that human tumours are more likely to resemble spontanous tumours that arise in animals than tumours that are induced by the injection of chemical carcinogens. This theory therefore relies on the majority of human tumours occurring spontaneously, rather than from induction by viruses or chemicals. Therefore, it would appear that spontaneously evolving human tumours are more likely to escape immune surveillance.

2.3 Immune Response to Tumours

From experimental work it would appear that there are a number of effector mechanisms involved in the destruction of tumour cells. Some of the main mechanisms are discussed below.

T-cells have been reported to be critical in the rejection of tumour cells and antigen-specific T-cells with antitumour activity have been generated in vitro using tumour infiltrating lymphocytes (TIL) extracted from melanoma (Topalian et al., 1989). T-cells are very much in evidence in tumours; however B lymphocytes are rarely observed except in melanomas (Ruiter et al., 1982). There are two main subpopulations of T-cells which may be distinguished according to their expression of CD4 or CD8 molecules. $CD4^+$ T-cells recognise antigen associated with MHC class-II molecules and may act as helper T-cells (Weaver and Unanue, 1990). $CD8^+$ T-cells recognise antigen associated with MHC class-I molecules and appear to mediate the cytotoxic destruction of cells (Yewdell and Bennink, 1992; Julius et al., 1993). As cytotoxic T cells are MHC class-I restricted, the levels of MHC expression may help determine the extent of the immune response to the tumour. Therefore, up-regulation of MHC molecules on tumour cells by cytokines, appears to enhance tumour destruction by cytotoxic T cells (Restifo et al., 1992).

Tumour infiltrations can also consist of phagocytic cells such as macrophages (Svennevig and Svaar, 1979). When isolated from tumour tissue these cells show spontaneous cytotoxic activity against tumour cells in vitro and macrophages can be modified to show enhanced anti-tumour activity through the administration of cytokines (Kos, 1989). Certain tumours such as colorectal cancer have a large infiltration of macrophages; however reports indicate that their presence is not always beneficial as macrophages can exhibit enhanced secretion of immunosuppressive prostaglandins which would reduce the effectiveness of the immune response

(Maxwell et al., 1988). Prostaglandins can also be associated with tumours exhibiting a high metastatic potential (Rolland et al., 1980; Karmali et al., 1983).

Tumour cells have also been found to contain small numbers of natural killer cells (Vose and Moore, 1985). Natural killer cells (NK) are large granular lymphocytes which have the ability to kill tumour cells and to prevent the establishment of metastases (Gorelik et al., 1982). Stimulation of peripheral blood lymphocytes with IL-2 leads to the maturation of NK cells into lymphokine-activated killer (LAK) cells. These cells have been shown to be capable of lysing tumour cells in vitro (Grimm et al., 1982). LAK cells are discussed in detail in section 2.6.2.

Therefore, for the rejection of tumour cells to take place it would appear that the host must possess an effective immune system. Indeed, patients receiving immunosuppressive therapy following organ transplantation have a higher incidence of all types of malignant disease (Mihalov et al., 1996). A number of theories exist as to the failure of the immune response in malignant diseases. Tumour cells have been reported to express tumour antigens or MHC molecules therefore, failure to do so would result in an ineffective immune response to these tumour types. This lack of MHC molecules has been reported in colorectal cancers and breast cancer (Goepel et al., 1991). It has also been observed that some tumours contain suppressor cells which can inhibit the effectiveness of the immune response (Werkmeister et al., 1981). Reports indicate that serum from cancer-bearing patients can suppress a range of immune functions in normal lymphocytes. In addition, specific defects have been identified in lymphocytes from cancer patients, including decreased responses to IL-2 (Monson et al., 1987). The ability of autologous tumour cells to trigger activation of lymphocytes but prevent their maturation into LAK cells has also indicated a tumour-mediated effect (Ting et al., 1987).

2.4 Tumour Infiltrating Lymphocytes

In the mid 1970s, it was proposed that the extent of mononuclear cell (MNC) infiltrations into solid tumours may bear a relationship to an improved prognosis (Ioachim, 1976; Underwood., 1974). From an immunologic point of view, a large cellular infiltration into tumours may represent a strong expression of immune surveillance. This theory has gained support from studies by Watt and House (1978) and Svennevig co-workers (1984) who reported an improved prognosis in colorectal tumours when accompanied with a strong inflammatory response. However, these correlations have not been reported in all cases (Brocker et al., 1984). These discrepancies in correlations between patient outcome and the extent of the inflammatory response could perhaps be explained in that cellular infiltrations into tumours need not be tumour-specific and could in fact be just inflammatory cells. Tumour cells have been reported to have receptors for cytokines such as IL-6 and interferon-beta (Baldwin et al., 1989; Kirnbauer et al., 1989). Since inflammatory cells are a possible rich source of cytokines, a strong inflammatory infiltration could be detrimental to the prognosis of the patient due to the many growth promoting properties cytokines possess (Schirrmacher, 1980). It has also been suggested that TILs which are present in the microenvironment may be influenced by any of the immunohibitory factors reported to be secreted from the tumour such as retroviral envelope protein p15E (Snyderman and Cianciolo, 1984) and prostaglandins (Botha et al., 1986).

The intensity of MNC infiltrations and their distribution varies between tumours. MNC are commonly found around the periphery of tumours and in the tumour stroma (Vose and Moore 1985; Whiteside, 1990). Squamous cell carcinomas of the head and neck and primary melanomas are well-infiltrated and this infiltration has been linked to a good prognosis in these patients (Poppema et al., 1983; Snyderman et al., 1989). Using immunohistological and in situ hybridization techniques, it has been possible to demonstrate the nature of the cellular infiltration into tumours. It has been reported

that the majority of the infiltrating cells are T-cells (Rowe and Beverley, 1984; Whiteside et al., 1986) which have the the ability to lyse tumour cells in an MHC-unrestricted manner (Whiteside et al., 1989). Although $CD3^+$ T-lymphocytes are the prominent cells present in tumours, macrophages can also be found (Svennevig and Svaar, 1979). NK cells have also been observed in small numbers (Vose and Moore, 1985) and B-cells are rarely found (Ruiter et al., 1982).

Observations of the locations of T-cells in tumours have indicated that $CD8^+$ T-cells are found in the parenchyma; therefore the cytotoxic T-cells are in close contact with the tumour cells (Vose and Moore, 1985, Cardi et al., 1989) whereas $CD4^+$ T-cells are usually located in the stroma (Whiteside et al., 1987). It has been suggested that the $CD8^+$ T-cell content may have an effect on prognosis in some tumours (Kornstein et al., 1983; Vose and Moore, 1985). Studies have indicated that the ratio of $CD4^+$ to $CD8^+$ T-cells is altered due to an enhanced content of $CD8^+$ T-cells in for example renal carcinomas (Finke et al., 1988), metastatic melanomas (Cardi et al., 1989), primary breast carcinomas (Bilik et al., 1989) and lung cancer (Rabinowich et al., 1987).

Histological analysis of the MNC infiltrations has given an indication of state of activation of these cells. Many of the T-cells have been reported to be activated as they express HLA-DR antigens (Lopez-Nevot et al., 1986; Van Duinen et al., 1988). In situ hybridization technology has also allowed the nature of cytokine gene expression to be determined (Vitolo et al., 1992) therefore giving an insight into the functional characteristics of these cells. Results from this study have indicated that TIL from squamous cell carcinomas of the head and neck expressed mRNA for TNF-α, IL-2 and IFN-γ whereas TIL from ovarian and ductal breast tumours rarely expressed mRNA for any of these cytokines.

From these reports it can be seen that with certain types of tumour, a strong inflammatory infiltration can indicate a better prognosis for the cancer patient. It would therefore be plausible that manipulating the immune response to increase the cellular infiltration into tumours, could lead to tumour destruction. One straight-forward method to increase the cellular population would be to expand the cells using cytokines. The clinical effectiveness of such an approach is discussed in section 2.6.

2.5 Cytokines

Cytokines are a heterogeneous family of proteins which are produced by a wide variety of cells in the body. They have been subdivided into classes which include interferons, interleukins, tumour necrosis factors and chemotactic factors. Together they appear to regulate the magnitude and duration of the immune and inflammatory responses by stimulating or inhibiting the activation, proliferation and differentiation of various cells and by regulating the secretion of antibodies or other cytokines. Cytokines share a number of common characteristics in that they are all low molecular weight proteins (15-40KDa) which interact with high-affinity cell surface receptors which are specific for each cytokine. They are extremely potent substances with only picomolar concentrations required to mediate an effect and they have a short half-life ensuring that they act for a limited time. Interaction with their receptors on the membrane of target cells results in an altered pattern of gene expression in the target cell to produce the required response (Balkwill and Burke, 1989; Kroemer et al., 1993; Coze, 1994).

Together these responses form the complexity of the cytokine network which may result in either synergistic, additive or antagonistic interactions. Two $CD4^+$ T_H subsets may be distinguished by the cytokines they secrete. The cytokines produced by the T_H1 and T_H2 subsets exhibit cross-regulation in that cytokines secreted by one subset appear to block the production or activity of the cytokines secreted by the other subset. T_H1 cells appear to produce predominantly IL-2, IFN-γ and TNF while in the

case of T_H2 clones, IL-4, IL-5, IL-6 and IL-10 (Mosmann et al., 1989; Cherwinski et al., 1989). It would appear that IFN-γ secreted by the T_H1 subset preferentially inhibits the proliferation of the T_H2 subset (Gajewski and Fitch, 1988) and IL-10 may down-regulate the secretion of IFN-γ and IL-2 (Moore et al., 1990; Vieira et al., 1991). More recently, IL-12 has been reported to augment the generation of T_H1 cells (Trinchieri, 1993). IFN-γ and IL-2 appear to promote IgG2a production by B-cells early in their activation, but inhibit IgG1 and IgE production (Snapper et al., 1988a). In contrast IL-4 secreted by the T_H2 subset promotes production of IgG1 and IgE and appears to suppress the production of IgG2a (Snapper et al., 1988b).

Therefore, it would appear with reference to cytokine production by T-lymphocytes there is a complex system of regulation which limits the inflammatory response. In general an acute inflammatory response is transient but, in some cases persistant immune activation can occur, resulting in pathologic consequences. The cytokines produced during an inflammatory reaction such as IL-1, TNF-α (Evans et al., 1989) and IL-6 (Snick, 1990) can ultimately lead to tissue damage if continually produced. This type of damage can be observed in diseases such as rheumatoid arthritis where excess TNF-α is reported to be a pivotal molecule in the disease progression (Leirisalo Repo et al., 1995) which has led to the initiation of clinical trials using an antibody to TNF-α (Lorenz et al., 1996). Raised pro-inflammatory cytokines such as TNF-α and IL-6 have been detected in the epithelium and lamina propria of patients with coeliac disease and is thought to play a role in the pathogenesis of this disorder (Przemioslo et al., 1994). There is also evidence that pro-inflammatory cytokines are implicated in a variety of other diseases such as peptic ulcers (Muller and Hunt, 1993), ankylosing spondilitis (Gratacos et al., 1994), Alzheimer's disease (Mrak et al., 1995), multiple sclerosis (Patterson, 1995) and myasthenia gravis (Link, 1994). It has been reported that serum levels of cytokines such as IL-1, IL-6 and TNFα are higher in cancer patients (Bauer and Herrman, 1991; Mantovani et al., 1994) and some studies demonstrate that high levels of serum cytokines may promote the mechanisms

involved in the pathogenesis of neoplastic cachexia (McNamara et al., 1992; Strassmann et al., 1992).

From these reports it could be concluded that cytokines are potent mediators of the inflammatory response. Therefore their ability to evoke aggressive anti-tumour responses is currently being thoroughly investigated. The clinical effectiveness of cytokines as possible anti-tumour agents is discussed in section 2.6.

2.6 Clinical Effectiveness of Cytokines

2.6.1 Interferons

The interferons were the first cytokines to be discovered and these were originally thought to be a single substance, but they are now recognised to be a group of proteins and glycoproteins which can be subdivided according to their differences in their antigenic, biologic and chemical properties (Rubin et al., 1980). The three types of interferons α, β and γ. IFN-α and IFN-β (Type-I) are stable at acid pH and may be induced in a wide range of cell types to activate cells by binding to a common receptor. IFN-γ (Type-II) appears to be induced in T-lymphocytes and may be neutralised at acid pH. It reportedly activates cells via a different receptor (Chebath et al., 1987). Together, the interferons have been reported to possess a range of biological properties such as antiviral, antiproliferative and immunomodulatory activities (Petska et al., 1987).

In 1980, Gutterman reported that a partially-purified preparation of IFN-α could induce regression of tumours and established metastasis in patients with breast carcinomas or well-differentiated B-cell neoplasms. To date, clinical trials have reported that IFN-α has been effective in the treatment of a range of neoplasms such as hairy-cell leukaemia (Quesda et al., 1984). The receptor for the type-I interferon has been demonstrated in neoplastic cell lines such as leukaemic cells from hairy-cell leukaemia (Platanias et al., 1992). IFN-α does not cure hairy-cell leukaemia, and up to

50% of patients will relapse after treatment discontinuation (Quesda et al., 1984). However, most relapsed patients will respond to retreatment with IFN-α, and it has been reported that continuous administration of IFN-α may prolong survival to 6 years in 82% of patients (Smith et al., 1991).

IFN-α has also been reported to induce haematological remission in patients with chronic myelogenous leukaemia (Talpez et al., 1983). Administration of IFN-α has been reported to eliminate Philadelphia-positive cells from the bone marrow of patients with chronic myelogenous leukaemia (Talpez et al., 1986). A recent clinical trial to test the effectiveness of IFN-α with chemotherapeutic agents reported that IFN-α in combination with Busulphan or Hydroxurea was effective in prolonging the median survival of patients with chronic myelogenous leukaemia from 41 months to 61 months (Allan et al., 1995).

Treatment of multiple myeloma using IFN-α has been reported to be effective. However, the response rates of multiple myeloma to chemotherapeutic agents is reported to be greater; therefore IFN-α has been used as an adjunct to chemotherapy for multiple myeloma in order to increase the response rate to chemotherapy (Montuoro et al., 1990). IFN-α may also be administered as a maintenance therapy for multiple myeloma following induction therapy with combined chemotherapy (Mandelli et al., 1990). IFN-α in combination with cytotoxic chemotherapy was also reported to prolong the time to treatment failure, and the duration of the complete response in patients with clinically aggressive, low grade and certain histological variants of intermediate-grade non-Hodgkin's lymphoma (Smalley et al., 1992).

As well as leukaemias, the interferons have also been reported to induce responses in some solid neoplasms. A response rate of up to 20% was reported when IFN-γ was administered for renal cell carcinoma (Recombinant Human Interferon-γ Research Group, 1987). The combination of IFN-α with IL-2 has resulted in greater response

rates than IFN-γ alone for the treatment of renal cell carcinoma and malignant melanoma. Rosenberg (1989a) and co-workers reported response rates of 43% with the combination of IL-2 and IFN-α for renal cell carcinoma and malignant melanoma. Similar results have also been reported by Kirchner and colleagues (1990). As these tumours are not responsive to chemotherapy, IL-2 and IFN-α in combination is currently the treatment of choice for these neoplasms.

IFN-α in combination with 5-fluorouracil was reported to increase the response rate relative to 5-fluorouracil alone for the treatment of disseminated colorectal cancer (Wadler et al., 1989). In vitro studies demonstrated that cytotoxicity was greater when 5-fluorouracil was combined with Leucovorin and IFN-α in several cell lines (Rastum, 1991). Partial responses using this combination have been reported in half of the patients with adenocarcinomas of gastrointestinal or unknown primary or neuroendocrine tumours (Seymour et al., 1994). Administration of IFN-α with 5-fluorouracil and cisplatin for the treatment of oesophageal carcinoma has resulted in major response rates in half of the patients, although this was with severe toxicity (Ilson et al., 1995). However, IFN-α in combination with cisplatin and 5-fluorouracil for the treatment of recurrent or metastatic head and neck cancer, resulted in poor response rates which were again associated with severe toxicity (Cascinu et al., 1994).

As discussed previously in this section, IFN-γ reacts with a different cell surface receptor than IFN-α and IFN-β, therefore it was proposed that IFN-α or IFN-β in combination with IFN-γ may potentiate the anti-tumour activity of the interferons. However, the addition of IFN-γ to IFN-α was not reported to improve the response rate of IFN-α for the treatment of renal cell carcinoma, and the combination may have been antagonistic to the efficacy of IFN-α (Demulder et al., 1995).

2.6.2 Interleukin-2 and LAK Cells

Interleukin 2 is a lymphokine produced by T-lymphocytes after antigen or mitogen stimulation (Smith, 1988). IL-2 was originally called T-cell growth factor because it was reported to to sustain the growth and proliferation of T-cells (Morgan et al., 1978). It has now been renamed because it appears to have effects on the proliferation and function of immunological cells such as T-lymphocytes (Robb, 1984) natural killer (NK) cells (Henney et al., 1981) and B-cells (Gordon and Guy, 1987). There have been two approaches to the administration of IL-2. The first route is that of bolus injection or continuous systemic administration. The second approach, which Lotze and his co-workers described in 1981, is that of adoptive therapy, which is the systemic transfer of immunologic reagents appearing to possess anti-tumour activity. Lotze reported that peripheral blood mononuclear cells (PBMC) from normal individuals and cancer patients could be activated in vitro using IL-2 and the cells generated were capable of killing fresh tumour target cells, but not normal cells. The cells with lytic activity were referred to as lymphokine activated killer cells (LAK). Experimental studies in this area were facilitated by the cloning of the IL-2 gene and its insertion into E. coli (Rosenberg et al., 1984). Therefore, large amounts of highly purified IL-2 with full biological activity were available for use in animal and human studies.

There have been numerous clinical trials using IL-2 and IL-2/LAK cells. The first of this type was carried out by Rosenberg and colleagues in 1985 at the National Cancer Institute (NCI) with a follow-up report in 1987. 157 patients with metastatic cancer for whom standard therapy had proved ineffective were treated with LAK cells plus IL-2 or high dose IL-2 alone. Of 106 patients receiving LAK cells plus IL-2, 8 were reported to have complete responses , 15 had partial responses and 10 had minor responses. Of 46 evaluable patients treated with high-dose IL-2 alone, 1 had a complete response, 5 had partial responses, and 1 had a minor response.

Approximately one-third of patients receiving LAK/IL-2 therapy had an anti-tumour response but IL-2 alone produced only half of that response.

In 1989, Rosenberg and co-workers (1989b) carried out further trials with IL-2 for the treatment of cancer patients which demonstrated the type of carcinoma that appeared to be responsive to IL-2 or IL-2/LAK therapy. With IL-2 alone some responses were reported with melanoma and renal cell carcinoma. However, no responses were observed with colorectal cancer, non-Hodgkin's lymphoma or breast cancer. Furthermore, treatment with LAK/IL-2 produced greater responses in melanoma and renal cell carcinoma compared to IL-2 alone and responses were also reported with non-Hodgkin's lymphoma and colorectal cancer which were not observed with IL-2 alone.

A more recent trial administering IL-2 with and without LAK cells reported improved long-term survival after intracavitary injection of IL-2 and LAK cells for adults with recurrent malignant glioma. LAK cells and IL-2 were reported to be administered safely within the CNS, resulting in improved long-term survival in patients with recurrent glioblastoma. This increased survival was apparently associated with significant biologic changes such as an eosinophil and lymphocyte infiltration (Hayes et al., 1995) A phase-III randomised trial of IL-2 with or without LAK cells was carried out in patients with advanced renal cell carcinoma (Law et al., 1995). However, there appeared to be no differences between the two treatment arms with there being no reports of increased response rates or greater length of survival with the addition of LAK cells to IL-2. When IL-2 was administered alone there appeared to be a low level of anti-tumour immunity which did not appear to be enhanced by the addition of LAK cells (Law et al., 1995).

There are many problems associated with the use of IL-2 alone or in combination with LAK cells. Firstly the use of LAK cells is expensive and the culture of lymphocytes

from patients is time-consuming. Secondly, the toxicity of IL-2 is a major problem (discussed in section 2.7). In order to avoid the toxicity associated with IL-2, low-dose IL-2 has been combined with cyclophosphamide (Mitchell et al., 1988) or dacarbazine (Flaherty et al., 1988). The response rate of this combination was reportedly greater than IL-2 administered alone. During the combination treatment with IL-2 and cyclophosphamide, induction of LAK cell activation was reported in all responding patients, and none of the patients without LAK activation was reported to have a remission from their disease (Mitchell et al., 1988).

Another approach is to use TIL rather than LAK cells to induce cytotoxic cells by IL-2. In 1986, Rosenberg and his group reported that TIL derived from murine tumours and grown in rIL-2 were 50-100 times more effective than LAK cells in eradicating established pulmonary or liver metastasis in a murine sarcoma model. Based on these observations, clinical trials of TIL were initiated. Results of early clinical trials using TILs were encouraging (Rosenberg et al., 1988). However, recent reports have not supported these results. In 1995, a phase-I trial of repeated TIL infusion was reported (Ravaud et al., 1995). This trial was not a success with no objective responses against melanoma being observed. Treatment was also complicated by a variety of side-effects which could perhaps be related to the increase in plasma tumour necrosis factor levels. Furthermore, the use of TILs is limited because of difficulties in obtaining TIL from tumours. Once obtained, the TIL then have to be grown for 4-5 weeks to obtain therapeutic quantities which may prove an unacceptable length of time for patients with advanced cancer.

2.6.3 Interleukin-4

IL-4 is reported as having stimulatory effects on the function of a number of leukocytes including having B and T-cell regulatory functions (Howard et al., 1982; Mitchell et al., 1989). Therefore, there has been some interest in its therapeutic potential *in vitro* and *in vivo*.

Several laboratories have reported that IL-4 inhibits the in vitro growth of a variety of neoplasms such as, renal cell carcinoma (Hoon et al., 1991a), melanoma (Hoon et al., 1991b), oestrogen-dependant breast cancer (Toi et al., 1992), non-Hodgkin's lymphoma (Defrance et al., 1992), non-small cell lung carcinomas (Topp et al., 1993), head and neck carcinomas (Topp et al., 1995) and glioblastomas (Topp et al., 1994). Reports have also indicated that the receptor for IL-4 is detectable on some tumours such as colon and lung cancer (Al Jabaari et al., 1989).

Data from clinical trials of IL-4 for the treatment of different neoplasms has resulted in conflicting reports. Preliminary data from phase-I trials reported clinical responses in patients with chronic lymphocytic leukaemia and low-grade lymphomas (Maher et al., 1990). However a phase-II trial of IL-4 for patients with metastatic melanoma or and advanced renal carcinoma proved to be unsuccessful (Margolin et al., 1994). Margolin and co-workers reported that one of the 30 patients with metastatic melanoma had a complete response following treatment with IL-4 and none of the 19 patients with advanced renal cancer was reported to have any response to IL-4 therapy.

From these studies it appears that IL-4 does not have substantial anti-tumour activity in humans; however IL-4 combined with IL-2 has been suggested as possibly having greater therapeutic potential as it has been reported to stimulate the proliferation of T-cells activated by exposure to IL-2 (Kawakami et al., 1988). IL-4 has also been reported to stimulate peripheral blood mononuclear cells in vitro from patients with solid tumours treated with IL-2 (Treisman et al., 1990) and to enhance antibody-dependant cellular cytotoxicity mediated by peripheral blood mononuclear cells and lymphocytes (Wersall et al., 1991). It appears to be important that LAK cell activity is first preactivated in vitro or in vivo with IL-2 (Higuchi et al., 1989), since IL-4 alone does not produce LAK cell activity and may inhibit LAK cell induction if cells are exposed to IL-4 before IL-2 (Spits et al., 1988).

2.6.4 Tumour Necrosis Factor

Tumour necrosis factor has been extensively tested in clinical trials in a variety of routes, doses and schedules. However reports are disappointing in that TNF appears to have little anti-tumour activity when administered systemically (Table 1, Kunkel et al., 1992). Naylor and co-workers (1990) reported that half of the ovarian cancers they studied appeared to be transcribing the TNF gene. They proposed that if tumour cells have the ability to produce TNF, the production could contribute to neoplastic progression and alter the response to therapy.

Table 2.1 - Clinical Trials of Recombinant Human Tumour Necrosis Factor

TNF Dose	Route	Schedule	No. of Patients	Complete Response	Partial Response
1-48 x 10^4 units/m^2	1h, IV infusion	Every 3 weeks	29	0	0
4.5-645 ug/m^2	24h, IV infusion	Every 3 weeks	50	0	1
5-200 ug/m2	30 mins IV infusion	Days 1-5 every 3 weeks	27	0	1
5-200 ug/m^2	IM	Days 1-5 every 2 weeks	19	0	0
40-400ug/m^2	24h, IV infusion	Once or twice weekly	15	0	0
5-150 ug/m^2	SC	Days 1-5 every other week	9	0	0
683-956 ug/m^2	30-60 mins IV	Every other week	22	0	0
5-250 ug/m^2	30 mins and 4h, IV	Days 1-5 every 2 weeks	39	0	0
5 x 10^4- 3 x 10^5 units/m^2	5 day continuous IV infusion	Every 4 weeks	19	0	0

(Kunkel et al., 1992)

It also appears that the efficacy of TNF is not improved when administered systemically in combination with other cytokines. For example TNF was combined

with IFN-γ for the treatment of metastatic melanoma (Retsas et al., 1989). Reports from this study indicate that TNF and IFN-γ have no activity against tumour growth when administered together and that the toxicity associated with TNF was considerable. The results from the clinical trials with TNF have been disappointing considering the success of the animal experiments (Havell et al., 1988; Tomazic et al., 1988). One of the limiting factors in the administration of TNF in humans is that the cytokine is associated with considerable toxicity (discussed in section 2.7).

2.6.5 Interleukin-1

Interleukin-1 is reported to be an important mediator in the immune system in that, it appears to control haematopoesis and may be capable of inducing the release of IL-2 and other cytokines from activated lymphocytes (Dinarello, 1988). IL-1 has been reported to have antitumour activity in murine tumour models when administered alone (Johnson et al., 1991) or in combination with other cytokines (Brunda et al., 1994). In the latter study, activity of IL-1 was reported to be enhanced by the addition of IFN-α.

The results from the above animal studies are similar to the results of IL-1 therapy in clinical trials in that when IL-1 was administered alone, there appeared to be little anti-tumour activity (Redman et al., 1994), but a trial of IL-1 in combination with another cytokine was reported as being more encouraging (Triozzi et al., 1995). Triozzi and co-workers combined IL-1 with IL-2 which appeared to result in a marked activation of T-cells and LAK cells. In addition, there appeared to be objective anti-tumour responses, including a complete response in a patient with colon cancer metastatic to the liver.

One of the major complications limiting the dose intensity of chemotherapy is myelosupression (section 2.7). Since IL-1 is reported to be a major co-ordinator of the haematopoietic system, it was proposed that if IL-1 was administered in combination

with chemotherapy, this myelosupression could be reduced or prevented. Clinical trials have been carried out to evaluate the ability of IL-1 to protect patients from chemotherapy induced myelosuppression. In one trial, 18 patients with colon cancer consecutively received IL-1β alone followed by chemotherapy with 5-FU alone, followed by 5-FU combined with IL-1 in consecutive cycles of therapy (Crown et al., 1991). Reports from this study indicate that patients developed only a transient neutropenia, monocytopenia, lymphopenia and they also appeared to develop an increase in their platelet count. Another aspect to this type of therapy is that it may allow for administration of higher doses of chemotherapy without the fear of myelosuppression.

2.6.6 Interleukin-6

Interleukin-6 may help in the regulation of the expression of other cytokines, and it has been reported to stimulate effector cells to produce antitumour effects in murine tumour model systems when administered both systemically and locally (Borden et al., 1994; Mule et al 1990). However, in phase-I/II trials of human malignancies, little evidence of antitumour activity was noted (Weber et al., 1994, Van Gameren et al., 1994; Weiss et al., 1995). Additionally, IL-6 may have a role in the growth or behaviour of a number of tumours, including myeloma, ovarian cancer, and mesothelioma, although the exact nature remains controversial (Ballester et al., 1994; Monti et al., 1994; vander Zee et al., 1995).

2.6.7 Other Uses of Cytokines In Cancer Therapy - The Colony Stimulating Factors

Interleukin-3, granulocyte-colony stimulating factor, granulocyte-monocyte stimulating factor and monocyte-stimulating factor have been classified as colony stimulating factors. Among these CSF's, recombinant GM-CSF and recombinant G-CSF have been approved for clinical use in patients in association with chemotherapy in order to prevent or decrease the severity of chemotherapy-induced neutropenia. Clinical trials of GM-CSF and G-CSF performed on cancer patients with associated

chemotherapy-induced neutropenia demonstrated that neutropenia was significantly less severe and shorter in the cycles with CSF's as compared with those without (Antman et al., 1988; Shulz et al., 1991; Morstyn et al., 1988; Gabrilove et al., 1988; Bronchud et al., 1989). The frequency of infectious complications was also reported to be significantly reduced following administration of CSF's in these trials and patients needed fewer days of antibiotic treatment. Addition of CSF's to the regimen appear to allow higher doses of chemotherapy to be administered.

Preliminary clinical trials have reported that the CSF IL-3 may increase peripheral blood thrombocytes and accelerate the recovery from chemotherapy-induced thrombocytopenia (Stahl et al., 1992). Recombinant human IL-3 has also been reported to dose-intensify the chemotherapeutic agents carboplatin and cyclophosphamide in a phase-I trial for the treatment of epithelial ovarian cancer (Veldhuis et al., 1995).

2.7 Side-Effects Of Cytokine Therapy

One of the major problems in the use of cytokine therapy is the toxicity of these compounds which may result in the patient suffering serious side-effects. Therefore, cytokines have to be administered in low-doses to prevent these side-effects which in turn, may reduce their efficacy as anti-cancer agents. Fever, chills, fatigue and other flu-like symptoms are common place in patients undergoing cytokine therapy. These symptoms are thought to result from the elaborate cascade of secondary cytokines such as IL-1 and TNF which are commonly produced with cytokine therapy (Gemlo et al., 1988).

One of the most serious side-effects associated with cytokine therapy, particularly with IL-2, TNF and IL-1 is hypotension (Gaynor and Fisher, 1993). This hypotension appears to be the result of cytokine-induced vasodilation. Marked reductions in systemic vascular resistance were observed within two hours of an IL-2 injection

(Gaynor and Fisher, 1993; Lee et al, 1989), an effect which appeared to be mediated by the vasodilator, nitric oxide (Lowenstein and Snyder, 1992).

Administration of cytokines such as IL-2 and IL-4 appears to result in an increased vascular permeability which may result in extravasation of plasma into the tissues therefore leading to weight gain, oedema, pleural effusions and ascites (Mier, 1993; Atkins et al., 1992). Complement activation is also thought to contribute to this vascular leak syndrome as high levels of the complement pathway fragment C3a, appear to be detected in the plasma of patients receiving IL-2. The levels of which appeared to correlate with the extent of the vascular-leak syndrome (Vachino et al., 1991; Thijis et al 1990).

Higher rates of bacterial infection have been reported in patients undergoing IL-2 therapy (Snydman et al., 1990), which is thought to be due to an acquired defect in neutrophil function (Klempner et al., 1990). It is proposed that this chemotactic defect in neutrophil function is the result of endogenous TNF production in patients treated with IL-2, since a similar defect has been observed by exposing neutrophils to TNF in vitro (Atkinson et al., 1988).

Treatment of cancer patients with IL-2 or IFN-α has been reported to result in the development of autoimmune hypothyroidism (Fentiman et al., 1985; Atkins, 1993). Examination of thyroid aspirates from patients treated with IL-2 or IFN-α has demonstrated an extensive infiltration of lymphocytes (Pichert et al., 1990; Karlsson et al., 1991). Therapy with IL-2 and IFN-α may also result in side-effects which are similar to rheumatoid arthritis (Massarotti et al., 1992) and Crohn's disease (Sparano et al., 1993).

Other common side-effects associated with cytokine therapy include, disorientation and confusion with IL-2 therapy (Denicoff et al., 1987) and depression and myelosuppression with high-dose IFN-α therapy (Spiegal, 1987).

Together these systemic side-effects limit the dose of cytokines that may be safely administered to cancer patients and therefore potentially reduce their maximum possible efficacy. Therefore, alternative means of delivering these cytokines have been proposed in order to reduce their toxicity. The administration routes of local cytokine therapy and cytokine gene therapy have come under intense investigation over the past few years in a bid to improve the immunotherapy of cancer.

2.8 Local Administration of Cytokines

One of the methods investigated in order to avoid toxicity of cytokines has been their local administration. Bosco and colleagues reported that when IL-4 was administered subcutaneously around tumour-draining lymph nodes of a chemically-induced fibrosarcoma (CE-2) and a spontaneous adenocarcinoma (TS/A) in BALB/c mice, the tumour growth was inhibited (Bosco et al., 1990). It also appeared that this therapy with IL-4 could lead to a state of long lasting immune memory in that, growth of a second contralateral tumour challenge was reported to be significantly impaired. IL-4 appeared to be more effective than other cytokines such as, IL-2, IL-1β and IFN-γ in producing an anti-tumour response and the combination of cytokines did not result in an additive anti-tumour response. However, the establishment of a tumour-specific memory appeared to be enhanced with the combination of IL-4 and IL-1β. Analysis of the possible effector mechanisms responsible for the anti-tumour response demonstrated that $CD4^+$ T-cells appeared to be important (Bosco et al., 1990). This approach could be of use clinically as the perilymphatic injection of cytokines could be easily performed around tumour draining lymph nodes of many cancer patients. Furthermore, the response to this cytokine therapy appeared to be immediately local, which may reduce the side-effects associated with cytokine therapy.

Peritumoural injections of cytokines is another approach that has been attempted in order to reduce the toxicity and improve the efficacy of cytokine therapy (Gallagher and Zaloom, 1992). When IL-4 was injected into a B.16 melanoma which had been induced in C57BL/6 mice, it appeared that IL-4 could inhibit tumour growth and in some animals result in a complete tumour regression. In addition, local administration of IL-4 appeared to result in a systemic immune response in that, when mice were innoculated with tumour cells in the neck and in the thigh, peritumoural treatment with IL-4 at the neck site appeared to result in tumour regression at both sites (Gallagher and Zaloom, 1992). Following these observations, combination therapy of cytokines was carried out in the same tumour model (Zaloom and Gallagher, 1993).

When IL-4 and IL-2 were administered together, IL-2 appeared to antagonise the anti-tumour effect of IL-4 alone, and IL-2 alone, or in combination with IL-4, was not reported to induce a systemic protection as had been reported with IL-4 alone. Peritumoural injections of cytokines is another method which may be easily performed clinically for the treatment of cancer patients, although the site of the tumour would have a bearing on the feasiblity of this treatment.

Clinical trials have been carried out to assess the efficacy of local administration of cytokines such as, the perilymphatic administration of IL-2 for head and neck cancer (Whiteside et al., 1993) and the intralesional treatment of melanoma with TNF and IFN-γ (Retsas et al., 1989). These trials have had little success. Perhaps the most encouraging responses observed with local therapy is the use of TNF in isolated limb perfusion for melanoma. It has been reported that high dose TNF-α with IFN-γ and melphalan for stage-III melanoma may be administered successfully with little toxicity. In this trial, 90% of patients experienced a complete remission and 10 % of patients were reported to have a partial response from their disease with a median survival of 28 months. Similar results were reported with TNF-α and melphalan using isolated limb perfusion again for melanoma. In seven of the nine patients studied, sloughing and necrosis of the superficial tumours were apparent within 48 hours of perfusion and all patients experienced a complete tumour response. There were no systemic side-effects but local side-effects such as oedema and joint stiffness were pronounced in some patients (Hill et al., 1994).

3: INTRODUCTION: CYTOKINE GENE CELL THERAPY OF CANCER

3.1 Introduction

Thre is another approach to deliver cytokines locally in order to improve efficacy and avoid toxicity. This is to use tumour cells that have been genetically engineered to secrete cytokines. It has been proposed that the presence of cytokine-engineered tumour cells may render the cells more immunogenic and therefore enhance their recognition, signalling the immune system to respond more vigorously to a poorly or non-immunogenic carcinoma.

The first experiment of this type was carried out by Tepper and co-workers in 1989. His strategy involved transfecting and expressing the IL-4 gene into the J558L plasmacytoma and the K485 adenocarcinoma cell-lines. These cells were then injected alone or mixed with non-transfected tumour cells into T-cell deficient (nu/nu) mice and their growth monitored. It was found that the control plasmacytoma cells formed very large tumours only 8-10 days after injection, however, the IL-4 expressing plasmacytoma line did not form tumours in over 100 days of observation. The results also suggested that the presence of the IL-4 gene substantially reduced tumour growth in the adenocarcinoma cell-line. When IL-4 secreting cells were mixed with non-secreting cells and injected into mice, there was again no tumour formation. Furthermore, as there was no tumour growth in nude mice, it may be concluded that T-cells were not an important effector mechanism for the tumour cell rejection. Histological examination of the injection site showed that this anti-tumour effect appeared to be mediated by a cellular infiltration composed of eosinophils and macrophages.

It was the result of this study that triggered extensive research into gene therapy of cancer using cytokines. The use of cytokine-secreting cells has been successful in a number of animal models (detailed in the following chapter) and, has given an insight into the many possible effector mechanisms involved in tumour cell destruction.

3.2 Cytokine Gene Cell Therapy Using IL-2

Perhaps the most extensively studied cytokine for gene transfer has been IL-2. To date, IL-2 has been successfully transferred into a variety of tumour models and has given an insight into the many possible effector mechanisms involved in tumour cell destruction. For example, the gene for human IL-2 has been introduced into CMS-5 cell line, which is a weakly-immunogenic mouse fibrosarcoma cell-line (Gansbacher et al., 1990a). Injection of these cells into mice resulted in a decreased tumourigenicity and protection from a subsequent challenge with CMS-5 cells. When IL-2 producing CMS-5 cells were co-injected with unmodified tumour cells, tumour formation was again prevented. This protective response against the parental tumour cells correlated with the appearance of tumour-specific cytotoxic T cells in the animal spleens indicating that these cells may be responsible for the rejection of tumour. Decreased tumourigenicity by insertion of the IL-2 gene has also been reported in a variety of other models such as HSNLV (a rat sarcoma cell line) clones which were induced to secrete different amounts of IL-2 (Russell et al., 1991). The IL-2 secretion of this cell line resulted in a reduced tumourigenicity and metastatic potential in rats, which was associated with a $CD4^+$ and $CD8^+$ T cell lymphocytic infiltration. However, when the growth of IL-2 secreting cells in T-cell deficient nu/nu rats was slowed, this pointed to the involvement of another non-T-cell effector mechanism (Russell et al., 1991). This theory was confirmed when IL-2 was introduced into the non-immunogenic spontaneous adenocarcinoma TS/A cell line (Cavallo et al., 1992). When these cells were injected into mice, the high-secreting IL-2 clones were quickly rejected. This rejection was associated with a neutrophil infiltration, with a smaller $CD8^+$ T-cell infiltration. NK cells and $CD4^+$ T-cells appeared not to have a role in tumour rejection but the neutrophil-dominated rejection appeared to require $CD4^+$ lymphocytes to have a persistant tumour-specific memory (Cavallo et al., 1992).

A further effector mechanism for IL-2 was observed when the cDNA for human IL-2 was introduced into the human renal cancer cell line SK-RC-29 cells (Gastl et al.,

1992). When injected into BALB/c mice, the SK-RC-29 IL-2 secreting cells showed no visible tumours at the injection site when examined macroscopically. However further examination of the injection site in these animals revealed small tumour cell aggregates which, by immunohistology, revealed a strong peritumoural infiltration of peroxidase positive cells, the majority of which appeared to be macrophages. However macrophages appeared not to be the effector cells involved when the non-immunogenic murine fibrosarcoma MCA102 cell line was transduced to secrete IL-2 (Karp et al., 1993). These cells failed to grow when injected into C57BL/6 mice. This inhibition of growth was halted when $CD8^+$ T-cells or NK cells were depleted, but not when CD4+ T-cells were depleted. However, when the IL-2 secreting cells were mixed with parental tumour cells, the growth of the tumour was delayed and histologic analysis revealed a dense lymphocytic infiltration of $CD4^+$ and $CD8^+$ T-cells (but not macrophages) into these tumours (Karp et al., 1993).

An involvement of NK cells was also observed when murine MBT-2 cells (derived from a carcinogen-induced bladder tumour) were transfected with IL-2 (Saito et al., 1994). These cells were rejected when injected into mice and also when injected into nu/nu mice. Therefore, T-cells appeared not to be the main effector mechanism involved, although histological analysis revealed that $CD4^+$ and $CD8^+$ T-cells were present at the site of injection of the IL-2 secreting cells. However, with the cells at the injection site, there also appeared to be large numbers of NK cells. As T-cells appeared not to be responsible for the tumour cell rejection, this would suggest that NK cells may have been an important effector mechanism (Saito et al., 1994).

It has been reported that a variety of effector mechanisms are required for the rejection of IL-2 secreting J558L cells (Hock et al., 1993). Cell depletion experiments indicate that $CD8^+$ T-cells were required for complete long-term tumour rejection, although transient tumour suppression was observed in the absence of $CD8^+$ T-cells. However,

NK cells were also involved in the anti-tumour response and histological analysis revealed a heavy infiltration of macrophages (Hock et al., 1993).

3.3 Gene Cell Therapy Using Combinations of Cytokines

From the above, it is clear that the effector mechanisms involved in tumour rejection cannot be predicted from the use of the same cytokine in different tumour models. Nevertheless, such mechanisms underlying cytokine-induced tumour rejection in specific tumour models are important in that they allow an insight into the combination of cytokines that may produce complementary effector mechanisms and further enhance tumouricidal effects.

A combined cytokine experiment of this type was carried out by Ohira and co-workers (1994) in which IL-2 and TNF were co-transfected into the Lewis Lung carcinoma. It was reported that C57BL/6 mice rejected an innocculum of the cytokine secreting tumours, with the effects of the co-transfectants being superior to that of the IL-2 and TNF transfectants alone. This is in contrast to the human studies where systemic TNF has been unsuccessful in the clinical treatment of most cancers (Table 1, Kunkel et al 1992). Furthermore when TNF was transfected into MCA102 cells and injected into C57/BL6 mice, tumours grew progressively (Karp et al., 1993). Therefore, it would appear that the combination of TNF with another cytokine such as IL-2 may have superior efficacy compared with that of TNF alone.

It has also been reported that IL-2 secreted by renal cancer cells is capable of enhancing cytotoxic lymphocyte recognition of TILs isolated from autologous patients (Schendel and Gansbacher, 1993). TILs were isolated from a patient with renal cell carcinoma and an autologous LAK cell population was generated by culturing the peripheral blood lymphocytes with high doses of rIL-2. The recognition of IL-2 secreting tumour cells by TILs was enhanced in comparison to TIL recognition of unmodified tumour cells. However, LAK cell recognition of the IL-2

secreting tumour cells appeared to be unchanged or slightly reduced (Schendal and Gansbacher, 1993). As discussed previously in section 2.6.2, TILs have proved more effective than LAK cells for the treatment of cancer in animal models. However in clinical trials, treatments using TILs have been disappointing whereas LAK-cell therapy appeared to be more effective. Perhaps the method as described by Schendal and Gansbacher (1993) could lead to an enhanced effectiveness of TILs in the clinical setting. Furthermore, Schendal and Gansbacher (1993) reported that the recognition of IL-2 secreting tumour cells by TILs appeared to be further enhanced when the tumour cells were pretreated with exogenous IFN-α. This effect was proposed to be due to an up-regulated expression of MHC class I molecules (Schendal and Gansbacher, 1993). In fact, cytotoxic lymphocytes appeared to be 50-150 fold more effective than LAK cells in lysing autologous tumour cell lines that had been transduced with both IL-2 and IFN-α (Schendal and Gansbacher, 1993).

3.4 Gene Cell Therapy Using IFN-γ

IFN-γ has also been transfected into cell lines such as the renal cancer cell line, SK-RC-29 (Gastl et al., 1992). When injected into mice these IFN-γ secreting cells formed large subcutaneous tumours. Microscopic examination of the tumour nodule showed that there was no cellular infiltration into these nodules, but the cells secreting IFN-γ demonstrated increased expression of MHC class I antigen, β2 microglobulin, ICAM-1 and MHC class II antigen expression. A lot of importance has been placed on upregulated MHC expression by IFN-γ as being important in the reduction of tumourigenicity. However, it has been demonstrated that modulated MHC expression is not the most important effector arm required for tumour cell destruction (Esumi et al., in 1991). When the spontaneous SP1 murine adenocarcinoma was transfected with IFN-γ, the cells failed to grow when injected into syngeneic hosts and nude mice. It has been suggested that this lack of tumourigenicity was a result of MHC class I expression. However, more than 64units/ml of IFN-γ was required to inhibit tumourigenicity whereas only 8units/ml were required to induce MHC class I

antigens. Therefore, it would appear that some other effector mechanism must be involved in the anti-tumour response. Furthermore, IFN-γ-secreting SP1 cells failed to protect against a challenge with parental cells, which may indicate that factors other than IFN-γ production or class I MHC expression are needed to induce a protective response (Esumi et al., 1991). Therefore, it appears that an additional factor may be needed to prevent growth other than MHC. MHC class I has been proposed to be an important factor in the reduction of tumourigenicity and metastatic growth since the poorly immunogenic 3LL-D122 clone engineered to secrete IFN-γ since IFN-γ secretors showed significant decrease in tumourigenicity and metastatic growth, whereas non-secretors retain tumourigenicity and metastatic potential (Porgador et al., 1993a). The IFN-γ cells manifested high expression of MHC class I which was concluded to be a major participant in cytotoxic lymphocyte induction. Furthermore high levels of IFN-γ secretion appeared to induce protective immunity in that a subsequent challenge of parental malignant cells was rejected (Porgador et al., 1993a). However support for the participation of non-T-cell-effectors in the response to IFN-γ secretors derives from the reduced tumourigenicity of these cells in nude mice (Porgador et al., 1993a). Further support for non-MHC effector mechanisms of IFN-γ were observed when the MBT-2 cells derived from a carcinogen-induced bladder tumour were infected with IFN-γ (Saito et al., 1994). The cells secreting IFN-γ grew slightly slower than the non-secreting cells when injected into mice. Histology in this case revealed that $CD4^+$ as well as $CD8^+$ T-cells were present at the site of secreting cells, but not unmodified cells. As well as the T-cells, macrophages were also found at the injection site. From this animal work it would appear that there are many possible effector mechanisms involved in tumour rejection.

3.5 Gene Cell Therapy Using IL-4

As previously discussed in section 3.1, macrophages (but not T-cells) were an important effector mechanism in the prevention of tumour cell growth when the J558L plasmacytoma and K485 adenocarcinoma cell lines were induced to secrete IL-

4 (Tepper et al., 1989). Macrophages were also involved when murine renal cell tumours were engineered to secrete large doses of IL-4 but, in this case T-cells also appeared to be required for tumour rejection (Golumbek et al., 1991). When these IL-4 secreting renal cancer cells were injected into BALB/c mice they were completely rejected. Furthermore, tumour growth was not observed for 2 months when renal cancer cells secreting IL-4 cells were injected into nu/nu or SCID mice suggesting that T-cells were required for the ultimate rejection of tumour cells. Histological examination of the injection site revealed an influx of macrophages and some granulocytes. When $CD8^+$ cells were eliminated from the animal the rejection of parental renal cancer cells at a distant site was prevented. Elimination of $CD4^+$ T-cells had a lesser effect although there were some late recurrences suggesting that $CD4^+$ T-cells participated in the long-term memory responses. Histological analysis of the rejecting challenge tumours revealed that $CD3^+$ T-cells were a major component of the infiltrating population during the early stages of rejection (Golumbek et al., 1991). The importance of T-cells for IL-4 induced tumour rejection was again evident when J558L cells were induced to secrete IL-4 (Hock et al., 1993). Cell depletion experiments revealed that $CD8^+$ T-cells were required for complete long-term tumour rejection, although effective, transient host-dependent tumour suppression was also observed in the complete absence of $CD8^+$ T-cells. Again, immunohistochemical analysis revealed that a heavy infiltration of macrophages and some eosinophils was also required (Hock et al., 1993).

3.6 Gene Cell Therapy Using IL-7

IL-7 plays a major role in the proliferation of T and B-cells (Goodwin et al., 1988; Henney, 1989). When J558L cells expressing IL-7 were injected into mice they were completely rejected (Hock et al., 1991). However, this tumour rejection was not observed in nude mice, indicating the importance of T-cells. Immunohistological analysis of tumour tissue revealed an infiltration by $CD4^+$ and $CD8^+$ T lymphocytes, and also type 3 complement receptor positive cells ($CR3^+$), which were

predominantly macrophages. Depletion of T-cell subsets in tumour-bearing mice, initiated at the same time as the tumour injection, demonstrated the dependance of the anti-tumour response on $CD4^+$ T-cells and $CR3^+$cells , whereas tumour rejection was unaffected by depletion of $CD8^+$ T-cells (Hock et al., 1991).

3.7 Gene Cell Therapy Using GM-CSF

The significance of T-cells in combination with other non-T-cell effectors was also observed when B16 melanoma cells expressing GM-CSF were injected into mice (Dranoff et al., 1993). It was reported that irradiated cells stimulated potent, long-lasting and specific anti-tumour immunity. The systemic immunity was long-lasting in that the majority of mice vaccinated with cells that express GM-CSF remained tumour free despite being subsequently challenged with non-transduced cells several months after vaccination. The systemic immunity was also specific in that GM-CSF expressing cells did not protect mice from a challenge of Lewis Lung carcinoma cells. When the vaccination site was examined an extensive influx of immature dividing monocytes, granulocytes and activated lymphoctes was observed. Depletion of $CD4^+$ and $CD8^+$ T-cells but, not NK cells prevented the reduced tumourigenicity. Similar results were reported when murine MBT-2 cells were also engineered to secrete GM-CSF (Saito et al., 1994). When injected into mice, GM-CSF cells grew more slowly than the controls and those that did not develop tumours were also resistant to a second tumour challenge. Histology revealed that $CD4^+$ as well as $CD8^+$ T-cells were again present at the injection site of cytokine-secreting cells, as were macrophages.

3.8 Gene Cell Therapy Using G-CSF

Neither T-cells, NK cells or macrophages were involved when C-26 murine colon carcinoma cells transduced with the human gene for G-CSF lost their tumourigenicity when injected into BALB/c mice (Colombo et al., 1992). Tumour rejection appeared to be mediated through a mechanism that involved increased numbers of neutrophils

at the site of tumour rejection. Electron microscope analysis of the infiltrated tumours showed that the neutrophils appeared to be in intimate contact with the tumour cells.

3.9 Gene Cell Therapy Using IL-12

IL-12 was originally identified as NK cell stimulatory factor (Kobayashi et al., 1989). It is secreted by macrophages and some B-cell lines and it appears to induce NK cells and T-cells to produce IFN-γ (Chan et al., 1992). In addition, IL-12 may also enhance NK activity and highly specific cytotoxic T-cell responses (Zeh et al., 1993). IL-12 also appears to facilitate a T_H-1 response but inhibits differentiation of T_H-2 lymphocytes (Manetti et al., 1993). The potential anti-tumour effects of IL-12 have also been studied experimentally. BL-6 cells (a poorly immunogenic murine melanoma cell line) were innoculated into C57BL/6 mice (Tahara et al., 1994). The tumour cells were admixed with NIHT3 cells which had been transfected with IL-12 or the neomycin phosphotransferase gene. Compared to mice which were given injections of BL-6 cells admixed with non-secreting cells, the emergence of tumours was significantly delayed in mice given injections of BL-6 cells admixed with IL-12 secreting cells. Histological examination of the tumour innoculum from high amounts of IL-12-secreting cells showed reduced tumour volume and necrosis in comparison to the control admixture. However, infiltration of the tumour by either macrophages or lymphocytes was not enhanced by IL-12 production, and high levels of IL-12 actually decreased the number of $CD4^+$ T-cells infiltrating into the tumour (Tahara et al., 1994). IL-12 secreted from fibroblasts also appeared to efficiently eliminate or suppress growth of an established MCA207 sarcoma when injected at the site of tumour growth (Zitvogel et al., 1995). It was reported that in less immunogenic tumours (MCA102, MC38), established lung metastasis could be significantly reduced following delivery of IL-12 secreting fibroblasts and systemic administration of low doses of IL-2. Histological analysis of the regressing tumours revealed an infiltration of $CD4^+$ and $CD8^+$ T-cells plus macrophages. Another encouraging finding from these experiments was the lack of toxic side-effects. Tests of renal and

liver function monitored during the treatments were within the normal range. Therefore, it would suggest that IL-12-secreting fibroblasts could serve as an effective, non-toxic anti-tumour treatment (Zitvogel et al., 1995).

3.10 Gene Cell Therapy : Summary

So far, tumour cell targeted gene transfer has proved successful in anti-tumour activity and useful in analyzing the effector mechanisms involved in tumour destruction in vivo in animals. These experiments indicate that the immunological mechanisms by which tumours are rejected after cytokine gene cell transfer are varied not only by the cytokine, but also by the tumour model. This may be because tumour cells differ in the susceptibility for host effector mechanisms because of different surface molecule expression (e.g. MHC, adhesion molecules), soluble mediators other than the transfected cytokine gene, growth kinetics and immunogenicity. Other parameters such as the mouse strain or the level of cytokine expression may also influence the mode of rejection. Nevertheless, these studies point to which gene products or combinations thereof are best able to stimulate anti-tumour immunity in a variety of animal tumour models and gene transfer may prove to be a powerful tool for preventing tumourigenicity in humans

3.11 The FS-29 Cell Line

The FS-29 cell line is a weakly immunogenic murine fibrosarcoma from which a panel of cell lines has been generated by infection with recombinant retroviruses to enable it to carry cytokine cDNAs initially for IL-2 and IL-4 (Patel et al., 1993a) and subsequently IFN-γ (Patel et al., 1993b). In order to generate a control cell line, FS-29 cells were infected with a retrovirus lacking any cDNA insert. This section details experiments carried out by Patel and co-workers to investigate the anti-tumour potential of this modified cell line (1993). Initial experiments were carried out to establish whether the growth rates of the cytokine secreting cells was different from the non-secreting cells. It was found that the in vitro growth rate of the cytokine-secreting clones was not different from the non-secreting clones. It was reported that the individual clones secreted varying levels of the same cytokine; therefore those clones secreting the highest amount of cytokine were chosen for the experiments.

When 10^6 FS-29 IL-2-secreting cells were injected into C57/BL6 mice, Patel and co-workers reported that tumour nodules formed which then completely regressed in most animals. In the tumours which did arise from these clones, it was reported that the tumour cells secreted considerably reduced, or no IL-2, which was attributed to a decrease in the IL-2 encoding retroviral integrant. FS-29 IL-4 secreting clones also have demonstrated slower growth rates and reduced tumourigenicity when injected into C57BL/6 mice. However, when tumours from the IL-4 injected mice were explanted, their IL-4 secretion was found to be unreduced, and they appeared to have retained the retroviral integrant encoding IL-4. When IL-2 and IL-4 were doubly-transfected into the one cell line, this combination resulted in optimal tumour rejection suggesting that the two cytokines might mobilize different and complementary effector mechanisms.

When the explanted tumours were examined histologically and morphologically, the tumours formed from the unmodified FS-29 cells appeared to have few infiltrating

host cells. Pronounced lymphocytic infiltrations were observed in the tumours which formed from the IL-2 secreting cells, the majority of which appeared to be $CD8^+$ T-cells, with a few $CD4^+$ T-cells and some B-cells. The tumours which formed from the IL-4 secreting cells were characterised by a macrophage and granulocyte infiltration, with a small number of $CD8^+$ and $CD4^+$ T-cells. Further studies indicate that neither IL-2, nor IL-4 secreting FS-29 cells resulted in any slowing of growth when injected into mice with an equal number of admixed non-secreting FS-29 cells. Furthermore, it was reported that tumours which formed from admixtures of IL-2 and parental cells excised at day 14, appeared to have lost their IL-2 secretion which correlated with a loss of IL-2-encoding retroviral DNA sequences. Similar analysis of admixtures of IL-4 secreting and parental cells demonstrated greatly reduced IL-4 secretion after 14 days growth in vivo, and undetectable IL-4-encoding retroviral DNA. A more rapid loss of IL-2-secreting cells was reported in an admixture of IL-2 and IL-4 secreting cells with parental cells.

An alternative application of retroviral cytokine gene delivery in cancer therapy would be the use of tumour cells cultured following primary lesion excision and infected with cytokine encoding retroviruses. These cells could be administered as an injection to enhance eradication of minimal residual disease. Patel and co-workers reported that animals which had rejected the IL-4 secreting tumour cells showed considerably delayed tumour incidence when challenged after 48 days with FS-29 control cells. However, the IL-2 secreting tumour cells did not protect animals against a delayed parental cell challenge. This data suggests that IL-4 secreting tumour cells, while unable to induce a rapid enough immune response to cause the rejection of admixed parental cells, may stimulate an effective long-term anti-tumour response.

Subsequently, the cDNA for IFN-γ has been inserted into the FS-29 cell line (Patel et al., 1993b). When these IFN-γ secreting tumour cells were injected subcutaneously into C57/BL6 mice, the clones secreting IFN-γ developed tumours, which in most

cases regressed. Furthermore, when 10^6 FS-29 control cells, IL-2, IL-4 or IFN-γ secreting cells were injected into nu/nu mice, only the IL-2 secreting cells were reported to have slowed tumour growth and subsequent regression. Since the other cytokine-secreting and non-secreting cells formed tumours, this indicates the importance of T-cells for the anti-tumour mechanisms of IL-4 and IFN-γ. When non-secreting cells were injected either alone or with IL-2, IL-4 or IFN-γ secreting cells, growth of those injected with IL-2 or IL-4 admixtures appeared to be slightly slower than the controls, but the IFN-γ admixtures demonstrated the most successful slowing of growth, with the mean tumour diameter being around half of the control tumour size.

From the above, it would appear that the FS-29 cell-line is to date one of the most characterised cell-lines and has been used successfully in a number of gene cell therapy studies in animals. This cell-line was therefore chosen to be used in studies of gene cell therapy described in this thesis.

3.12 Aims

Based on the previous reports of the FS-29 cell line, the aims of the work were to further examine the effectiveness of this cell line by addressing the following questions;

1) When the animals are given a two-site injection of cells, is there a systemic reaction that prevents growth of the tumour cells and is this systemic protection dependant on the cytokine that the tumour cell secretes? Furthermore, is the ability to promote systemic protection dependant on the tumour burden?

2) Can those animals that have previously received secreting or non-secreting cells, which then grew slowly or did not grow at all, transfer immune memory to a naive animal and therefore protect against a subsequent tumour challenge? Is this protection again dependant on the original tumour burden?

3) Are the cytokine-secreting cell lines able to slow or regress growth of an already established tumour? Furthermore, are there any additive or antagonistic interactions of the cytokine secreting cells when administered in various combinations?

Furthermore, it proposed to examine the possible effector mechanisms involved in the anti-tumour responses of each of the cytokine-secreting cells for each of the above studies.

4.METHODS: DNA ANALYSIS OF THE FS-29 CELL-LINE

4.1 Introduction

The FS-29 cells were a gift from Dr. M. Collins (Institute of Cancer Research, London, U.K.) and their previous use experimentally has been discussed in detail in section 3.11. In order to use these cell-lines for further animal experiments, they had to be cultured and passaged in the appropriate medium to produce adequate stocks that could be harvested for in vivo experiments. This process is detailed in section 4.2.

Before the cells could be used for animal experiments, it was important to check that there was no cross-contamination of the cell lines. Therefore, DNA analysis of each cell line was carried out to check that there was only one cytokine gene present in each cell line or, in the case of the control cells, that there was no cytokine DNA insert present. DNA was isolated from each of the cell lines and amplified using the technique of polymerase chain reaction (PCR). The products were then run on an agarose gel in order to detect the possible presence of the genes for the cytokines IL-2, IL-4 and IFN-γ in each cell-line. The methods of DNA extraction and amplification are detailed in sections 4.3 and 4.4, and the results from these techniques are described in sections 4.5 and 4.6.

4.2 Culture of the FS-29 Cell Lines

The FS-29 cell lines were continually cultured and passaged throughout the duration of the animal experiments in Dulbecco's modified eagles medium (DMEM). This also contained penicillin-streptomycin (pen:100 units/ml, strep:0.1mg/ml), L-glutamine (2mM) and 10% v/v foetal calf serum (All from Biological Industries Ltd. Cumbernauld, U.K.). The cells were cultured in an incubator at 37°C with 5% CO_2 and harvested by firstly removing the culture medium and then washing in phosphate buffered saline (PBS, pH7.4). The cells were dissociated from the culture flask by the addition of 0.02% EDTA in PBS under incubation at 37°C for 15 minutes. In order to obtain a cell pellet, the cell suspension was centrifuged at 350g for 10 minutes (Jouan

Ltd., Ilkeston., U.K.). The supernatant was decanted and the cells were washed in PBS for 10 minutes in a centrifuge at 350g.

When the cells were required for animal experiments they were counted using an "improved Neubauer" haemocytometer (Sigma Chemical Co. Dorset, U.K.) and the cell density was adjusted by the addition of PBS.

4.3 Isolation of Genomic DNA

A sample of each of the cell lines was harvested using the method described in section 4.2. The cell pellet obtained was resuspended in 500µl of PBS and stored at -20°C until required. The previously-isolated cells were allowed to thaw and sodium acetate was added to give a final concentration of 0.3M in a final volume of 1ml and the solutions were mixed gently. 150µl of a 10% solution of sodium dodecyl sulphate (Sigma Chemical Company, Dorset U.K.) was added to burst open the cells. 150µl of a 1mg/ml solution of proteinase-K (Stratagene, Cambridge, U.K.) and 200µl of sterile water were then added to digest the excess protein. The solutions were mixed gently and incubated at 37°C for 48 hours.

After incubation, the resultant viscous solution was split between 2 x 1.5ml microcentrifuge tubes and an equal volume of Tris/EDTA-saturated phenol:chloroform was added (pH8) to extract the protein. The tubes were vortexed briefly and the aqueous and solvent layers were separated by centrifugation at 13,500rpm for 10 minutes in a microcentrifuge (Scotlab Ltd. Coatbridge, U.K.). The upper layer containing the genomic DNA was removed by pipetting and placed into a new microcentrifuge tube and an equal volume of Tris/EDTA saturated phenol:chloroform added. The tubes were vortexed briefly and the aqueous and solvent layers were separated by centrifugation in a microcentrifuge at 13,500rpm for 10mins. The upper layer containing the genomic DNA was removed by pipetting and placed into a new microcentrifuge tube and an equal volume of chloroform was

added. The tubes were vortexed briefly and the aqueous and solvent layers were again separated by centrifugation as previously carried out. The upper layer containing the genomic DNA was removed by pipetting and placed into a new microcentrifuge tube and a 1/10th volume of 5M ammonium acetate (pH 8) and 1ml of ice-cold ethanol were added to precipitate the DNA. Genomic DNA was removed from the mixture by spooling onto sealed glass pasteur pipettes and allowed to dry for 2-3 hours.The resultant DNA pellet was washed firstly in ethanol then sterile water and left to dissolve in fresh sterile water.

4.4 PCR of the DNA Samples

PCR of the DNA samples was carried out according to the method described by Sambrook and co-workers (1989). Primers for this experiment comprised; mouse IL-2 which has an expected product size of 451 b.p., mouse IL-4 with an expected product size of 279 b.p., and mouse IFN-γ which has an expected product size of 405 b.p (Stratagene, Cambridge, U.K.). The primers were reconstituted in 100µl of 5mM tris, 0.1m EDTA to give a working stock of 25µM. For PCR the primers were used at a final concentration of 1µM.

For each PCR reaction the following mix was prepared which was added to each reaction; 1 unit of Taq polymerase - (Advanced Biotechnologies, Surrey, U.K.), 5µl of 10 x buffer (Advanced Biotechnologies, Surrey, U.K.), 5µl of 10 x dNTP's (Pharmacia Biotech. Ltd., St. Alban's, U.K.), 1.5mM magnesium chloride (Advanced Biotechnologies, Surrey, U.K.) and 2µl of target DNA. This mixture was made up to 50µl using sterile water, and 2 drops of mineral oil were added to each tube to prevent evaporation of the solution.

The PCR reaction was carried out in a thermoblock (Biometra Ltd., Kent, U.K.) under the following conditions; 5 minute denaturation at 94^{o}C, 5 minute annealing at 60^{o}C,

35 cycles of 1.5 minutes at 72°C, 45 seconds at 94°C, 45 seconds at 60°C, and a final extension of 10 minutes at 72°C.

Following PCR, 10µl of orange G (30% v/v solution of glycerol and 0.2mg/ml of orange G - Sigma Chemical Co., Dorset, U.K.) was added to give weight to each sample and 10µl of the final sample was added to each well. The samples were run along a mini-gel rig (Pharmacia Biotech. Ltd., St. Alban's, U.K.) 70mA for 1 hour on a 2% agarose gel with 1 x TAE buffer (0.04M tris acetate, 0.001M EDTA, pH 8). 10µl of ethidium bromide was added to stain the DNA. In this case the ladder used was φX174 RFDNA/Hae III fragments which is suitable for sizing linear double-stranded DNA from 72-1353 bp (Gibco BRL., Paisley, U.K.). The gel was developed using ethidium bromide and examined under UV light and photographed.

4.5 Results

Table 4.1 and figure 4.1 demonstrate the results from the products of the PCR reactions. Gel 1, lane 1 contained the marker which was used to estimate the size of the DNA bands. Lane 2 contained DNA from untransduced parental FS-29 cells and the primer for IL-2, there was no band present in this lane. The primer for IL-2 and and the DNA extracted from the control cells was present in lane 3 which also remained blank. Lane 4 contained the DNA from the IL-2 secreting cells and the IL-2 primer. There was a band present in this lane which corresponded to the size of the IL-2 insert. No bands were present in lane 5 which contained the IL-2 primer and the DNA extracted from the IL-4 cells or in lane 6 which contained the IL-2 primer and DNA from the IFN-γ cells. Lanes 7 and 8 were also blank. Therefore it can be concluded that only the IL-2 cell-line contained the IL-2 insert.

Gel 1, lane 9 contained the IL-4 primer and untransduced control FS-29 cells. This lane remained blank as did lane 10 which contained the IL-4 primer and DNA extracted from the control FS-29 cells. Lane 11 contained the IL-4 primer and DNA extracted from the IL-2-secreting cells. This lane did not contain any bands. However, a band corresponding to the size of the IL-4 gene was present in gel 2, lane 2. This lane contained the IL-4 primer and DNA from the IL-4-secreting cells. No bands were present in gel 2, lane 3 which contained the IL-4 primer and DNA extracted from the IFN-γ-secreting cells. Lane 4 only contained the primer and no DNA sample, and there was no band in this lane. Gel 2, lane 5 was blank. From these results it can be concluded that only the IL-4-secreting cells contained the gene for IL-4.

Gel 2 , lanes 6-11 contained the primer for IFN-γ along with DNA extracted from each of the cell-lines. No band was present when the IFN-γ primer was present with DNA extracted from the untransduced parental cells or the FS-29 control cells (lanes 6 and 7). However, bands were present in gel 2 lanes 8 and 9 which contained the IFN-γ primer but DNA extracted from the IL-2 and IL-4-secreting cells. The bands were

close to the size of the IFN-γ gene, so it would seem that these lanes may have been contaminated. Another band was present in gel 2, lane 10 which contained the IFN-γ primer and DNA extracted from the IFN-γ-secreting cells. This band corresponded to the size of the IFN-γ gene. Lanes 11 only contained the IFN-γ primer and no DNA sample, and there was no band in this lane. Lane 12 was blank and there was also no band in this lane. From these results it can be concluded that the IFN-γ-secreting cell-line contained the gene for IFN-γ.

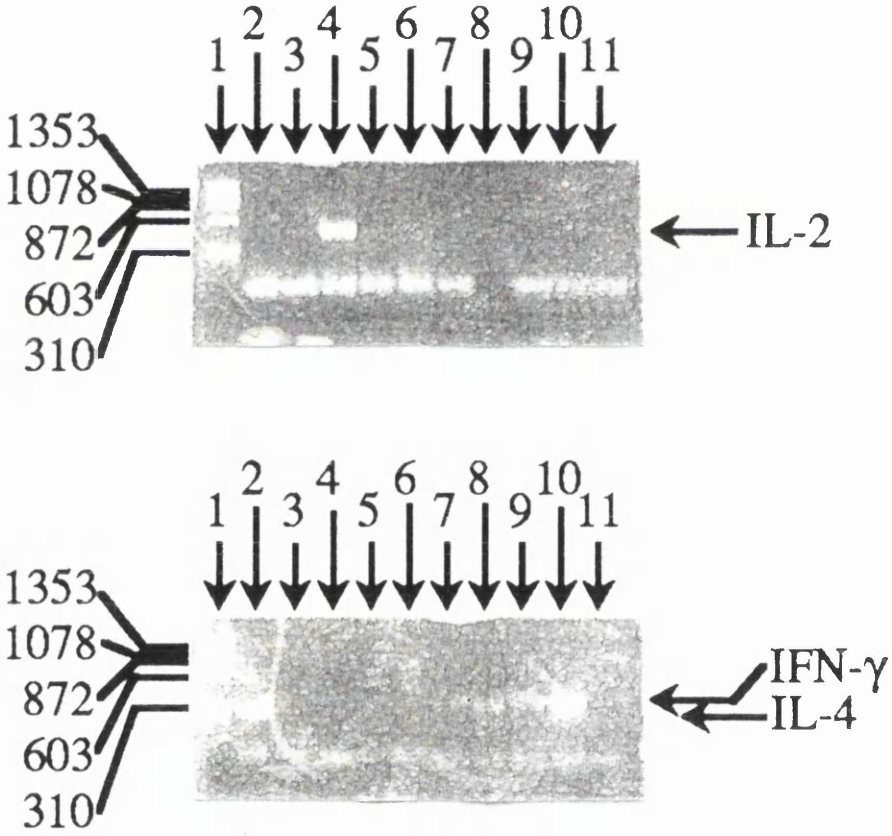
4.6 Discussion

Examination for the presence of the cytokine genes using DNA electrophoresis indicated that, none of the cell-lines were contaminated with the IL-2 and IL-4-secreting cells. However, there was evidence that both the IL-2 and IL-4 cell-lines appeared to be contaminated with DNA for IFN-γ. From the results of the gel the non-transfected cell-line did not appear to contain the genes for either IL-2, IL-4 or IFN-γ. Furthermore, it is unlikely that DNA for IFN-γ could be inserted in to the parental cells during their transfection with IL-2 and IL-4 (Viewig and Gilboa, 1995). Therefore, it would appear unlikely that the contaminated bands were due to IFN-γ DNA from the IL-2 and IL-4-secreting cells. It may be that these bands were a result of spillage from the IFN-γ cell lane.

Clearly, the fact that these cell-lines contain the gene for the relevant cytokine does not establish that these cells are actually secreting cytokines. In order to do this the presence of mRNA expression could be determined as an indicator of whether the cytokine DNA was being translated. This method was attempted in the lab, but was abandoned due to a number of technical difficulties and the lack of time available. In order to further establish that the cell-lines were secreting cytokines and to determine the level of the cytokine secretion it would be possible to use bioassays or ELISAs. However, these techniques were not available for use within this project. Furthermore there are problems associated with the use of bioassays (Chirmule et al., 1991) Therefore, it was assumed from the previous work carried out by Patel and co-workers (1993a, and personal communication from Dr. M. Collins) that the level of cytokine expression remained relatively constant for the cell-lines used in the present work. Indeed Patel and co-workers (1993a and personal communication) have defined the amounts of IL-2, IL-4 and IFN-γ expressed by these cell lines as 25000U/10^6cells/48hrs, 74500U/10^6cells/48hrs and 50U/10^6cells/48hrs respectively. There was no cytokine secretion by the parental cell-line and the transfected cell-lines only secreted one type of cytokine (1993a and personal communication).

Table 4.1

Gel 1		Gel 2	
Lane	1 - Marker.	Lane	1 - Marker.
	2 - IL-2 + parental cells.		2 - IL-4 + IL-4 cells.
	3 - IL-2 + control cells.		3 - IL-4 + IFN-γ cells.
	4 - IL-2 + IL-2 cells.		4 - IL-4 + blank.
	5 - IL-2 + IL-4 cells.		5 - Blank.
	6 - IL-2 + IFN-γ cells.		6 - IFN-γ + parental cells
	7 - IL-2 + blank.		7 - IFN-γ + control cells.
	8 - Blank.		8 - IFN-γ + IL-2 cells.
	9 - IL-4 + parental cells.		9 - IFN-γ + IL-4 cells.
	10 - IL-4 + control cells.		10 - IFN-γ + IFN-γ cells.
	11 - IL-4 + IL-2 cells.		11 - IFN-γ + blank.
	12 - Blank.		12 - Blank.


1 2 3 4 5 6 7 8 9 10 11
1353
1078
872
603
310
IL-2
1 2 3 4 5 6 7 8 9 10 11
1353
1078
872
603
310
IFN-γ
IL-4

5: METHODS: IMMUNOHISTOLOGICAL ANALYSIS OF EXCISED TUMOURS

5.1 Introduction

An indication of the extent of an immune response to a tumour is the nature of the lymphocytic infiltration. Patel and co-workers (1993a) examined the nature of the cellular infiltration into the tumours that formed during their studies (discussed in section 3.11). The presence of a lymphocytic infiltration has implications for the possible effector mechanisms that may be involved in tumour rejection, since rejection in many tumour models appears to be dependant on a lymphocytic infiltration (discussed in section 2.3). The tumours excised from the animal studies in this present work were analysed in order to examine the composition of the T and B-cell infiltration between the treatment groups. Structural differences in the tumours due to differing cytokine expression was also examined by routine histological staining.

5.2 Preparation of Cryostat Sections

All reagents were from Sigma Chemical Co. (Dorset, U.K.) unless otherwise stated. Glass slides were treated to increase their adhesiveness to sections by coating them with 3-aminopropyl-triethoxy-silane. The coated slides were prepared by firstly washing in acetone for five minutes followed by another five minutes in a 2% 3-aminopropyl-triethoxy-silane in acetone. The slides were thoroughly washed in running water for 30 minutes and allowed to dry. The tumour samples were removed from their storage in liquid nitrogen and 5μM tumour sections were cut in a cryostat (Bright Instrument Company Ltd., Huntingdon, England.) at -20^{o}C and thawed onto the coated slides. The tumour samples were returned to liquid nitrogen storage as soon as sections were cut to minimise the risk of tissue damage. The sections were air-dried for thirty minutes, before being wrapped in tin-foil and stored at -70^{o}C until required. Prior to immunohistochemistry, the wrapped slides were brought to room temperature before opening to prevent condensation forming on the sections. The sections were circled with a water impermeable pen in order to contain the solutions used during

immunohistochemical staining and prevent leakage across the slide (Dako Ltd., High Wycombe, U.K.).

5.3 Non-Specific Staining

Preliminary experiments were performed to examine the extent of non-specific binding in the tumour samples. Sections were processed without primary or secondary reagents and just with the chromogen diaminobenzidine (DAB). In this way the extent of endogenous peroxidase activity could be assessed. In a proportion of cases, endogenous peroxidase activity was present in single cells within the sections. This was considered to be present in a significant number of cases and was of sufficient intensity to make the distinction between specifically stained lymphocytes and cells with endogenous peroxidase activity difficult. The following methods, which have been established for particular tissues, were used in an attempt to suppress the activity in these tissues (Polak and Van Noorden, 1986).

a) 1% hydrogen peroxide in tris-buffered saline (TBS, 0.01M Tris(hydroxymethyl)methylamine, pH 7.6 with HCL, 0.14M Sodium Chloride) for 30 minutes.

b) 10% hydrogen peroxide in TBS. for 10 minutes.

c) 0.1% phenylhydrazine in TBS. for 1 hour at 37^{o}C.

d) 1% hydrogen peroxide in methanol for 30 minutes.

Unfortunately none of the methods was sufficiently successful, either having no effect or being too strong a treatment resulting in removal of the section from the slide. An alternative solution to this problem was devised by staining the endogenous peroxidase black at the beginning of the procedure. In this way, the black cells with endogenous peroxidase activity could readily be distinguished from the specific antibody labelled cells which were ultimately stained red. This was done using a nickel-DAB solution (0.025% diaminobenzidine, 0.07 % $NiCl_2.7H_2O$, 0.01% H_2O_2 in PBS (0.01M sodium phosphate, 0.14M sodium chloride, pH 7.6)). DAB forms a

brown precipitate on contact with peroxidase when catalysed by hydrogen peroxide. However, in the presence of nickel ions, this reaction results in a black precipitate. This phenomenon was used to differentiate between the endogenous peroxidase and the positively stained cells. This protocol is a modification of a double immunohistochemical staining technique developed by Hsu and Soban (1982).

Further preliminary studies carried out suggested that the tumour sections may also contain endogenous biotin-like activity. This was distinct from the endogenous peroxidase and was apparent in sections incubated with the streptavidin conjugate alone in the absence of primary antibodies indicating a specific binding of the streptavidin to the tissues. Biotin is a vitamin and coenzyme present in many tissues, and it binds to avidin and streptavidin with high affinity. This can result as non-specific staining when using a streptavidin-bound enzyme complex such as streptavidin horse-radish peroxidase (Wood and Warnke, 1981). This binding activity is quite pronounced when using cryostat sections, but can be overcome by adding avidin to block the endogenous biotin. As avidin is tetravalent for biotin, it is then necessary to block the unoccupied sites with free biotin which would otherwise be able to bind the biotinylated antibodies used in the immunohistochemical procedures.

Methods have been described for the blocking of endogenous biotin using purified avidin. However, purified avidin may be replaced by egg white which is a rich source of avidin. Therefore, before application of the primary antibodies the sections were incubated with a 50% solution of egg white in PBS (a rich source of avidin) for 60 minutes followed by a solution of 0.02% free biotin included in the serum blocking solution (Reeves et al., 1994).

A serum blocking solution was employed to reduce any further non-specific binding. 25% non-immune rabbit serum and 25% normal human serum with 10% of each

serum type included in the primary antibodies and streptavidin peroxidase complex resulted in sections with low non-specific staining.

5.4 Primary Antibodies

The availability of monoclonal antibodies made it possible to stain for $CD3^+$ T-cells and its subsets of $CD4^+$ and $CD8^+$ T-cells, and also for B-cells. All antibodies were supplied conjugated with biotin (Cambridge Bioscience, Cambridge, U.K.). The optimum concentration of primary antibodies (the highest dilution possible without reduction of the signal) were pre-determined.

a) Hamster anti-mouse CD3ε (IgG) - It reacts with the 25kDε chain of the receptor-associated CD3 complex expressed on all T-cells. It was used at a concentration of 0.01mg/ml.

b) Rat anti-mouse CD4 (L3T4) (IgG2a) - It reacts with the CD4 (L3T4) antigen found on a subpopulation of T cells. It was used at a concentration of 0.02mg/ml.

c) Rat anti-mouse CD8α (Ly-2) (IgG2a). This antibody is specific for the α chain of the CD8 differentiation antigen (Ly-2 or Lyt-2) found on most thymocytes and a subpopulation of mature T cells (T suppressor/cytotoxic cells). It was used at a concentration of 0.02mg/ml.

d) Rat anti-mouse CD45R/B220 (IgG2a). It reacts with pre-B lymphocytes and lytically-active subsets of lymphokine-activated killer cells expressing the B220/CD45 phenotype . It was used at a concentration of 0.02mg/ml.

e) Isotype matched control antibodies were used in place of these primary antibodies at similar concentrations as negative controls in parallel sections.

5.5 Immunohistochemical Labelling of Sections

The sections were fixed in absolute acetone for 15 minutes and washed in PBS before the endogenous peroxidase activity was revealed with a 10 minute incubation in nickel-DAB (Section 5.3). After 3 x 5 minute washes in PBS, the endogenous biotin was suppressed as described in section 5.3. The serum blocking solution (containing free biotin) was applied for 10 minutes and this was aspirated from the sections and replaced with the primary or control antibody solutions (section 5.4). After a 60 minute incubation, the excess antibody was rinsed from the slide followed by 3 x 5 minute washes in PBS. The sections were then incubated with streptavidin horseradish peroxidase (Dako Ltd., High Wycome, U.K.) for 30 minutes followed by 3 x 5 minute washes in PBS. The peroxidase signal was developed using 3-amino-9-ethyl carbazole (AEC) as the substrate. An AEC tablet (20mg) was dissolved in 2.5ml of dimethylformamide. To this was added 47.5ml of 50mM acetate buffer (pH5) and 25µl of fresh 30% hydrogen peroxide. The slides were incubated in AEC for 5 minutes then, washed in PBS. for 5 minutes, followed by washing in running tap water for 5 minutes. They were counterstained in Harris's haematoxylin (Sigma Chemical Co., Dorset, U.K.) and dehydrated in alcohol. As AEC is alcohol soluble, an aqueous mounting medium was used. This was carried out by baking the sections with a thin layer of Crystal Mount (Biogenesis Ltd., Poole, U.K.) at 56^{o}C for 20 minutes. The sections were then mounted with a synthetic mounting medium (DPX, BDH, Glasgow, U.K.). Negative control parallel sections were processed for each specimen using control antibody in the place of the primary antibody (section 5.4). A positive control section of mouse spleen (known to contain large numbers of T and B-cells) was processed with each batch of samples in order to ensure that staining was consistant throughout the studies.

5.6 Results and Discussion

A method was developed that allowed immunohistological analysis of the lymphocytic infiltration to be carried out on the tumours excised from each animal. Each tumour sample was stained for $CD3^+$, $CD4^+$, $CD8^+$ T-cells and B-cells. The $CD3^+$ T-cell staining was unsatisfactory since this antibody cross-reacted with the matrix tissue of the tumour resulting in large amounts of non-specific staining (Figure 5.1). A number of attempts to remove this non-specific staining such as biotin removal, reduction in antibody concentration, and addition of blocking serum were unsuccessful. Nevertheless, the extent of the T-cell response could still be obtained through the $CD4^+$ and $CD8^+$ T-cell staining. Conditions were established such that staining using the $CD4^+$ and $CD8^+$ T-cell antibodies and also the B-cell antibody were easily visualised in positive cells therefore allowing the samples to be scored.

Many of the studies which have examined the lymhocytic infiltration into the tumour nodules following cytokine or cytokine gene therapy, have not used a scoring system to determine the extent of the lymphocytic infiltration, but have qualitatively determined whether a particular cell type has infiltrated the tumour nodule (Tepper et al., 1989; Golumbek et al., 1991; Hock et al., 1991; Gallagher and Zaloom, 1992; Gastl et al., 1992; Tahara et al., 1994). Further studies have denoted the presence of a lymphocytic infiltration as being a positive and the absence of any lymphocytic infiltration as being negative (Hock et al., 1993; Saito et al., 1994). Therefore, in order to give a better indication as to the extent of the lymphocytic infiltration into the tumours that formed during the course of the present studies; the sections were coded and scored blind using a semi-quantitative four-point scoring system. This is a modification of the five-point scoring method used by Patel and colleagues (1993a) and Karp and co-workers (1993). However, the scorer did not observe a firm distinction between the ++ and +++ points in Patel's scoring method therefore, the five-point method was reduced to a four-point scoring system in the present studies. The four-point scoring system, used to analyse the tumours that formed from the

following studies, was defined as follows; tumours which had no positively stained cells were given a negative score (-) as demonstrated on figure 5.2. Sections that had a poor infiltration of positively stained cells were given a + score (Figure 5.3). Tumours that had a moderate infiltration of positive cells were assigned a ++ score (Figure 5.4). Those tumours that had a strong infiltration of positive cells were given a +++ score as demonstrated on figure 5.5. The reproducibility of immunohistochemistry scoring can be questioned because of its qualitative nature, and has been rarely addressed in immunohistochemistry studies. In the present work, the reproducibility of the scoring system was assessed by the blind scoring of 30 slides on two separate occasions at least six months apart. There was agreement in scoring in 76% of the slides and those slides that were discordant from the original score differed by only one point on the four-point scoring system. Furthermore, none of the slides that scored negative on the first occasion scored positive the second time they were scored, and similarly, none of the positively scored slides were scored negative on the second scoring occasion. The discordant scoring results may have been due in part to the positive staining fading with time. From the above, the scoring system used was considered to give a reproducible, semi-quantitative measure of the degree of lymphocytic infiltration.

The establishment of the above staining and scoring methods allowed the examination of the nature of the lymphocytic infiltration and morphology of the tumours in the following animal experiments (see chapters 6, 7, and 8).

Table 5.1 - Description of figures 5.1 - 5.5.

Figure	Tumour Type	Stain	Score
5.1	IL-4 Cells.	$CD3^+$ T-cells	–
5.2	10^5 IL-4	T-cells	–
5.3	10^5 IL-2	T-cells	+
5.4	10^5 IL-2	T-cells	++
5.5	10^5 IL-2	T-cells	+++

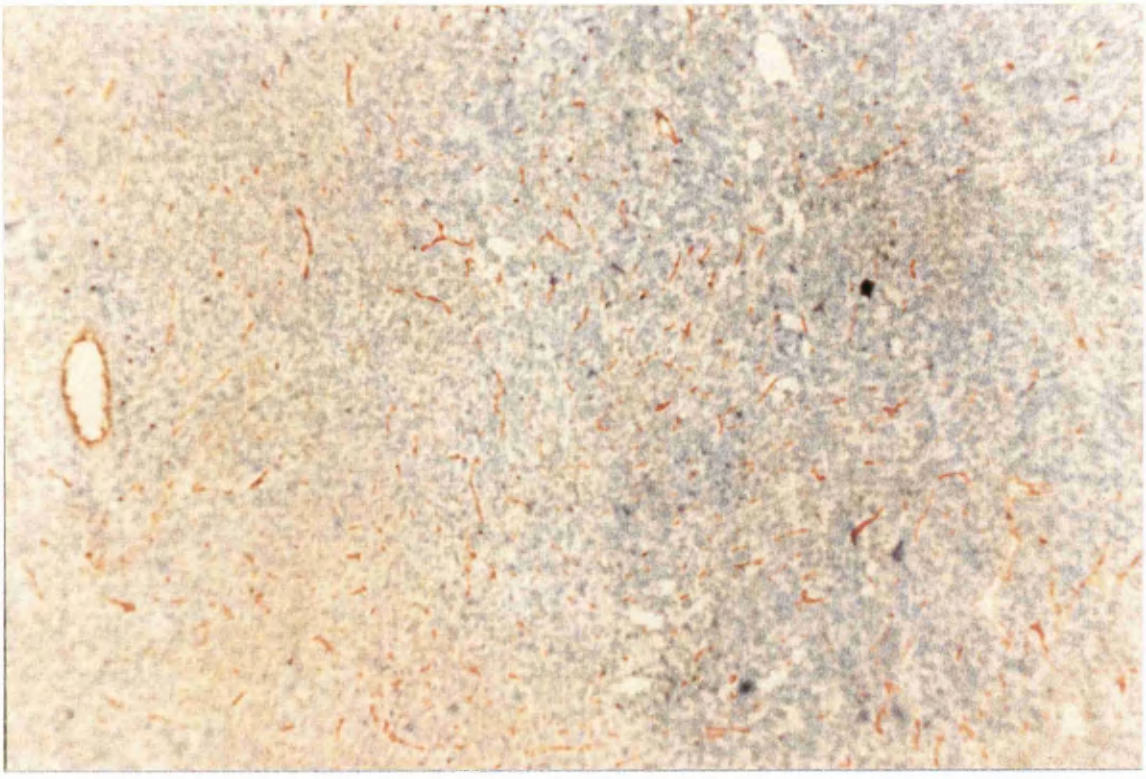


Figure 5.1 - 5µm tumour section from an animal that had received IL-4-secreting FS-29 cells. This section has been stained for CD3+ T-cells using AEC (red) and counterstained with Harris's haematoxylin. Staining using this antibody was unsuccessful, as can be seen from the large amounts of non-specific staining. Attempts to remove the background staining was unsuccessful.
x10 magnification.

Figure 5.2 - 5μm tumour section from an animal that received 10^5 IL-2-secreting FS-29 cells. This section has been stained for T-cells using AEC (red) as the substrate and Harris's haematoxylin as the counterstain. Nickel-DAB has stained the endogenous peroxidase black as indicated by the arrow. There are no positive cells present in this section and it has therefore been given a negative score (-). x10 magnification.

Figure 5.3 - 5μm tumour section taken from an animal that received 10^5 IL-2-secreting FS-29 cells. This section has been stained for T-cells using AEC (red) as the substrate and Harris's haematoxylin as the counterstain. Nickel-DAB has been used to stain the peroxidase-positive cells black. There is a poor cellular infiltration into this tumour sample and it has therefore been given a + score. x 10 magnification.

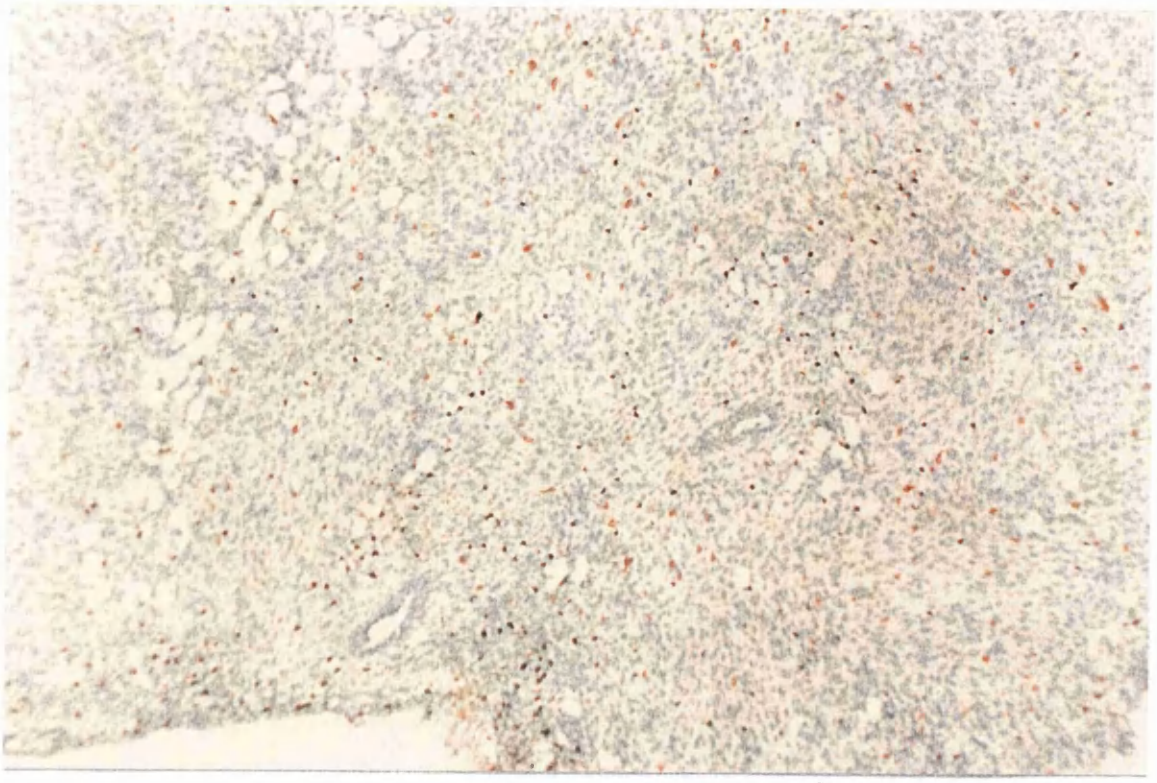


Figure 5.4 - 5μm tumour section taken from an animal that had received 10^5 IL-2-secreting FS-29 cells. This section has been stained for T-cells (red). There is a moderate cellular infiltration into this sample and it has therefore been given a ++ score. x 10 magnification.

Figure 5.5 - 5μm tumour section taken from an animal that received 10^5 IL-2-secreting FS-29 cells. This section has been stained for T-cells (red). There is a strong cellular infiltration into this tumour sample which is therefore given a +++ score. x10 magnification.

6: TUMOUR GROWTH AND SURVIVAL IN GENE CELL THERAPY

6.1 Introduction

Several authors have reported that tumour cells, which have been genetically altered to secrete cytokines, may prevent tumour growth in an number of animal models (Tepper et al., 1989, Gansbacher et al., 1990a, Russell et al., 1991, Hock et al.,1993). It has also been reported that tumour rejection at one site may induce systemic protection in that growth of a second tumour may be prevented (Dranoff et al., 1993, Saito et al., 1994). Furthermore, studies indicate that tumour cells altered to secrete cytokines may also reduce the number of metastases (Porgador et al., 1993a).

Most studies that have been conducted to examine the extent of systemic protection in animals involve firstly, innoculating the animal with tumour cells and secondly, administering a subsequent tumour challenge only if the original tumour cells were rejected. However, it may be that at the time the primary tumour has been detected, dissemination may have already taken place. Therefore, such protocols do not entirely mimic the natural process of cancer dissemination. In order to take this confounding factor into account, one approach is to give a two-site injection of parental or cytokine-secreting tumour cells which would better mimic the metastatic process. The extent of the systemic protection induced by the different cytokine-secreting FS-29 cells could therefore be examined. Patel and co-workers (1993b) reported that neither the IL-2 or IL-4-secreting cells were capable of slowing growth of an equal number of admixed non-secreting FS-29 cells. However, when the IL-2 or IL-4-secreting cells were present in a tenfold excess, there was an initial delay in the growth of the unmodified cells, but rapidly growing tumours were eventually formed in all animals. When an equal number of parental cells were admixed with IFN-γ-secreting cells, there appeared to be significant slowing of tumour growth. Furthermore, when the IFN-γ secreting cells were present in a tenfold excess, tumour rejection appeared to be induced in two of the seven animals. This would indicate that the original tumour burden is important in determining the extent of the anti-tumour response.

The aim of the present study was to examine the extent to which cytokine-secreting FS-29 cells were capable of inducing systemic protection and so reduce the metastatic process in mice. Furthermore, in the present study the animals received two different amounts of cytokine-secreting or control cells which would indicate the effect of tumour burden on the ability to evoke a systemic anti-tumour response. Histological examination of any tumour nodules formed during the present study was also carried out to give an indication as to the effector mechanisms that may implicated in a systemic anti-tumour response.

6.2 Materials and Methods

Cells were cultured and harvested as described in section 4.2.The cell density was adjusted to give stocks of 10^7/ml and 10^6/ml in PBS. Thirty-one female 8-week old immunocompetent C57BL/6J mice (Harlan U.K.) were randomized into 8 treatment groups (7 groups of 4, 1 group of 3). Mice were given at least a two-day settling in time before going on procedure. Four of the treatment groups received 10^5 FS-29 cytokine-secreting or control cells and the other four groups received 10^6 control or cytokine-secreting FS-29 cells as a 100µl injection through a 26-gauge needle into both flanks (illustrated in figure 6.1).

Animals were checked daily and tumour growth was measured by palpation between the thumb and first finger. When tumours reached approximately 1cm in diameter, the animals were killed by neck dislocation. The excised tumours were frozen in liquid nitrogen immediately and stored in liquid nitrogen until required for histological analysis. Immunohistochemical analysis of the individual tumour sections was carried out as detailed in chapter 5. After 57 days the experiment was terminated and any surviving animals were examined by autopsy for signs of tumour growth.

Statistics: Data are presented as the median of the tumour diameter and where appropriate, comparisons of data from different groups were analysed using the Kruskall-Wallis test, which is a non-parametric equivalent of a one-way analysis of variance (Minitab Inc., Pasadena, CA, U.S.A.). When the Kruskall-Wallis test showed a significant overall difference between groups, the groups with cytokine-secreting tumours were compared with the control group on a pair-wise basis using the Mann-Whitney test. Significance was assessed at the 5 percent. level.

6.3 Results

Tumour growth and survival: The animals injected with 10^5 tumour cells demonstrated varying rates of tumour growth and survival (Table 6.1). Of the animals injected with 10^5 control cells, 2 were killed on day 24 and the remaining two were still tumour-free at the end of the experiment on day 57. In comparison with the control cells, the animals with IL-2-secreting cells were killed on days 21, 24 and 29. None of these animals survived until the end of the experiment.

Of the four animals injected with the IL-4-secreting cells, one was killed after 37 days, another 2 after 38 days, with one animal surviving until the end of the experiment at day 57. However, this surviving animal did have evidence of a tumour at autopsy. Of the four animals injected with IFN-γ-secreting cells, one was killed on day 38 however, the remaining 3 animals survived until the end of the experiment. However, one of the remaining three animals did have a substantial tumour when examined at autopsy.

Tumour growth rates and survival for the animals that received 10^6 tumour cells is documented in table 6.2. Of the four animals injected with 10^6 control cells, all developed tumours and were killed on day 23. Of the animals injected with 10^6 IL-2-secreting cells, two mice were killed on day 22, one on day 23 and the last on day 24.

Similarly the animals injected with 10^6 IL-4-secreting were killed on days 24, 38, and the last 2 on day 41. In contrast, of the four animals injected with 10^6 IFN-γ-secreting cells, all survived until day 57 when the experiment was terminated. Two of these animals did however have some evidence of ascites. The remaining two appeared to be completely tumour-free.

Statistical analysis of the groups using the Kruskall-Wallis test indicated that there was no significant difference between the treatment groups for the number of days of

survival for the animals that received a 10^5 cell inoculum ($p = 0.107$). However the groups that received a 10^6 cell inoculum showed a significant statistical difference between groups for the number of days of survival ($p = 0.006$). Further analysis comparing individual treatment groups with control for the animals that had received a 10^6 cell inoculum was carried out using a Mann-Whitney U-test. Results showed a significant difference between the IL-4 group and control group ($p = 0.026$) and the IFN-γ group and control group ($p = 0.023$). However, there was no significant difference between the IL-2 group and control ($p = 0.757$) for the number of days of survival.

Immunohistological analysis: This was carried out on the tumours excised from each animal (Table 6.3). Each sample was stained for $CD4^+$and $CD8^+$ T-cells and B-cells. The resultant sections were scored using a four-point system (see section 5.6)

The tumours excised from the 10^5 control animals scored a strong $CD4^+$ T-cell response, moderate $CD8^+$ T-cell response and a poor B-cell response. The tumours from the 10^6 control animals scored poor $CD4^+$and $CD8^+$ T-cell and B-cell responses (Figures 6.2, 6.4 and 6.6). Tumours from the animals that had received 10^5 or 10^6 IL-2-secreting cells scored strong $CD4^+$ and $CD8^+$ T-cell responses with a poor B-cell response (Figures 6.3, 6.5 and 6.7).

Of the IL-4-secreting tumours 7 scored a moderate $CD4^+$ T-cell response and one tumour sample scored a poor $CD4^+$ T-cell response. The $CD8^+$ T-cell and the B-cell responses were poor in one half of the tumours and negative in the other. Although the degree of infiltration was low, the tumour structure was also very poor. In comparison to the other cell-lines, where the tumour had a definite structure (Figure 6.8), there were large areas of tissue necrosis and some holes in the IL-4 tumours (Figure 6.9). It was also noted that these tumours contained large amounts of peroxidase-like activity, particularly around the periphery which was not observed in the other treatment

groups (Figures 6.10 and 6.11). As mentioned previously, the peroxidase was stained black using nickel-DAB and could therefore be easily identified (Section 5.2). Peroxidase may be released from macrophages therefore, a large amount may be an indication of a macrophage infiltration.

The tumours that formed from the IFN-γ-secreting cells were also stained. One sample scored a moderate response for $CD4^+$ and $CD8^+$ T-cells with a negative B-cell response. The second sample scored a poor $CD4^+$ T-cell response and negative $CD8^+$ T-cell and B-cell responses.

6.4 Discussion

The aim of this study was to assess the growth rates and survival of cytokine-secreting tumour cells in vivo using a two-site injection technique which has not been previously reported. The small number of animals examined in this study limited the power of the statistical analysis of the results and therefore most of the observations of the present study are of a qualitative nature. Two of the animals that received 10^5 control FS-29 cells failed to develop tumours, while the other two were killed at 24 days. This divergance in results for the control group further limited the possibility of detecting differences associated with the cytokine-secreting cells in the 10^5 cell injection group.

When 10^5 or 10^6 IL-2-secreting FS-29 cells were injected into both flanks of C57/BL6 mice, tumour nodules developed in all animals, which did not regress. There did not appear to be any systemic effect from the two-site injection with there being similar numbers of tumours formed from the IL-2-secreting cells as from the non-secreting control FS-29 cells. Survival time of these animals which received 10^5 IL-2-secreting cells was poorer than the control cells with the 10^5 injection size having shorter survival rates than the 10^5 control cell injection. Therefore, from this study, it appeared that the IL-2-secreting cells did not have reduced tumourigenicity. This was unexpected since Patel and co-workers (1993a) reported that when 10^6 IL-2-secreting FS-29 cells were injected into C57/BL6 mice, the initial tumour nodules which formed completely regressed in most animals. However Patel's study was only with one injection site and not with 2 sites as used in the present study. Therefore, it may be that the tumour load was too great for the animal to mount a significant enough anti-tumour response.

It is of interest that the IL-2 secreting cells in the present study afforded no protection from tumour growth since this is contrary to the results observed with many other IL-2-secreting cell-lines. For example, CMS-5 cells engineered to secrete IL-2 lost their

tumourigenicity and a long-lasting protective response against a subsequent challenge with parental CMS-5 cells was induced (Gansbacher et al., 1990a). Decreased tumourigenicity with IL-2 transfectants has also been observed with a variety of other cell-lines (Russell et al., 1991, Cavallo et al., 1992, Gastl et al., 1992 and Saito et al., 1994), which was usually accompanied by a characteristic T-cell infiltration. In general, these experiments were performed with weakly immunogenic tumours for which IL-2 markedly suppressed tumour growth in vivo in correlation with the increased amount of the secreted IL-2. For example, when IL-2 was retrovirally transduced into a highly malignant and poorly immunogenic 3LL-D122 cell clone, both high and low IL-2 producers showed an elimination of tumourigenicity in syngeneic immunocompetent mice. However, in the nude mice, only the high IL-2 producers showed a reduced tumourigenicity in comparison with the parental 3LL-D122 cells (Porgador et al., 1993b). A correlation between the increased amount of IL-2 produced from transduced tumour cells and the capability of tumour rejection was further demonstrated in a spontaneous adenocarcinoma cell-line (TS/A; Cavallo et al., 1992). While clones that released low amounts of IL-2 grew in 60% of syngeneic mice, clones that released large amounts of IL-2 were promptly rejected. Therefore, it may be that the IL-2-secreting cell-line used in the present study did not secrete large enough amounts of IL-2 to reduce the tumourigenicity of the cell-line. Furthermore, Patel and co-workers (1993a) also reported that tumours which arose from the IL-2-secreting FS-29 cells secreted considerably reduced or no IL-2 which was attributed to a decrease in the IL-2 encoding retroviral integrant. Therefore, if the retroviral integrant for the IL-2-secreting cells is unstable, perhaps the retroviral integrant was lost from the IL-2-secreting cells in the present study, resulting in a lack of IL-2 secretion and as a consequence, any anti-tumour response.

When the tumours that formed from the IL-2-secreting FS-29 cells were examined histologically, a strong $CD4^+$ and $CD8^+$ T-cell infiltration with a poor B-cell infiltration was observed. This is in accordance with the results from Patel and co-

workers (1993a) who reported that a pronounced lymphocytic infiltration was present within the tumours that formed from the IL-2-secreting cells the majority being $CD8^+$ T-cells with some $CD4^+$ T-cells and B-cells. There was a strong cellular infiltration into the tumours in the present study but, in the case of the two-site injection experiment performed, these effector mechanisms were not able to prevent growth of the tumours. However, it has been reported that effector cells other than T-cells are important in the rejection of IL-2-secreting tumour cells. Rat sarcoma cells which were transduced with the human IL-2 gene and secreted IL-2 following transfer, were reported to have reduced tumourigenicity and metastatic potential both in athymic and immunocompetent rats which would indicate that effector cells other than T-cells are important for rejection (Russell et al., 1992). Other studies have reported that IL-2 producing tumour cells show infiltration with T and NK lymphocyte populations, as well as neutrophils and eosinophils (Cavallo et al., 1992, Karp et al., 1993).

The survival of the animals injected with 10^5 IL-4-secreting cells into both flanks, was not significantly different from that of the control animals. However, the animals injected with 10^6 IL-4 cells had significantly longer survival times in comparison to the control group suggesting a retardation of tumour growth. Despite the prolonged survival, more animals developed 2 tumours suggesting that there was no systemic anti-tumour effect of IL-4. Patel and co-workers (1993a) also reported that FS-29 IL-4-secreting clones showed slower growth rates and reduced tumourigenicity in comparison to the control cells. A possible explanation for the IL-4-secreting cells being more effective than the IL-2-secreting cells could lie in the stability of their respective retroviral integrants. Patel and colleagues (1993a) reported that when tumours from IL-4 injected mice were explanted, their IL-4 secretion was found to be unreduced and the cells had retained the retroviral integrant encoding IL-4. The IL-2 integrant was lost more quickly from the IL-2-secreting cells than the IL-4 from the IL-4-secreting cells. This may explain why animals receiving IL-4-secreting FS-29 cells had longer survival times than those that had received the IL-2-secreting cells.

Histological analysis of tumours from IL-4 treated animals showed that lymphocytic infiltrations were not as strong as those seen in the IL-2 treated animals. Most of the IL-4 secreting tumours had a moderate $CD4^+$ T-cell response. The $CD8^+$ T-cell and the B-cell response was poor in one half of the tumours and negative in the other. This is in accordance with Patel and co-workers (1993a) who reported that tumours that formed from the IL-4-secreting cells contained a small number of $CD8^+$ and $CD4^+$ lymphocytes. Patel and co-workers (1993a) also reported that these tumours contained a characteristic macrophage and granulocyte infiltration. Although tumours from the present study were not stained for macrophages, it was found that there were large amounts of peroxidase-positive cells present, particularly around the periphery of the tumours. Peroxidase may be released from macrophages, and a large amount of peroxidase may be an indication of a heavy macrophage infiltration. In order to substantiate this, the tumours would have to be stained specifically for macrophages. This would be difficult as it was found to be impossible to remove this peroxidase, which would mask any positive staining. One alternative would be to have a pathologist examine the morphology of these cells to indicate the extent of the macrophage and granulocyte infiltration. Tepper and colleagues (1989) reported that a macrophage infiltration was present when IL-4-secreting J558L plasmacytoma cells were injected into nu/nu mice, thus indicating that macrophages and not T-cells were an important effector mechanism for preventing tumour growth. Golumbek and co-workers (1991) reported that IL-4-secreting renal cancer cells were completely rejected in immunocompetent mice. However, tumour growth was observed after two months when the cells were injected into SCID mice, indicating that T-cells were required for the absolute rejection of tumour cells even though there was the characteristic influx of macrophages and granulocytes. Treatment of tumour-bearing immunocompetent mice with anti-$CD8^+$ T-cell antibodies to eliminate $CD8^+$ T-cells, prevented the rejection of parental renal cancer cells, and elimination of $CD4^+$ T-cells had a much lesser effect, although there were some late recurrences suggesting that $CD4^+$ T-cells participated in long-term memory responses. Hock and co-workers

(1993) also reported that $CD8^+$ T-cells were required for complete long-term tumour rejection of IL-4-secreting J558L cells and that this was associated with a heavy infiltration of macrophages and some eosinophils.

In the present study, the tumours that formed in those animals that had received IL-4-secreting FS-29 cells had a very poor general structure. In comparison to the other cell-lines, which produced tumours with a definite structure, there were large areas of necrotic tissue and some holes in these IL-4-secreting tumours. This difference could be due to the fact that IL-4 induces different genes, such as lipase, in activated cytotoxic lymphocytes than IL-2 does and this difference appears to correlate with increased lytic function of the lymphocytes by IL-4 (Grusby et al., 1990). Therefore, although the IL-2-secreting cells encouraged an extensive infiltration of lymphocytes, their cytotoxic activity may not have been as powerful as that of the lymphocytes induced by IL-4-secreting cells. Other actions of IL-4 which may enable it to elicit an enhanced immune response include its ability to upregulate the expression of MHC class-II. IL-4 has been shown to induce macrophages to express MHC class-II (Stuart et al., 1988) and to activate them to kill tumour cells (Crawford et al., 1987). Loss of tumourigenicity of IL-4-secreting tumour cells has also been associated with a massive infiltration of activated macrophages and eosinophils (Tepper et al., 1989, Golumbek et al., 1991).

IL-4 has been reported to induce adhesion molecules such as VCAM on endothelial cells (Thornhill et al., 1991). Through the expression of these adhesion molecules, the endothelium becomes activated to allow binding and migration of inflammatory cells. For example, IL-4 has been shown to induce specifically the expression of the adhesion molecule VCAM-1 on endothelium (Thornhill et al., 1991). The cell surface ligand for this adhesion molecule is the leukocyte integrin VLA-4. VLA-4 is expressed on both T and B lymphocytes, monocytes and eosinophils (Walsh et al., 1991). It has been reported that VCAM-1 activation of human endothelium in vitro

promotes the adhesion of purified eosinophils (Schleimer et al., 1992). These findings suggest that an eosinophilic inflammatory infiltration induced by IL-4 may relate to the specific introduction of VCAM-1 on the endothelial surface. Furthermore, it has been reported that depletion of eosinophils using a cytotoxic antibody renders mice no longer able to kill IL-4-expressing tumour cells (Tepper et al., 1992). Therefore, in the present study eosinophils and macrophages may have been implicated in the in the anti-tumour response observed with the IL-4-secreting cell-line.

In the present study, when 10^5 IFN-γ secreting FS-29 cells were injected into either flank of C57/BL6 mice, three of the four animals survived until the end of the experiment, although one of these animals did have a tumour. All four of the mice injected with 10^6 IFN-γ secreting FS-29 cells survived until the end of the experiment although two had very slight ascites. The survival times of the animals injected with 10^6 IFN-γ secreting cells were significantly different from the controls and this cell-line appeared to have a more reduced tumourigenicity than the other two transfected cell-lines.

Similar results were reported when the weakly immunogenic IFN-γ-secreting murine fibrosarcoma lost its tumourigencity and induced a persistent anti-tumour immunity in animals. The local IFN-γ release caused a T-cell mediated immunity against the tumour in association with up-regulation of MHC class-I antigens (Gansbacher et al., 1990b). Comparable effects were found in mouse neuroblastoma and highly metastatic Lewis lung carcinoma cells transduced with the IFN-γ gene. These tumour cells had a strongly suppressed tumour growth when inoculated in animals and induced a specific anti-tumour activity (Watanabe et al., 1989).

Histological analysis of the tumours from the IFN-γ secreting cells resulted in one sample scoring a moderate $CD4^+$ T-cell and $CD8^+$ T-cell response and a negative B-cell response. The second sample scored a poor $CD4^+$ T-cell response and negative

$CD8^+$ T-cell and B-cell responses. Since there appeared not to be a strong lymphocytic infiltration into these tumours, perhaps there may have been other effector mechanisms responsible for the decreased tumourigenicity observed with this cell-line.

One of the mechanisms involved in altering tumour growth is believed to be differing MHC class-I and-II expression between parental cells and transfectants. It has been reported that the expression of MHC antigens can dramatically enhance the immunogenicity of tumour cells (Gastl et al., 1992). Patel and colleagues (1993b) reported that the IFN-γ secreting cells expressed high levels of MHC class-I and-II molecules, and the supernatant from these cells could upregulate MHC expression on parental FS-29 cells. Porgador and co-workers (1993a) reported that 3LL-D122 cells secreting IFN-γ showed decreased tumourigenicity and metastatic growth. These IFN-γ cells appeared to have a high expression of MHC class-I, which was thought to be a major participant in the cytotoxic lymphocyte induction and anti-tumour response. However, Esumi and colleagues (1991) proposed that an enhanced MHC expression was not sufficient to produce an anti-tumour response. When SP1 cells secreting IFN-γ failed to grow in syngeneic hosts and nude mice it was thought that this abrogated tumourigenicity was due to MHC class-I expression, but greater amounts of IFN-γ were required to inhibit tumourigenicity than was needed to raise MHC class-I expression; therefore another effector mechanism may be involved. Expression of the IFN-γ gene in poorly immunogenic tumour cells resulted in tumour suppression in euthymic but not athymic mice, suggesting a major role for T-cells in the IFN-γ anti-tumour response (Watanabe et al., 1989; Gansbacher et al., 1990b). When the bladder cancer cell-line MBT-2 was infected with murine IFN-γ using a retroviral system, no growth inhibition was observed in vitro, but MHC class-I expression was increased. However, when the transfected cells were injected subcutaneously, specific anti-tumour immunity was generated and was proposed to involve CD8+ T-cells (Hiura et al., 1994).

The mechanism of the anti-tumour response induced by IFN-γ may also be related to macrophage activation. Varesio and co-workers (1984) reported that potent activation of mouse macrophages was possible by recombinant IFN-γ. One effect of IFN-γ stimulation in macrophages is to increase the expression of both MHC class-I and class-II antigens (Wallach et al., 1982). In addition to MHC antigens, IFN-γ up-regulates the expression of cell-surface receptor (FcR) for IgG and thus increases antibody-mediated cellular cytotoxicity by macrophages, neutrophils and eosinophils (Petroni et al., 1988). One of the main functions of IFN-γ appears to be to prime macrophages to mount a respiratory burst i.e. rapid production of oxygen radicals and hydrogen peroxide (H_2O_2) and this contributes to their toxic activities towards bacteria and intracellular pathogens (Flesch and Kaufmann, 1987). IFN-γ has also appears to potentiate the respiratory burst of neutrophils to different stimuli (Cassatella et al., 1988). Further implications for eosinophils in the anti-tumour effects of IFN-γ was reported when high doses of IFN-γ were incorporated into gelatin chondroitin microspheres. When the microspheres were mixed with irradiated tumour cells and injected subcutaneously, an inflammatory infiltration predominantly consisting of eosinophils was present at the injection site (Golumbek et al., 1993).

From this study it appears that the IL-4 and IFN-γ-secreting, but not the IL-2-secreting, FS-29 cells can reduce tumourigenicity. The IFN-γ-secreting cells were the most successful in reducing tumourigenicity; however from the histological data there is little evidence of the mechanism of this anti-tumour response. With respect to the IFN-γ-secreting cells, an indication of the effector mechanisms involved could perhaps be observed if larger cell numbers were injected into animals such that more tumours were formed. In order to establish the efficacy of these regimes, further animal studies are required.

Table 6.1: Tumour Growth In Gene Cell Therapy In Mice (10^5Cells)

Animal No.	Control		IL-2		IL-4		IFN-γ	
	Survival (Days)	Tumour Size (mm)	Survival (Days)	Tumour Size (mm)	Survival (Days)	Tumour Size (mm)	Survival (Days)	Tumour Size (mm)
1	24	6.7x5.8	21	6.2x9.6	37	5.6x4.5	38	4.0x2.0
2	24	5.3x6.4 7.8x6.2	24	5.6x4.4	38	6.8x6.4 5.8x3.2	57	12.3x 10.6
3	57	N.T.	29	10.2x5.7	38	7.9x4.3 4.6x4.3	57	N.T.
4	57	N.T.			57	7.6x6.2	57	N.T.
Median	40.5		24		38		57	

Table 6.2: Tumour Growth In Gene Cell Therapy In Mice (10^6Cells)

Animal No.	Control		IL-2		IL-4		IFN-γ	
	Survival (Days)	Tumour Size (mm)	Survival (Days)	Tumour Size (mm)	Survival (Days)	Tumour Size (mm)	Survival (Days)	Tumour Size (mm)
5	23	9.1x7.4	22	3.6x3.5 17.8x9.9	24	4.5x3.5 6.8x3.4	57	Ascitic
6	23	6.3x4.5 6.7x5.3	22	6.8x6.8 4.6x5.0	38	3.6x3.6 8.6x6.4	57	Ascitic
7	23	6.7x5.4 8.5x5.5	23	5.6x4.5 6.3x4.5 5.7x4.6	41	9.4x8.5 21.4x4.3	57	N.T.
8	23	10.1x7.8	24	10.1x6.6	41	7.3x5.8 5.7x3.7 5.9x5.2	57	N.T.
Median	23		22.5		39.5		57	

N.T. = No Tumour.

Table 6.3 - Histological Analysis

Treatment	CD4	CD8	B220
10^5 Control	+++	++	+
10^5 Control	+++	++	+
10^5 Control	N.T.	N.T.	N.T.
10^5 Control	N.T.	N.T.	N.T.
10^5 IL-2	+++	++	+
10^5 IL-2	+++	+++	-
10^5 IL-2	+++	+++	+
10^5 IL-4	++	+	-
10^5 IL-4	++	-	+
10^5 IL-4	++	-	-
10^5 IL-4	++	-	-
10^5 IFN-γ	++	++	-
10^5 IFN-γ	+	-	-
10^5 IFN-γ	N.T.	N.T.	N.T.
10^5 IFN-γ	N.T.	N.T.	N.T.
10^6 Control	+	-	+
10^6 Control	++	+	+
10^6 Control	+	+	-
10^6 Control	+	+	-
10^6 IL-2	+++	+++	+
10^6 IL-2	+++	+++	+
10^6 IL-2	+	+++	++
10^6 IL-2	++	+++	+
10^6 IL-4	++	+	+
10^6 IL-4	++	+	+
10^6 IL-4	++	+	-
10^6 IL-4	+	+	+
10^6 IFN-γ	N.T.	N.T.	N.T.
10^6 IFN-γ	N.T.	N.T.	N.T.
10^6 IFN-γ	N.T.	N.T.	N.T.
10^6 IFN-γ	N.T.	N.T.	N.T.

+ Poor Response. ++ Moderate Response.

+++ Strong Response. - No Response.

N.T. - No Tumour.

Table 6.4 - Description of Figures 6.2 - 6.11.

Figure	Tumour Type	Stain	Score
6.2	10^6 Control	$CD4^+$ T-cells	+
6.3	10^5 IL-2	$CD4^+$ T-cells	+++
6.4	10^6 Control	$CD8^+$ T-cells	+
6.5	10^5 IL-2	$CD8^+$ T-cells	+++
6.6	10^6 Control	B-Cells	+
6.7	10^5 IL-2	B-Cells	+
6.8	10^6 Control	Haematoxylin + Eosin	Well-defined tumour structure.
6.9	10^6 IL-4	Haematoxylin + Eosin	Poor tumour structure.
6.10	10^5 Control	Nickel-DAB	Few peroxidase-positive cells.
6.11	10^5 IL-4	Nickel-DAB	Many peroxidase-positive cells.

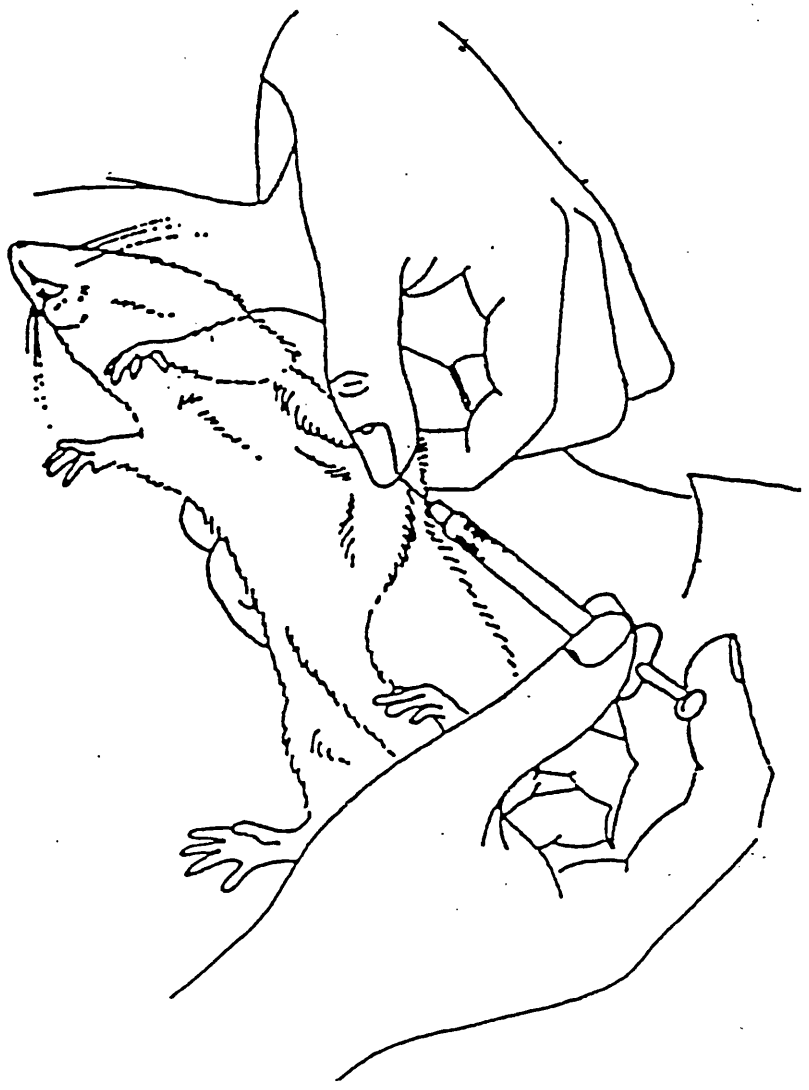


FIGURE 6.1

The mouse - subcutaneous injection into the flank

Figure 6.2 - 5µm section of tumour taken from an animal that had received 10^6 control cells into either flank. The section has been stained for CD4+ cells using AEC as the substrate (red) and counterstained with Harris's haematoxylin. There is a poor (+) CD4+ infiltration into this tumour. x10magnification.

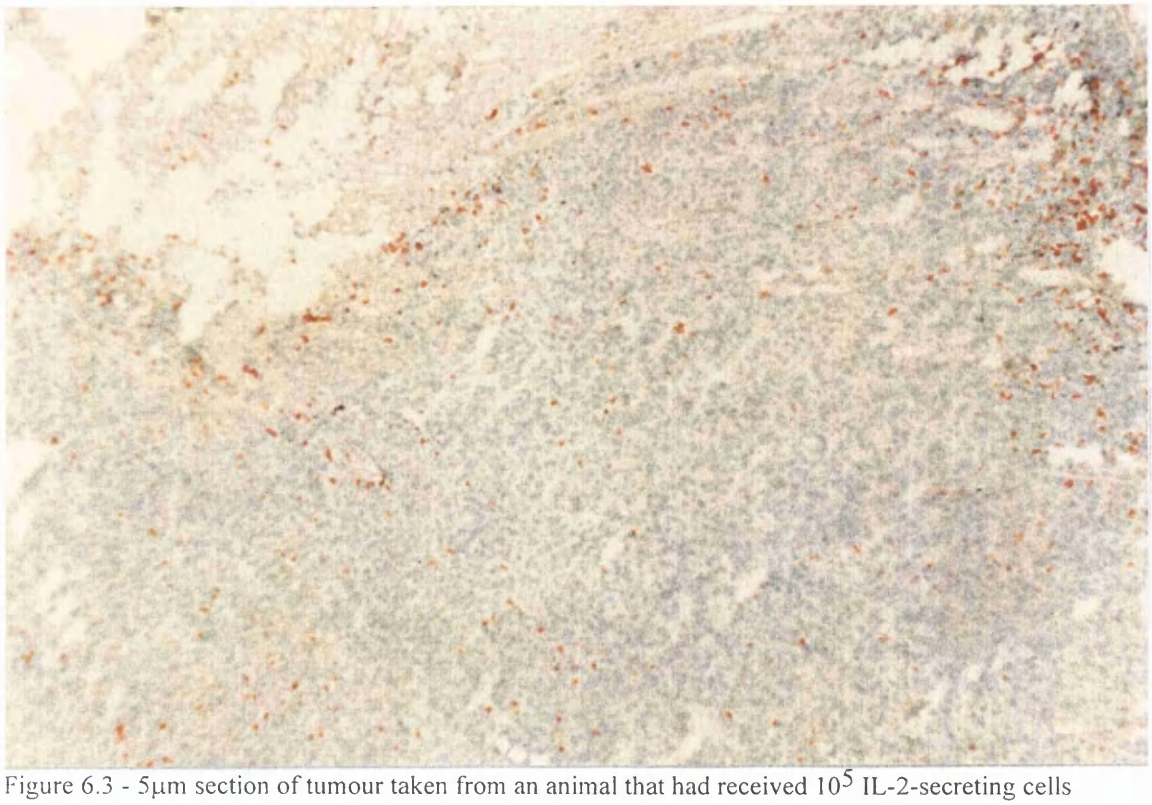


Figure 6.3 - 5µm section of tumour taken from an animal that had received 10^5 IL-2-secreting cells into either flank. This section has also been stained for CD4+ cells using AEC as the substrate (red) and Harris's haematoxylin as the counterstain. There is a strong (+++) CD4+ infiltration into this tumour sample. x10 magnification.

Figure 6.4 - Tumour section taken from an animal that had received 10^6 control cells into either flank. This 5µm section was stained for CD8+ T-cells using AEC as the substrate (red) and Harris's haematoxylin as the counterstain. There is a poor (+) CD8+ infiltration into this tumour. x10 magnification.

Figure 6.5 - 5µm tumour section from an animal that had received 10^5 IL-2-secreting FS-29 cells. There is a strong (+++) CD8+ infiltration into this tumour. x10 magnification.

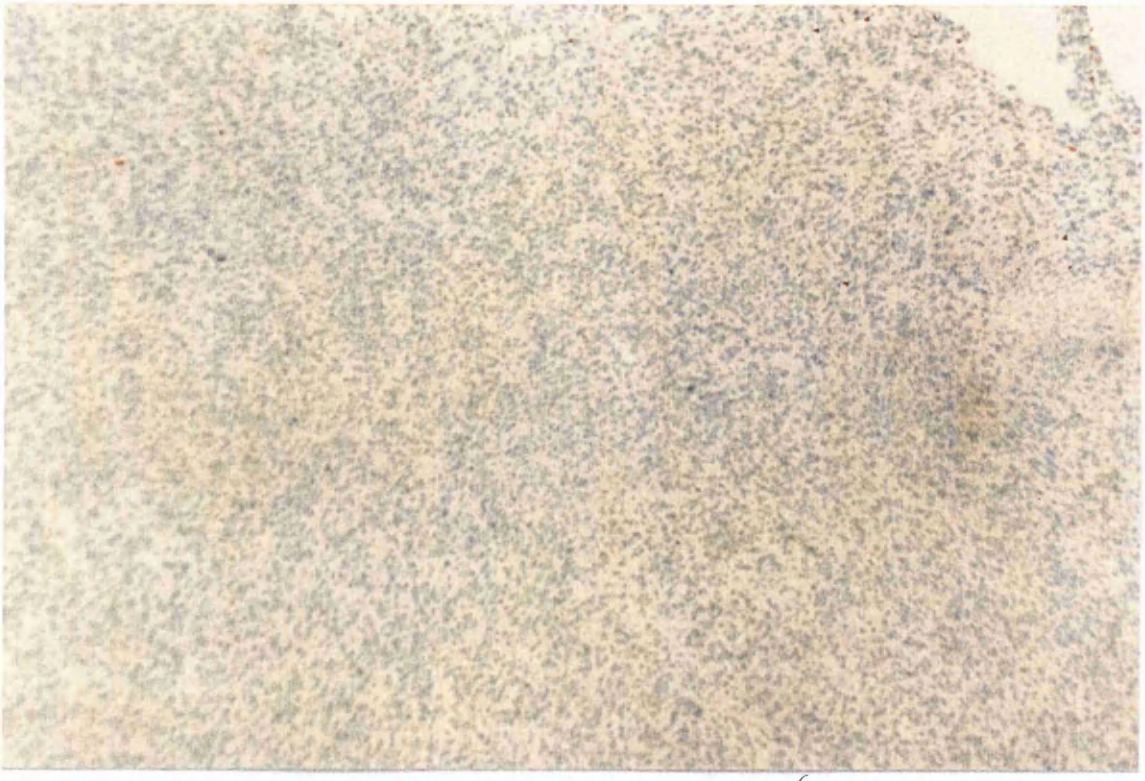


Figure 6.6 - This tumour was excised from an animal that had received 10^6 control cells into either flank. The 5µm section has been stained for B-cells. The photograph shows a poor (+) B-cell infiltration into this tumour nodule. x10 magnification.

Figure 6.7 - 5µm section from a tumour excised from an animal that had received 10^5 IL-2 secreting-cells into either flank. This photograph is indicative of all the treatments, in that poor (+) B-cell infiltrations were observed with all FS-29 cell types. x10 magnification..

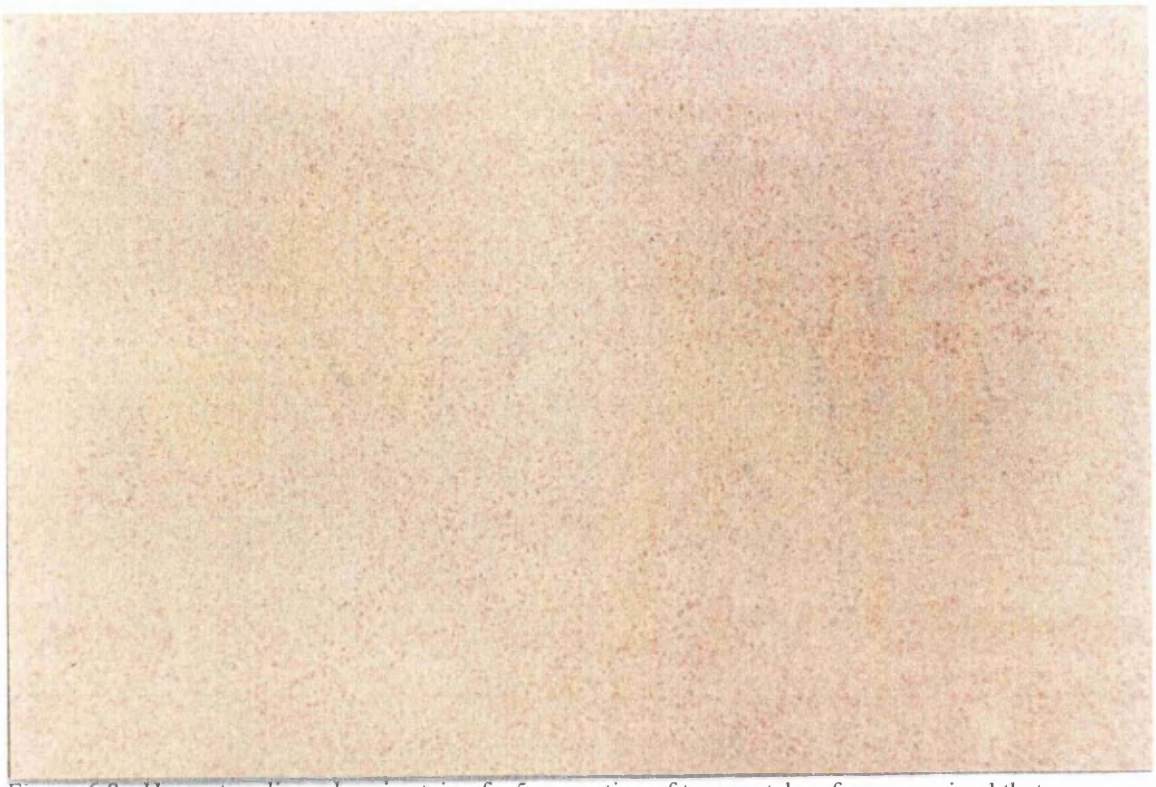


Figure 6.8 - Haematoxylin and eosin stain of a 5μm section of tumour taken from an animal that received FS-29 control cells. The photograph shows the well defined structure of the tumour sample. x10 magnification.

Figure 6.9 - Haematoxylin and eosin stain of a 5μm section of tumour taken from an animal that received IL-4-secreting FS-29 cells. In comparison to the above well defined structure of the control, the structure of this tumour section is poor, with with there being some necrosed areas and holes present (indicated by the arrows). x10 magnification.

Figure 6.10 - 5µm section of tumour taken from an animal that had received 10^5 control cells into either flank. The section has been stained with a control antibody and Nickel-DAB which stains peroxidase positive cells. The section has again been counterstained with Harris's haematoxylin. This photograph shows little peroxidase-positive cells which indicates a poor macrophage infiltration. x10 magnification.

Figure 6.11 - 5µm section from a tumour taken from an animal that had received 10^5 IL-4-secreting FS-29 cells into either flank. This photograph shows a heavy infiltrate of peroxidase-positive cells into the tumour. This indicates that IL-4 may induce a strong macrophage infiltration into the tumour. x10 magnification.

7: SPLENOCYTE TRANSFER IN GENE CELL THERAPY

7.1 Introduction

It has been reported that when animals have rejected cytokine-secreting cells, they may subsequently reject a challenge of control cells (Gansbacher et al., 1990a, Dranoff et al., 1993). This process indicates that the host immune system appears to have developed memory for the tumour cells. One such model of this type of immune recognition has been reported by Gansbacher and co-workers (1990a). In this study CMS-5 cells modified to secrete IL-2, demonstrated decreased tumourigenicity and protection against a subsequent challenge with CMS-5 non-secreting cells. Furthermore, this protective response appeared to correlate with tumour specific cytotoxic T-cells in the spleen of the treated animals.

The aim of this study was to attempt to transfer immune memory against tumour cells by transferring splenocytes from animals, that had previously received a tumour challenge and had survived, into naive hosts. The naive host would then be challenged with control cells, and the ability to reject this tumour would be assessed. Animals that survived from the first study had their spleens removed and their splenocytes harvested for use in this present study.

Histological examination of any tumour nodules that formed during the present study was also carried out to give an indication as to the effector mechanisms that may be implicated in the anti-tumour response.

7.2 Materials and Methods

When the first study was terminated after 57 days, some animals had survived until this point with tumours that had not yet reached 1cm in diameter, or which had no tumour growth. These remaining mice were killed and their spleens excised and their splenocytes used in the present study. The spleens were teased open with forceps and the splenocytes were harvested into PBS and counted as described in section 5.2.

Thirty-four female immunocompetent C57/BL6J mice were randomised into seven groups (6 groups of 5, 1 group of 4). Each animal received $1x10^7$ splenocytes intra-peritoneally through a 26 gauge needle. Each group received splenocytes from the following animals;

Group 1 received splenocytes from a naive mouse i.e. a mouse that had not previously received any form of treatment. Group 2 received splenocytes from a mouse which had previously received 10^5 control FS-29 cells and did not develop a tumour. Group 3 was injected with splenocytes from a mouse that had previously received 10^5 IL-4-secreting FS-29 cells and had developed a tumour. Group 4 contained mice which received splenocytes from a mouse that had previously been injected with 10^5 IFN-γ-secreting FS-29 cells and had developed a tumour. Group 5 received splenocytes from a mouse that had previously been injected with 10^5 IFN-γ-secreting FS-29 cells and did not develop a tumour. Group 6 received splenocytes from an animal that was previously injected with 10^6 IFN-γ-secreting FS-29 cells and did develop a tumour. Group 7 received splenocytes from an animal that previously received 10^6 IFN-γ-secreting FS-29 cells and did not develop a tumour.

FS-29 non-secreting cells were cultured and harvested as described in section 5.2. Two days after injection of splenocytes all mice received 10^6 control FS-29 cells subcutaneously into the left flank through a 26 gauge needle. Animals were checked daily and tumour growth was monitored by palpation between the thumb and first finger. When tumour growth reached 1cm in diameter, animals were killed by neck

dislocation. The excised tumours were placed into liquid nitrogen immediately and stored until required for histological analysis. Immunohistochemical analysis of the individual tumour sections was carried out as detailed in chapter 5. After 56 days the experiment was terminated and any surviving animals were examined by autopsy for tumour growth.

Statistics: Data are presented as the median and where appropriate, comparisons of data from different groups were analysed using the Kruskall-Wallis test which is a non-parametric equivalent of analysis of variance (Minitab Inc., Pasadena, CA, U.S.A.).

7.3 Results

Splenocyte transfer: The results from the splenocyte transfer study are shown in tables 7.1 and 7.2. The first treatment group contained 5 naive animals, in that they received splenocytes from mice that had not received any gene cell therapy. Following injection of control cells, three of the animals were killed on days 33, 40 and 47. The remaining two animals survived until day 56; however one of these animals did have a tumour.

The mice in group 2 received splenocytes from animals that had previously received 10^5 control cells. Following challenge with the parental tumour cell-line, the animals were killed on days 27, 30, 35 and 42. None of the members of this group survived until the end of the experiment. Group 3 received splenocytes from mice that had previously received 10^5 IL-4-secreting cells and had not developed a tumour. Subsequent challenge with 10^6 non-secreting cells resulted in survival times of 31, 31, and 34 days and the remaining two animals survived until the end of the experiment on day 56. One of the surviving animals had developed a tumour, but the other appeared to be tumour-free.

Administration of splenocytes from animals who had previously received 10^5 IFN-γ cells and had developed a tumour, resulted in survival rates of 27, 35, 35 and 52 days in group 4. Group 5 contained animals that received splenocytes from mice that had been injected with 10^5 IFN-γ-secreting cells animals and did not develop tumours. Following tumour challenge, all of the animals survived until the end of the experiment. Three of the animals appeared to be completely tumour free, but one animal had a small tumour.

Group 6 received splenocytes from mice that were previously injected with 10^6 IFN-γ -secreting cells and had not developed tumours. All of the animals in this group did develop tumours when challenged with non-secreting cells and none survived until the

end of the experiment. The animals were killed on days 18, 38, 38, 40 and 55. Group 7 contained animals that received 10^7 splenocytes from mice that had previously received 10^6 IFN-γ-secreting cells and had not developed tumours. When animals in this group were challenged with control cells, one animal was tumour free after 56 days, but the remainder of the group all developed tumours and were killed on days 27, 35, 39 and 40.

Statistical analysis of these groups using the Kruskall-Wallis test showed that there was no significant differences between the treatment groups for the number of days of survival ($p = 0.113$).

Histological Analysis: The histology results are detailed in table 7.3. The first treatment group, which had received naive splenocytes, scored a strong $CD4^+$ T-cell response (Figure 7.1), a moderate $CD8^+$ T-cell (Figure 7.3) and a poor B-cell response. Similar responses were also observed with animals injected with splenocytes from mice that has received 10^5 control cells and did not develop a tumour. These samples also scored a strong $CD4^+$ T-cell response, moderate $CD8^+$ response and a poor B-cell response.

Group 3 contained animals that received splenocytes from mice that had previously been injected with 10^5 IL-4-secreting cells and had developed a tumour and group 4 contained animals that received splenocytes from an animal that was injected with 10^5 IFN-γ-secreting cells and also developed a tumour. Both of these groups scored a strong $CD4^+$ T-cell response, a moderate $CD8^+$ T-cell response and a poor B-cell response.

In group 5 there was only one animal that developed a tumour. This animal received splenocytes from a mouse that was previously injected with 10^5 IFN-γ-secreting cells and had not developed a tumour. This tumour sample scored a moderate $CD4^+$ T-cell

response, (Figure 7.2) a poor $CD8^+$ T-cell response (Figure 7.4) and a poor B-cell response.

The two remaining groups received splenocytes from animals that had previously been injected with 10^6 IFN-γ-secreting cells. Both of these groups scored a moderate $CD4^+$ and $CD8^+$ T-cell responses and a poor B-cell response.

There appeared to be little or no peroxidase activity present in tumours that formed from any of the treatment groups. Tumours that formed in each of the treatment groups had necrotic areas, but the extent of tumour necrosis appeared to be more striking in tumours that formed from animals that had received splenocytes from mice previously treated with 10^5 or 10^6 IFN-γ-secreting cells. The majority of these tumours had large necrotic areas and a poor general structure in comparison with the tumours that formed in mice that received splenocytes from a naive mouse (Figures 7.5 + 7.6).

7.4 Discussion

This study was carried out to assess the ability of anti-tumour immunological memory to be transferred between animals through the transfer of splenocytes. It was proposed that if animals have previously received tumour cells and survived, the animals may have antigen-specific cells in their spleens. Furthermore, if splenocytes from these animals were transferred to naive animals, it was proposed that protection against tumour growth may also be transferred in that a challenge with control tumour cells may be rejected.

In this study mice received 10^7 splenocytes intra-peritoneally and two days later were challenged with 10^6 control FS-29 cells. The first treatment group received splenocytes from a naive mouse followed by control FS-29 cells. In comparison with the survival rates from the first study when the animals received 10^6 control FS-29 cells, the survival rates from the present study were increased, perhaps indicating that in general, increased numbers of lymphocytes may enhance the immune response and perhaps help reduce tumourigenicity. In the second treatment group animals received splenocytes from an animal that had previously received 10^5 control FS-29 cells and had not developed a tumour. Following challenge with parental cells all animals developed tumours and none survived until the end of the experiment which may indicate that there was little if any transfer of immunological anti-tumour memory in this group.

The third group received splenocytes from an animal that had previously received 10^5 IL-4-secreting cells and had not developed a tumour. When animals in this group were challenged with control cells, two of the four animals survived until the end of the experiment, with one animal appearing to be completely tumour-free when examined at autopsy. Although none of the treatment groups demonstrated statistically significant differences, and given the small numbers of animals in the experiment, it would be of interest to compare the groups with greater animal numbers. Perhaps in

this group, there may have been a slight transfer of protection against tumour growth, in that one of the treated animals did not develop a tumour. Histological analysis of the tumours that formed in this treatment group revealed that there was a strong $CD4^+$ T-cell infiltration, a moderate $CD8^+$ T-cell infiltration and a poor B-cell response. Previous studies have indicated that T-cells are important for a long-term anti-tumour response against IL-4-secreting cells. For example, when renal cancer cells were altered to secrete IL-4 and injected into nu/nu mice, tumour growth was not observed for 2 months which suggested that T-cells were required for the ultimate rejection of tumour cells. Furthermore, when $CD8^+$ T-cells were eliminated from the animal, the rejection of parental renal cancer cells at a distant site was prevented. When $CD4^+$ cells were eliminated, there were some late recurrences which suggests that $CD4^+$ cells participated in the long-term memory responses (Golumbek et al., 1991). Golumbek and co-workers (1991) reported that macrophages may also be involved in the anti-tumour response. However, there was little or no peroxidase staining in the present tumour samples which would suggest that there was little macrophage infiltration into the tumour nodules.

Group 4 contained animals that had received splenocytes from an animal that was inoculated with 10^5 IFN-γ-secreting cells and had developed a tumour. Following challenge with 10^6 control FS-29 cells, three animals developed tumours and none survived until the end of the experiment. One animal in this group was killed on day 35 because it was thought to be ascitic. However, examination at autopsy failed to reveal any indication of tumour growth therefore, this animal may have remained tumour-free until the end of the experiment. There did not appear to be any transfer of anti-tumour memory in this group through the transfer of splenocytes. Similar results were observed with group 6 animals. Mice in this group received splenocytes from an animal that had previously received 10^6 IFN-γ-secreting cells and had developed a tumour. Following a challenge with parental cells all animals in this group developed

tumours which indicated that there was little transfer of anti-tumour memory between these animals.

Animals in Group 5 received splenocytes from mice that had previously received 10^5 IFN-γ-secreting cells and had not developed a tumour. Only one animal in this group developed a small tumour and all animals survived until the end of the experiment. Perhaps if this group had contained more animals or the experiment was allowed to run for longer, more information about the mechanisms of tumour rejection would have been obtained. However, the result of this study is encouraging as it may indicate that protection against tuumour growth may be transferred between animals in this group. Group 7 contained animals that received splenocytes from a mouse that had been injected with 10^6 IFN-γ-secreting cells and had not developed a tumour. Following a challenge with control FS-29 cells, four of the five animals in this group developed tumours. Therefore, there appears to be little transfer of immunological anti-tumour memory between these animals. As the group that received splenocytes from an animal that was inoculated with 10^5 IFN-γ-secreting cells contained more surviving animals than the group inoculated with splenocytes from an animal that received 10^6 IFN-γ-secreting cells, it may be proposed that the initial tumour burden is important in determining the effectiveness of transfer of immunological anti-tumour memory.

Results from this study are encouraging and it may be possible to transfer protection from tumour growth between animals. Several authors have reported that when animals have rejected an initial injection of cytokine-secreting cells, the animals may subsequently reject an inoculum of non-secreting control cells either at the same site or at a different site (Gansbacher et al., 1990a; Dranoff et al., 1993). Therefore, these animals appear to have developed immunologic anti-tumour memory for the tumour cells. Gansbacher and colleagues (1990a) reported that when CMS-5 cells were altered to secrete IL-2, these cells demonstrated decreased tumourigenicity and

subsequent protection against a challenge with CMS-5 non-secreting control cells. Furthermore, it appeared that this protective response correlated with tumour-specific cytotoxic T-cells in the spleen.

Histological analysis of the tumour nodules that formed in this study was carried out in order to assess the possible effector mechanisms that may be involved in the anti-tumour response. It was found that all tumours that formed in animals that had received splenocytes from animals that had previously been injected with the IFN-γ-secreting FS-29 cells appeared to have large necrosing areas and a poor general structure. Therefore, tumours that formed in these groups may have undergone a vigorous immunological anti-tumour response and may be dying tumours. Further histological analysis revealed that tumours that formed in animals that received splenocytes from mice that had previously been injected with 10^5-IFN-γ-secreting FS-29 cells and did develop a tumour, had a strong $CD4^+$ T-cell response, moderate $CD8^+$ T-cell response and a poor B-cell response. The groups that received splenocytes from animals that were previously treated with 10^6 IFN-γ-secreting cells had a moderate $CD4^+$ and $CD8^+$ T-cell response and a poor B-cell response. As there was only one tumour that formed in the group that received splenocytes from an animal that was inoculated with 10^5 IFN-γ-secreting cells and did not develop a tumour, a firm indication of the IFN-γ anti-tumour response cannot be concluded. However, results from histological analysis indicate that $CD4^+$ T-cells may be important with there being a moderate infiltration of this cell type into the tumour nodule. There appeared to be little involvment of $CD8^+$ T-cells or B-cells in the anti-tumour response as there was a poor infiltration of these lymphocytic groups into the tumour nodule.

It is an exciting possibility that protection against tumour growth may be transferred between animals through the transfer of lymphocytic cells. Unfortunately numbers

were small in the treatment groups, but the approach used in this study warrants further investigation.

Table 7.1: Splenocyte Transfer In Gene Cell Therapy

Animal No.	Naive		10^5Control N.T		10^5 IL-4 W.T.		10^5 IFN-γ W.T.	
	Survival (Days)	Tumour Size (mm)	Survival (Days)	Tumour Size (mm)	Survival (Days)	Tumour Size (mm)	Survival (Days)	Tumour Size (mm)
1	33	8.9x5.1 ascitic		N.T.R.D	31	12.9x8.6 ascitic	27	10.0x7.6
2	40	11.6x8.5	27	12.6x9.8	31	9.7x12.9	35	9.4x7.0
3	47	8.4x10.9	30	5.3x4.4 10.3x8.6	34	ascitic	35	N.T.
4	56	N.T.	35	10.1x8.5	56	N.T.	52	ascitic
5	56	7.5x6.9	42	8.5x8.5	56	9.5x6.6		
Median	47		32.5		34		35	

Table 7.2: Splenocyte Transfer In Gene Cell Therapy

Animal No.	10^5 IFN-γ N.T.		10^6 IFN-γ W.T.		10^6 IFN-γ N.T.	
	Survival (Days)	Tumour Size (mm)	Survival (Days)	Tumour Size (mm)	Survival (Days)	Tumour Size (mm)
1		N.T.R.D	18	7.5x6.1	27	13.7x 12.2
2	56	4.1x6.6	38	10.7x7.6	35	10.3x 10.9
3	56	N.T.	38	ascitic	39	ascitic
4	56	N.T.	40	6.1x6.0*	40	ascitic
5	56	N.T.	56	12.6x 10.5	56	N.T.
Median	56		38		39	

N.T.- No Tumour. W.T.- With Tumour.

N.T.R.D. - Non-tumour related death. * Ulcerated Tumour.

Table 7.3 - Histological Analysis

Treatment	CD4	CD8	B220
Naive	++	++	+
Naive	+++	++	+
Naive	+++	++	+
Naive	N.T.	N.T.	N.T.
Naive	+++	++	+
10^5 Control N.T.	++	++	-
10^5 Control N.T.	++	+	+
10^5 Control N.T.	+++	++	+
10^5 Control N.T.	+++	++	+
10^5 Control N.T.	+++	++	+
10^5 IL-4 W.T.	+++	++	+
10^5 IL-4 W.T.	++	++	+
10^5 IL-4 W.T.	+++	++	+
10^5 IL-4 W.T.	N.T.	N.T.	N.T.
10^5 IL-4 W.T.	+++	+++	+
10^5 IFN-γ W.T.	+++	+++	-
10^5 IFN-γ W.T.	+++	++	+
10^5 IFN-γ W.T.	N.T.	N.T.	N.T.
10^5 IFN-γ W.T.	++	+	+
10^5 IFN-γ N.T.	++	+	+
10^5 IFN-γ N.T.	N.T.	N.T.	N.T.
10^5 IFN-γ N.T.	N.T.	N.T.	N.T.
10^5 IFN-γ N.T.	N.T.	N.T.	N.T.
10^5 IFN-γ N.T.	N.T.	N.T.	N.T.
10^6 IFN-γ W.T.	+++	++	+
10^6 IFN-γ W.T.	++	++	+
10^6 IFN-γ W.T.	+	+	-
10^6 IFN-γ W.T.	+++	++	+
10^6 IFN-γ W.T.	++	++	+
10^6 IFN-γ N.T.	++	++	+
10^6 IFN-γ N.T.	++	++	+
10^6 IFN-γ N.T.	++	+	+
10^6 IFN-γ N.T.	+	+	+
10^6 IFN-γ N.T.	N.T.	N.T.	N.T.

+ Poor Response ++ Moderate Response +++ Strong Response

-ve No Response N.T.R.D. - Non-tumour Related Death

Table 7.4 - Description of Figures 7.1 - 7.6.

Figure	Tumour Type	Stain	Score
7.1	Naive splenocytes + control cells	$CD4^+$ T-cells	+++
7.2	10^5 IFN-γ N. T.+ control cells	$CD4^+$ T-cells	++
7.3	Naive splenocytes + control cells.	$CD8^+$ T-cells	++
7.4	10^5 IFN-γ N. T.+ control cells	$CD8^+$ T-cells	+
7.5	Naive splenocytes + control cells	Haematoxylin + Eosin	Well defined tumour structure.
7.6	10^5 IFN-γ N. T.+ control cells	Haematoxylin + Eosin	Poor tumour structure.

Figure 7.1 - 5μm section taken from an animal that received 10^7 splenocytes from a naive mouse, followed 2 days later with 10^6 FS-29 control cells. This section has been stained for CD4+ T-cells using AEC as the substrate (red) and Harris's haematoxylin as the counterstain.This photograph shows the strong (+++) CD4+ infiltration which was observed in most of the excised control tumours. x10 magnification.

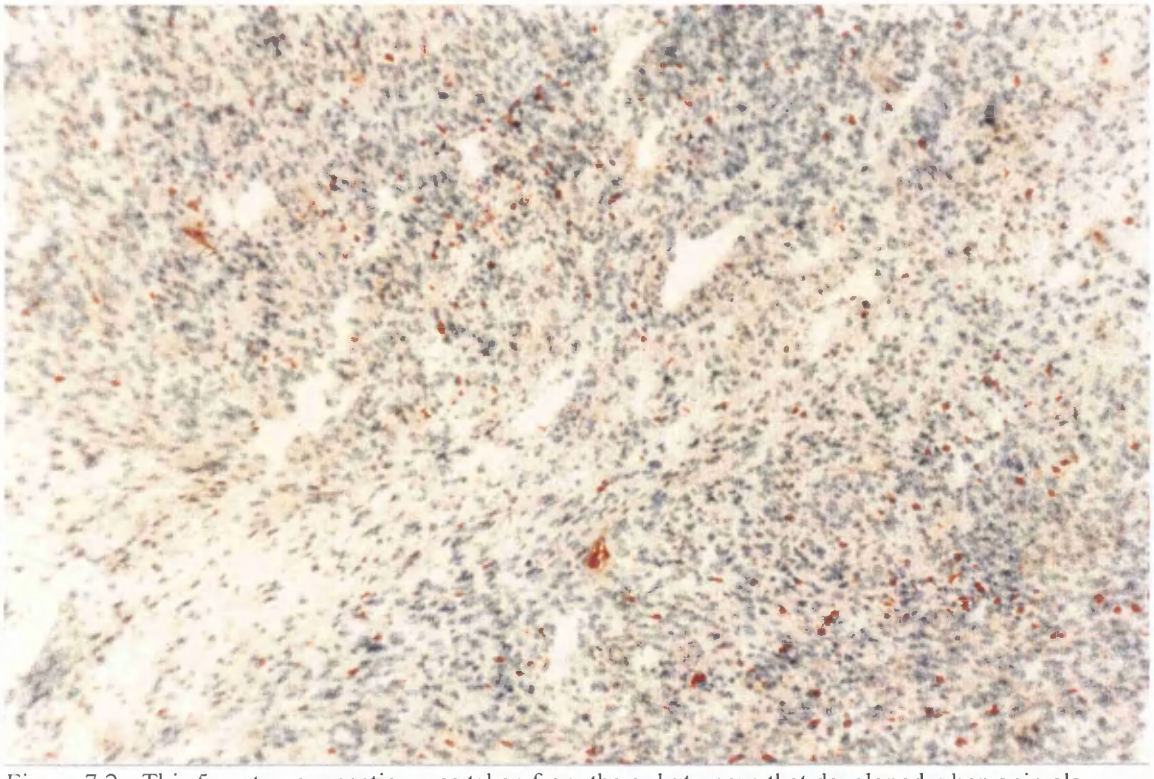


Figure 7.2 - This 5μm tumour section was taken from the only tumour that developed when animals received splenocytes from 10^5IFN-γ treated animals who did not develop tumours. This section was also stained for CD4+ cells and unlike the strong infiltration observed with the control animals, this treatment produced a moderate (++) CD4+ infiltration. However, as there was only one sample, this result cannot be conclusive for this treatment. x10 magnification.

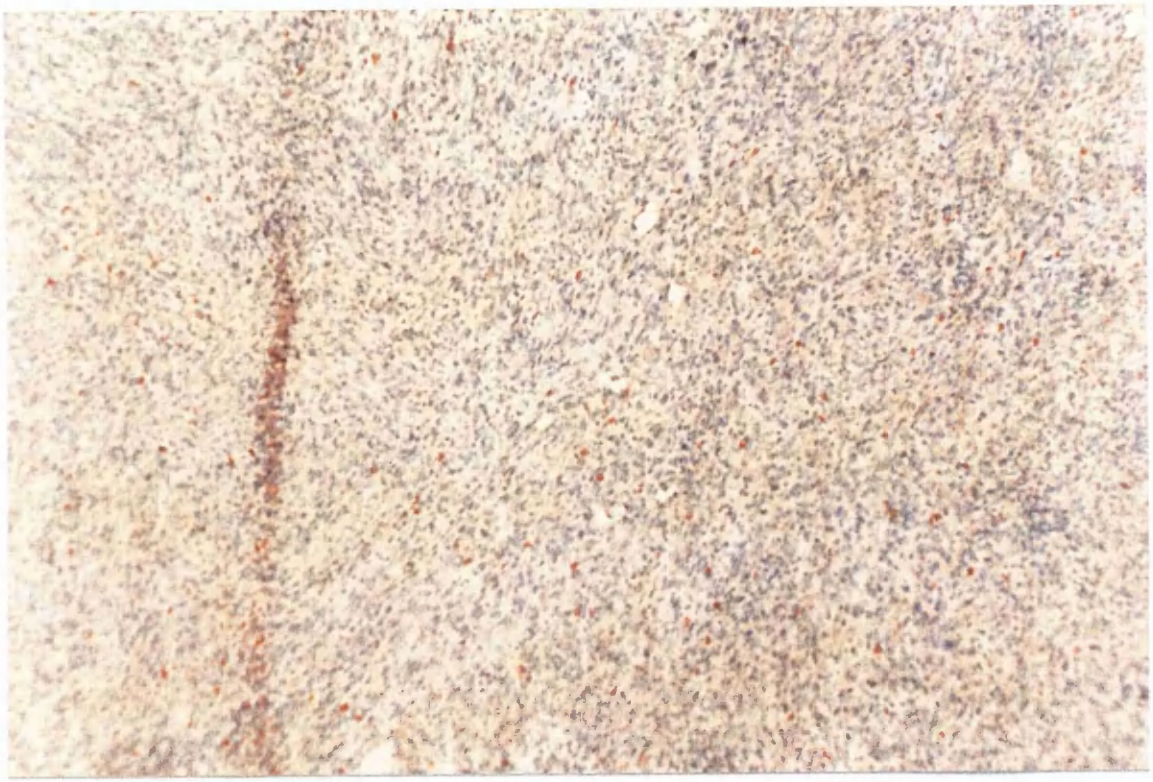


Figure 7.3 - 5μm tumour section taken from an animal that had received 10^7 splenocytes from a naive animal, followed 2 days later by 10^6 control FS-29 cells. This section, which has been stained for CD8+ T-cells, shows that there is a moderate (++) CD8+ infiltration into this tumour nodule. x10 magnification.

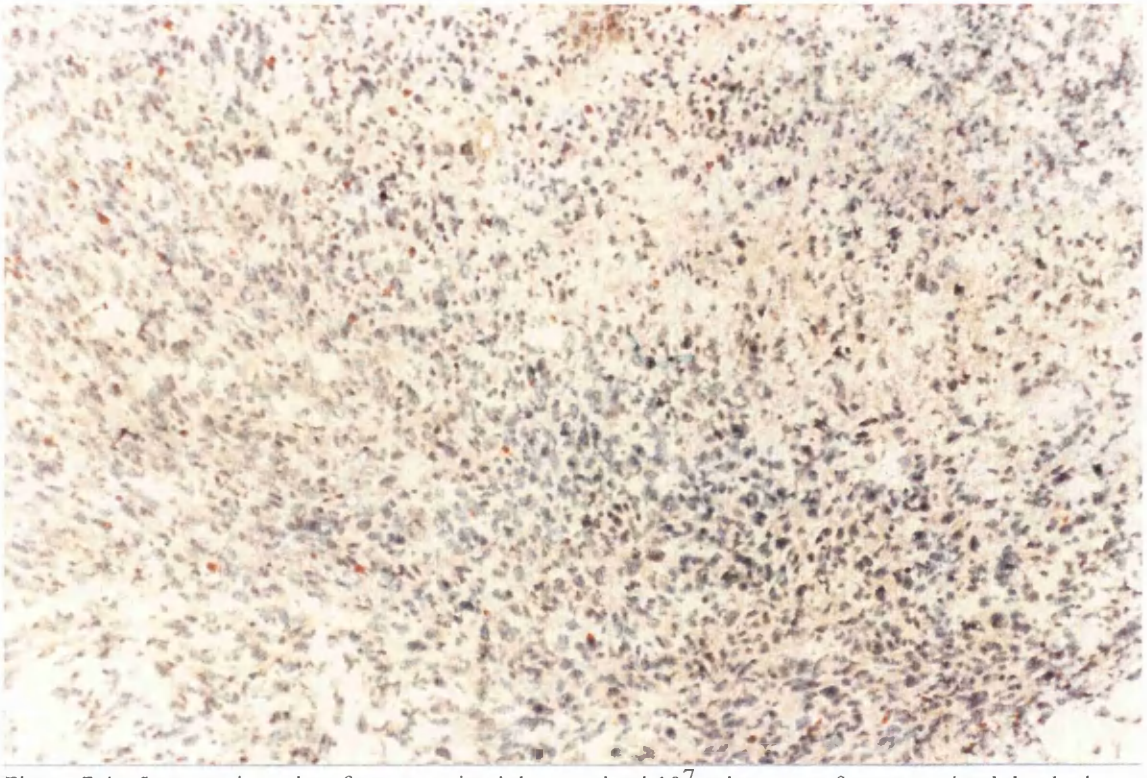


Figure 7.4 - 5μm section taken from an animal that received 10^7 splenocytes from an animal that had previously received 10^5 IFN-γ cells and did not develop a tumour. 2 days later this animal received 10^6 FS-29 control cells into the left flank. This section, which has been stained for CD8+ cells, shows a poor (+) CD8+ infiltration into this tumour. However this cannot be a conclusive result as only one tumour developed in this treatment group. x10 magnification.

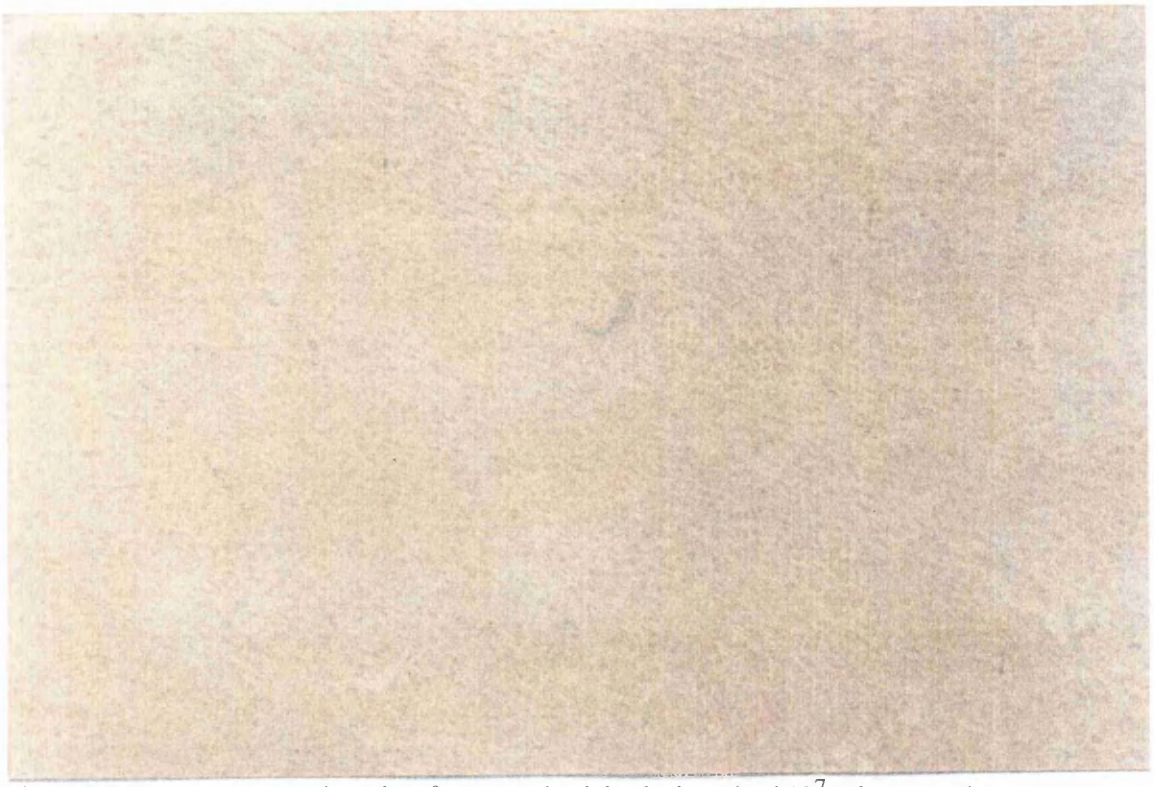


Figure 7.5 - 5μm tumour section taken from an animal that had received 10^7 splenocytes intra-peritoneally from a naive animal. 2 days later the animal received 10^6 FS-29 control cells into the left flank. This haematoxylin and eosin stain shows quite well defined structure of this tumour nodule. x10 magnification.

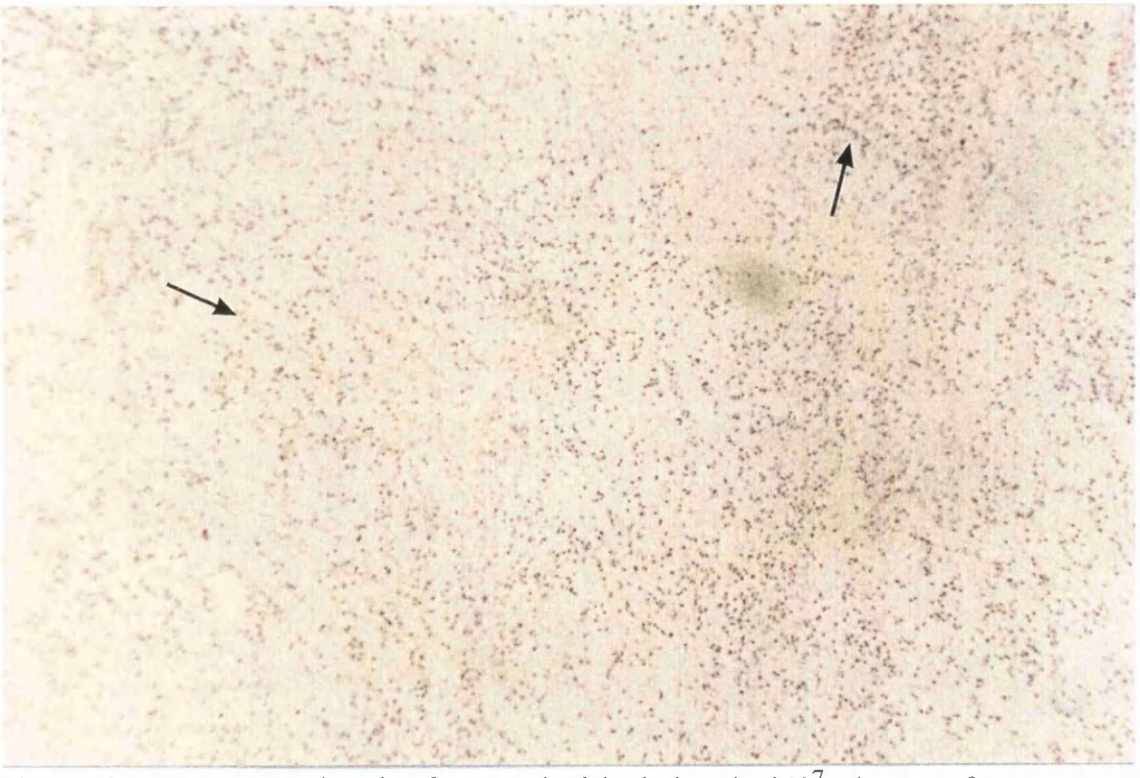


Figure 7.6 - 5μm tumour section taken from an animal that had received 10^7 splenocytes from an animal that had previously received 10^6 IFN-γ cells and did not develop a tumour. This haematoxylin and eosin stain is indicative of those animals that received splenocytes from animals that had previously been given 10^5 or 10^6 IFN-γ cells. Of the animals that developed tumours they all appeared to have a very necrosed, undefined structure (indicated by arrows). x10 magnification.

8: REGRESSION OF AN ESTABLISHED TUMOUR

8.1 Introduction

It has been reported that when genetically altered tumour cells are admixed with control tumour cells and injected into animals, tumour growth can be slowed or prevented (Patel et al., 1993a, 1993b.). Although these studies examine the ability of cytokine-secreting cells to reduce growth of admixes of cells, the ability of cytokine-secreting cells to inhibit growth of an already established tumour appears to have been examined only once previously. This point seems fundamental in that gene cell therapy clinical trials are being carried out in patients with advanced cancer in order to regress growth of their primary tumour or of metastatic disease.

Studies have indicated that the effects of cytokine therapy may be enhanced if different cytokines are administered in combination (Ohira et al., 1994). The aim of this experiment was to examine the ability of the cytokine-secreting cells, alone or in various combinations, to regress growth of an already established tumour. Therefore, animals were given an injection of control cells into the left flank. One week later the mice were also injected into the right flank with the cytokine-secreting cells.

Histological examination of any tumour nodules that formed during the present study was also carried out to give an indication as to the effector mechanisms that may be implicated in the anti-tumour response.

8.2 Materials and Methods

This experiment was carried out to assess the ability of the cytokine-secreting cells to inhibit the growth of an already established tumour nodule. The control cell-line was cultured and harvested as described in section 5.2. The cell density was adjusted to give 10^7/ml. Fourty-two female immunocompetent C57Bl/6 J mice were randomised into 8 treatment groups (7 groups of 5, 1 group of 6). Each treatment group received 10^6 control cells as a 100µl injection subcutaneously into the left flank using a 26 gauge needle. One week later the mice received a 100µl injection subcutaneously of control cells or cytokine secreting cells, either alone, or in various combinations, into the right flank using a 26 gauge needle. One mouse was killed before it received a second injection as it appeared to be in poor health.

Animals were checked daily and tumour growth was measured by palpation between the thumb and first finger. When tumours reached approximately 1cm in diameter, the animals were killed by neck dislocation. The excised tumours were frozen in liquid nitrogen immediately and stored in liquid nitrogen until required for histological analysis. Immunohistochemical analysis of the individual tumour sections was carried out as detailed in chapter 5. After 28 days the experiment was terminated with the death of the last remaining animal.

Statistics: Data are presented as the median and where appropriate, comparisons of data from different groups were analysed using the Kruskall-Wallis test which is a non-parametric equivalent of analysis of variance (Minitab Inc., Pasadena, CA, U.S.A.).

8.3 Results

Regression of an already established tumour: In this study all of the animals received an initial injection of 10^6 control cells into the left flank. One week later they were re-challenged with control cells or cytokine-secreting cells alone or in various combinations into the right flank. The results from this study are shown in tables 8.1 and 8.2. Animals that had received a second injection of control cells all developed tumours and were killed on days 14, 16, 17, 17 and 21. The animals injected with IL-2-secreting cells had survival rates of 14, 15, 15, 20 and 21 days. The mice treated with IL-4-secreting cells also developed tumours which were initially slow-growing, but then rapidly reached 1cm. These animals were killed on days 16, 16, 16, 17 and 20.

Those animals injected with IFN-γ-secreting cells were killed on days 14, 15, 18, 26 and 28. The animal which survived until day 28 had a tumour but also had another tumour growing behind it which was not initially detected. So, the number of days of survival in this case should have been less.

When IL-2 and IL-4-secreting cells were administered together, all animals developed tumours and were killed on days 12, 17, 17, 18, and 26. All animals developed tumours when they received an injection of IL-2 and IFN-γ-secreting cells together. Animals from this group were killed on days 16, 16, 20, 26 and 27. The last animal had a very large tumour which was not easily detectable and should have been killed before 27 days.

All animals that received an injection of IL-4 and IFN-γ-secreting cells together, developed tumours and were killed on days 11, 14, 15, 16 and 19. The final group received IL-2, IL-4 and IFN-γ cells in one injection. Again all animals developed tumours and were killed on days 15, 16, 16, 16, 19 and 20.

Statistical analysis of this experiment using the Kruskall-Wallis test showed that there was no significant differences between groups for the number of days of survival ($p = 0.585$).

Immunohistological Analysis: The results are detailed in table 8.3. The animals that received a second injection of control cells scored a moderate $CD4^+$ and $CD8^+$ T-cell response with a poor B-cell response (Figures 8.1 + 8.3). Animals that received an injection of IL-2-secreting cells also scored moderate $CD4^+$ and $CD8^+$ T-cell responses with a poor B-cell response.

Those animals treated with IL-4-secreting cells scored a moderate $CD4^+$ T-cell response with poor $CD8^+$ T-cell and B-cell responses. An injection of IFN-γ-secreting cells resulted in a mainly strong $CD4^+$ T-cell response, moderate $CD8^+$ T-cell response and a poor B-cell response. Tumours that formed in animals that received IL-2 and IL-4-secreting cells scored moderate $CD4^+$ T-cell, $CD8^+$ T-cell and B-cell responses. An IL-2 and IFN-γ-secreting cell combination scored a moderate $CD4^+$ and $CD8^+$ T-cell response with a poor B-cell response.

When IL-4 and IFN-γ were injected in combination, the B-cell score was poor but, there was a strong $CD4^+$ T-cell infiltration (Figure 8.2) and a moderate $CD8^+$ T-cell response (Figure 8.4). A strong $CD4^+$ T-cell infiltration was observed when IL-2, IL-4 and IFN-γ-secreting cells were injected in combination. The $CD8^+$ T-cell response was moderate and the B-cell response poor.

In this study, there appeared to be little or no peroxidase activity in the different treatment groups. The tumour structure in most groups ranged from poor to good. The most significant structural difference was in the IL-2 and IFN-γ combination group. Four of the five tumours appeared to have large necrosed areas in comparison to the control group (Figures 8.5 + 8.6).

8.4 Discussion

The aim of this study was to assess the ability of the cytokine-secreting cells to regress growth of an established tumour nodule. The ability of the cytokine-secreting cells to perhaps produce additive anti-tumour actions when administered in combination was also examined in this study. Animals received 10^6 FS-29 control cells into the left flank. One week later, the animals received 10^6 IL-2-secreting cells alone, or in combination with IL-4 or IFN-γ-secreting cells into the right flank. It was found that the IL-2-secreting cells either alone, or in combination with IL-4 or IFN-γ-secreting cells could not regress or slow growth of an established tumour nodule. Therefore, the combination of cytokine-secreting cells did not result in an enhanced anti-tumour response. Patel and co-workers (1993a) reported that when IL-2 and IL-4-secreting FS-29 cells were doubly transfected, this combination resulted in an optimal tumour rejection when injected into mice. Further evidence of the beneficial effects of cytokines administered in combination was reported by Ohira and co-workers (1994). He reported that when IL-2 was co-transfected with TNF into the Lewis Lung carcinoma and injected into mice, the anti-tumour effects of the co-transfectants was superior to that of the IL-2 and TNF transfectants alone. In contrast it has been reported that IL-2-secreting FS-29 cells could not prevent growth of an equal number of admixed non-secreting FS-29 cells (Patel et al., 1993a). This work is consistent with the present study in that the IL-2-secreting FS-29 cells were not able to reduce growth of a tumour nodule.

Patel and co-workers (1993a) reported that the tumours that formed from admixtures of IL-2-secreting cells and parental cells, had lost their ability to secrete IL-2. It was proposed that this loss of secretion may have been due to loss of the IL-2-encoding retroviral DNA sequences. Furthermore, a more rapid loss of IL-2 secretion by the FS-29 cells was observed when IL-2 and IL-4-secreting cells were admixed and injected together into mice. Therefore, it is perhaps unlikely that there was an additive anti-tumour response when IL-2 and IL-4-secreting FS-29 cells were injected together in

this study. Histological analysis of the tumour nodules that formed in animals that had received IL-2-secreting cells either alone or in combination with IL-4 or IFN-γ-secreting cells, revealed that these groups had a similar nature of lymphocytic infiltration in that they had a moderate $CD4^+$ and $CD8^+$ T-cell response and a poor B-cell response. Vaccination of mice with tumour cells engineered to express cytokines has been successful in limited cases in causing regression of established tumours. For example, it has been reported that IL-2-producing MBT-2 cells could regress growth of an orthotopically implanted MBT-2 tumour (Connor et al., 1993).

The ability of the IL-4-secreting cells either alone or in combination with IL-2 or IFN-γ-secreting cells to reduce growth of an established tumour nodule was also investigated. It was found that IL-4-secreting cells alone or in combination with other cytokine-secreting cell-lines could not prevent growth of a contralateral tumour nodule. Again, it may have been unlikely that the IL-4-secreting cells would be capable of regressing growth of an established tumour nodule, since the IL-4-secreting cells could not prevent growth of an equal number of non-secreting cells when admixed together and injected into mice (Patel et al., 1993a). However, Dranoff and co-workers (1993) reported that GM-CSF-secreting B16 melanoma cells could cause the rejection of a low inoculum (5×10^4) of non-secreting B16 melanoma cells. Therefore, the size of the primary tumour appears to be important in determining whether regression with cytokine-secreting cells is possible.

Examination of the cellular infiltration into tumours that formed during the course of the experiment revealed that, IL-4-secreting cells resulted in a moderate $CD4^+$ T-cell response with poor $CD8^+$ T-cell and B-cell responses. However there appeared to be a stronger lymphocytic infiltration into the tumour nodules that formed when the animals received a combination of IL-4 and IFN-γ-secreting cells. The B-cell response was still poor but, there appeared to be a strong CD4+ T-cell response and a moderate $CD8^+$ T-cell response. This strong $CD4^+$ T-cell response was again observed when

IL-2, IL-4 and IFN-γ-secreting cells were injected together. A strong $CD4^+$ T-cell infiltration may indicate that the IFN-γ and IL-4 combination may enhance the anti-tumour immune response. There was little peroxidase activity present in these tumours which may indicate that macrophage infiltration into the tumours was minimal.

The ability of the IFN-γ-secreting cells to regress growth of an established tumour nodule was also examined in this study. When animals received IFN-γ-secreting cells alone or in combination with IL-2 or IL-4-secreting cell-lines, growth of the primary tumour could not be prevented. It was proposed that the IFN-γ-secreting cells may reduce tumour growth, as Patel and co-workers (1993b) reported that the IFN-γ-secreting cells demonstrated significant slowing of growth of an equal number of admixed non-secreting cells. However, a tenfold excess of IFN-γ-secreting cells regressed tumours in only two of the seven tested animals. In the present study, animals received 10^6 control cells followed one week later by 10^6 IFN-γ-secreting cells. Therefore, perhaps this study should be repeated using at least a tenfold increase in the quantity of cytokine-secreting cells.

Examination of the cellular infiltration into the tumours that formed in the animals that had received IFN-γ-secreting cells alone, revealed that there was a strong $CD4^+$ T-cell response, moderate $CD8^+$ T-cell response and a poor B-cell response. When IFN-γ and IL-2-secreting cells were injected together a moderate $CD4^+$ and $CD8^+$ T-cell response and a poor B-cell response was observed. Furthermore, in the tumours that formed in the animals that had received an IL-2 and IFN-γ-secreting cell combination, four of the five tumours had large necrotic areas. This may indicate that the combination of the effector mechanisms in this group may lead to a more destructive anti-tumour response. There was little peroxidase activity in any of the groups which may indicate that macrophages were not a main effector mechanism involved in the immune response.

From the present study, it appears that the cytokine-secreting cell-lines cannot regress growth of an established tumour nodule. When the cytokine-secreting cells were injected in various combinations into mice, there did not appear to be any additive interactions that could enhance regression of the primary tumour nodule. However, there did appear to be an enhanced overall lymphocytic infiltration and extensive necrosis of the primary tumour nodule with some cytokine combination treatments. Results from other studies indicates that the original tumour burden is important in determining whether regression of the primary tumour will take place. Therefore, a more appropriate method of using these cytokine-secreting cells may be to inject the cells into animals when the primary tumour has been excised in order to prevent growth of residual tumour cells. Furthermore, administration of the cytokine-secreting cells in various combinations may enhance the cellular infiltration into the tumour resection area and therefore promote a more vigorous anti-tumour response.

Table 8.1: Regression of an Established Tumour

Animal No.	Control		IL-2		IL-4		IFN-γ	
	Survival (Days)	Tumour Size (mm)	Survival (Days)	Tumour Size (mm)	Survival (Days)	Tumour Size (mm)	Survival (Days)	Tumour Size (mm)
1	16	12.3x7.5	15	12.8x5.9	16	ascitic	15	8.6x6.6 9.8x6.5
2	17	6.7x10.0	21	10.1x8.4	17	9.2x8.3	14	10.9x4.6
3	21	9.7x8.3	15	10.0x6.6	20	5.2x4.1 8.8x5.2	28	7.7x6.2 4.2x4.6
4	17	ascitic	20	9.2x5.2 6.1x5.0	16	ascitic	26	10.8x 12.9 7.7x7.5
5	14	ascitic	14	6.6x4.1 8.2x6.4	16	ascitic	18	7.2x8.3
Median	17		15		16		18	

Table 8.2: Regression of an Established Tumour

Animal No.	IL-2/IL-4		IL-2/IFN-γ		IL-4/IFN-γ		IL-2/IL-4/IFN-γ	
	Survival (Days)	Tumour Size (mm)	Survival (Days)	Tumour Size (mm)	Survival (Days)	Tumour Size (mm)	Survival (Days)	Tumour Size (mm)
1	17	7.2x10.1	16	ascitic	19	3.3x3.2 1.0x1.0 ascitic	15	12.7x4.1 7.3x5.6
2	17	4.0x6.0 4.1x5.6 ascitic	16	ascitic	11	11.9x5.5	20	7.6x3.3 6.4x4.2 ascitic
3	18	9.9x7.4	20	7.3x5.7 10.7x8.0 ascitic	15	8.7x7.5	16	10.4x7.9
4	12	ascitic	26	14.4x 11.8 ascitic	16	ascitic	19	11.1x5.2
5	26	12.3x 11.5	27	8.2x8.1 8.9x8.5	14	3.4x13.5	16	8.6x6.9 5.8x4.5
6							16	10.3x6.8
Median	17		20		15		16	

N.T. - No Tumour.

Table 8.3 - Histological Analysis

Treatment	CD4	CD8	B220
Control + Control	+++	+++	+
Control + Control	++	++	+
Control + Control	++	+	+
Control + Control	++	++	+
Control + Control	++	++	+
Control + IL-2	+++	++	+
Control + IL-2	++	++	+
Control + IL-2	++	++	+
Control + IL-2	++	+	+
Control + IL-2	++	+	+
Control + IL-4	++	+	+
Control + IL-4	++	++	+
Control + IL-4	++	+	+
Control + IL-4	++	+	+
Control + IL-4	+	+	+
Control + IFN-γ	+++	+++	+
Control + IFN-γ	+++	++	+
Control + IFN-γ	+	+	+
Control + IFN-γ	++	++	++
Control + IL-2/IL-4	++	++	+
Control + IL-2/IL-4	+	++	+
Control + IL-2/IL-4	++	+	+
Control + IL-2/IL-4	++	+	+
Control + IL-2/IL-4	++	++	+
Control + IL-2/IFN-γ	++	++	+
Control + IL-2/IFN-γ	+++	+++	+
Control + IL-2/IFN-γ	+	+	+
Control + IL-2/IFN-γ	++	+	+
Control + IL-2/IFN-γ	+++	++	+
Control + IL-4/IFN-γ	+++	+++	++
Control + IL-4/IFN-γ	+++	+++	+
Control + IL-4/IFN-γ	+++	++	+
Control + IL-4/IFN-γ	++	++	+
Control + IL-4/IFN-γ	++	+	+
Control + IL-2/IL-4/IFN-γ	+++	+++	++
Control + IL-2/IL-4/IFN-γ	+++	++	+
Control + IL-2/IL-4/IFN-γ	+++	++	+
Control + IL-2/IL-4/IFN-γ	+++	++	+
Control + IL-2/IL-4/IFN-γ	+	+	-
Control + IL-2/IL-4/IFN-γ	+	+	+

+ Poor Response. ++ Moderate Response. +++ Strong Response.

- No Response

Table 8.4 - Description of Figures 8.1 - 8.6.

Figure	Tumour Type	Stain	Score
8.1	Control cells	$CD4^+$ T-cells	++
8.2	IL-4 +IFN-γ cells.	$CD4^+$ T-cells	+++
8.3	Control cells	$CD8^+$ T-cells	++
8.4	IL-4 +IFN-γ cells.	$CD8^+$ T-cells	++
8.5	Control cells	Haematoxylin + Eosin	Well defined tumour structure.
8.6	IL-2 +IFN-γ cells.	Haematoxylin + Eosin	Poor tumour structure.

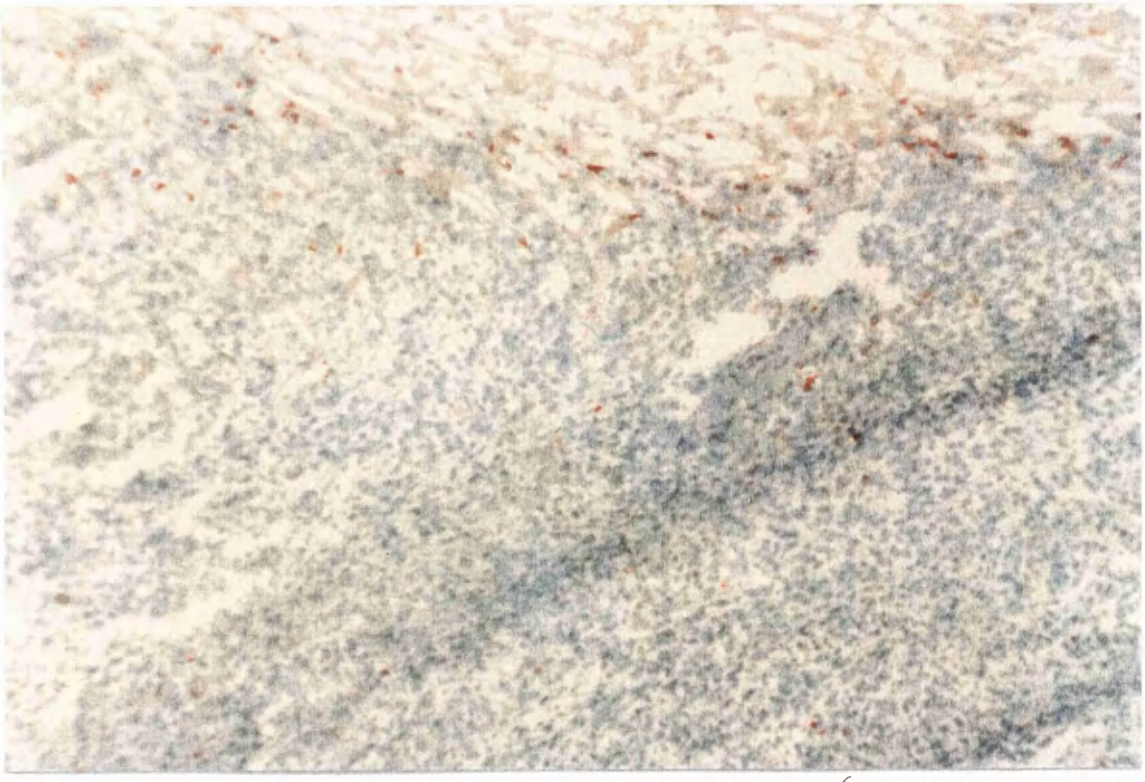


Figure 8.1 - 5μm section of tumour taken from an animal that had received 10^6 control FS-29 cells into the left flank, followed 7 days later with 10^6 control cells into the right flank. This section has been stained for CD4+ T-cells using AEC as the substrate and Harris's haematoxylin as the counterstain. The photograph shows that there is a moderate (++) CD4+ infiltration into the tumour nodule. x10 magnification.

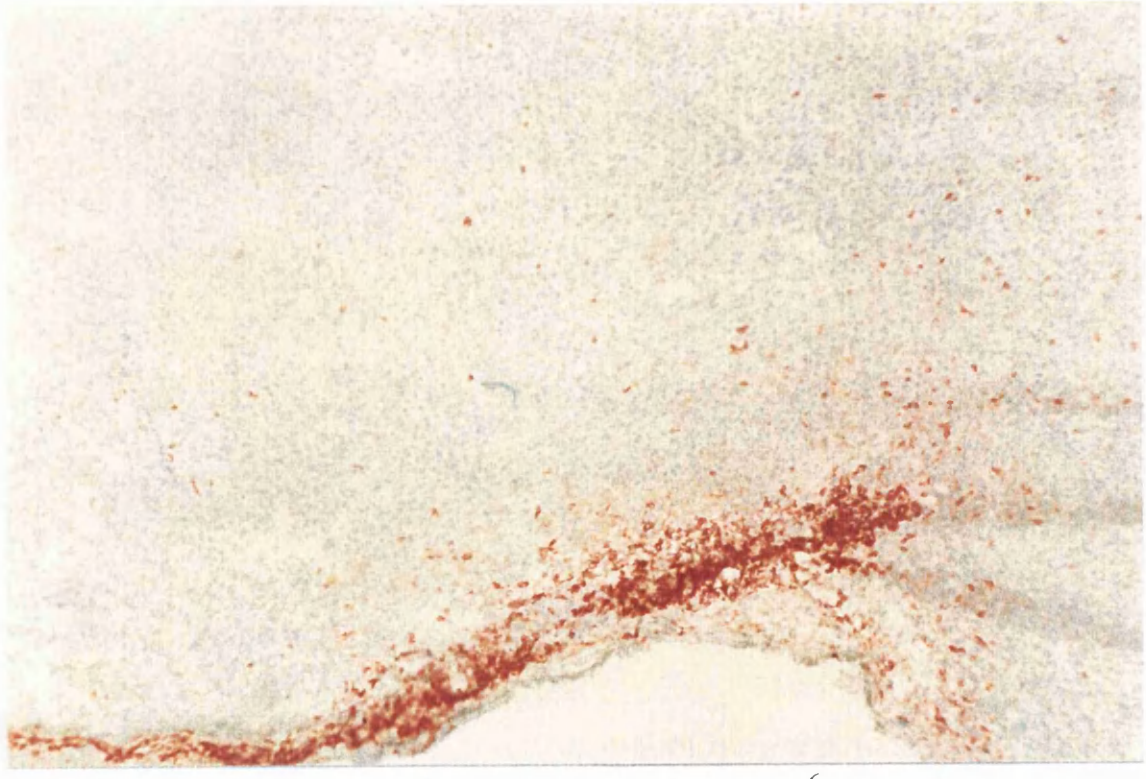


Figure 8.2 - 5μm tumour section taken from an animal that had received 10^6 control cells into the left flank, followed 7 days later with 10^6 IL-4/IFN-γ FS-29 cells into the right flank. Use of this cytokine combination resulted in a strong (+++) CD4+ infiltration into the tumour nodule. x10 magnification.

Figure 8.3 - 5μm section from a tumour nodule excised from an animal that received an injection of 10^6 control FS-29 cells, followed one week later with 10^6 control cells into the right flank. This sample was stained for CD8+ T-cells using AEC as the substrate and Harris's haematoxylin as the counterstain. The control cells induced a moderate (++) CD8+ response into the tumour. x10 magnification.

Figure 8.4 - 5μm tumour section from an animal that had received 10^6 control cells into the left flank followed one week later with 10^6 IL-4/IFN-γ secreting FS-29 cells into the right flank. With this cytokine combination there appears to be an overall stronger (+++) CD8+ T-cell response into the tumour. x10 magnification.

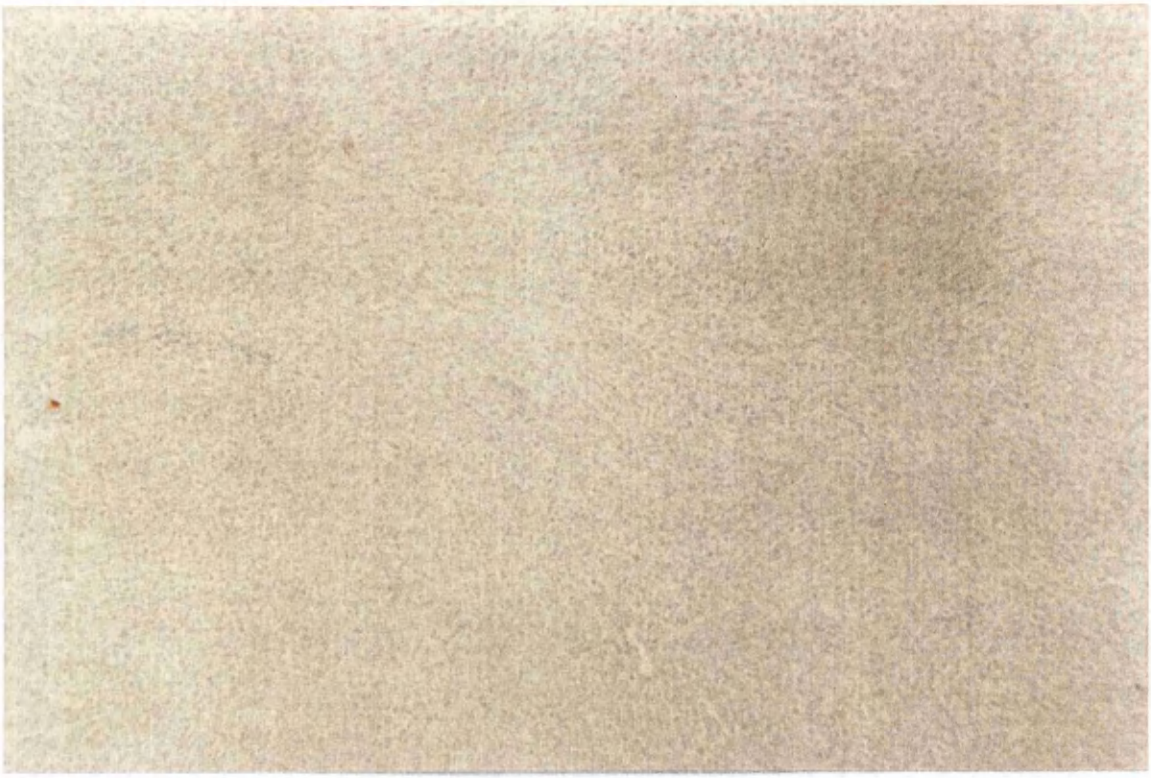


Figure 8.5 - Haematoxylin and eosin staining of a tumour excised from an animal that had received 10^6 control FS-29 cells into the left flank, followed 7 days later with an injection of 10^6 control cells into the right flank. This 5μm section of tumour shows a well defined structure with no necrosed areas. x10 magnification.

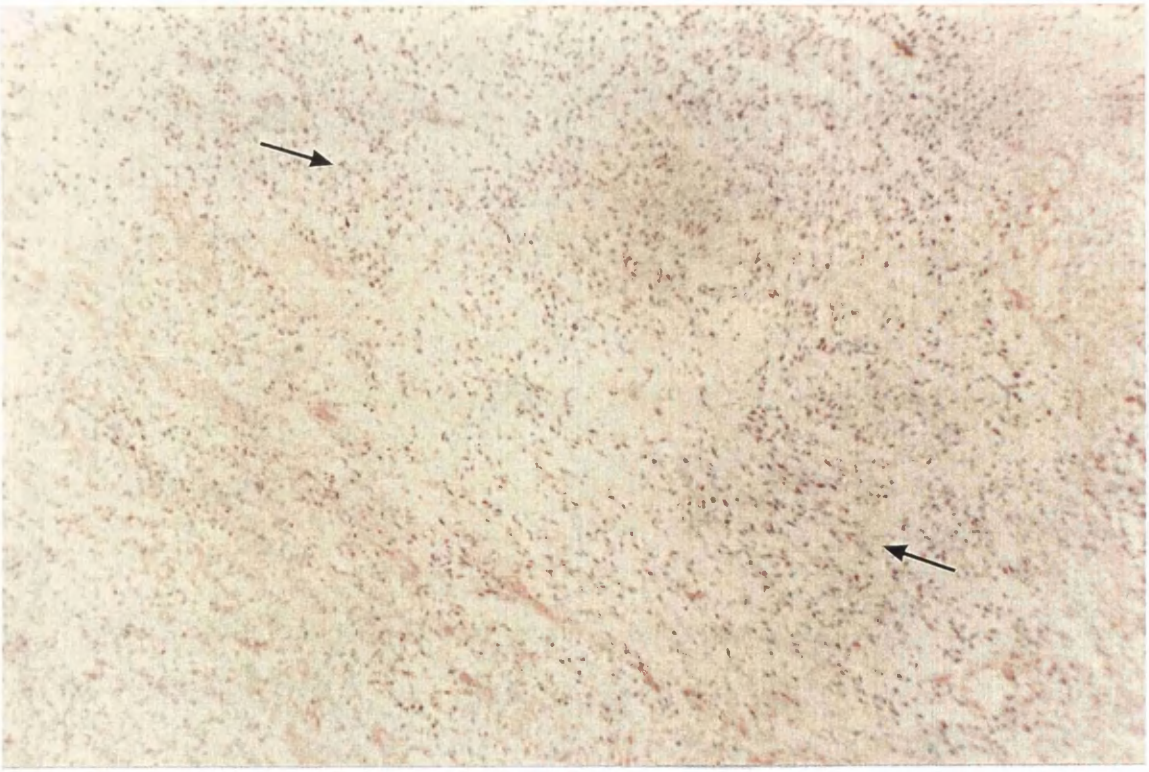


Figure 8.6 - Haematoxylin and eosin staining of a tumour excised from an animal that had received an initial injection of 10^6 control FS-29 cells into the left flank, followed by 10^6 IL-2/IFN-γ secreting FS-29 cells into the right flank. The structure of this tumour is very poor in comparison to the control. There are large areas that appear to be necrosed tissue (indicated by the arrows). x 10 magnification

9: Conclusion

In section 3.12 the aims of this thesis were defined as follows;

9.1 Aim 1

This was to address the following questions in an animal tumour model. When animals are given a two-site injection of cells is there a systemic anti-tumour reaction that prevents growth of the tumour cells at both sites and is this systemic protection dependant on the cytokine that the tumour cell secretes? Furthermore, is the ability to promote systemic protection dependant on the tumour burden?
What are the possible effector mechanisms involved in the anti-tumour response in this study?

There have been several attempts over the years to exploit cytokines as an immunotherapeutic treatment for cancer. However, success with cytokines has been limited mainly due to the toxic side-effects of these compounds. Therefore, several other routes of administration have been employed in an attempt to reduce toxicity and improve efficacy with various degrees of success (Retsas et al., 1989; Hill et al., 1994). It was the first successful cytokine gene cell therapy study by Tepper and co-workers in 1989 that triggered more studies into the effectiveness of rendering tumour cells more immunogenic by enabling them to secrete cytokines. The aim of the first study was to further assess the growth rates of the FS-29 cell-line which has been engineered to secrete cytokines (Chapter 6). The animals in this study received a two-site injection of either 10^5 or 10^6 control FS-29 cells or cytokine-secreting cells into each flank. It was observed that the IL-2-secreting cells did not grow more slowly in either the 10^5 or 10^6 cell injection groups. However, this cell-line appeared to induce a stronger T-cell response than the non-secreting cells into the tumour nodules that formed but, this infiltration did not promote an effective anti-tumour response. The IL-4-secreting cells grew significantly slower than the non-secreting cells, however this was only in the animals that received the larger tumour cell number. Histological analysis of the tumour nodules that formed in the IL-4 treatment group revealed that

there was a moderate T-cell infiltration into the tumour nodule. Furthermore, the tumour nodules that formed from the IL-4-secreting cells appeared to have a strong infiltration of peroxidase-positive cells into the tumour nodule, which may be macrophages and therefore, macrophages may be an important effector mechanism in the IL-4 anti-tumour response. The IFN-γ-secreting cells grew significantly slower than the control cells but, again this was only in the animal treatment group that had received 10^6 cells. As there were only two tumours that formed in the IFN-γ treatment group, little indication of the effector mechanisms that may be involved in the IFN-γ anti-tumour response could be gained. However, T-cells may be an important effector mechanism as there was a moderate infiltration of $CD4^+$ and $CD8^+$ T-cells into these tumour nodules. Therefore, from this work it would appear that the IFN-γ and the IL-4-secreting FS-29 cells but, not the IL-2-secreting FS-29 cells, demonstrate reduced tumourigenicity in vivo. Furthermore, it would appear that large numbers of cytokine-secreting cells are required for an effective anti-tumour response and that the possible effector mechanisms involved in the anti-tumour response remain unclear.

9.2 Aim 2

This was to address whether animals that have previously received secreting or non-secreting cells, which then grew slowly or did not grow at all, transfer immune memory to a naive animal and therefore protect against a subsequent tumour challenge?

Furthermore, what are the possible effector mechanism involved in the transfer of anti-tumour immune memory between animals?

It has been reported that when animals have rejected a challenge with cytokine-secreting cells and have subsequently rejected a challenge of non-secreting cells that this protective response appeared to correlate with tumour-specific cytotoxic T-cells in the spleens of these animals (Gansbacher et al., 1990a). This study was carried out to assess the ability of tumour-specific T-cells to transfer anti-tumour memory

between animals (Chapter 7). Unfortunately none of the animals that had received either cytokine-secreting or control FS-29 cells appeared to transfer anti-tumour protection to naive animals. However, the treatment groups were small in this study and as some of the animals did reject tumour growth, it may be that further studies with larger animal group numbers may give a better indication as to the potential of splenocyte transfer.

It was of interest that the group that received 10^7 splenocytes from an animal that had previously received 10^5 IFN-γ-secreting cells and had not developed a tumour, demonstrated slower tumour growth rates when challenged with 10^6 control FS-29 cells. Only one of the four animals developed a small slow growing tumour in this treatment group. However, the animals that received splenocytes from an animal that had previously received 10^6 IFN-γ-secreting cells and did not develop a tumour had more animals in this group developing tumours, with four of the five animals developing tumours. Therefore, this study suggests that the original tumour burden of the animal may be important when determining whether splenocyte transfer is capable of transferring anti-tumour memory. Little information has been gained as to the effector mechanisms that may be involved in the transfer of anti-tumour memory as only one tumour formed in the most successful group. However, there was a moderate $CD4^+$ T-cell infiltration and a poor $CD8^+$ T-cell infiltration into this tumour nodule.

9.3 Aim 3

This was to address whether the cytokine-secreting cell-lines are able to slow or regress growth of an already established tumour? Furthermore, are there any additive or antagonistic interactions of the cytokine secreting cells when administered in various combinations?

What are the effector mechansim involved in regressing growth of an established tumour nodule and is the inflammatory infiltration enhanced or reduced when cytokines are administered together?

It has been reported that when cytokine-secreting tumour cells are admixed with control tumour cells and injected into animals, tumour growth may be slowed or prevented (Patel et al., 1993a, 1993b). Studies have indicated that the efficacy of cytokine gene therapy may be enhanced if cytokines are administered in combination (Ohira et al., 1994). However, in the present study none of the IL-2, IL-4 or IFN-γ-secreting cell-lines either alone or in various combinations was capable of regressing growth of an established tumour nodule (Chapter 8). However, there did appear to be an overall stronger T-cell infiltration into the tumour nodules that formed when the IL-4-secreting cells and the IFN-γ-secreting cells were administered together. The present study and the work reported by Patel and colleagues (1993a, 1993b) indicates that the initial tumour burden is important in determining the extent of the anti-tumour response, in that cytokine-secreting cells are capable of reducing growth of an equal number of admixed tumour cells at most. Patel and colleagues (1993b) reported that the IFN-γ-secreting cells had to be present in a tenfold excess in comparison to the control cells in order for complete tumour regression to take place. Therefore, it is likely that a large number of cytokine-secreting cells would have to be administered in order to regress tumour growth in humans and in particular those with advanced disease.

From the present studies and those of Patel and co-workers (1993a and 1993b) it can be concluded that the IFN-γ-secreting FS-29 cells are capable of producing a more effective anti-tumour response in comparison to that of the IL-2 or IL-4-secreting cells. However, it is not known which effector mechanism is responsible for the anti-tumour response, whether it be cytotoxic lymphocytes or macrophages. Clearly, experimental work to determine this effector mechanism is required and such information may enable the development of the optimum anti-tumour response in this animal model.

9.4 Clinical Studies Using Cytokine Gene Cell Therapy

Although the animal studies using cytokine gene modified tumour cells are encouraging, there are a number of considerations that have to be taken into account that will determine whether this approach to cancer therapy will have value in clinical trials. For example most human tumours are difficult to establish as cell-lines and autologous cell lines will have to be established from each patient to be treated. There is also the problem that after extended passage, the antigenic composition of the tumour cell may change in comparison to the primary tumour from which the cell originated. Once the patients cell-line has been generated, high efficiency gene transfer systems are required to genetically alter these primary tumour cell-lines. These systems must be capable of consistently transferring the gene into the cell and must also allow the continual expression of the cytokine. The use of retroviral vectors appears to overcome some of these problems, and may allow efficient gene transfer into primary tumour explants (Jaffee et al., 1993).

Future vaccination of patients with cytokine-secreting tumour cells will require methods to prevent tumour formation by gene-modified tumour cells in vivo. However, it is important that any procedures used should not diminish the capability of gene-modified tumour cells to secrete cytokines, and should not abolish their immunogenicity. High dose γ-irradiation has been successfully tested in some cell lines. Dranoff and co-workers (1993) reported that GM-CSF-secreting melanoma cells were still effective at stimulating potent, long-lasting and specific anti-tumour immunity when irradiated. When the renal cancer cell line, SK-RC-29, was irradiated, growth of the cytokine gene-modified cells was prevented in vivo, and their ability to release cytokines and express antigenic determinants, such as HLA-DR, appeared to be unaffected (Gastl et al 1992).

A problem of this new type of therapy is that these cytokine-secreting cells may be given to patients with advanced disease when established chemotherapy regimens

have failed. If this is the case it would appear likely that the tumour burden will be too large for the cytokine-secreting cells to have a measurable effect. Therefore, it may be that the most efficacious regimen for this type of therapy is to administer cytokine-secreting cells to patients who have received their primary surgery, in order to eradicate any remaining local disease.

This present study and that of Patel and co-workers (1993b) indicates that IFN-γ-secreting tumour cells may be an effective anti-tumour therapy. Previous clinical trials administering IFN-γ subcutaneously to metastatic renal cancer and non-small cell lung cancer patients demonstrated varied clinical responses and were associated with severe toxicity (Weiner et al., 1994; Shaw et al., 1995). Therefore, it is hoped that the use of cytokine-secreting tumour cells may increase the efficacy and reduce the toxicity associated with IFN-γ. A trial using IFN-γ-secreting tumour cells for the treatment of metastatic melanoma is underway and it is hoped that the clinical results will have similar success to that of the animal studies (Seigler et al., 1994). From the literature it would appear that such studies in cancer patients are in their infancy, and it may be some time before the therapeutic merits of cytokine gene cell therapy are known.

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