THE ROLE OF CYTOKINES, COAGULATION AND FIBRINOLYSIS IN LEUCOCYTE AND LAK CELL CYTOTOXICITY OF TUMOUR CELLS

A Thesis submitted for the degree of Doctor of Philosophy

by

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ABSTRACT

Interleukin-2 activates lymphocytes to become highly cytotoxic for a wide range of tumour cell types *in vitro* (lymphokine activated killer or LAK cells), and in animal models. However, only limited therapeutic benefit was observed in clinical trials of LAK cell therapy. This project aimed to investigate the molecular and cellular interactions involved in the production and effector functions of LAK cells, to identify factor(s) which might be responsible for the poor clinical responses observed in LAK cell therapy.

Tumour cell lines were heterogeneous in their response to killing by cytokines (TNFα, LT, IFNγ and IL-1β), and purified monocytes or lymphocytes, but were consistently highly sensitive to LAK cell cytotoxicity. Autologous monocytes and lymphocytes were not killed by LAK cells, in contrast to human umbilical vein endothelial cells and fibroblasts. Supernatants from LAK cells were considerably less cytotoxic than the effector cells, and physical separation of effector and target cells resulted in inhibition of killing. Lymphocyte and LAK cell cytotoxicity was associated predominantly with the CD8+ (cytotoxic T-cell) lymphocyte sub-population, and was significantly inhibited by anti-TNFα and anti-LT, demonstrating that these cytokines were the primary effector molecules in this system. LAK cells and A375 melanoma cells showed procoagulant activity, predominantly via the tissue factor pathway, and LAK cells also possessed surface factor V. In addition, A375 cells were highly fibrinolytic. Tumour cell killing by LAK cells was inhibited by plasma, and further experiments determined that polymerised fibrin, but not fibrin monomer was responsible.

From these results it was suggested that culture of small numbers of cells from tumour biopsies, and the determination of their sensitivity to cytotoxic drugs, cytokines and effector cells may lead to more effective treatment protocols for immunotherapy of individual tumours. In order to enhance the efficacy of immunotherapy, further *in* vivo research is required to elucidate the interactions between immune effector cells and the coagulation/fibrinolytic systems.

CHAPTER 1

INTRODUCTION

1.1.1 Epidemiology of Cancer

The treatment of most cancers has changed little in the past twenty years. Radiation therapy, surgery and cytotoxic drugs, or a combination of these, continue to be the most widely used and are thus considered to be the most effective treatments available. Some success has been achieved using these therapeutic approaches with a number of the rarer cancers, but the overall death rate (USA) due to cancer continues to rise. Although the large increase in lung cancer incidence is primarily responsible for the observed rise in death rate, other cancers such as non-Hodgkin's lymphoma, multiple myeloma, and cancers of the prostate, brain, kidney, oesophagus and breast have shown considerable increases in incidence and death rate in recent years (Bailar and Smith, 1987).

1.1.2 Malignant Melanoma

Cutaneous malignant melanoma, which is still one of the rarer cancers, is increasing at a faster rate than any other cancer in the USA, Australia, northern Europe and Canada (Muir and Nectoux, 1982). The incidence of melanoma in the USA has doubled every 10-20 years since 1950 (Rigel *et al.*, 1987). Exposure to sunlight appears to be the most significant factor involved in the observed increase. This is probably due to changes in lifestyle which include altered dress habits, more holidays in sunny climates and possibly depletion of the ozone layer. Other risk factors, such as fluorescent lights (Maxwell and Elwood,1983), exposure to chemicals and ionising radiation (Wright *et al.*, 1983; Austin and Reynolds, 1984; Holman *et al.*, 1986), steroid hormones (Prentice and Thomas, 1987) and diet (Mackie *et al.*, 1980) would appear to be considerably less important than exposure to the sun. Unlike most cancers, the incidence of melanoma does not increase linearly with age. The incidence curve rises steeply prior to age 50, and increases less

rapidly in the older age groups (Devesa *et al.*, 1987). World-wide, the incidence of melanoma varies over 100-fold, from a low of 0.2 per 100,000 person years in parts of Japan, to approximately 40 per 100,000 person years in Queensland, Australia (Green, 1984; Muir *et al.*, 1987) . Although still comparatively rare, melanoma is rapidly becoming an important cause of disease and death among white populations. This epidemiological data would suggest that current treatment protocols are not sufficient in themselves to influence significantly the increasing death-rate due to the majority of cancers (Bailar and Smith, 1987).

1.1.3 Clinical Presentation of Melanoma

Malignant melanoma is a neoplastic disease characterised by uncontrolled proliferation of melanocytes, the pigment producing cells of the skin, meninges, substantia nigra, and the eye. There are various forms of cutaneous malignant melanoma (Table 1), but the progression of disease in each form follows a similar pattern of growth and clinical deterioration (Roses *et al.*, 1983).

1. Superficial Spreading	♦ **Represents 70% of cutaneous melanomas** ♦ **Amelanotic regression areas often seen** ♦ **More prevalent in women than men**
2. Nodular	♦ **15 -30% of cutaneous melanomas** ♦ **More aggressive than superficial spreading and quicker developing** ♦ **Incidence greater in men than women**
3. Lentigo Maligna	♦ **Less likely to metastasise** ♦ **4 - 10% of patients**
4. Acral Lentiginous	♦ **Occurs principally on palms and soles** ♦ **More frequently presents in older patients**

TABLE 1.1. Growth patterns of cutaneous melanomas.

The most benign types of melanoma are very slow growing and, apart from a visible skin lesion, symptomless. An example of this is lentigo maligna, which commonly presents in the sixth and seventh decades of

life and may take several years to become invasive. Generally, the first sign of invasion by melanoma in terms of growth pattern is an increase in melanoma cell proliferation in the horizontal or radial dimension across the surface of the skin. An example of a primary melanoma in the radial growth phase is superficial spreading melanoma. Further neoplastic progression occurs by melanoma cell proliferation in the vertical plane of the skin, a feature seen in the presentation of nodular melanoma. Acral lentiginous and mucosal melanomas are, in most cases, identical to the other three main forms of primary skin-associated melanoma. These melanomas develop in stratified squamous epithelium but the sites are on palms and soles, or on mucosal surfaces respectively. Table 2 shows the clinical staging of melanoma progression as agreed by the American Joint Committee on Cancer.

Stage	**Description**
1a	**Localised Melanoma <0.75mm**
1b	**Localised Melanoma).76mm - 1.5mm**
2a	**Localised melanoma 1.5mm - 4mm**
2b	**Localised Melanoma >4mm**
3	**Limited nodal metastases involving only one regional lymph node, or less than 5 in transit metastases but without nodal metastases**
4	**Advanced regional metastases or any patient with distinct metastases**

TABLE 1.2. American joint committee on cancer staging scheme. Distances in millimetres represent the depth of tumour invasion into the dermis. Higher stage numbers indicate worsening prognosis for the patient.

1.1.4 Metastasis of Melanoma

The final growth patterns observed in the progression of melanoma include the formation of local and distant metastases which tend to proceed rapidly from a vertical growth phase. Frequently, a diagnosis of melanoma is not made until metastases are already in abundance and some degree of primary tumour site regression may have occurred as well. The onset of metastasis in malignant melanoma indicates a very poor prognosis for treatment and recovery. Melanoma cells initially metastasise locally, forming starburst-like satellite nests of neoplastic cells around the primary tumour, and then distant metastases are established via the lymph vessels or skin capillaries. In general, distant metastasis is likely to have occurred if neoplastic cells have invaded the reticular dermis (about 0.75mm from the dermo-epidermal junction; Roses *et al.*, 1983). Distant metastases then develop very rapidly, involving most internal organs in a diffuse fashion. As yet, no discrete patterns of secondary involvement have been observed. In a study of 216 post-mortems with histologically proven metastatic melanoma, involvement of nearly all organs was observed (Patel *et al.*, 1978). The organs which were most commonly involved were the lung (71.3%); liver (53.8%); brain (54.6%) and bone (48.6%). Lymph nodes were involved in 75% of cases . An intermediate stage of metastasis is often described as in-transit metastasis. This classification describes lesions greater than 5cm from the primary tumour and is believed to precede distant metastasis, although the presence of these lesions usually indicates that distant metastatic spread has already occurred.

1.1.5 Spontaneous Regression of Melanoma

Melanoma is an unusual cancer in that it exhibits a relatively high level of spontaneous regression of the primary growth (7-8% (Smith and Stehlin, 1965; McGovern.,1972). The only other malignant tumour that has demonstrated this phenomenon to any significant degree is renal cell carcinoma, which has a spontaneous primary regression rate of 1-2% (de Riese *et al.*, 1991). Regression of melanoma may occur to

varying degrees, from a slight decrease in the size of the tumour with accompanying hypopigmentation and fibrosis, to complete destruction of the primary melanoma with residual fibrosis and melanosis (presence of numerous melanophages filled with melanin). Unfortunately, primary melanoma regression is not usually considered a good prognostic sign, as metastases are often observed concurrently or shortly after regression. This phenomenon may account for the presentation of malignant melanoma in a disseminated form with no detectable primary site. A study published in 1972 (McGovern.,1972) reported 7% of 613 cases of melanoma where metastases were diagnosed with no evidence of a primary tumour, showing how commonly complete primary tumour regression may occur.

Histological observations of melanoma regression include a lymphocyte infiltration of the papillary dermis that ranges from a sparse perivascular population to a full thickness band of lymphocytes in the papillary dermis beneath the melanoma (*i.e.* melanoma still confined to the epidermis). Observations indicate that these lymphocytes are capable of cytotoxicity to melanoma cells that breach the dermo-epidermal junction and enter the papillary dermis and to a lesser extent to the lesion still confined to the epidermis. Other accompanying changes following regression are thickening and fibrosis of the papillary dermis with dilatation of the vascular spaces and variable numbers of dermal melanophages. The lymphocyte infiltrate also disappears following regression, implying that the response is specifically mounted against the melanoma cells. Table 3 summarises the clinical manifestations of melanoma regression (McGovern, 1972).

♦ Inflammatory nodule with or without pigmentation.
♦ Scarring in a primary cutaneous melanoma.
♦ Melanoma composed of separate pigmented lesions.
♦ Pigmented focus with depigmented halo.
♦ Pigmented scar with or without microscopic islands of surviving melanoma cells.
♦ Metastatic melanoma with no demonstrable primary tumour.

TABLE 1.3. Clinical Manifestations of Melanoma Regression.

1.1.6 Conventional Treatment of Melanoma

Conventional treatment of melanoma falls into two categories. Firstly, the treatment of the primary lesion and secondly, the treatment of metastatic disease. Primary lesions in the earlier stages of growth without metastatic capability are treated surgically with wide excision (usually at the same time as biopsy). This is by far the most effective means of treatment provided early detection has been possible. In the presence of secondary deposits and/or a large primary that makes adequate surgery impractical, chemotherapeutic protocols have been used to attempt cure or provide palliative treatment. Chemotherapy has included systemic administration or local perfusion (arterial cannulation) of limbs with Actinomycin-D. Treatment of advanced metastatic melanoma is still one of the biggest problems in clinical practice. In terms of chemotherapy Actinomycin-D with or without Dacarbazine demonstrates some palliative efficacy (Hochster *et al.*, 1985). Other cytotoxic drugs used in the treatment of advanced melanoma include Melphalan (Coit, 1994) and Vinblastine (Elliott *et al*, 1994).

1.2.0 Immunity and Cancer

1.2.1 Background

Immunological responses directed against tumours have been recognised for over a century, when it was observed by surgeons that tumour necrosis and regression occasionally follows bacterial infection in cancer patients. In 1893, William Coley, a New York surgeon, began to

investigate this phenomenon by producing a filtrate of bacterial cultures termed "Coley's Mixed Toxins", and injecting it into patients with various types of advanced cancer. The treatment was continued for six months to a year for each patient (Coley-Nauts *et al*, 1953). He noticed that more effective responses to this treatment were obtained when a marked fever was induced. Coley carried out clinical trials with his toxins, evaluating their efficacy as compared to radium treatment and amputation. In one study concerning 170 operable cases of long bone malignancy, he found that more effective clinical responses were observed when amputation was followed by toxin therapy, which gave a 50% survival rate for 3-33 years. Amputation alone allowed a maximum survival of 3 years (Coley and Coley, 1926).

Coley's work was later reviewed by his daughter, where she detailed 30 complete remissions, which were representative of 270 complete remissions from 1,200 patients treated with toxins (Coley-Nauts *et al.*, 1953). However, by the late 1930's this form of treatment was largely abandoned in favour of radiotherapy and chemotherapy, which together with surgery are still the major forms of cancer treatment today.

Since the studies of Coley, research in tumour immunity has progressed in animal models, and more recently in human cell lines to identify specific tumour antigens which are recognised by either the humoral or cellular immune systems, which have been extensively reviewed. (Ferrone, 1990, Oettgen, 1989)

1.2.2 Cellular Immunity and Cancer

1.2.2.1 Background

In 1959, Thomas expanded upon an earlier theory of "aberrant germs" previously proposed by Ehrlich, to propose that the specific, adaptive immune response may have evolved so as to protect the body from neoplasia. This theory, termed the immune surveillance theory (Burnet, 1970) stated: "*The thesis is that when aberrant cells with proliferative potential arise in the body they will carry new antigenic determinants on their cell surfaces. When a significant amount of new antigen has*

developed, a thymus-dependent immunological response will be initiated and eventually eliminates the aberrant cells in essentially the same way as an allograft is destroyed." According to this theory cancer would only develop due to some failure of immune function, such as immunosuppression, or by adoption of immune evasion mechanisms by the tumour. Tumour-specific antigens have been relatively difficult to identify for most tumour types. Of the few tumours which express specific antigens, the majority are associated with virally induced cancers, so that immune surveillance is currently considered to be directed primarily against virus infected cells, rather tnan nascent tumours (Martz and Howell, 1989). Only the minority of cancers expressing appreciable levels of specific antigen (*e.g.*, melanoma, renal cell carcinoma) are considered true candidates for immune surveillance (Klein and Klein, 1977), although it appears that the immune system 'selects' for the least immunogenic clones of a tumour, which then progresses unhindered by further immune involvement. Current strategies for cancer treatment therefore focus on augmenting the immune system, so as to increase its surveillance for, and subsequent destruction cancer cells. In addition to the specific cellular immune response to cancer described above, certain cytotoxic lymphocytes, lymphokine-activated killer (LAK) cells, natural killer (NK) cells and macrophages recognise and kill tumour cells by a mechanism which does not require sensitisation to antigen, and is not major histocompatibility complex (MHC) restricted.

In the late 1980's, clinical trials were undertaken by Rosenberg's and other groups (Rosenberg *et al.,* 1986; Kawakami *et al*, 1988; Heo *et al*, 1987), in which tumour infiltrating lymphocytes (TIL) were removed from excised tumours and stimulated with Interleukin-2 (IL-2) in the presence of cellular material from the tumour (to stimulate antigen-specific lymphocytes) for weeks to months. In the majority of tumours tested, the predominant population of lymphocytes present were CD3+, CD8+ T-cells, which were highly cytotoxic for autologous, but not allogeneic, tumour cells *in vitro*. After expansion of this cell population, the cells were reinjected into the cancer patient in order to promote tumour regression.

1.2.3 Effector Cells of the Cellular Immune Response to Cancer

1.2.3.1 Cytotoxic T-Cells

Cellular cytotoxicity by lymphocytes was first shown by Govaerts in 1960 where he described killing of donor kidney epithelial cells *in vitro* by thoracic duct lymphocytes from dogs which had previously rejected a kidney allograft. These observations were expanded by other workers to include lymphocyte cytotoxicity against virally infected cells (Gardner *et al*, 1974) and autologous, as well as, transplanted tumours (Clark, 1988). The effector cells in these experiments are now known as cytotoxic T lymphocytes.

Cytotoxic T-cells (Tc) are defined as a heterogeneous subpopulation of lymphocytes which are able to recognise and kill suitable target cells in several distinct ways. The majority of Tc are CD8+ and recognise antigen in the presence of MHC Class I molecules, whereas CD4+ lymphocytes (comprising approximately 10% of the Tc population) recognise antigen in the presence of MHC Class II (Erb *et al.*, 1990). Specific recognition of antigen by T-cells is achieved via the αβT-cell receptor (TCR). A major difference observed between CD8+ Tc and CD4+ Tc was that in a population of two or more different target cells CD8+ Tc would only kill those target cells which express specific antigen, whereas CD4+ Tc also killed other cells mixed with cells bearing specific antigen ("bystander killing"; Gromkowski *et al.*, 1988). More recent evidence has demonstrated that both specific (cell contact dependent), and bystander killing (release of soluble effector molecules), can be induced by both CD8+ (Burrows *et al.*, 1993; Hakim *et al.*, 1991) and CD4+ T-cells (Gravelle and Ochi, 1989; Yasukawa, *et al.*, 1993).

Some CD8+ Tc may also kill target cells in an non-MHC restricted fashion, particularly after stimulation with IL-2 in tissue culture (see section 1.3.3). These cells are cytotoxic to a wide range of tumour cell lines, including many which are insensitive to NK cell killing. Cytotoxicity is not mediated by the TCR, but by the induction of cell surface NK-like receptors (*e.g.*, CD56) on these cells in culture. In addition, killing of NK

resistant target cells may be mediated via LFA-1, CD2 (Chan *et al*, 1989) and CD45 (Starling, *et al.*, 1989; Bell *et al.*, 1993)

Another T-cell subpopulation possessing the γδTCR (γδT-cells), is cytotoxic for allogeneic target cells after recognition via their TCR. Although this would seem to be antigen specific MHC dependent killing, recent reports suggest that γδT-cells recognise a limited number of antigens which are associated with CD1c (Faure *et al.*, 1990), which resembles MHC Class I molecules and binds to β2-microglobulin. In addition, some γδT-cells are cytotoxic for tumour cells in an MHC unrestricted fashion, triggered directly via the γδTCR, or alternatively by the recognition of non-polymorphic MHC-related molecules (*e.g.* CD1). These functions of γδT-cells may represent distinct functional subpopulations within the γδT-cell subset.

1.2.3.2 Natural Killer Cells and K Cells

Natural killer cell (NK cells) comprise about 15% of human blood lymphocytes, and constitute the large granular lymphocyte (LGL) population in peripheral blood. They are characterised by their expression of CD16 (FcγRIII) and CD56, and are distinguished from T- and B-cells by the absence of either surface immunoglobulin or TCR. Little is known about the lineage of NK cells, though they and T-cells are thought to arise from a common precursor in the bone marrow (Sanchez, *et al*, 1994). The main functions of NK cells include inhibition of the early stages of viral infection, as well as resistance to tumours and other infectious diseases. They adhere to antibody coated target cells via CD16-Fc binding, and induce cell death by antibody-dependent cellular cytotoxicity (ADCC, Perussia *et al*, 1984). A subpopulation of LGL are only able to mediate cytotoxicity via ADCC and are defined as K-cells (Wahlin and Perlmann, 1983). In addition to ADCC, the majority (95%) of LGL also kill target cells via a CD16 independent pathway (*i.e.*, independent of Fc receptor binding to antibody coated target cells), which has not been fully characterised, although recent evidence suggests that a specific NK receptor exists. (Daniels *et al.*, 1994). The

molecular nature of the ligand(s) for this receptor are unknown, but there is evidence that NK cells bind to specific carbohydrate residues on cell surface glycoproteins (Bezouska *et al.*, 1994), as well as laminin (Gismondi *et al.*, 1992) and sulphated glycolipids (Young *et al.*, 1980; Yogeeswaran *et al.*, 1981). Although NK cells are cytotoxic for a wide variety of tumour cells, some target cell lines are resistant to NK activity, implying a degree of specificity. Thus, it has been postulated that various subpopulations of NK cells exist, with different target cell specificities. Since natural killer cells are rarely seen in solid tumours, their main antitumour activity may involve the suppression of metastatic tumour cells entering the circulation (Hanna and Fidler, 1980).

1.2.3.3 Lymphokine Activated Killer (LAK) Cells

Lymphokine activated killer cells were first described by Yron in 1980, and were originally thought to be a new class of lymphocyte derived cytotoxic cell (Grimm *et al.*, 1982). Incubation of lymphoid cells with IL-2 results in non-antigen dependent, non-MHC restricted cytotoxicity against a wider range of tumour cells than observed for NK cells or Tc. Since LAK cells are derived from a variety of lymphoid precursors, the term LAK is a description of a cellular activity rather than a specific cell type, *i.e.*, lymphokine-activated killer (LAK) cell activity (Maghazachi *et al.*, 1988). Other cytokines such as tumour necrosis factor-α (TNFα), lymphotoxin (LT) and interleukin-1 (IL-1) augment LAK activity by synergising with IL-2, probably by upregulating the α-chain (p55) of the IL-2 receptor. Lymphokine activated killer cell production is inhibited in man (but augmented in mice) by IL-4, possibly by the inhibition of transcription of a number of genes including that coding for the α-chain of the IL-2 receptor (Peace *et al.,* 1988). Lymphokine activated killer cells differ from NK cells in their cytotoxicity for NK resistant cells (*e.g.*, Daudi, Raji), including freshly isolated tumour cells (which are rarely susceptible to NK cell cytotoxicity). In addition, LAK cells require induction for at least 24-48h (but will remain active for months if continuously cultured in the presence of IL-2) to show optimal activity,

whereas NK cells are cytotoxic to suitable target cells immediately after isolation from peripheral blood.

1.2.3.4 Monocytes and Macrophages

Mononuclear phagocytes perform a wide variety of functions ranging from microbicidal, phagocytic, immunoregulatory (Unanue *et al* 1984) and tumouricidal (Colotta *et al.*, 1985). They can also modulate the function of other immune cells such as T- and B-cells, *i.e.* in antigen presentation, the production of mitogenic factors, suppression of cell proliferation (Varesio *et al.*, 1979), cytokine production (Varesio and Holden, 1980a) and protein synthesis in lymphocytes (Varesio and Holden, 1980b). However, the mechanisms by which macrophages exert these effects are not fully understood. There may be several macrophage subpopulations, as differences in cell morphology, surface markers and density have been observed (Fishman, 1980). However this heterogeneity may also be ascribed to differences in anatomical location and the various states of macrophage activation (Springer *et al.*, 1979).

Cells of the macrophage lineage have the ability *in vitro* to become cytostatic or cytotoxic to certain tumour cell lines but are not cytotoxic to non-transformed cells. These effects may either be spontaneous (Tagliabue *et al.*, 1979) or may be stimulated by specific activation agents. Tumouricidal activity has been reported for non-activated monocytes and macrophages (Tagliabue *et al.*, 1979), but there is some controversy as to the validity of these claims (Adams, 1980). It has been proposed: (1.) that monocyte preparations might be contaminated with NK cells (Freundlich *et al.*, 1984); (2.) that the effector cells are unintentionally activated by the cell separation procedure chosen, or (3.) that the reagents used in the assays are contaminated with endotoxin.

Monocytes and macrophages can be activated to the tumouricidal state by a variety of substances such as supernatants from mitogen stimulated lymphocytes, lipopolysaccharide (LPS) (Kildahl-Andersen and Nissen-Meyer, 1984; Horwitz *et al.*, 1979; Chen *et al.*, 1986), muramyldipeptide (MDP) (Lopez-Berestein et al., 1983), interferons (Jett *et al.*, 1979;

Fischer *et al* 1983; Feinman *et al.*, 1985), interleukin 1 (IL1) (Onozaki *et al.*, 1985), interleukin-2 (Malkovsky *et al.*, 1987), double stranded RNA (dsRNA) and the calcium ionophore A23187 (Drysdale *et al.*, 1983).

From studies into the mechanisms of action of these compounds four discrete stages of macrophage activation have been proposed (Johnson *et al.*, 1983) (Adams and Marino, 1981): (1.) resident macrophages which do not possess markers of inflammatory macrophages, (low phagocytosis of immunoglobulin coated erythrocytes, secretion of plasminogen activator and increased levels of 5' nucleotidase) and which do not secrete cytotoxic proteases. These cells are not tumouricidal; (2.) in response to a stimulant the macrophages express the markers of inflammatory macrophages and can be triggered to become tumouricidal; (3.) macrophages will bind to tumour cells but are still unable to kill them. At this stage cytotoxic activity can be triggered by LPS; (4.) activated cells which will kill tumour cells without further stimulation. An example of primed (stage 3) cells are pyran copolymer-induced macrophages which require LPS in order to become tumouricidal, whereas Bacillus Calmette-Guerin (BCG) elicited peritoneal macrophages are cytotoxic without the addition of LPS (stage 4) (Johnson *et al.*, 1983). However, this pathway of macrophage activation may not be the only one which leads to tumouricidal activity. It has been shown that polyinosinic:polycytodylic acid (poly(I,C)), but not cytokine is able to stimulate tumouricidal activity in a few hours in the absence of LPS (Taramelli and Varesio, 1981). Cycloheximide can also inhibit the activation of macrophages by α- and β- but not γ-IFN or cytokines (Blasi and Varesio, 1984).

Macrophage cytotoxicity is a relatively short lived event, and cells cultured *in vitro* for longer than 3-4 days lose their cytolytic activity, although this time may vary according to the activator used and/or the assay conditions employed (*e.g.*, lymphokine-activated macrophages retain cytolytic activity longer than LPS activated cells (Russel *et al.*, 1977; Fidler *et al.*, 1976; Ruco and Meltzer, 1978; Taffet and Russell, 1981a,b). After this period the cells cannot be re-stimulated to become

tumouricidal, implying that there is a regulatory mechanism which leads to the suppression of tumouricidal activity. The molecular mechanisms involved in this decline are not known, but prostaglandin-E2 (PGE2) has been implicated in abolishing the tumouricidal activity of LPS-stimulated macrophages (Taffet and Russell, 1981a,b).

Corticosteroids have also been found to inhibit tumour cell killing by macrophages (Dimitriu, 1976), and tumour cells may produce factors which inhibit macrophage function (Szuro-Sudol and Nathan, 1982). The mechanism may also involve other cells such as T-cells since lymphokine-activated macrophages become suppressor cells which inhibit T-cell activation and hence further production of lymphokine. This may be the basis of a possible negative control mechanism of tumouricidal activity (Taramelli *et al.*, 1980).

There is evidence that the activation of macrophages *in vivo* to become tumouricidal has some effect on the tumour cells. Treatment of tumour (metastasising) bearing mice with macrophage activating factor (MAF) or muramyl dipeptide (MDP) enclosed within liposomes led to a significant regression in tumour load of the animals (Fidler *et al.,* 1982; Lopez-Berestein *et al.,* 1983). The reason for the use of liposomes was two-fold: (1.) MDP is rapidly excreted from the system (<60min) and encapsulation prolongs the retention time; (2.) the monocytes and macrophages phagocytose these particles and subsequently become activated by them (Fidler *et al.*, 1982). Liposome encapsulated MDP has also been demonstrated to activate alveolar macrophages *in vitro* (Sone and Tsubura, 1982).

The nature and relative significance of the role of macrophages in tumour cell killing independent of, or in concert with other tumouricidal effector cells is still not clear. The numbers of macrophages present in tumours may vary considerably with the tumour type, but few macrophages are usually present in most murine tumours (Fidler *et al.*, 1982), and some tumours may be able to prevent macrophage infiltration by producing factors which inhibit macrophage chemotaxis. As a further complication, macrophage products (IL-1, TNFα) have been reported to

stimulate the growth of some tumour cell lines *in vitro* (Lachman *et al.*, 1987).

1.2.4 Mechanisms of Tumour Cell Destruction

There are three processes by which tumour cells may be destroyed. These are necrosis, lysis and apoptosis. Necrosis primarily occurs as a result of anoxia within the tumour mass, due to inadequate vascularisation of the tumour. Tumour cells adjacent to the necrotic areas (but still distant from capillaries) become hypoxic, resulting in their relative insensitivity to conventional therapies such as radiation (Thomlinson and Gray, 1955). In contrast to normal tissues, tumour capillaries are often spaced more than 2mm apart, resulting in some degree of necrosis in nearly all tumours. Agents which inhibit blood flow to tumours, by destruction of tumour capillaries (*e.g.*. flavone acetic acid causes selective endothelial cell destruction and haemorrhage in some tumours (Murray *et al.*, 1989)), inhibition of tumour angiogenesis or blockage of tumour capillaries (*e.g.,* by induction of coagulation) induce necrosis of tumour cells. Although these treatments often kill over 95% of cells within the tumour, the remaining 5% are often sufficient to re-establish tumour growth.

Cell lysis occurs when the integrity of the target cell membrane is compromised. Membrane damage occurs in two main ways. (1) Release of reactive molecules such as reactive oxygen intermediates (ROI), proteases, hydrogen peroxide into the intercellular space, or directly onto the target cell membrane from granules within the effector cells (*e.g.*, macrophages, granulocytes), resulting in irreversible membrane damage. This process may be non-specific; (e.g., secretion of cytolytic molecules into solution by activated macrophages, which will attack all cells in their vicinity), or specific ; as in ADCC, where macrophages or NK cells initially adhere to antibody coated target cells via their Fc receptors and subsequently release cytolytic molecules into the intercellular junctions. In this case the process of tumour cell recognition and adherence is non-specific, but only antibody coated tumour cells are

lysed. (2) Tumour cells lysis is also induced by pore forming proteins. In the humoral immune system pores are formed in antibody coated target cell membranes by the complement components. A membrane attack complex (MAC, a polymeric complex of the complement proteins C5b-C9) is assembled within the membrane forming a pore which allows free movement of water and solutes across the membrane, resulting in osmotic swelling and cell lysis. In the cellular immune system adherence of effector to target cells results in the release of granules containing perforins into the intercellular space. The structure of perforin is homologous with that of C9 and, on polymerisation, forms pores in membranes or lipid vesicles 5-20nm in diameter (smaller than those produced by the C9 MAC) which act as permanently open ion channels. Unlike the C9 MAC, perforins do not require the presence of pre-formed elements on the target cell surface to exert their effect and are Ca^{++} and pH dependent.

Apoptosis, or programmed cell death can be induced by immune effector cells. and is thought to be the main way in which the body remodels (*e.g.*. during embryogenesis) and processes tissues (*e.g.*. clonal selection of T-cells in the thymus) during normal life. It is, therefore, an important mechanism which maintains correct cell numbers in the body by balancing cell death with cell production. Apoptosis may also be a control mechanism in cellular positioning in the body (Meredith *et al* 1993, Frisch and Francis, 1994). Cells undergoing apoptosis *in vitro* first round up, and the endoplasmic reticulum breaks up into vesicles which may fuse with the plasma membrane. The chromatin forms dense aggregates and there is invagination of the nuclear membrane. At this point the plasma membrane also convolutes resulting in the characteristic 'blebbing' of cells seen during apoptosis. Membrane bound segments of cell (apoptotic bodies) are then budded off into the environment (zeiosis). The earliest event in apoptosis is the fragmentation of nuclear DNA into approximately 200 base pair fragments (corresponding to the number of base pairs between nucleosomes), probably caused by calcium dependent endonucleases (McConkey *et al.*, 1988; Kizaki *et al.*, 1989). The mechanism by which

apoptosis is triggered has recently become an important area of research. Apoptosis is induced in susceptible target cells by cytokines such as TNFα, IL-1β and IFNγ.

1.2.5 Immunotherapy of Cancer

The various forms of immunotherapy may be classified into two main groups: active and passive. Non-specific active immunotherapies include activation of the immune system with agents such as BCG or Corynebacterium parvum (adjuvant immunotherapy). Specific active immunotherapy involves the inoculation of cancer patients with vaccines derived from killed tumour cells or tumour cell extracts. Alternatively, purified tumour antigens may be injected to induce a specific immune response to the tumour. Reinjection of tumour infiltrating lymphocytes which have been cultured in the presence of tumour antigen is also a form of specific immunotherapy.

Passive immunotherapy may be non-specific, specific or a combination of both. Non-specific passive immunotherapy includes treatment with cytokines or LAK cells which are dealt with in more detail in the later sections. Specific passive immunotherapy involves treatment with monoclonal antibodies (whole molecules or Fab fragments) directed against tumour antigens. Antibodies coupled to cytotoxic drugs, toxins (such as ricin) or radioisotopes provide specific delivery systems for these cytotoxic effectors.

1.2.5.1 Cytokine Immunotherapy

Cytokines are low molecular weight proteins (<80-kDa), which function as cell regulators in an autocrine or paracrine manner, and are involved in the regulation of the amplitude and duration of the immune response. They bind to high affinity receptors which are specific for individual cytokines or groups of cytokines, and alter cellular behaviour (of immune and other normal and neoplastic cells) by changing the patterns of DNA, RNA and protein synthesis in cells. Cytokines often have a number of regulatory activities, which differ according to their concentration, the

cellular composition of their environment, and the presence of other cytokines. There are, therefore, a number of ways in which cytokines can be used in the treatment of cancer: (1.) direct regulation of cell growth (cytostatic, cytotoxic, *e.g.* the tumour necrosis factors), or synergy with other cytokines to modulate cancer cell growth and behaviour (*e.g.* Interferons); (2.) some cytokines cause alterations in the tumour vasculature, resulting in nutrient deprivation and tumour cell necrosis (*e.g.*, TNFα); (3.) stimulation of a host immune response against cancer (*e.g.*, IL-2); (4.) therapy with cytokines such as the colony stimulating factors has proved useful in the recovery of haemopoiesis after conventional treatment with cytotoxic drugs and/or radiotherapy (Brandt *et al.*, 1988); (5.) some cytokines (*e.g.*, IL-1, TNFα (Lachman *et al.*, 1987; TNFα is an example of a cytokine which exhibits multiple, and in some cases opposite, biological activities), TGFβ (Lee *et al.*, 1987) and oncogene products possess autologous tumour growth promoting activity. This autocrine growth hypothesis of cancer suggests that malignancy may result from uncontrolled production of, or response to, these autocrine growth factors. Alternatively, neoplasia may arise from the deletion or mutation of a gene for a growth inhibitory protein. In either case, cytokine therapy may have a role in the modulation of tumour cell growth mediated via these mechanisms.

The following sections will outline the molecular properties and the role of several major cytokines in cancer therapy.

1.2.5.2 Tumour Necrosis Factor-α and Lymphotoxin

Following Coley's observations, it was found that endotoxin was the factor responsible for inducing necrosis of a range of transplantable tumours in mice (Shear and Perrault, 1944), and subsequent experiments by O'Malley in 1962 showed that tumour necrosis activity could be transferred in the serum of endotoxin treated animals. A factor produced by activated T-cells in response to bacterial toxin, which was cytotoxic for a number of tumour cell lines, was termed tumour necrosis factor (TNF) by Carswell and co-workers (1975). Another tumouricidal

product of activated T-cells, called lymphotoxin (LT, also known as TNFβ) had also been characterised by Granger and Williams (1968). Subsequently, recombinant forms of TNFα (Pennica *et al.*, 1984) and LT (Gray *et al.*, 1984) became available and the cytotoxic activities of these molecules was confirmed.

Tumour necrosis factor-α is a trimer of non-glycosylated 17-kDa subunits, but there is also a 26-kDa transmembrane form of the molecule (Wingfield *et al.*, 1987). It is produced by macrophages, activated T-cells and NK cells. Lymphotoxin is a 25-kDa molecule which does not have a transmembrane form. Both molecules are coded for on the short arm of chromosome 6. A wide range of normal and neoplastic cells have high affinity receptors for these cytokines ranging from 1000-10,000 per cell. The same receptor is shared between TNFα and LT, but its expression does not seem to correlate with the susceptibility of cells to the action of these cytokines (Tsujimoto *et al.*, 1985; Immamura *et al.*, 1987). The tumour necrosis factors have a large number of separate activities on normal and neoplastic cells in addition to their tumouricidal activity, including protection of cells from viruses, neutrophil activation (Shalaby *et al.*, 1985), promotion of osteoclastic bone resorption (Bertolini *et al.*, 1986), alteration of the haemostatic and inflammatory properties of endothelial cells (Bevilacqua *et al.*, 1986). On endothelial cells TNFα inhibits the activity of thrombomodulin, augments the secretion of inhibitors of plasminogen activators, and induces the expression and cell surface expression of tissue factor procoagulant activity, resulting in a shift in endothelial cell function from a normally anticoagulant to a procoagulant state. Thus an important role of TNFα treatment may be to induce restricted blood flow to tumours.

The tumour necrosis factors activate phospholipases, particularly phospholipase A2 and proteases, which may directly cause cell damage or activate other enzymes (Suffys *et al.*, 1988). In addition, breakdown of DNA occurs (apoptosis) in TNFα and LT susceptible cells, and this action is augmented by IFNγ. Recent evidence suggests that signal transduction by TNFα occurs via the sphingomyelin pathway (Kolesnick

and Golde, 1994). This pathway involves the conversion of plasma membrane sphingomyelin to ceramide, which acts as a second messenger, via mitogen activated protein kinase (MAP) and NF-κB (a transcription factor).

Tumour necrosis factor-α has undergone several clinical trials, but LT has not been investigated as a therapeutic agent. After i.v. bolus injection the half-life of TNFα was only 15-30min, and circulating levels were only seen with doses above 150μg/ml (Asher *et al.*, 1987). Continuous infusion protocols produced very low but measurable circulating levels of TNFα. Little clinical benefit was observed, possibly because low doses of cytokine were used to treat patients with advanced cancer, who had already undergone extensive chemotherapy. Administration of recombinant TNFα directly into the tumour was of greater clinical benefit. In one such trial, 10 of 22 treated patients developed partial responses (greater than 50% reduction in tumour size) to therapy (Taguchi, 1987). This is in agreement with experiments in which TNFα was injected into mice bearing human tumour xenografts (Balkwill *et al.*, 1987).

1.2.5.3 Interferons

The term interferon was first used by Isaacs and Lindenmann in 1957 to describe certain proteins produced by vertebrate cells in response to viral infection, which were able to induce resistance in cells of the same species to a wide range of viruses. It was subsequently found that these molecules could inhibit the growth of experimental tumours, and purified extracts of cellular supernatants were injected into cancer patients, with some therapeutic success (Goldstein and Laszlo, 1986).

The interferons are subdivided into 2 main groups; the type I interferons which include interferon-α (IFNα), interferon-β (IFNβ), and type II interferons or Interferon-γ (IFNγ). Other Interferon subtypes have recently been reported (Li and Roberts, 1994; Charlier *et al.*, 1993). Interferon-α and -β are produced by leucocytes and fibroblasts

respectively in response to viral infections, whereas IFNγ is produced by antigen- or mitogen-activated T-lymphocytes. The interferons exhibit a range of biological activities, some of which are listed in table 4.

Interferon Type	Biological Activity
IFNα, IFNβ, IFNγ	Enhance NK cell activity
IFNγ	Induce or enhance MHC Class II expression
IFNα, IFNβ, IFNγ	Induce or enhance MHC Class I expression
IFNα, IFNβ, IFNγ	Protection of cells from viruses and parasites
IFNγ	Induction of cytokine secretion (IFNs, TNFs, IL-1, CSFs*)
IFNγ (+IL-2)	Enhance B-cell proliferation and IgG production
IFNα, IFNβ, IFNγ	Inhibit normal and transformed cell growth
IFNα, IFNγ	Influence Cellular Differentiation

TABLE 1.4. Biological Functions of the Interferons. *CSFs - Colony Stimulating Factors
Reviewed by Balkwill, 1989

The IFNα family consists of 23 genes coding for at least 15 proteins of molecular mass 18- to 20-kDa, which have a high degree (~90%) of sequence homology. It is not known why so many similar proteins are produced, all of which seem to exert a similar range of biological activities. There are 2 main classes of a-interferons. Class I IFNαs are composed of 165-166 amino acid residues and are non-glycosylated, whereas class II IFNαs have 172 amino acids and are glycosylated (Capon *et al.*, 1985). Interferon-α has been tested extensively in clinical trials, in which thousands of cancer patients have been treated (Balkwill and Smyth, 1987; Spiegel *et al.*, 1987), with encouraging results on some tumour types. Among those cancers which responded well to treatment were hairy cell leukaemia, chronic myelogenic leukaemia and low grade lymphomas, in which complete remissions were often observed (Goldstein and Lazlo, 1986; Talpaz *et al.*, 1987). In contrast,

melanoma, renal cell carcinoma and breast cancers responded poorly to IFNα.

Interferon-β is a glycoprotein of Mr 23-kDa which, like IFNα, is coded for on chromosome 9. Fewer clinical trials have been performed using this cytokine than with IFNα, but some remissions have been reported. In one study, 2 out of 18 patients with renal cell carcinoma underwent partial remissions (Rinehart *et al.*, 1986). The best response rate to IFNβ treatment was obtained in a study of 6 patients with adult T-cell leukaemia, in which 3 partial remissions were observed (Tamura *et al.*, 1987).

Interferon-γ is a glycosylated protein of Mr 20-kDa which is coded for on chromosome 12. It has no sequence homology to the other IFNs. More than a thousand cancer patients have been treated with IFNγ in phase I (Kurzrock *et al.*, 1986; Vadhan-Raj *et al.*, 1986) and phase II (Kurzrock *et al.*, 1987; Tamura *et al.*, 1987) clinical trials. The results of these studies suggested that IFNγ had some antitumour activity, but was not as effective as IFNα. As a therapeutic agent, IFNγ showed a similar pattern of clinical responses IFNα (*e.g.*, chronic myelogenic leukaemia, renal cell carcinoma). In contrast to IFNα and IFNβ, IFNγ induces upregulation of class II MHC molecules on target cells, activate immune cells, and stimulate the release of other immunomodulatory and cytotoxic effector cytokines from macrophages, NK cells and T-Cells (Table 3). It has been proposed that IFNγ functions mainly through its effect on immune cells, rather than by direct action on tumour cells (Aulitzky *et al.*, 1987).

1.2.5.4 Interleukin-1

There are currently two proteins in the interleukin-1 (IL-1) family, IL-1α and IL-1β, which consist of 271 and 269 amino acid precursors and are processed into 159 and 153 amino acid proteins (17.5-kDa) respectively (March *et al.,* 1985). There is only 26% amino acid homology between these molecules, but Interleukin-1β also has some sequence homology with both basic and acidic forms of fibroblast growth factor (Dinarello, 1986). Many biological activities previously thought to be controlled by discrete factors are now attributed to IL-1. These include endogenous pyrogen, certain B-cell and T-lymphocyte activating factors, catabolin (which catalyses the breakdown of collagen) and an osteoclast activating factor. Interleukin-1 has multiple biological activities, many of which overlap with those of TNFα and LT, even though the only similarity between these molecules is the lack of a hydrophobic signal peptide (the presence of which is common to many other secreted proteins). Almost every cell type in the body is capable of IL-1 synthesis, induced by antigen, toxins, injury, inflammatory reactions and other cytokines (mainly IFNs and TNFs). In turn, IL-1 can induce the production of other cytokines from cells (including IL-1 itself from particular cell types *i.e.* auto-stimulation), and this pattern of induction is the same as for TNF (*i.e.*, IFNα, IFNβ, IL-6 and the colony stimulating factors). Another similarity between IL-1 and TNF is the ability to be mitogenic for some cells (Lachman *et al.*, 1987), whilst being cytostatic or cytotoxic for others, although these cytokines do not necessarily have the same actions on a particular cell type. Several tumour cell lines have been reported to synthesise IL-1 *in vitro* (Griffin *et al.*, 1987), and it acts as an autocrine growth factor on some leukaemia cell lines (Sakai *et al.*, 1987). An inhibitor of IL-1, termed IL-1 receptor antagonist (IL-1RA), has been cloned (Hannum *et al.,* 1990). It has an Mr of 18-kDa, and binds to the IL-1 receptor with a similar affinity to IL-1, but has no IL-1 activity. The inhibitor has a sequence homology of 19% and 26% for IL-1α and IL-1β respectively, and appears to be produced by the same cells which produce the active cytokines.

IL-1α and IL-1β exert their biological effects by binding to two distinct IL-1 receptors. Both receptors belong to the Ig superfamily of receptors. The type I receptor (IL-1RI) is an 80-kDa protein, possessing three extracellular Ig-like domains, a short transmembrane and a relatively large (215 amino acids) cytoplasmic domain (Tate *et al.,* 1992). The second IL-1 binding protein, type II IL-1 receptor (IL-1RII), is also a member of the Ig superfamily, and, like the type I receptor, possesses three extracellular Ig-like domains and a short transmembrane domain. Unlike the type I receptor, it has a short cytoplasmic domain of 29 amino acids (MacMahan *et al.,* 1991), and is unable to transduce signals. The two receptors bind to IL-1α, IL-1β and IL-1ra with different affinities. The extracellular domain of IL-1RII is released in soluble form at sites of local inflammation and into the serum during systemic inflammation. This soluble form of IL-1RII binds IL-1β more strongly than to IL-1α or IL-1RA, and is possibly an endogenous inhibitor of IL-1β at inflammatory sites.

In animal models, IL-1 seems to be less active than the tumour necrosis factors. At high doses, it failed to cause haemorrhagic necrosis in the TNFα sensitive Meth A sarcoma. (Palladino *et al.*, 1987). However, when given intramuscularly every 3 days to mice bearing Meth A sarcoma, complete responses were observed in 86% of cases, and this effect was further augmented by treatment with indomethacin (Nakata *et al.*, 1988). Indomethacin also augmented B16 melanoma cell, adenocarcinoma cell, and lung metastases of the Lewis lung tumour cell killing by IL-1α. No clinical trials using ether IL-1α or IL-1β have yet been undertaken.

1.2.5.5 Interleukin-2

Human IL-2 (previously known as T-cell growth factor, TCGF) is a 15-kDa (133 amino acids) glycosylated protein which is structurally unrelated to the interferons, tumour necrosis factor or other interleukin families of cytokines. The IL-2 gene is on the short arm of chromosome 4 and the protein is produced as a 153 amino acid precursor which is subsequently processed to the mature form containing 133 amino acids. There is a disulphide bond between amino acids 58 and 105, which is

required for the activity of the molecule. Although carbohydrate is present on the IL-2 molecule, it does not seem to be involved in its biological function (Robb, 1984).

Interleukin-2 is only produced by activated T-cells and functions primarily in the generation of cell proliferation and induction of cytotoxic activity in activated T-cells (CD4+, CD8+ and γδT-cells), macrophages and NK cells. T-cells which have not been activated by antigen (or mitogenic signals such as IL-1, IL-6, phytohaemagglutinin (PHA), Concanavalin-A (Con-A), anti-CD3) and a co-stimulatory signal such as CD28 binding to it's ligand (b7) do not proliferate in response to IL-2, but cytotoxic activity can still be induced (LAK activity) in these cells.

There are three proteins which serve as receptors for IL-2, the α-chain (p55, CD25; Smith, 1988), the β-chain (p75; Nakarai *et al.,* 1994), and the γ-chain (64kDa; Nakarai *et al.,* 1994). These bind to IL-2 with relatively low affinity, but they can combine together to form high affinity IL-2 receptors (Robb *et al.*, 1987; Nakamura *et al.,* 1993). Intermediate affinity receptors are also formed by combination of the β- and γ-chains. High affinity IL-2 receptors are generally expressed by activated T-cells and B-cells (IL-2 induces proliferation and antibody production by antigen stimulated B-cells), but low affinity receptors (either α- or β-chains) have been reported on NK cells (β-chain only), oligodendrocytes (Saneto, 1986) and some tumour cell lines (Rimoldi *et al.*, 1993). Unstimulated blood monocytes express IL-2 receptors upon activation by IFNγ.

In experimental animal models, high dose IL-2 therapy has been reported to induce marked regression of pulmonary metastases and subcutaneously inoculated tumours (Rosenberg *et al.*, 1985): an effect that was thought to arise from the induction of LAK cell activity. Based on these encouraging results, clinical trials were undertaken using high dose IL-2 on patients with various advanced cancers. However, the toxicity of IL-2 in man was considerably greater than that observed in any of the animal models, and treatment with this cytokine was often performed in hospital intensive care units. The high toxicity of IL-2 often

required a reduction in dose, and hence efficacy of treatment, and patients had to be carefully selected for their tolerance to IL-2, which resulted in a decrease in the potential usefulness of IL-2 therapy as a general treatment for terminal cancers. There are many toxic side effects of IL-2 therapy, the most common of which are fevers, chills gastro-intestinal problems, and reversible hepatic dysfunction characterised by hyperbilirubinaemia (Rosenberg *et al.*, 1987; Thompson *et al.*, 1987). In addition, there is an increase in vascular permeability and a decrease in systemic vascular resistance, resulting in hypotension that requires fluid replacement and vasopressor treatment. Associated with this , a common effect of IL-2 therapy is an increase in body weight of up to 10% during a single treatment course (Lotze *et al.*, 1986)

Since the generation of LAK cells was the most probable mechanism by which IL-2 induced it's anti-tumour activity, new protocols were devised whereby leucocytes were removed from the patients, stimulated with very high doses of IL-2, and reinjected into the patients, together with much lower maintenance doses of IL-2 (Rosenberg *et al.*, 1987). The following section describes the clinical results obtained using IL-2/LAK cell therapy.

1.2.6 Adoptive Immunotherapy (IL-2/LAK Cell Therapy)

In recent years several new approaches to the treatment of cancer have been proposed, and one of these, adoptive immunotherapy has been studied extensively in clinical trials over the past decade. Several types of malignancy responded to this treatment, including non-Hodgkin's lymphoma and colon cancer, but malignant melanoma and renal cell carcinoma were more responsive to IL-2/LAK cell therapy than other cancers. In some clinical trials approximately 25% of patients responded to treatment, with some partial, (reduction in tumour mass of >50%) but few complete remissions (Rosenberg *et al.*, 1989; Parkinson *et al.*, 1991). Other trials however, reported lower response rates of between 0-20% combined complete and partial responses for melanoma and renal cell carcinoma (Dutcher *et al.*, 1991; Abrams *et al.*, 1990). Adoptive

immunotherapy is still a treatment undergoing development and is only used on patients who are refractory to all conventional treatments, and in whom the prognosis is very poor. Recent research suggests that immune cell interaction with tumour cells is improved when the tumour load is small (Ingram *et al.*, 1990). Immunotherapy may, therefore, be more beneficial to patients who have undergone surgery to reduce their tumour load, or in patients who have been recently diagnosed with early tumours. In addition, the efficacy of adoptive immunotherapy has been reported to be enhanced by concomitant treatment with chemotherapeutic drugs such as cyclophosphamide (Mitchell, 1992).

Adoptive immunotherapy initially involves the injection of moderate doses of IL-2 into cancer patients to boost leucocyte numbers. Mononuclear cells are then leucopheresed from the patient, stimulated *in vitro* with IL-2 for 2-7 days, and reinjected into the patient's circulation, where they must (1.) detect a tumour recognition signal, (2.) bind to and diapedese across the endothelium adjacent to the tumour, (3.) infiltrate the tumour and (4.) adhere to and kill its cells. These stages represent several distinct environments with which activated leucocytes must interact successfully in order to bring about tumour cell destruction.

Following continuous i.v. infusion of IL-2 (prior to LAK cell therapy) into cancer patients, increased tumouricidal activity, *in vitro,* by peripheral blood leucocytes has been reported (Thompson *et al.*, 1987). In most of the early studies this increased cytotoxic activity was measured against an NK sensitive cell line (typically K562), an NK resistant cell line (typically Daudi or Raji), and rarely against a cell line of the same type as that of the patient. Later trials included a wider variety of more relevant cell lines as target cells, sometimes including cells grown from the patients own tumour. Furthermore, high levels of cytotoxicity were still observed *in vitro* when LAK cells were reintroduced into the patients, together with low doses of IL-2. When treatment was discontinued, LAK cell activity (measured by killing of Daudi cells) decreased rapidly. Unfortunately, the increased killing observed *in vitro* was rarely associated with improved clinical benefit.

1.3.0 Haemostasis, Immunity and Cancer

1.3.1 Background

A relationship between cancer and abnormalities of the coagulation system has been recognised for over 100 years. In 1865, Armand Trousseau noticed that deep-vein thrombosis of the extremities often accompanied visceral cancer and concluded that *"spontaneous coagulation is common in cancerous patients because of a special crasis of the blood, which irrespective of inflammation, favours intra-venous coagulation"* (Trousseau's sign). Another of his observations, later restated by Irving Wright in 1952, was that thromboembolic disease (usually of unknown aetiology) refractory to anti-coagulant therapy, was an early detectable sign of an underlying cancer, which could precede the onset of observable cancer by months or years (Wright, 1952). Whether patients presenting with idiopathic venous thrombosis should be rigorously tested for the presence of cancer is still a subject of debate (Luzatto and Schafer, 1990; Levine and Hirsh, 1990). However, there is growing evidence to support Wright's observations. In a recent study 250 patients with no known cancer, who presented with a first episode of deep-vein thrombosis of the lower extremity, were followed up for 2 years to determine if any cancer was evident. There was a statistically significant association between idiopathic venous thrombosis and the subsequent development of clinically overt cancer, especially in patients with recurrent thromboembolism (Prandoni *et al.*, 1992). Other studies have also shown similar results with up to 23% incidence of cancer during 2 year follow-up periods following idiopathic deep-vein thrombosis (Monreal *et al.*, 1991; Aderka *et al.*, 1986).

Although idiopathic venous thrombosis may be an early diagnostic sign for the presence of undetectable cancer, patients with established cancer may suffer from a wide variety of haemostatic disorders, ranging from thromboembolic episodes to haemorrhagic disease. Haemostatic abnormalities in cancer include thromboembolic disease, abnormal coagulation, overcompensated intravascular coagulation with fibrinolysis

(ICF), disseminated intravascular coagulation (DIC), qualitative and/or quantitative platelet defects, and interference of malignant paraproteins with coagulation factors. Generalised bleeding, although less common than thrombosis, may also occur in cancer. This usually happens in the later stages of cancer when tumour cells have extensively invaded tissues which are responsible for the production of elements of the haemostatic system. These include tumour invasion of the bone marrow, resulting in reduced platelet numbers; and cancer (or cancer therapy) induced liver damage, causing decreased levels of circulating coagulation factors. These problems are also common side effects of radiation treatment and chemotherapy. Haemostatic abnormalities account for the second highest death rate among cancer patients (Ambrus *et al.*, 1975, Levine and Hirsh, 1990).

1.3.2 Laboratory Investigation of Coagulation Abnormalities in Cancer

Although many cancer patients exhibit clinically significant haemostatic abnormalities, about 50% of all patients (>90% with metastases) have abnormal laboratory coagulation parameters (Luzzatto and Schafer, 1990). The most commonly reported abnormalities are elevated fibrinogen and fibrinopeptide A (FPA) levels, raised platelet count and prolonged prothrombin time. One study reported up to 27% of patients with cancer were thrombocytopaenic (Kies *et al.*, 1980), whereas others have reported thrombocytosis in as many as 60% of cancer patients studied (Davis *et al.* 1969; Hagedorn *et al.*, 1974). A more recent study of 431 cancer patients, prior to their entry into an anticoagulant trial, again demonstrated increased levels of plasma FPA, fibrinogen and platelets, together with a steady increase in fibrinogen and platelet count prior to death (Edwards *et al.*, 1987). Levels of FPA also correlated with the progression of disease, but other coagulation parameters such as PT, APTT, FDPs or ELT showed no consistent abnormalities during this study. In addition, these workers previously demonstrated that certain

forms of chemotherapy and radiotherapy also caused increased levels of FPA.

Disseminated intravascular coagulation may be subdivided into three main categories (Owen and Bowie, 1974). If the coagulation system is significantly activated, coagulation factors and platelets will be depleted, with a resultant increase in fibrinolysis, this is termed uncompensated DIC. When activation of coagulation is less overt, clotting factors and platelets are replaced at the same rate as their consumption, resulting in compensated DIC. However, these elements may be replaced at a faster rate than their consumption, and elevated levels of fibrinogen, other clotting factors and platelets are seen. Fibrinopeptide-A levels are also be increased, indicating that the presence of an underlying coagulopathy. This form of overcompensated DIC is, thus, a possible explanation of the results obtained in the studies mentioned above.

1.3.3 Extravascular Coagulation and Cancer

Although the subject of controversy for many years, it is currently accepted that fibrin is present in the majority of solid tumours (Dvorak *et al*, 1984; Brown *et al.*, 1988a). The relative distribution and amount of fibrin varies both between tumour types and between similar tumours in different patients, but is characteristic for individual human tumours and their metastases, as well as murine tumours transplanted several times (Brown *et al.*, 1988b). However, the distribution of fibrin within tumours does not correlate with tumour progression (Harris *et al.*, 1982). For example, in carcinomas, fibrin is generally present on the periphery of the tumour and surrounding tumour cell clusters but not in older areas of tumour where collagenous connective tissue has been laid down (Dvorak *et al.*, 1981). In contrast, fibrin deposition within lymphomas is generally around individual tumour cells as well as in areas of tumour sclerosis (Harris *et al.*, 1982).

Most tumours (with the exception of hepatomas) do not synthesise fibrinogen, so tumour fibrin must therefore be derived from plasma fibrinogen. Extravasation of fibrinogen (and other plasma proteins) is

very slow into normal tissues (in the absence of inflammation or delayed (type IV) hypersensitivity reactions, in which leucocyte mediated fibrin deposition is a normal process of wound healing), but is considerably greater in tumours implying that the tumour capillary endothelium is more permeable to plasma proteins than normal endothelium. Tumour cells are able to increase vascular permeability in several ways (*e.g.*, stimulation of mast cells adjacent to the tumour to produce histamine (Tanooka *et al.*, 1982), induction of a cellular immune response (Dvorak *et al.*, 1980) kinin generation (Matsumura *et al.*, 1988) and the production of vascular permeability factor(s)). The most potent of these is vascular permeability factor (VPF, Dvorak *et al.*, 1992), which is a polypeptide (Mr 34- to 43-kDa), and increases vascular permeability in skin at concentrations of as low as 1×10^{-13} M (50,000 times more active than histamine). Vascular permeability factor is produced by many tumour cell types. It's mechanism of action is not known, but it increases intracellular calcium levels (an indication of cellular activation) in human endothelial cells (Ferrara *et al.,* 1992).

1.3.4 Procoagulant Activities of Tumour Cells

Tumour cells have been reported to express a number of procoagulant activities. O'Meara in 1958 observed that malignant tissue was able to shorten the clotting time of recalcified plasma more than most normal tissues. Tissue factor (TF, tissue thromboplastin), is a non-protease membrane protein which enhances the ability of factor VII to activate factor X. It is present in most normal tissues, and was initially shown to be expressed by leukaemic cells (Gralnick and Tan, 1974), gastric carcinomas (Sakuragawa *et al.*, 1977) and adenocarcinomas (Dvorak *et al.*, 1983). A wide range of tumour cells are now known to express tissue factor

Another procoagulant factor produced by tumour cells was first detected by Gordon *et al*, 1975, termed cancer procoagulant (CP). Unlike TF, CP did not require the presence of factor VII for its activity, but activated factor X directly. Cancer procoagulant was characterised in 1985 as a

68-kDa protein with cysteine protease activity (Falanga and Gordon, 1985), and has since been found in a variety of human and animal tumours. It has an amino acid structure different from that of other cysteine proteases such as cathepsin B, and may be a useful marker in the detection of human malignancy. Although CP induces fibrin formation in solid tumours, other evidence suggests that the majority of cancer procoagulant activity generated by tumour cells *in vitro*, is attributable to TF activity (Edwards *et al.*, 1981).

Prothrombin conversion to thrombin is mediated by the association of prothrombin, FVa and FXa (in the presence of calcium ions) on a suitable phospholipid surface (the prothrombinase complex). In normal blood coagulation, platelet membranes generally provide the phospholipid component of the prothrombinase complex, but this activity has also been reported for other normal cells such as leucocytes and endothelial cells. Assembly of the prothrombinase complex on tumour cells was first described on a guinea-pig hepatocarcinoma cell line (Van deWater *et al.*, 1985) and later on human tumour cells. Clot formation induced by the release of thrombin-like enzymes has been reported in patients with pancreatic cancer, but this was attributed to the release of trypsin into the blood stream by the pancreas (Semerano and Donati, 1981).

The procoagulant activities of tumour cells are predominantly exhibited via the extrinsic or the common pathway of coagulation. However, direct activation of factors XII and XI (the intrinsic pathway) by the abnormal vasculature of some tumours has been reported (Semerano and Donati, 1981). In this case activation of factor X is initiated by factor VIIIa and requires the presence of calcium ions and platelet phospholipid.

1.3.5 Procoagulant Activities (PCA) of Leucocytes in Cancer

Monocytes and to some extent lymphocytes and endothelial cells are responsible for extravascular coagulation in a wide range of inflammatory conditions. These reactions may be specific, as in delayed-type-hypersensitivity where activated T-cells induce monocyte procoagulant activity, mostly via TF expression, (Edwards and Rickles, 1980), or non-specific, as in activation of monocytes by LPS. In cancer, tumour infiltrating leucocytes contribute procoagulant activity in addition to that produced by tumour cells and their endothelium. Monocytes also synthesise several molecules with direct procoagulant activity, such as TF, Factor V, Factor VII, Factor XIII (McGee *et al.*, 1990; Rothberger *et al.*, 1984; Weisberg *et al.*, 1987), and high affinity receptors for Factor X /Xa and fibrinogen via Mac1 (CD11b/18; Sherman and Lee, 1977). In addition, monocytes secrete cytokines, such as IL-1, TNFα and IFNγ, which upregulate the procoagulant activities of monocytes, tumour cells and endothelial cells (Moon and Geczy, 1988; Carlsen *et al.*, 1988; Nawroth and Stern, 1986). Monocyte procoagulant-inducing factor (MPIF) is another cytokine that is chemotactic for monocytes (Gregory *et al.*, 1986). Activated T-cells could induce monocyte procoagulant activity in the tumour either by releasing cytokines (*e.g.*, IFNγ and TNFα) which activate monocytes, or via antigen presentation.

Monocytes and NK cells are also been implicated in the generation of blood clotting abnormalities in cancer patients. Mononuclear cells isolated from cancer patients exhibited greater procoagulant activity than mononuclear cells from normal donors (Edwards and Rickles. 1980). In a murine tumour model macrophages derived from tumours which were poorly immunogenic or metastatic, exhibited relatively low PCA, comparable to the levels found in peritoneal exudate macrophages from normal mice. Macrophages derived from the MSV sarcoma (which is a highly immunogenic tumour), however, were strongly procoagulant (and tumouricidal) (Guarini *et al.*, 1984).

1.3.6 Interactions of Tumour Cells with Platelets

Platelets are rarely present within the stroma of solid tumours. The main platelet-tumour cell interactions are believed to occur within the vasculature during the process of tumour metastasis. Some tumour cells and membrane bound vesicles shed from them activate and aggregate platelets directly, whereas others neoplastic cells only cause aggregation and secretion by platelets previously activated with ADP or serotonin (Paschen *et al.*, 1979). It is not clear how tumour cells activate platelets, but it has been reported that close cell-cell contact is required together with the release of tumour products such as ADP (Gasic *et al*, 1980), or cathepsin B (Sloane, 1981). In addition, release of procoagulants such as TF from circulating tumour cells may cause thrombin generation, and hence platelet activation.

On entering the circulation, tumour cells adhere to platelets by mechanisms which are thought to involve the tumour cell cytoskeleton, integrin binding, large molecular weight glycoproteins and products of the lipoxygenase system (Chopra *et al.*, 1988). Several platelets are often able to bind to one tumour cell forming clusters which travel through the blood until the platelets adhere to damaged endothelium. Platelet/tumour cell clusters adhere weakly to intact endothelium but very strongly to damaged endothelium, (Mehta, 1984). After adherence to damaged endothelium, the clusters interact with the subendothelial layers and fibrin deposition is stimulated. The tumour cells are then able to migrate into the distant tissue and form secondary tumours. In the absence of disrupted endothelium the cell aggregates continue moving in the circulation until they block a small capillary in a distant organ, resulting in platelet/tumour cell induced endothelial damage, and subsequent tumour cell extravasation (Warren, 1981). In either case it would appear that the adherence of platelets to tumour cells promotes metastasis (Mehta, 1984; see section on coagulation therapy of cancer). In addition, the attachment of platelets to tumour cells may physically inhibit immune cytotoxicity in the circulation, again increasing the

likelihood of successful metastatic spread. A number of platelet products have been implicated in metastatic spread, such as serotonin (increases vascular permeability), the α-granule adhesive proteins fibronectin, thrombospondin, von Willebrand factor and endoglucosidases which degrade heparan sulphate. Similar molecules are produced by some tumours and break down the subendothelial matrix glycosaminoglycans (Wasteson *et al.*, 1977).

Platelets have been reported to be directly cytotoxic for tumour cells after 18h in culture (Ibele *et al.*, 1985, Sagawa. *et al.*, 1990). *In vivo*, however, it is likely that tumour cell extravasation predominates over the slower process of platelet cytotoxicity (Crissman *et al.*, 1988).

Tumour metastasis is inhibited by treatment of tumour bearing animals with drugs which reduce platelet aggregation or activation, such as aspirin (Gatspar, 1970), dipyridamole (a platelet aggregation inhibitor; Gatspar *et al.*, 1984), and prostacyclin (PGI2; Honn *et al.*, 1981). The calcium channel blockers nimodipine and nifedipine reduce pulmonary metastasis in experimental animals, possibly by inhibition of tumour-platelet-endothelial cell interactions (Onoda *et al.*, 1984). Thrombocytopaenia induced by neuraminidase (which removes negatively charged sialic acid residues from cell surface carbohydrates), or anti-platelet antibodies, also results in a marked reduction in secondary tumour production in animal models (Gasic *et al.*, 1984).

1.3.7 Anticoagulation Therapy of Cancer

The function of fibrin in the tumour is unclear, but some reports suggest that fibrin deposits can promote tumour growth and neovascularisation of growing tumours, protect tumours from immune or chemotherapeutic attack, and aid tumour cell adhesion to endothelial cells during metastasis (Donati and Poggi, 1980; Dvorak *et al.*, 1981; Gralnick and Tan, 1981; Gorelick *et al.*, 1984). Alternatively, fibrin deposition around solid tumours may protect the host from tumour cell invasion by blocking their entrance into the circulation (Colucci *et al.*, 1981).

Few clinical trials of anti-coagulation therapy of cancer have been undertaken. Most experiments were performed in animal models to determine the effects of anticoagulants on metastatic tumour progression. Early studies using isotope labelled tumour cells showed that tumour cell deposition occurred irrespective of whether the animals were anticoagulated, but that subsequent metastatic growth was decreased by anticoagulant therapy, implying a role for fibrin in metastatic tumour growth (Fisher and Fisher, 1967).

Warfarin reduces metastasis in animals (McCulloch and George, 1987), and Zacharski and co-workers (1984) have undertaken clinical trials using warfarin treatment of patients with head and neck, lung, colon and prostate cancer. A significant survival advantage was seen only with small cell carcinoma of the lung (SCCL), and this finding was later confirmed by others (Chahinian *et al.*, 1989). The mechanism of warfarin action in SCCL may not be entirely due to it's anticoagulant activity. Warfarin also affects fibrinolysis by interfering with vitamin K-dependent post-translational carboxylation of glutamic acid residues on Protein C (activated protein C neutralises factor V and factor VIII, thereby inducing fibrinolysis) and other biologically active proteins. Warfarins have also been reported to cause a decrease in tumour cell adherence to endothelial cells (Orme and Ketchum, 1967), and to inhibit primary experimental tumour growth and metastasis (Hilgard *et al.*, 1977), and other biological activities, any of which could also be involved in the reduction of metastatic tumour spread (Zacharski *et al* 1984).

The efficacy of heparin treatment in metastatic inhibition in animal models was variable, but was consistently less effective than warfarin. In some cases an increase in tumour growth was observed (Chan and Pollard, 1980). The heparins comprise a large range of negatively charged glycosaminoglycans which have many other biological activities in addition to their anticoagulant properties, such as interference with lymphocyte recirculation and regulation of smooth muscle growth.

Significant reduction of metastatic tumour growth has also been observed using a number of other anticoagulants such as salivary gland extracts from *Haementeria* leeches, and coumarin derivatives (Gasic *et*

al., 1983). Desulphatohirudin, a low molecular weight protease (thrombin) inhibitor, normally present in the peripharangeal glands of medicinal leeches inhibits the production of lung metastases of B16 murine melanoma (Esumi *et al.*, 1991).

Anticoagulant therapy has been combined with chemotherapy with some success. Significant reduction in metastasis was observed using combinations of warfarin and adriamycin or warfarin and 5-fluorouracil (5FU) or cyclophosphamide in the presence of bleomycin (Hilgard *et al*, 1977), compared to each treatment alone Warfarin may interfere with 5FU degradation, thus enhancing the antitumour activity of the drug (Kirsch *et al.*, 1969).

1.3.8 Fibrinolytic Activities of Tumour Cells and Leukocytes

Tumour cells produce a number of proteolytic enzymes which degrade various components of their stroma, including collagen (type I in the extracellular matrix and type IV in the basement membrane), laminin, elastin, and fibrin. Some enzymes (collagenases, cathepsins and elastase) have a direct activity on tumour associated fibrin, but in the majority of cancers, tumour cells produce plasminogen activators (PA). These activate plasminogen (extravasated together with fibrinogen from the plasma) to plasmin which in turn degrades fibrin, thus allowing tumour cell motility though the extracellular matrix. There are three main PA produced by tumour cells, but the urokinase type plasminogen activator (uPA) which has several forms of 33, 55 and 100-kDa MW (Evers, 1982) is the most commonly expressed form. The tissue type plasminogen activators (tPA, 70 and 100-kDa, Rijken and Collen, 1981) and another high molecular weight PA are also produced by some tumours (Vetterlein *et al.*, 1979). The plasminogen activators may also promote tumour growth and metastasis, by the induction of morphological changes associated with tumour promoter action in virally transformed chicken fibroblasts (O'Donnelli-Tormey and Quigley, 1981). The production of PA by tumour cells does not seem to correlate with metastatic potential. A possible explanation for this is that fibrinolysis

enhances tumour cell release into the vasculature from established tumours, but also inhibits their adhesion (via platelets and fibrin deposition) to endothelial cells, thus preventing tumour cells from leaving the blood stream (Donati *et al.*, 1981). This hypothesis is supported by data from experimental animal models. Treatment of animals with anti-fibrinolytic agents such as epsilon amino caproic acid (eACA) or tranexamic acid increased the number of metastases when tumour cells were injected intravenously suggesting that fibrin was important in tumour cell extravasation. In contrast, a decrease in metastasis was seen when the same drugs were given to animals with transplanted primary tumours (*i.e.*, the presence of extravascular fibrin prevented tumour cell migration into the circulation). In one clinical study, a combination of tranexamic acid with cytostatic treatment and radiation was claimed to be beneficial (Astedt *et al.*, 1977).

Unlike tPA, uPA is not dependent on the presence of fibrin for its ability to activate plasminogen, thus providing the tumour cells with the ability to generate plasmin whether in the presence or absence of fibrin. This is important since plasmin activates procollagenases (Paranjpe *et al.*, 1980) such as type IV procollagenase which breaks down Type IV collagen in the basement membrane, and also laminin, another extracellular matrix component (Liotta *et al.*, 1981). Plasminogen activator activity has also been implicated in several other normal physiological processes such as ovulation and spermatogenesis, embryonic development, prohormone processing and keratinocyte differentiation (Saksela, 1987).

Macrophages, B-cells, thymocytes and large granular lymphocytes (NK cells) also synthesise PA (Chapman *et al.*, 1979, Goldfarb *et al.*, 1984). T-cells are not thought to produce plasminogen activator activity (Maillard and Favreau, 1981). Tumour infiltrating macrophages are therefore able to contribute to fibrin degradation in the tumour matrix. This may allow cytotoxicity of neoplastic cells by macrophages and T-cells and act as a host protection mechanism.

1.4 Aims of the Project

Non-specific immunotherapy of cancer may be subdivided into several treatment regimes. These are: (1.) infusion of a single cytokine; (2.) infusion of a combination of two or more cytokines; (3.) combination cytokine therapy and chemotherapy and; (4.) adoptive immunotherapy with LAK cells alone or combined with maintenance doses of cytokine (usually IL-2). The reaction of the host immune cells, cytokine network, vascular system, tumour cells and tumour stroma to these therapies is highly complex. The main aim of this project, therefore, was to isolate *in vitro* the interactions between these forms of treatment, and representative cellular or stromal components present within the cancer patient, in order to identify possible synergistic interactions or inhibitory factors involved in the process of tumour cell destruction. To this end, the project was subdivided into several major objectives. These were:-

1. To investigate the interactions of several of the major cytokines (TNFα, LT, IFNγ IL-1β and IL-2) used in cancer therapy with a range of tumour cell lines *in vitro*, in order to assess their direct cytotoxic or growth promoting activities.

2. To study the effect of combinations of cytokines and cytotoxic drugs (Act-D and cycloheximide) on their ability to kill tumour cells, and to identify any synergistic or inhibitory interactions which may arise.

3. To examine the cytotoxic activities of unstimulated and cytokine activated peripheral blood leucocytes (lymphocytes and monocytes) for untreated or cytokine and/ or cytotoxic drug pre-treated tumour cells, and to investigate the mechanism of these interactions.

4. To determine the cytotoxicity of IL-2 activated leucocytes (LAK cells) against autologous lymphocytes and monocytes, and against human umbilical vein endothelial cells or fibroblasts.

5. To investigate the effect of components of the coagulation system on LAK cell cytotoxicity for tumour cells, and to identify any factors which may augment or inhibit cytotoxicity.

CHAPTER 2

MATERIALS AND METHODS

2.1 Preparation and Culture of Tumour and Normal Cells

2.1.1 Adherent Tumour Cell Lines

Four human melanoma cell lines (A375, DX3, SK23 and LT5.1) were provided by Dr Ian Hart (ICRF, Lincoln's Inn Fields, London). The promonocyte cell line U937 was supplied by Dr N. Hogg (ICRF, Lincoln's Inn Fields, London) and the K562 (Erythroblastoma) and Daudi (EBV transformed B cells) cell lines were already established in the Immunology Department at St Thomas' Hospital.

2.1.2 Tissue Culture of Adherent Tumour Cell Lines

The four adherent melanoma cell lines were kept in continuous cell culture in $75cm^2$ tissue culture flasks (Costar Ltd., Berks., U.K.). On reaching confluence, cells were detached from plastic by incubation for 2-3 min with 3ml trypsin (0.5g/l) and EDTA (0.2g/l) in Ca^{2+}, Mg^{2+} free HBSS (Life Technologies, Renfrewshire, Scotland). After centrifugation at 200*g* for 10min the supernatant was removed and 5ml RPMI/FCS (appendix III) was added to the cells. A 1ml aliquot of cells was added to each of five $75cm^2$ tissue culture flasks and the total volume in each flask adjusted to 35ml with RPMI/FCS. The flasks were then returned to the incubator until required for experiment or until they reached confluence.

In some experiments, A375 cells were required to be grown to confluence in the wells of 96 well microtitre plates. They were detached from culture flasks and washed, as described above. The final cell pellet was reconstituted to 20ml with RPMI/FCS and 100µl added to all wells of one or two 96 well microtitre plates and incubated at 37^0C for 24-48h, until the cells had reached confluence.

2.1.3 Tissue Culture of Non-Adherent Tumour Cell Lines

The non-adherent cell lines (K562, Daudi and U937) were maintained in continuous cell culture as 20ml volumes in upright 25cm^2 tissue culture flasks. Cells were passaged every 2-3 days by resuspension with gentle agitation and diluting 1:1 with fresh RPMI/FCS. The cells were returned to the incubator until required for experiments or until further subdivision was necessary.

2.1.4 Cytokine Pre-treatment of A375 Cells

In some experiments, A375 cells were pre-incubated with cytokines prior to their inclusion in the cytotoxicity assay. The supernatant was decanted from the cells and the flasks were rinsed twice with warm (37^{0}C) RPMI. Dilutions of recombinant IL-2 (500-2000U/ml), TNFα (100-500U/ml) IFNγ (100-1000U/ml) or a combination of two of these cytokines were prepared in 10ml of RPMI/FCS, and added to the flasks for 3-72h. In all experiments, control A375 cells were incubated in 10ml of RPMI/FCS alone for an equivalent time to those flasks containing cytokine dilutions.

2.1.5 Pre-treatment of Tumour Cells with Protein Synthesis Inhibitors

Culture flasks containing fresh or cytokine pre-treated adherent tumour cells were rinsed twice with RPMI, and 10ml of RPMI/FCS alone or containing either actinomycin-D (0.0001-1μg/ml; Sigma, Dorset, U.K.) or cycloheximide (0.01-10μg/ml; Sigma, Dorset, U.K.) added to appropriate flasks for 3h. The cells were then detached from the flasks using Trypsin/EDTA (section 2.1.2), and a cell count and viability performed (section 2.1.15), prior to their use in cytotoxicity experiments.

Flasks containing K562 cells (non-adherent) were decanted into 30ml universal containers and centrifuged at 200*g* for 10min. The supernatant was discarded and the cells resuspended in 1-2ml of RPMI/FCS. A cell count and viability was performed, and aliquots containing 1x10^7 cells were again centrifuged and the pellets resuspended in 10ml RPMI/FCS

alone or RPMI/FCS containing actinomycin-D (0.1μg/ml), and incubated for 3h at 37^{0}C. The cells were washed twice in RPMI/FCS prior to their inclusion in cytotoxicity experiments.

2.1.6 Peripheral Blood Collection

Peripheral venous blood (50-100ml) was withdrawn from normal healthy donors into 60ml syringes containing 10U/ml preservative free heparin (Leo Laboratories, Princes Risborough). The syringe was inverted several times to mix the blood and anticoagulant, and decanted into a 100ml glass bottle under sterile conditions.

2.1.7 Preparation of Mononuclear Cells by Lymphoprep Density Gradient

Heparinised blood was diluted 1:1 with warm (room temperature) RPMI, and 15ml layered over 8ml Lymphoprep (Nycomed (U.K.), Sheldon, Birmingham) in plastic universal containers. These discontinuous density gradients were centrifuged for 25min at 450*g* and the mononuclear cell interface removed using a sterile plastic pasteur pipette. The cells were washed three times by resuspension and recentrifugation at 200*g* for 10min in RPMI/FCS so as to remove platelets. The final cell pellet was resuspended in 2-5ml RPMI/FCS (or 2-5ml RPMI for coagulation experiments; section 2.2.6) and a cell count and viability performed as described in sections 2.1.15.

2.1.8 Monocyte Purification by Nycodenz Monocytes Density Gradient

For this procedure human peripheral blood (9 parts) was collected into glass tubes containing 1 part 2.7% (w/v) EDTA (pH 7.4). Blood (3ml) was then layered onto 3ml Nycodenz Monocytes in a 12ml tube and centrifuged at 600*g* for 15min. After centrifugation the clear plasma was removed until 3-4mm remained above the interface. The remaining plasma together with approximately half of the separation medium was collected and washed three times in calcium- and magnesium-free buffer

containing RPMI/AB (appendix III) serum. The purified monocytes were resuspended in RPMI/AB and a count and viability performed prior to assay.

2.1.9 Lymphocyte and Monocyte Purification Using Discontinuous Percoll Density Gradients

Percoll (polyvinylpyrollidone coated silica beads (Pharmacia Biosystems Ltd, Milton Keynes) was diluted 9 parts to one part medium 199 (Life Technologies, Renfrewshire, Scotland) containing phenol red to obtain an isotonic solution with a density of 1.1294, which will be referred to as 100% percoll. Subsequently, 100% percoll was further diluted with RPMI (1x) to 70% (density 1.09), 60% (density 1.077), 50% (density 1.067), 40% (density 1.056) and 30% (density 1.043), in aliquots of 2ml/gradient. The 70%, 50% and 30% aliquots were slightly acidified by the addition of a drop of 0.1mol/L HCl to help in the identification of the interfaces between the percoll fractions. Two millilitres of 100% percoll were placed into a 15ml round bottomed Falcon tube (these tubes provided a smooth flow of percoll, resulting in cleaner interfaces between percoll fractions), and 2ml aliquots of 70%, 60%, 50% and 40% percoll were sequentially layered on top using a glass pasteur pipette. Mononuclear cells obtained from Lymphoprep density gradients (section 2.1.7; not more than 5 x 10^7cells/gradient) were pelleted by centrifugation and resuspended in 2ml of 30% percoll. The cell suspension was layered onto the top of the percoll gradient and the tube centrifuged at 450*g* for 25min. Cells were removed from the gradient interfaces using a pasteur pipette, and washed twice in RPMI to remove any remaining percoll.

2.1.10 Lymphocyte and Monocyte Purification by Plastic Adherence

Mononuclear cells obtained from the Lymphoprep density gradient (section 2.1.7) were adjusted to 2 x 10^6 cells/ml, and aliquots of 5 x 10^7 cells added to 75cm^2 tissue culture flasks. The flasks were incubated at 37^{0}C, in 5% CO_2, for 1h in order to allow monocyte adherence to the plastic. The non-adherent cells (lymphocytes) were decanted into universal containers and washed twice with RPMI. A cell count (section

2.1.15) was performed on the final cell suspension and cytocentrifuge preparations were made for analysis of cell purity. Cell purity was determined by differential counting of lymphocytes and monocytes using May-Grunwald/Giemsa stain. The flasks containing the remaining adherent cells (monocytes) were rinsed twice with RPMI and 10ml of 3mmol/L EDTA in RPMI/FCS was added. The flasks were incubated at 37^0C for 12min, or until the cells had detached from the plastic (determined by observation under an inverted microscope). The cells were removed from the flasks using a 5ml pipette, transferred into universal containers, and washed three times in RPMI/FCS to remove any residual EDTA. A cell count (section 2.1.15) was performed on the final cell suspension and cytocentrifuge preparations were made for determination of cell purity. It was important that the monocytes were used in experiments immediately to avoid re-adherence to the plastic, which would result in a decreased cell yield.

2.1.11 Choice of Purification Technique for Monocytes and Lymphocytes

In order to obtain highly purified preparations of lymphocytes and monocytes from peripheral blood, several separation techniques were tested for their yield and purity of these cells. Two established methods were used to separate lymphocytes and monocytes from a mononuclear cell population obtained from lymphoprep density gradients (section 2.1.7): discontinuous density gradient separation using percoll (section 2.1.9; Brandslund *et al.*, 1982), and adherence of monocytes to serum coated plastic, and subsequent elution using EDTA (section 2.1.10). In addition, monocytes alone were purified from whole blood using a Nycodenz density gradient (section 2.1.8.; Boyum, 1983). Cell purities were measured on cytocentrifuge preparations stained for either non-specific esterase (monocytes positive; Tucker *et al.*, 1977) or May-Grunwald Giemsa stain (morphological identification of lymphocytes and monocytes) The cell yield was represented as the number of cells ($x10^6$) derived from 50ml of whole blood.

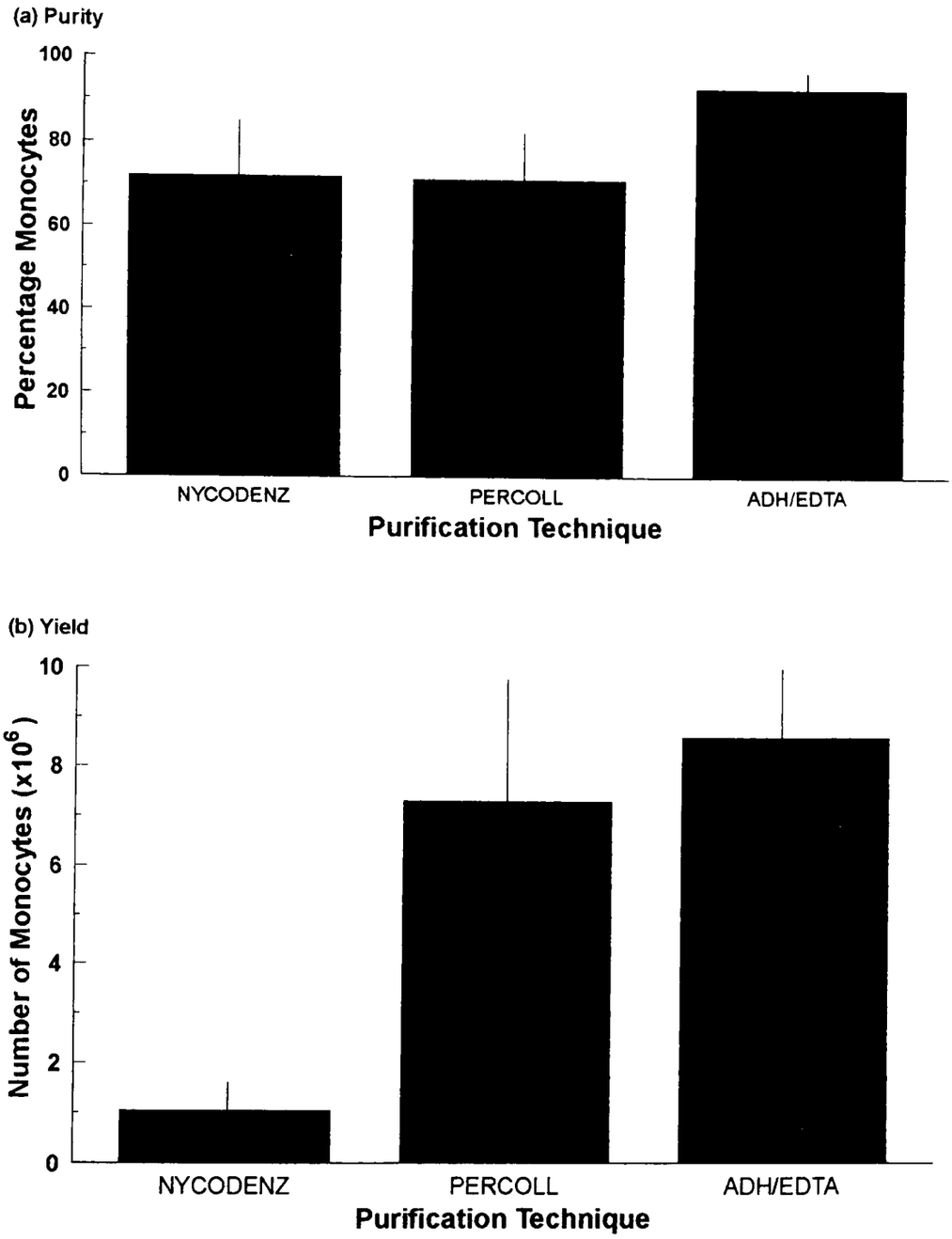

FIGURE 2.1. Isolation of monocytes by three techniques. Means and SEMs from 5 (Nycodenz), 6 (Percoll) or 8 (Adherence/EDTA) experiments, showing the purity (figure 2.1a) and yield of monocytes from 60ml peripheral blood. Monocyte purity by adherence was significantly greater ($p<0.05$) than either of the other separation methods, by MWU. The monocyte yield from plastic adherence was significantly greater ($p<0.05$) than that from Nycodenz, but not from Percoll ($p>0.05$).

Figures 2.1a and 2.1b show the purity and yield respectively of monocytes using Nycodenz Monocytes, Percoll or adherence/EDTA. The purity of monocytes obtained from Nycodenz gradients was highly variable (72 +/- 7%, n=5), and the final yield of monocytes was very low (1 +/- 0.4 $x10^6$ cells, n=5). For these reasons Nycodenz was not used in further experiments.

Monocyte separation using Percoll resulted an equivalent purity (71 +/- 5%, n=5; $p>0.05$), but a significantly greater yield (7 +/- 3%, n=5; $p<0.05$), than Nycodenz (13 +/- 8 $x10^6$ cells, n=5). Highly purified lymphocytes were present at both the 50-60% (96 +/- 3%, n=5) and 60-70% (94 +/- 2%, n=5) percoll interfaces, although the majority of cells were found at the 50-60% interface (27 +/- 7 $x10^6$ cells, n=5). Two problems were found using percoll as a separation medium. Firstly, contamination of the final monocyte population with approximately 30% lymphocytes made it impossible to determine the contribution of monocytes to tumour cell cytotoxicity. Secondly, the lymphocyte population was split into three strata (the monocyte layer (40-50%), and two lymphocyte layers (50-60% and 60-70%), each of which was unrepresentative of the donor whole blood lymphocyte population. Since these could not be completely reconstituted due to the presence of monocytes in the 40-50% layer, percoll was rejected as a separation technique in this study.

Adherence/EDTA treatment produced a very high purity of monocytes (92 +/- 5%, n=8) which was significantly greater ($p<0.05$) than that obtained using either Nycodenz or Percoll separation. Monocyte yield (9 +/- 2 $x10^6$ cells). from adherence/EDTA was similar ($p>0.05$) to that of Percoll, but significantly greater ($p<0.05$) than Nycodenz. The non-adherent fraction contained 92 +/- 1% (n=8) lymphocytes with a mean yield of 38 +/- 6 $x10^6$ cells (n=8; Figures 2.2a and 2.2b), and purity of 92 +/- 1% (n=8), which were not significantly different ($p>0.05$) from the values seen for Percoll. Adherence/EDTA was, therefore, chosen as the method for lymphocyte and monocyte purification in this study.

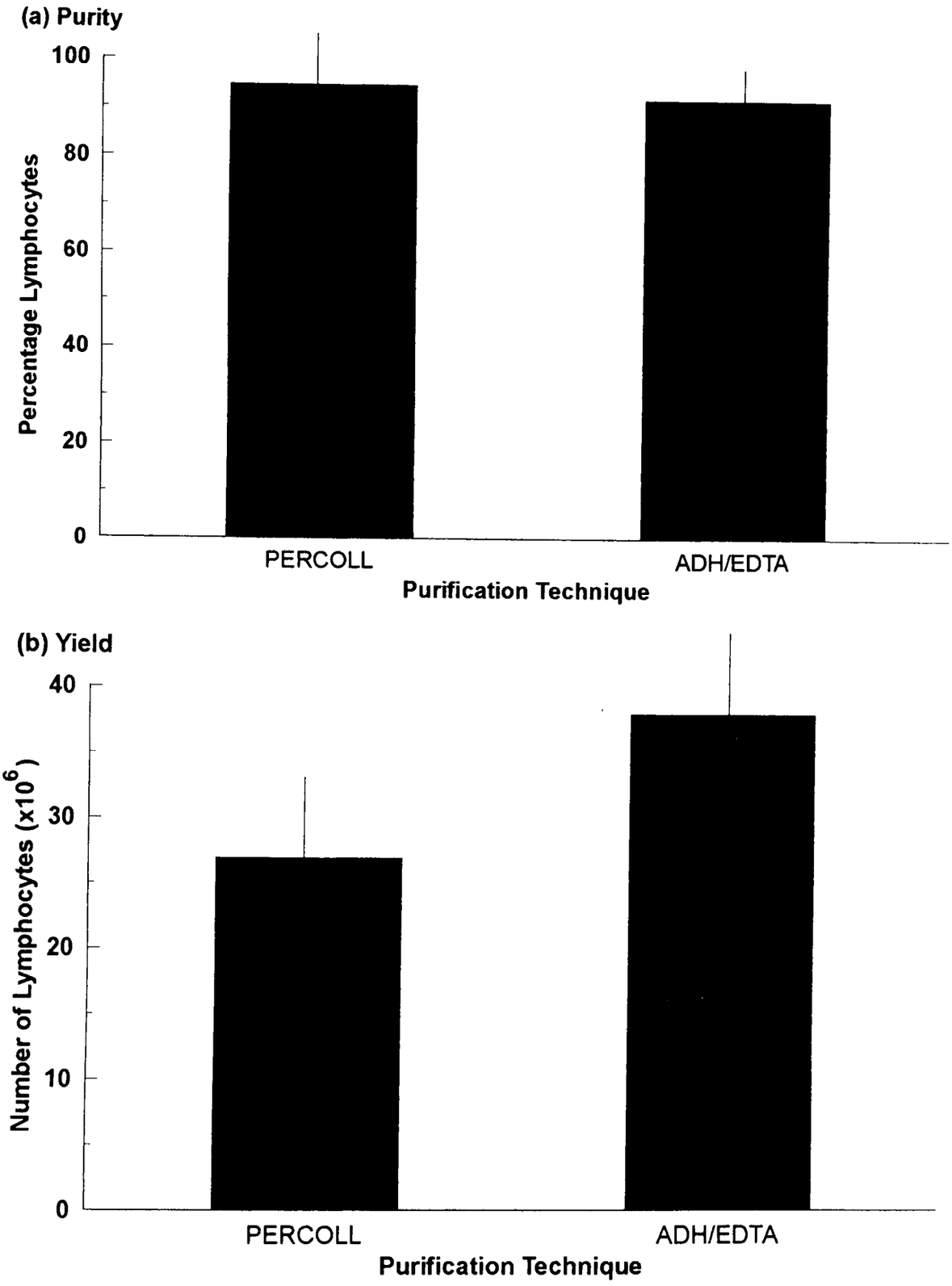

FIGURE 2.2. Isolation of lymphocytes by two techniques. Means and SEMs from 6 (Percoll 50-60% interface) or 8 (Adherence/EDTA) experiments. Mononuclear cells from 60ml blood were either layered onto percoll gradients or fractionated into lymphocytes and monocytes by plastic adherence. Lymphocyte purities and yields were not significantly different ($p>0.05$) by either method, as determined by MWU.

2.1.12 Purification of Lymphocyte Subsets by Cell Sorting

Peripheral blood mononuclear cells prepared as described in section 2.1.7, were incubated for 18h in 75cm^2 tissue culture flasks at a density 10^6/ml in RPMI/FCS. The cells were washed once in azide free flow cytometry sheath fluid (Facsflow; Becton-Dickinson Immunocytometry Systems, Oxford, U.K.) and the cell concentration adjusted to 5 x 10^6/ml in HBSS. The cell preparation was incubated with 2.5µl of FITC labelled anti-CD8 monoclonal antibody per 100µl cells for 20min at room temperature, and washed twice in HBSS. An aliquot (100µl) of unlabelled lymphocytes was also labelled with 2.5µl isotype IgG1 FITC control, incubated for 20min, and washed twice in HBSS. All cell preparations were resuspended in 400µl HBSS per 100µl cells. The cells were then passed through the cell sorter according to the following procedure.

After preparation of the FACStar plus for sterile sorting, the isotype control labelled cells were passed through the flow cytometer to determine the negative to positive delineator for FITC. A "sort gate" was placed around the unlabelled cells, which resulted in the collection of lymphocytes depleted of CD8+ cells. Approximately 5-10 x 10^6 cells were collected for inclusion in the cytotoxicity assay. In all experiments depletion of CD8+ cells was >95%.

Three experiments were performed to determine if the passage of the cells through the cell sorter alone had any effect on subsequent lymphocyte activity (figure 2.3). Unlabelled lymphocytes were passed through the cell sorter and their cytotoxicity compared with that of fresh matched lymphocytes (*i.e.* cells which had not been passed through the cell sorter) which were added directly onto the target cells (untreated A375 melanoma cells). The assay was set up according to the method described in section 2.2. Figure 2.3 shows that passage through the cell sorter did not significantly affect lymphocyte killing of A375 cells ($p>0.05$ by MWU and PTT).

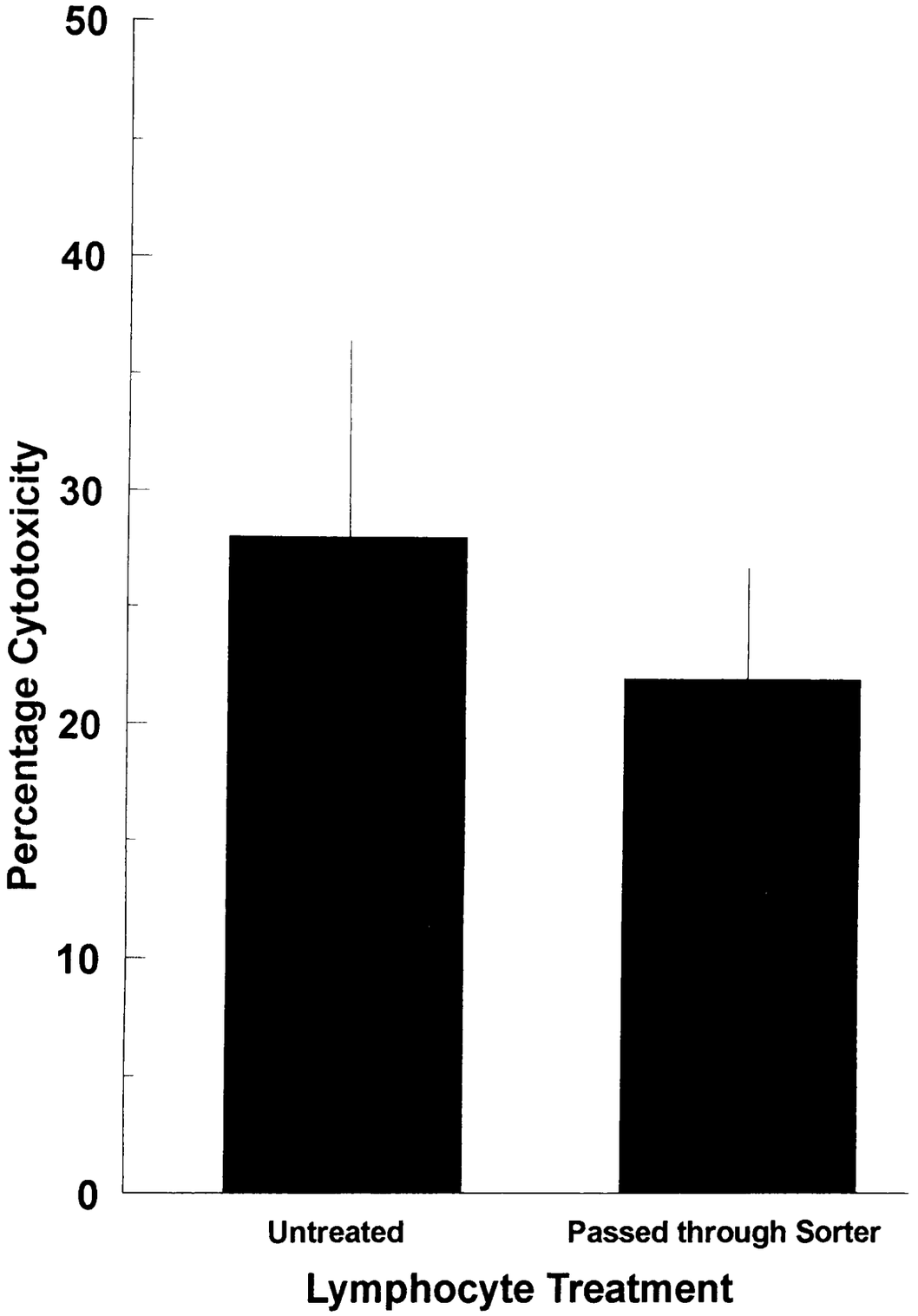

Figure 2.3. Effect of cell sorting on the cytotoxicity of lymphocytes against A375 cells. Freshly isolated lymphocytes were split into 2 aliquots. One aliquot was incubated at 37^{0}C (untreated) and the other passed through the cell sorter. Lymphocytes were added to A375 cells at an effector:target cell ratio of 50:1 for 18h at 37^{0}C. The results are expressed as the mean and SEM from 3 such experiments. No significant effect ($p>0.05$) on lymphocyte cytotoxicity was observed by passage of lymphocytes through the cell sorter, as determined by MWU and PTT.

2.1.13 Preparation of LAK and Control Cells

Non-adherent lymphocytes derived from 60ml of blood were divided into two aliquots containing equal numbers of cells. The cells were centrifuged and the pellets resuspended to 2 x 10^6 cells/ml in either RPMI/FCS (control cells) or RPMI/FCS containing graded concentrations of IL-2 (LAK cells). Lymphocytes were incubated at 37^0C upright in $25cm^2$ (not more than 25ml/flask) tissue culture flasks for 4h to 7d, decanted into universal containers, washed three times in RPMI to remove any residual IL-2, and a cell count and viability assessment (sections 2.1.15) performed prior to their inclusion in cytotoxicity experiments.

2.1.14 Isolation and Growth of Endothelial Cells and Fibroblasts

Human umbilical cords were collected into physiological saline by staff in the St. Thomas' Hospital delivery suite and stored at 4^0C for up to 16h. In the laboratory, the cords were cut into 10-15cm lengths, and one vein of each cut piece cannulated using 16 gauge stainless steel cannulae. The veins were flushed through with 10ml RPMI to remove any blood, and then filled with 10ml RPMI containing 300U/ml type IV collagenase (Sigma, Dorset, U.K.). After 10min incubation at 37^0C the collagenase solution containing endothelial cells was removed into universal containers. RPMI (30ml total volume) that was flushed though each vein to remove any remaining endothelial cells was also collected. The cell suspensions were centrifuged at 200*g* for 10min, and the pellets resuspended in 20ml of RPMI/FCS. An aliquot of cells was examined under the microscope to check for the presence of endothelial cells. The cell suspension was then placed in $25cm^2$ tissue culture flasks and incubated at 37^0C. After 6-18h the flasks were examined for adherent cells. The culture medium was changed every 2 days until the cells had formed a confluent monolayer. On attaining confluence, the cells were detached by the addition of 2ml trypsin/EDTA solution (as used for the detachment of tumour cells; section 2.1.2) followed by gentle swirling of

the flask. Using an inverted microscope cell detachment was seen as sheets of endothelial in suspension. Twenty millilitres of RPMI/FCS were then added to each flask and repeatedly pipetted in and out of the flask to break up the cell monolayers. The cells were either decanted into 2 fresh flasks (10ml each) together with 10ml RPMI/FCS for further growth, or 200μl aliquots pipetted into flat bottomed 96 well microtitre plates (Costar Ltd., Berks., U.K.) for use in the cytotoxicity assays. Plates were regularly checked for cell attachment and growth, and were introduced into cytotoxicity experiments on reaching confluence.

The concentration and incubation time of collagenase in the umbilical veins was critical. Increasing either or both of these factors resulted in fibroblast detachment from the vessel walls, which subsequently overran and replaced the endothelial cells in culture. In this study, fibroblast cultures were treated in the same way as the endothelial cultures for inclusion in the cytotoxicity assay.

2.1.15 Assessment of Cell Numbers and Cell Viability

Tumour, leucocyte or normal cell numbers or viability were determined by the addition of 20μl of cell suspension to 180μl of either crystal violet solution (0.1% w/v crystal violet (Merck Ltd, Lutterworth, Leics.) in a solution of 3% acetic acid (v/v) in distilled water, or (0.5%) w/v of the vital stain trypan blue (Merck Ltd., Lutterworth, Leics.) respectively. The cell suspensions were placed in an Improved Neubauer haemocytometer chamber. The number of cells occupying the central and four peripheral large fields was counted and divided by 50. This represented the concentration of cells in 10^6cells/ml.

2.2 Cytotoxicity Assays

2.2.1 Cytotoxicity of Tumour Cells

Cytotoxicity of tumour cells was determined using a standard $Na^{51}CrO_4$ (Amersham International PLC, Aylesbury, Bucks) release assay. All assays were performed in triplicate. Target cells (fresh tumour cells; sections 2.1.2 and 2.1.3) or cytokine (section 2.1.4) and/or Act-D pretreated tumour cells (section 2.1.5) were washed once in RPMI to remove any FCS, the supernatant discarded and 150μCi of added to the pellet and mixed. The cells were then incubated at 37^0C for 45min. After this period, the cells were washed twice with RPMI/FCS (to remove any free $Na^{51}CrO_4$), followed by a single wash in RPMI. The final pellet was resuspended in 1ml RPMI and a cell count and viability (section 2.1.15) performed. The cell concentration was adjusted to 1 x 10^5/ml or 2 x 10^5/ml and 100μl or 50μl respectively added to the appropriate wells of a 96 well round bottomed microtitre plate. The ratio of effector molecules and cells varied according to experimental design. The details of preparation and addition of these effectors to the tumour cells are outlined in the following subsections.

2.2.2 Cytotoxicity Mediated by Soluble Effector Molecules and Cytokines

In experiments investigating the cytotoxic activity of various factors (*e.g.* cytokines, LPS ; see chapter 4), serial dilutions (usually 6) were made from the stock solutions (appendix IV) in RPMI/AB or RPMI/FCS (depending on the experiment) and 100μl added to tumour cells in the microtitre plate.

2.2.3 Inhibition of Cytokine Cytotoxicity by Monoclonal Antibodies

In these experiments, tumour cells were initially diluted in RPMI to four times the final dilution required and 50μl added to each of the assay wells. Monoclonal antibodies to cytokines (TNFα and LT) were diluted to four times the required final concentration in RPMI, and 50μl added to relevant wells in the microtitre plate. Cytokines were prepared and added to the wells as in the previous section.

2.2.4 Cytotoxicity Mediated by Effector Cells

To investigate the cellular cytotoxicity of tumour cells, effector cells (control, LAK, monocytes) were adjusted to 5 x 10^6 cells/ml in RPMI/AB or RPMI/FCS (depending on the experiment), and 5 serial two-fold dilutions prepared. Effector cells (100μl) were then added to the appropriate wells containing tumour cells.

Spontaneous and total $Na^{51}CrO_4$ release were determined by adding 100μl RPMI/AB (FCS) and 100μl 6% sodium dodecyl sulphate (SDS) respectively to wells (in triplicate) containing only tumour cells. Spontaneous counts were thus a measure of non-specific $Na^{51}CrO_4$ release from the target cells, and total counts represented the total amount of radioactivity in 100μl of target cells.

2.2.5 Inhibition of Cellular Cytotoxicity by Monoclonal Antibodies

Tumour cells were initially diluted in RPMI to four times the final dilution required and 50μl added to each of the assay wells. Monoclonal antibodies to cytotoxic effector molecules (TNFα or LT; appendix IV) were diluted to four times the required final concentration in RPMI, and 50ml added to relevant wells in the microtitre plate. Lymphocytes or LAK cells were prepared and added to the wells as described in section 2.2.4.

2.2.6 Inhibition of Cellular Cytotoxicity by Coagulation Factors and Their Inhibitors

In experiments investigating the inhibition of cytotoxicity by plasma (chapter 5) the following modifications were made to the cytotoxicity assay. Target cells (A375) were adjusted to 2 x 10^5/ml and 50μl added to relevant wells. Plasma/serum (normal or coagulation factor deficient) were diluted to twice the final concentration required and 100μl added to relevant wells. The coagulation factor deficient plasmas (factor-V; factor-VII, factor-VIII, factor-IX, factor-X), were obtained from Sigma Chemical Co. Ltd., Dorset, U.K.; and factor XIII deficient plasma was obtained from a patient with hereditary severe factor XIII deficiency, who had been referred to the Haemophilia Centre by Dr Osier at Bournemouth General Hospital. Effector cells were adjusted to 1x10^7/ml and 50μl added to relevant wells. Additional constituents of the assay such as graded concentrations of eACA or Gly-Pro-Arg-Pro (Sigma Chemical Co., Ltd., Dorset, U.K.) were included in the serum/plasma dilution. In some experiments effector or target cells were mixed with serum/plasma dilutions and incubated for 30min prior to addition of the other cellular component(s). Total counts were measured as described above, but spontaneous counts were determined by the addition of 100μl of the relevant plasma/serum dilution and 50μl RPMI to the tumour cells . After addition of both target cells and effector (cells or soluble factors and/or inhibitors), the plates were incubated at 37^{0}C for 4-18h, depending on the experiment. At the end of the incubation, the supernatants were harvested from the wells using a filter based supernatant collection system (Skatron Ltd) (In experiments investigating the inhibition of cytotoxicity by plasma, almost all of the supernatant could be collected from the wells using this harvesting system, even when clots were present). The tubes were then counted on a gamma spectrometer (Wallac (U.K.) Ltd.,Crownhill, Milton Keynes; model number 1282). The mean values from the triplicate counts was calculated.

Results were expressed as:- $\frac{\text{cpm(test) - cpm(spontaneous)}}{\text{cpm(total) - cpm(spontaneous)}} \times 100\%$

2.2.7 Cytotoxicity by Soluble Factors from Effector Cells.

To investigate whether cell-cell contact was necessary for cellular cytotoxicity to occur, effector cells were physically separated from target cells in the culture wells by polycarbonate culture well inserts, which were porous to soluble molecules, but would not allow the passage of cells. Cytotoxicity observed under these conditions was compared with that in which the membranes were absent.

Culture well inserts are only available for 24 well and larger tissue culture plates. The microcytotoxicity assay described in section 2.2 was therefore scaled up from a total final volume of 200µl using 96 well plates to 2ml in 24 well plates (Costar Ltd., Berks., U.K.). A375 cells were detached from plastic and labelled with $Na^{51}CrO_4$ (Section 2.2.1), and assessed for cell number and viability (section 2.115). The cell concentration was adjusted to 1 x 10^5cells/ml in 10%FCS and 1ml added to the relevant wells of a 24 well microtitre plate.

Control and LAK cells were prepared as described in section 2.1.13, and adjusted to 5 x 10^6cells/ml in RPMI/FCS. The effector cells (1ml) were either added to wells containing tumour cells alone (thus mixing effector and target cells), or to the inside of polycarbonate membranes (0.8µ pore size, Nunc, Life Technologies, Renfrewshire, Scotland) which had been inserted above the tumour cells in the wells, thus preventing cell-cell contact. The volume of target cells (1ml) ensured that the polycarbonate inserts were surrounded by culture medium, in order to allow the passage of soluble molecules across the membranes. Spontaneous and total counts were determined by the addition of 1ml RPMI/FCS or 1ml 6% SDS respectively to the tumour cells. The plates were incubated at 37^{0}C for 18h and the polycarbonate membranes removed. The plates were then centrifuged at 400*g* for 5min in a Jouan

CR422 centrifuge (with swingout adaptors for microtitre plates), and 0.5ml of supernatant transferred to LP2 tubes (Luckham Ltd, Burgess Hill, Sussex). The radioactivity was counted, and the results calculated as described in the previous section.

2.2.8 Control and LAK Cell Cytotoxicity of Autologous Leucocytes

Lymphocytes were purified from 60ml heparinised blood (section 2.1.6) as described in section 2.1.10, and control and LAK cells prepared (section 2.1.13). After 48h the control and LAK cells were washed three times in RPMI/FCS to remove any residual IL-2, and a differential cell count and cell viability determined (section 2.1.15). The effector cell concentration was adjusted to 5 x 10^6/ml and added to the relevant wells of a microtitre plate together with target cells where appropriate.

After the 48h incubation required for the preparation of control and LAK cells, a second blood sample (30ml) was collected from the same donor and lymphocytes and monocytes prepared (section 2.1.10). An aliquot of cells (3-5x10^6 cells depending on cell yield) was centrifuged at 200*g* for 10min, resuspended in its own volume, 100μCi $Na^{51}CrO_4$ added and the cells incubated for 45min at 37^{0}C. After this time the cells were washed twice in RPMI/FCS and a differential cell count and viability test (section 2.1.15) performed. The cell concentrations were adjusted to 1x10^5 cells/ml and 100μl added to the relevant wells of the microtitre plate. Total and spontaneous counts were determined as described for tumour cells in section 2.2.4.

2.2.9 Control and LAK Cell Cytotoxicity of Endothelial Cells and Fibroblasts

Microtitre plates containing confluent endothelial cells or fibroblasts were washed twice with RPMI and 100μl of RPMI added to all cells except 1 row of cells to which was added trypsin/EDTA for 5 min. The 12 wells containing trypsin/EDTA treated cells were gently pipetted repeatedly several times using a plastic pasteur pipette to dislodge any remaining

adherent cells and their contents pooled into a 7ml plastic bijou bottle. The cells were counted using crystal violet (section 2.1.15), and the mean concentration of cells/well calculated. The RPMI in the plate was decanted and 100μl of RPMI containing 2μCi $Na^{51}CrO_4$ added to each well and incubated for 45 min at 37°C. The cells were washed twice in RPMI/FCS and 200μl of RPMI/FCS containing either control or LAK cells were added to appropriate wells. The highest density of control or LAK cells was adjusted to 50 times the mean concentration of target cells, and doubling dilutions were made from this preparation. To six wells were added 200μl 10%FCS which acted as a medium control, and 200μl 6% SDS were also added to 6 wells, from which the total radioactivity present in each well was determined. After 1, 2, 4 or 18h incubation at 37°C the supernatants from individual wells were collected onto absorbant filters using the Skatron Macrowell™ harvesting system (Skatron Ltd., Newmarket, Suffolk), and the radioactivity counted on a gamma spectrometer. Results were calculated according to the formula described in section 2.2.6

2.3 Coagulation Assays and Preparative Techniques

2.3.1 Preparation of Normal Plasma and Serum

Sixty millilitres of venous blood was obtained from normal healthy donors, of which 30ml was placed into 6 vacutainer tubes containing sodium citrate (9 parts blood to 1 part 0.109M trisodium citrate) for plasma, and 30ml placed in 3 glass vacutainer tubes for serum. All tubes were centrifuged at 600*g* for 15min and the serum or plasma removed into separate 30ml universal containers, which were re-centrifuged at 600*g* for 15min. Serum and plasma were then divided into 1ml aliquots in 7ml plastic bijou bottles and stored at -70^{0}C. For use, vials of plasma were removed from the freezer, together with vials containing donor matched serum. These were thawed at 37^{0}C in a water bath in order to minimise cryoprecipitate formation.

2.3.2 Preparation of Aluminium Hydroxide Adsorbed Plasma

A stock solution of Aluminium Hydroxide was prepared by adding 3g $Al(OH)_3$ to 4ml distilled water in a universal container. The resulting saturated solution of aluminium hydroxide (1 part) was added to fresh citrated plasma (5 parts), and the mixture incubated for 3min at 37^{0}C. The mixture was centrifuged at 600*g* for 10min and the supernatant (aluminium hydroxide adsorbed plasma) was removed for use in the cytotoxicity assay.

2.3.3 Preparation of Plasminogen Depleted Plasma

Plasminogen depleted plasma had already been prepared in the coagulation research unit and was therefore available for use in this study. It was prepared by passing whole plasma though a chromatography column packed with sepharose 4B, to which lysine had been covalently coupled (Cuatrecasas, 1980). As a result, plasminogen in plasma bound to the lysine groups on the matrix, and the run-through volume of the column contained plasma which had been depleted of plasminogen (as previously assessed by ELISA).

2.3.4 Visual Assessment of Clot Formation

Coagulation induced in normal and coagulation factor deficient plasmas (V, VII, VIII, IX and X) in the presence of RPMI, control cells, LAK cells, A375 cells, control + A375 cells and LAK + A375 cells was observed in 24 well tissue culture plates. The final leucocyte and tumour cell concentrations (and thus the effector:target cell ratio of 50:1) were the same as those used in the cytotoxicity assay (section 2.2.1). Control and LAK cells were adjusted to $1x10^6$cells/ml (when added to the plate in the absence of A375 cells) or $2x10^6$/ml (when added to the plate with A375 cells) and 1ml or 0.5ml respectively added to relevant wells of a 24 well tissue culture plate. The A375 cell concentration was adjusted to 1 $x10^5$cells/ml (in the absence of effector cells) or 2 x 10^5/ml (in the presence effector cells) and 1ml or 0.5ml respectively added to the wells. Plasma was diluted 1:9 with RPMI and 1ml added to the relevant wells. All assays were performed in triplicate. Where multiple time points were required, plates were set up in parallel (one for each time point). The plates were incubated at 37^0C for 0,1,2 or 4h, and were visually examined for clot formation. A score from - to +++ was assigned to each well, depending on the solidity of the clot. A score of: (-) indicated no observable clot formation; (+) - partial clotting, resulting in increased viscosity of the cell suspension; (++) - a solid clot which disintegrated on agitation; (+++) - a solid clot which was not dispersed by agitation. Supernatants (0.5ml) were taken from each well and snap frozen at - 70^0C in cryovials (Costar Ltd., Berks., U.K.) for later analysis of thrombin and D-dimer production.

2.3.5 Chromogenic Determination of Thrombin Generation

Supernatants from experiments described in section 2.3.4 were thawed and 50µl added in triplicate to the relevant wells of a 96 well microtitre plate. To this was added 50µl of diluent buffer from a commercial thrombin test kit (Quadratech Ltd, Epsom, Surrey.), together with 100µl of S2238 thrombin

specific chromogenic substrate. The plate was incubated for 4h at 37^{0}C and the reaction was stopped by the addition of 100µl of 50% acetic acid (B.D.H. Laboratory Supplies, Lutterworth, Leics.) in distilled water. The absorbance values were read at 405nm on a microtitre plate reader. Results were expressed as the mean absorbance (from the triplicates) for each sample.

2.3.6 Chromogenic Determination of Factor X Activation by A375 Cells

Lyophilised purified Factor X (Alpha Therapeutic) was reconstituted with 10ml distilled water, diluted to 1U/ml (1U is the amount of Factor X present in 1ml of normal plasma), and 100µl added in quadruplicate both to the A375 cells in the wells of a 96 well microtitre plate, and in parallel to empty wells of a separate microtitre plate. The plates were incubated for 4h at 37^{0}C and 100µl of the chromogenic substrate S2222 (specific for Factor Xa; Quadratech Ltd., Epsom, Surrey) was added to each well which contained Factor X. After a further incubation at 37^{0}C the plates were read at 405nm on a microtitre plate reader. Absorbances in wells containing reagents but not A375 cells (blank) were subtracted from absorbances obtained from wells which contained cells.

2.3.7 Chromogenic Determination of Plasmin Activity on A375 Cells

To confluent A375 cells in a microtitre plate (section 2.1.2) was introduced ,in triplicate, 100µl of RPMI or plasminogen depleted plasma (section 2.3.3), normal plasma or matched serum which had been diluted to 20, 10, 5, 2.5, 1.25 and 0.63% in RPMI. The plate was incubated at 37^{0}C for 4h, washed three times with RPMI and 100µl of the plasmin specific chromogenic substrate S2251 (Quadratech Ltd, Epsom, Surrey: S2251 does not show activity with any of the collagenases, elastase or cathepsin-G: personal communication from Graham Jones, Quadratech Ltd.) added to each well. The plate was incubated at 37^{0}C overnight and the absorbance of the wells measured at 405nm in a microtitre plate reader

2.3.8 Measurement of D-Dimer Production

Supernatants obtained in experiments described in section 2.3.4 were thawed and D-dimer measured using a commercial Enzyme Linked Immunosorbant Assay kit (ELISA; Quadratech Ltd., Epsom, Surrey). Microplate wells coated with an anti-D-dimer monoclonal antibody were incubated with samples or D-dimer standard solutions. During the incubation period, D-dimer present in the sample bound to the antibody. The wells were then washed with buffer to remove unbound substances, and an enzyme labelled anti-D-dimer antibody was added to the wells and a further incubation performed. This conjugate bound to the antigen-antibody complexes which in turn were bound to the plate. The plate was again washed to remove excess labelled antibody and the enzyme substrate was added for a further incubation period. The absorbance of the product in the wells was read in a microtitre plate reader at 450nm. A standard curve was obtained by plotting the concentrations of the D-dimer standard solutions against their absorbance readings. The concentration of D-dimer present in the samples was interpolated from the standard curve.

2.3.9 Gel Filtration of Fibrinogen

One vial of commercial fibrinogen concentrate (Centre de Regionale Transfusion de Lille, France. Batch No. 1020030.0) was reconstituted with 20ml distilled water according to the manufacturer's instructions. The fibrinogen concentration was 18.62mg/ml (as measured by ELISA, section 2.3.10). A chromatography column containing sepharose CL-6B was equilibrated with RPMI and 12ml fibrinogen concentrate (223.44mg) added. The flow rate through the column was adjusted to 60ml/h and fractions (10ml) were collected until the absorbance at 280nm had returned to baseline values A total of 31 fractions were collected and stored at -70^{0}C. The fibrinogen concentration of each fraction was determined as described in section 2.3.10. Figure 2.4 shows that the

peak fibrinogen concentration in the fractions corresponded to the peak absorbance. The original fibrinogen preparation and fractions 13, 14, 15 and 16 (containing the highest concentrations of fibrinogen) were assayed for functional (clottable) fibrinogen by the clinical coagulation laboratory. The fibrinogen in the samples was clotted by addition of thrombin in 96 well microtitre plates. The plates were centrifuged and a protein determination performed on the supernatants. The detection of protein in the supernatants suggested the presence of non-clottable fibrinogen. Fibrinogen was reported to be >95% clottable by this technique, indicating that the fibrinogen retained its biological activity after gel filtration.

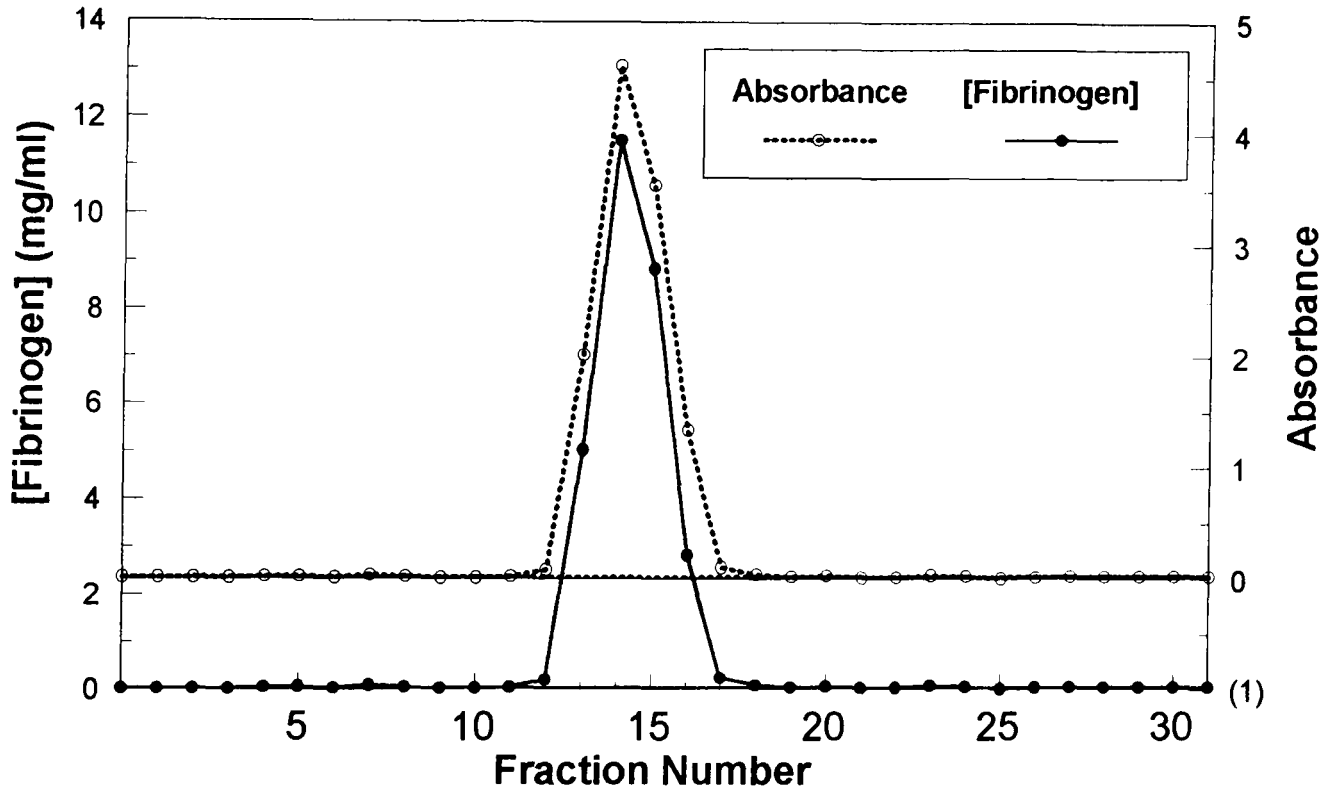

FIGURE 2.4. Absorbance and protein concentration of fractions from gel filtered fibrinogen. The absorbance at 280nm was measured during gel filtration of fibrinogen, until 310ml (31 fractions) had passed through the column. A peak in absorbance was observed in fractions 13-16. Fibrinogen concentration (measured by ELISA) showed a peak in the same fractions, indicating that the absorbance peak corresponded to eluted fibrinogen. The Fibrinogen concentrations in these fractions were: fraction 13 (5.032mg/ml); fraction 14 (11.52mg/ml); fraction 15 (8.85mg/ml) and; fraction 16 (2.83mg/ml).

2.3.10 Fibrinogen ELISA

The fibrinogen concentrations of the fractions collected from the gel filtration column were measured using an "in-house" ELISA specific for fibrinogen.

Rabbit anti-human fibrinogen polyclonal antibody (Immunotech Ltd.) was diluted 1:1000 in carbonate-bicarbonate buffer (0.05mol/L) and aliquots (100μl) added to each well of a 96 well microtitre plate, for 1h at room temperature in a damp chamber. During this period the supernatants from the gel filtration column were thawed and the lyophilised plasma standard (NIBSC) and control plasma (Immuno Ltd, Sevenoaks, Kent) reconstituted. These were then diluted 1:8000, 1:16000, 1:32000 and 1:64000 in 1ml/L BBS. After incubation the plate was washed three times with BBS, with a 1-2min wait before discarding each wash. The diluted standards or test samples, in duplicate, (buffer was added to each well of row 1 as a sample blank) were added to the plate, incubated for 1h at room temperature in a damp chamber, and washed three times with BBS. Peroxidase conjugated rabbit anti-human fibrinogen was diluted to 1:2000 with BBS and 100μl added to each well of the plate, for a further one hour incubation. This was followed by two washes with BBS, one wash with citrate buffer, and 100μl of the substrate added to each well. The plate was incubated at room temperature until the absorbance of the control at a 1:8000 dilution reached 0.15 at 492nm. The enzyme reaction was stopped by addition of 100μl 1.0mol/L sulphuric acid to each well and the plate read at 492nm. The relative potencies of the samples were calculated using a bioassay computer program (Williams *et al.*,1975).

2.3.11 Phast Gel Electrophoresis of Fibrinogen (Reduced)

Samples were diluted in reducing buffer (4% sodium dodecyl sulphate, 2% mercaptoethanol, 20% glycerol, 0.0082% bromophenol blue, 0.125mol/L tris. pH 6.8), heated in a boiling water bath for 10min and centrifuged at 12000g for 3min. Parafilm was placed over an electrophoresis stencil and pressed to form 8 indentations into which small (4μl) volumes of the samples were placed in positions 2-7. The

same volume of the molecular weight marker solution (MW range 14 to 94-kDa, Pharmacia Biosystems Ltd., Knowhill, Milton Keynes) was added to positions 1 and 8. The separation bed of the phast system was wetted with water and 20% Phast gels (resolution 2 to 150-kDa, Pharmacia Biosystems Ltd, Knowhill, Milton Keynes) placed on the bed within the boundaries and any air bubbles removed. SDS buffer strips (Pharmacia Biosystems Ltd., Knowhill, Milton Keynes) were inserted into the frames and placed in the electrophoresis chamber on either end of each gel. The electrodes were lowered onto the buffer strips, and the electrophoresis program set to run for 20% gels. Reagents were prepared as described in appendix VII and connected to the appropriate inlet and outlet ports of the machine. The waste collection bottle was connected to port 0. A sample applicator with 8 teeth was lowered onto the samples and placed in the sample holder. The lid of the phast gel electrophoresis unit was then closed and the separation program activated.

After separation, the gels were inserted into the development unit and stained with coomassie blue, after which they were dried and scanned on a computing densitometer (Molecular Dynamics Ltd., Sevenoaks, Kent) to obtain computerised images (figure 2.5).

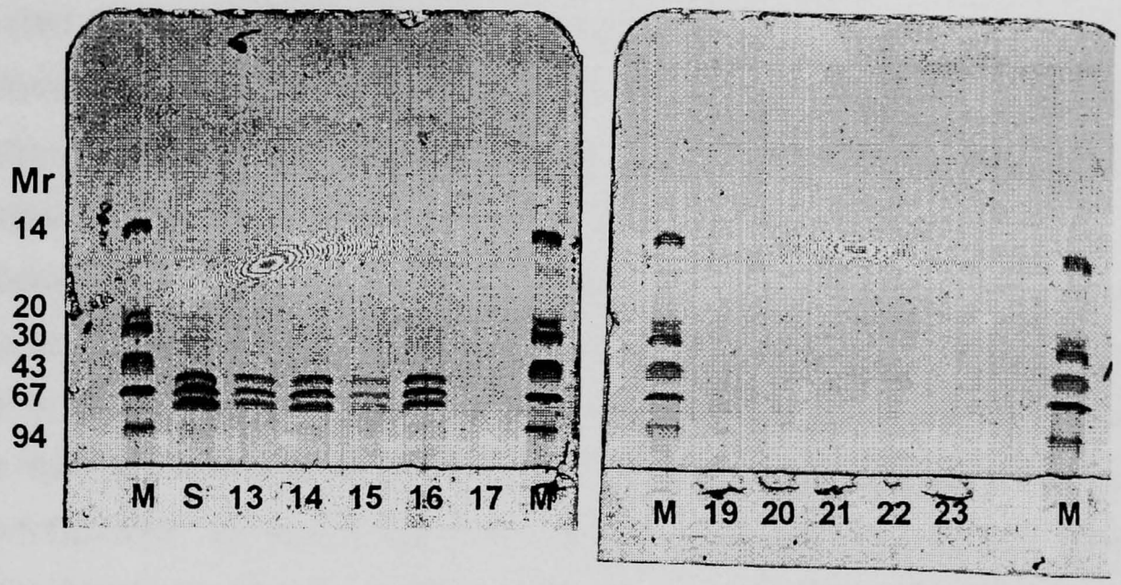

FIGURE 2.5. Phast gel electrophoresis of fibrinogen fractions 13-23. Fibrinogen fractions (numbers under lanes) and the unfractionated commercial product (S) were diluted in reducing buffer, boiled, centrifuged at 10,000*g* and applied to the gels. Molecular weight markers (M) were added to the first and last lanes of each gel. The two gels were run together on the phast system. After staining with Coomassie blue, the characteristic 3 bands of reduced fibrinogen were observed in the unfractionated material, *i.e.*, the Aα (66.5-kDa), Bβ (52-kDa) and γ (46.5-kDa) chains, as well as fractions 13-16 (corresponding to the absorbance and fibrinogen ELISA peak), but not in any subsequent fractions.

In the starting material and fractions 13-16, only the fibrinogen bands were present, indicating that these preparations were not contaminated with proteinaceous material with an Mr below 100kDa. The molecular weight markers in the right hand lane of the first gel gave closer molecular weight comparisons for the fibrinogen chains to the published data.

2.4 Flow Cytometry

2.4.1 Detection of Cell Surface Antigens (Direct Labelling)

Mononuclear cells, lymphocytes, monocytes, LAK cells or tumour cells were adjusted to 5 x 10^6 cells/ml in Facsflow™ (Becton-Dickinson, San Jose, California, USA). Cells were aliquotted (100μl) into Falcon™ FACS tubes (Becton-Dickinson, San Jose, California, USA)., and 20μl of fluorophore labelled (FITC, PE) monoclonal antibody (see individual experiments for antibody specificity) or isotype control added, for 20min at room temperature. In some experiments, two different antibodies were added to the same tube in order to distinguish more than one antigen on the same cell, *e.g.*, monocytes were distinguished from lymphocytes in the same cell preparation by addition of anti-CD14PE, as well as the antibody of interest (*e.g.*, anti-CD25). Where Becton-Dickinson directly labelled antibodies were used, equivalent volumes of antibody and isotype control could be used. For other antibodies the volume of isotype control was calculated to achieve an equivalent final immunoglobulin concentration to that of the monoclonal antibody. Two millilitres of Facsflow were added to each tube and the tubes were centrifuged at 400*g* for 5min. The supernatant was discarded and the cells resuspended in 400μl of Facsflow. Cell surface markers were measured on the cells using a Becton-Dickinson FACScan and calculated on Lysys II and QuickCal software. Ten thousand events were acquired from each tube, and compared with an equal number of events acquired from the isotype (negative) control.

2.4.2 Detection of Cell Surface Antigens (Indirect Labelling)

The procedure for the labelling of cells with unlabelled primary antibody (*e.g.*, anti-human Factor V (Sigma, Dorset, U.K.) was essentially the same as in the previous section, with the exception that the volume of isotype control was adjusted to equal the concentration of IgG1 in respective tubes. After the initial incubation and wash step, 5μl of FITC labelled goat anti-mouse Fab" antibody were added to the cell pellets (total volume ~100μl) and incubated at room temperature for 20min. The cells were again washed and resuspended in 400μl Facsflow. Cells were counted as described in the previous section.

2.4.3 Quantification of Cell Surface Markers

2.4.3.1 Instrument calibration.

The fluorescence intensities of QuickCal™ FITC labelled latex beads carrying six different known amounts of FITC (Flow Cytometry Standards Corporation (FCSC), Leiden, The Netherlands) were measured on a FACScan using the same instrument settings as for cellular acquisition. This allowed the instrument to be calibrated for FITC. The data was then loaded into the QuickCal™ software program which created a Levey-Jennings plot of histogram channel number against the known values of molecules of equivalent soluble fluorophore (MESF) for FITC.

2.4.3.2 Fluorochrome to protein (F/P) ratio calculation.

Goat anti mouse coated latex beads ("Simply Cellular", FCSC, San Juan, PR) were incubated with 5µl of relevant FITC labelled monoclonal antibody (McAb) at RT for 15min. Subsequent analysis of gated singlet bead population on FSC Vs SSC dot plot and histogram analysis of the FITC peak produced a median channel value for the fluorescent peak for the McAb. The MESF values for the McAb were calculated from the QuickCal FITC Levey-Jennings plot by loading the median channel values into the MESF calculator software programme. Since "Simply Cellular" beads have a quoted number of mouse McAb binding sites, the F/P Ratio for each McAb was calculated by dividing the MESF values by the bead binding site number. Autofluorescence of latex beads was subtracted at all times from test MESF values by acquiring certified blank latex beads (FCSC, Leiden, The Netherlands;) and calculating the MESF value due to latex beads alone.

2.4.3.3 Determination of Cell Surface Antibody Binding Site Numbers.

Histogram FL-1 analysis of the cell population produced a median channel value for the relevant McAb. This channel value was converted to an MESF value via the MESF calculation software, as were cellular

autofluorescent median channel values. The mean antibody binding site number (MBSN) for the McAb was calculated by first subtracting the MESF value of autofluorescent certified blank latex beads from the autofluorescence of unlabelled cells. This value was then subtracted from the MESF value of labelled cells, thus accounting for any MESF values contributed by cellular autofluorescence. Since the F/P ratio is known for the McAb, the MBSN could be calculated for antibody binding sites on the cell populations.

2.5 Statistical Methods

In most of the groups of experiments in this study an insufficient number of data points were present to establish whether the data was normally distributed. As a result, non-parametric statistical procedures were applied to the data to test the null hypotheses that the results to be compared originated from the same population, and to determine the significance of any deviations observed. Error bars on figures represent standard error of the mean (SEM),

The Mann-Whitney U-test (MWU) was used to test unpaired data, and the Wilcoxon signed rank test (WSR) was used to test paired data, when the number of observations (n) exceeded 5. When n was less than 5 the paired t-test and Mann-Whitney U-tests were used. Probabilities less than 0.05 ($p<0.05$) were considered to be significant. These tests were performed using the Unistat statistical software package (Unistat Ltd., Maida Vale, London).

2.6 Cytotoxicity Assay Development

2.6.1 Time Course of Spontaneous ^{51}Cr Release from Tumour Cells

All cells spontaneously release $Na^{51}CrO_4$ with time after loading. Therefore the incubation time for cytotoxicity assays is limited by the rate at which radiolabel is lost from the cells. Typically, $Na^{51}CrO_4$ release assays continue up to 24h, after which, for most cell types, the spontaneous $Na^{51}CrO_4$ release becomes too high to give meaningful results. The cell lines K562 and Daudi were tested for their spontaneous $Na^{51}CrO_4$ release at 4, 18, 48 and 72h (Figure 2.6).

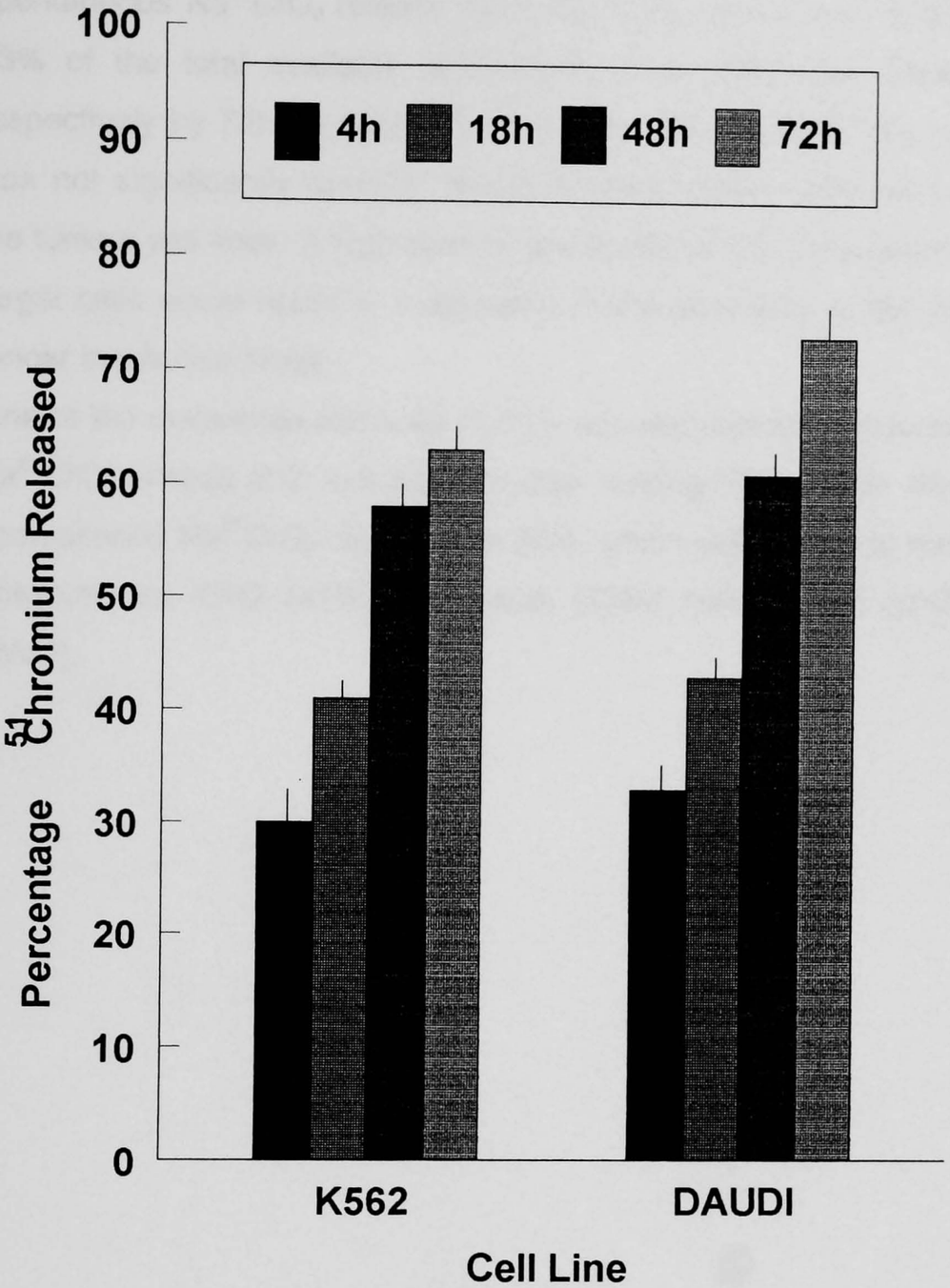

FIGURE 2.6. Time course for spontaneous $Na^{51}CrO_4$ release from K562 and Daudi cells. Tumour cells (K562 and Daudi) were loaded with excess $Na^{51}CrO_4$ (150μCi / $5x10^6$ cells for 45min), washed and added to 4 microtitre plates (1×10^4 cells/well). After incubation for 4, 18, 48 or 72h incubation supernatants were removed from the appropriate plate and the radioactivity counted. Three experiments were performed, and the means and SEMs are shown. After 4h 30% (K562) and 33% (Daudi) of the $Na^{51}CrO_4$ was released from the cells and there was a time-dependent increase in spontaneous $Na^{51}CrO_4$ release from the tumour cells ($p<0.05$ by MWU and PTT), reaching 63% (K562) and 73% (Daudi) after 72h.

Figure 2.7 shows that there was a time-dependent increase in the spontaneous $Na^{51}CrO_4$ release from the cells, with a loss of 63% and 73% of the total available radioactivity from K562 and Daudi cells respectively by 72h. At each time point, the level of $Na^{51}CrO_4$ released was not significantly ($p>0.05$, Mann-Whitney U-test) different between the tumour cell lines. A high level of spontaneous $Na^{51}CrO_4$ release from target cells would result in a decrease in the sensitivity of the assay at longer incubation times.

One of the melanoma cell lines (A375) was also tested for spontaneous $Na^{51}CrO_4$ release at 2, 4, 6 and 18h after loading (Figure 2.8). After 18h, spontaneous $Na^{51}CrO_4$ release was 38%, which was similar to the levels observed for K562 (41%) and Daudi (43%) cells at 18h ($p>0.05$ by MWU).

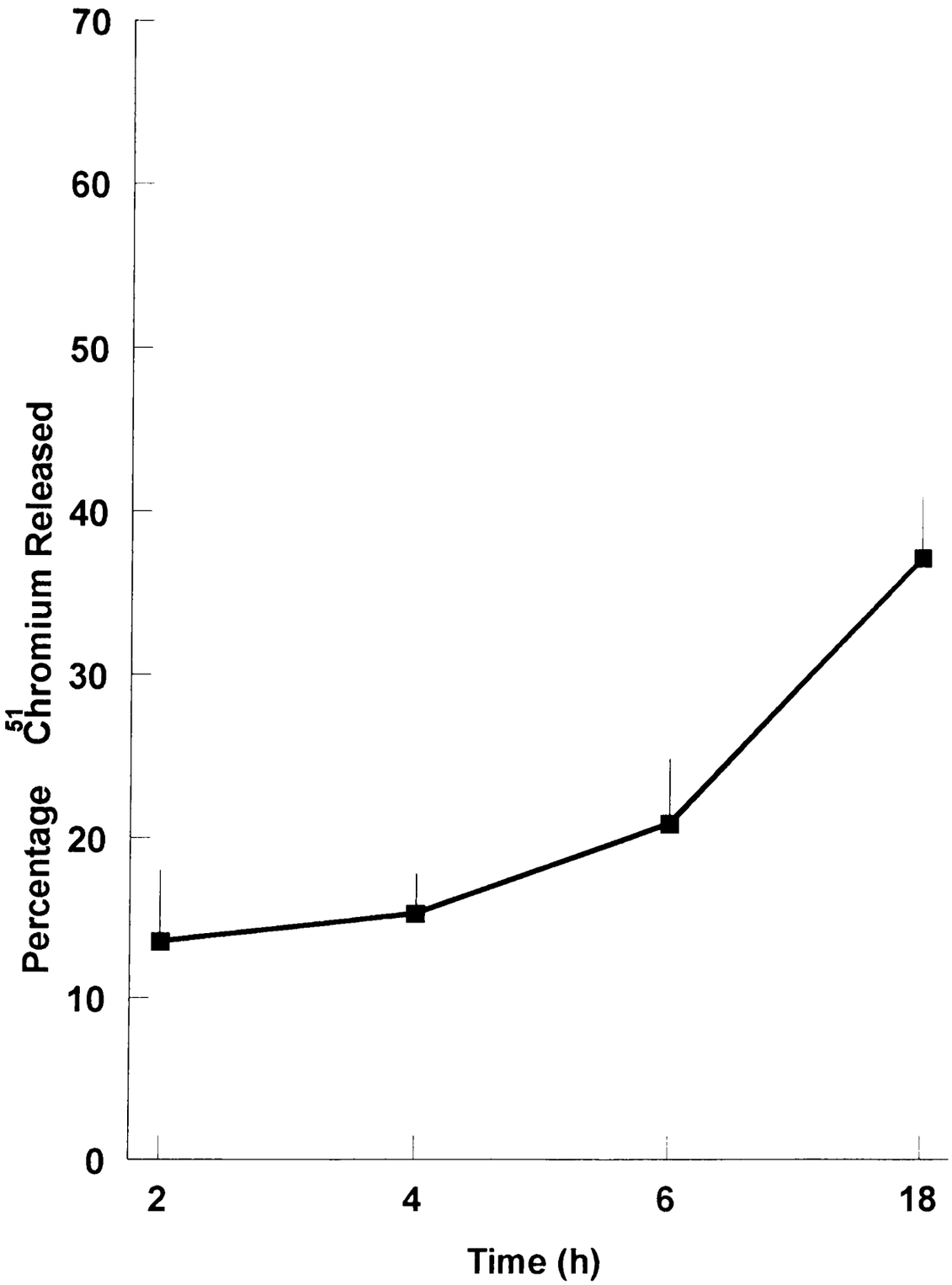

FIGURE 2.7. Time course for spontaneous $Na^{51}CrO_4$ release from A375 cells. After detachment from plastic, A375 cells were treated as described in figure 2.7. The means and SEMs from 6 experiments are shown After 2h the spontaneous $Na^{51}CrO_4$ release was 14%, which rose to 38% after 18h ($p<0.05$ by WSR). The observed increases in spontaneous $Na^{51}CrO_4$ release at 4 and 6h were not significantly different to the value at 2h (WSR).

2.6.2 Cytotoxic Activity of Actinomycin-D on A375 Melanoma Cells

Actinomycin-D is a cytotoxic drug used in the treatment of various malignant diseases including melanoma, and has been extensively reported to sensitise tumour cells *in vitro* to the tumouricidal activity of cytokines and leucocytes (Colotta *et al.,* 1984). A375 cell susceptibility to killing by Act-D was investigated at drug concentrations of 0, 0.0001, 0.001, 0.01, 0.1 and 1μg/ml. Figure 2.8 shows that concentrations of Act-D above 0.01mg/ml significantly increased ($p<0.05$ by Wilcoxon signed rank test) $Na^{51}CrO_4$ release from A375 cells, in a dose dependent manner, up to the highest concentration tested (1.0μg/ml). In the absence of Act-D pre-treatment, spontaneous $Na^{51}CrO_4$ release was 34%, which was similar to the level observed in the previous section (38%, $p>0.05$ by MWU and WSR)

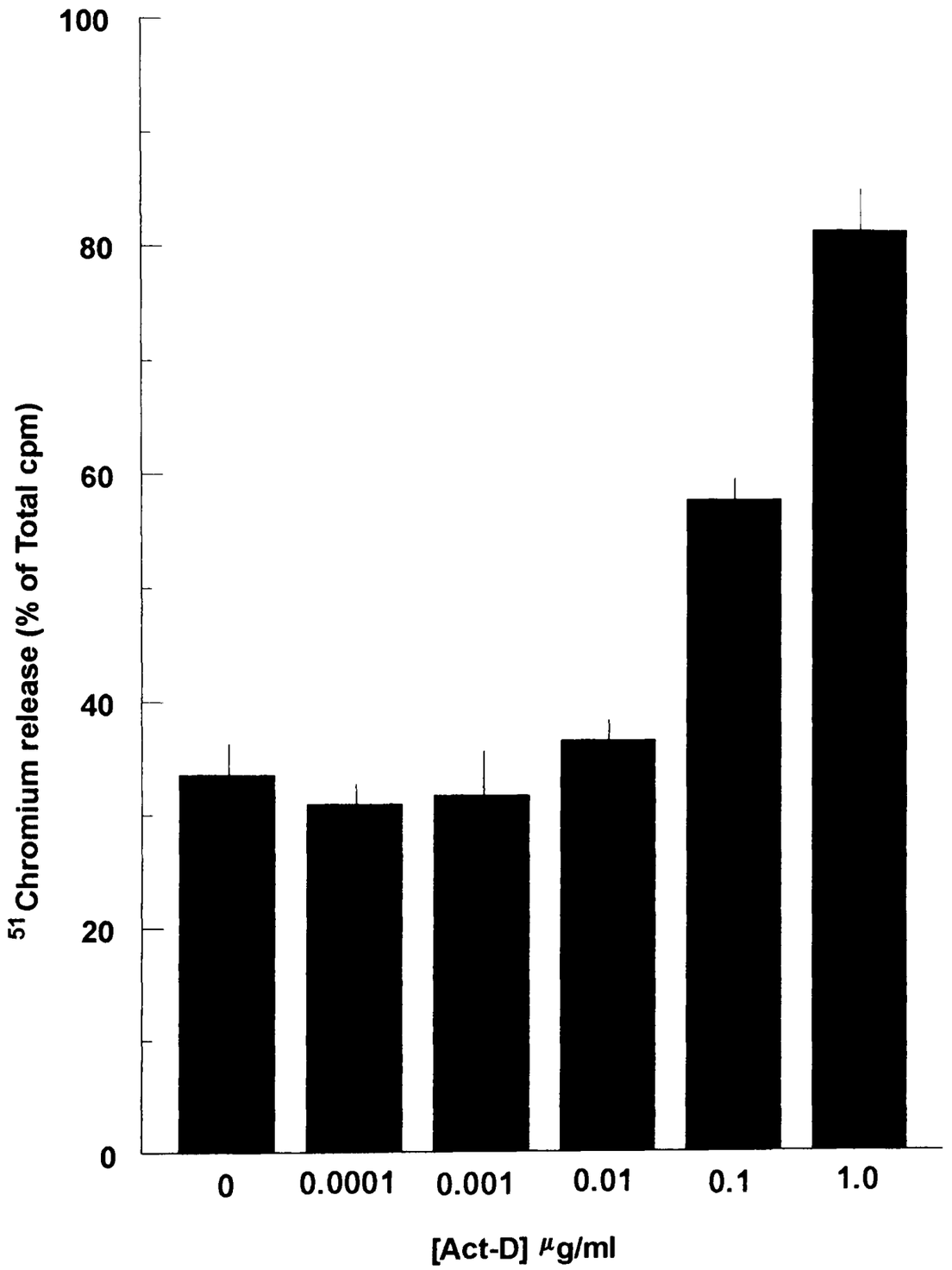

FIGURE 2.8. Actinomycin-D induced $Na^{51}CrO_4$ release from A375 cells. Actinomycin-D (0-1.0μg/ml, in triplicate) was added to $Na^{51}CrO_4$ labelled A375 cells ($1x10^4$cells/well) in microtitre plates, incubated for 18h at 37^0C, and the radioactivity in the supernatants counted. The results shown are means and SEMs from 4 such experiments. A dose dependent increase in $Na^{51}CrO_4$ release was induced by Act-D, to a maximum of 82% ($p<0.05$ by PTT and MWU) at the highest concentration tested. A significant increase ($p<0.05$) in $Na^{51}CrO_4$ release was also observed at 0.1μg/ml.

2.6.3 Cytotoxic Activity of Cycloheximide on A375 Melanoma Cells

Cycloheximide is another protein synthesis inhibitor which acts at a post-transcriptional level. In 3 experiments the direct cytotoxic activity of cycloheximide (0.01, 0.1, 1.0 and 10μg/ml) against K562 and A375 cells was investigated at 18h in the $Na^{51}CrO_4$ release assay (section 2.2.1). Specific cytotoxicity was not greater than 4% on either cell line at any concentration of cycloheximide tested, indicating that the drug was not cytotoxic for these cell lines.

2.6.4 Effect of Target Cell Confluence on Lymphocyte Cytotoxicity

Actively growing A375 cells reach confluence within 2-3 days after subculture (section 2.1.2). Extending the incubation time results in the cells growing over each other. At this point there may be changes in the expression of various surface markers which could influence susceptibility to killing by leucocytes. To determine whether the state of confluence affected tumour cell cytotoxicity by lymphocytes, cells which had been grown for 24h after subculture (non-confluent) were harvested at the same time as cells which had been grown for 72h (confluent) for inclusion in the cytotoxicity assay. Figure 2.9 shows the mean lymphocyte cytotoxicity (E:T ratio 50:1) of confluent and non-confluent A375 cells obtained from 3 experiments. The state of confluence of the cells had no significant effect on lymphocyte killing (figure 2.9). Accordingly, all future experiments were performed with 2-3 day cultured A375 cells, because of the greater cell yield after this time.

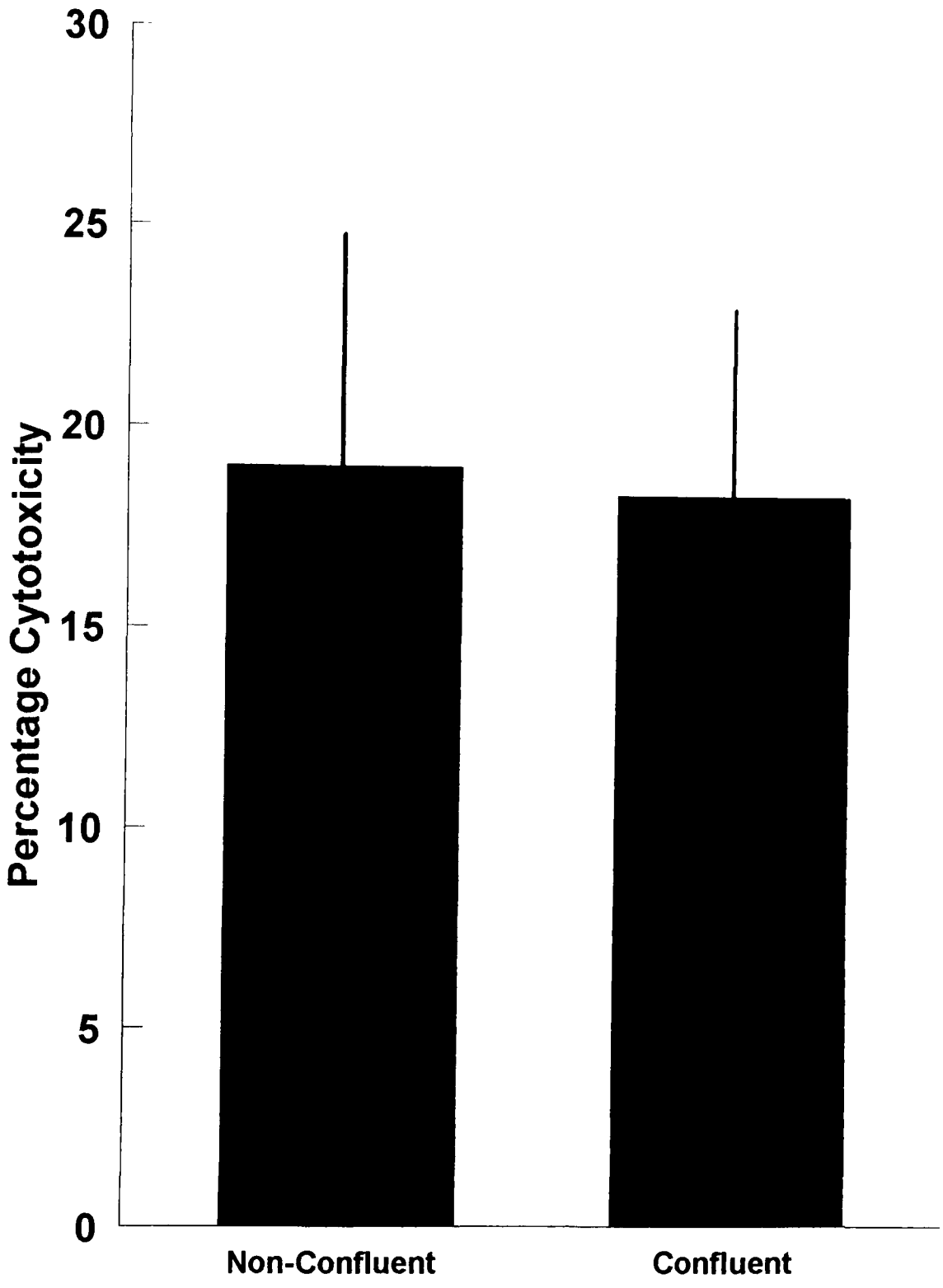

FIGURE 2.9. Effect of A375 cell confluence on their susceptibility to killing by lymphocytes. A375 cells were harvested from flasks which had been subcultured 24h (non-confluent) or 72h (confluent) previously, labelled with $Na^{51}CrO_4$ and $1x10^4$ cells added to a microtitre plate, together with $5x10^5$ freshly isolated lymphocytes. The plate was incubated for 18h and the radioactivity in the supernatants counted. Cytotoxicity was calculated as described in section 2.2. In 3 experiments (means and SEMs are shown), lymphocyte cytotoxicity against 24h cultured tumour cells was not significantly different ($p>0.05$ by MWU and PTT) from that of tumour cells grown for 72h prior to inclusion in the cytotoxicity assay.

2.6.5 Effect of Serum on Lymphocyte Killing of A375 Cells

Initial experiments on lymphocyte cytotoxicity of tumour cells were performed using 10% human AB serum in RPMI 1640 (RPMI/AB), because AB serum is not mitogenic for human mononuclear cells. Foetal calf serum (FCS) had been used in previous studies (Lamph *et al.*, 1988). Consequently, a comparison of lymphocyte cytotoxicity against A375 cells in tissue culture medium containing either RPMI/AB serum or RPMI/FCS was performed. In three comparative experiments no significant difference in lymphocyte killing was seen using AB serum or FCS (Figure 2.10). Subsequent experiments were carried out using RPMI/FCS as the tissue culture medium in the cytotoxicity assay.

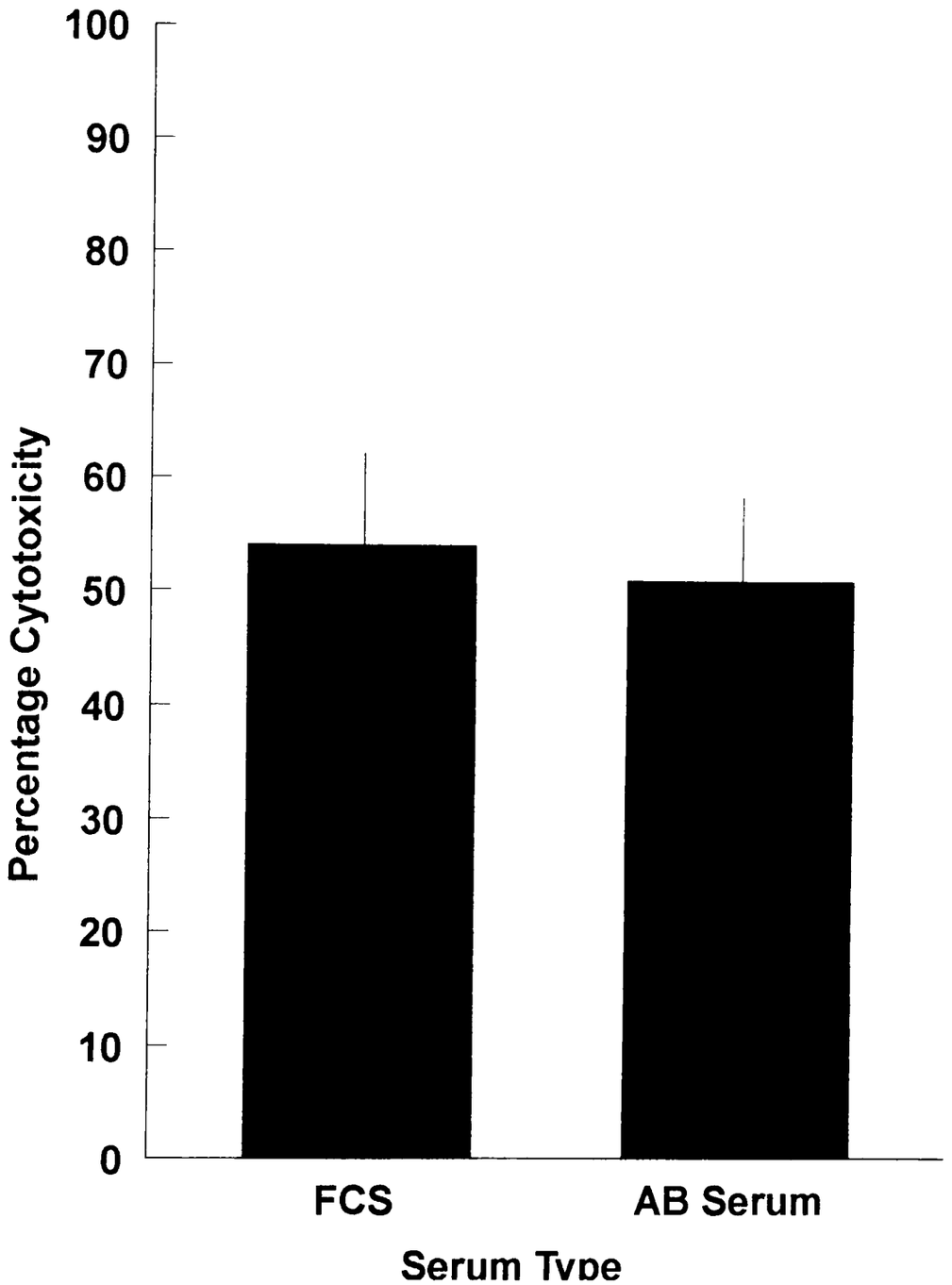

FIGURE 2.10. Comparison of FCS with AB serum on lymphocyte killing of Act-D pre-treated A375 cells. Freshly isolated lymphocytes were added to A375 cells (effector:target cell ratio 50:1), in the presence of either RPMI/FCS or RPMI/AB, and incubated for 18h. The supernatants were harvested, counted and cytotoxicity calculated. In 3 experiments, there was no significant difference ($p>0.05$ by MWU and PTT) between lymphocyte killing of A375 cells in the presence of either serum type.

CHAPTER 3

CYTOTOXICITY OF TUMOUR CELLS BY CYTOKINES AND LEUCOCYTES.

3.0 Introduction

The interactions between immune cells, cytokines, the extracellular matrix, and tumour cells *in vivo* are highly complex. Leucocytes adhere to tumour cells and kill them using a variety of cytolytic mechanisms. Among these are production of reactive oxygen metabolites, and secretion of pore forming proteins (perforins). Leucocytes also secrete cytokines, some of which are directly cytolytic to tumour cells. Other cytokines influence tumour growth by their interaction with leucocytes or tumour cells to modulate other mechanisms of immune killing. In addition, some tumours produce cytokines which either influence leucocyte reactions to the tumour or possess an autoregulatory growth function.

Over the past twenty years, several cytokines have been found to be therapeutically beneficial when given to tumour bearing animals or patients with terminal cancer. These primarily include the interferons (Balkwill and Smyth, 1987), tumour necrosis factor-α (Blick *et al.*, 1987) lymphotoxin (animal experiments; Funahashi *et al.,* 1993); and interleukin-1 (animal experiments, Nakata *et al.*, 1988), which also have direct anti-tumour activity *in vitro.* Cytokines such as interleukin-2 and the colony stimulating factors, are not directly cytotoxic to tumour cells, but act by leucocyte activation or growth promotion, and thus play a secondary, but important, role in tumour cell destruction.

In addition to the multiple biological functions exhibited by most cytokines, the presence of more than one cytokine in a cellular environment, which would normally be expected *in vivo*, often results in activities not attributable to an individual cytokine. For example, the cytotoxic activity of TNFα was augmented by addition of any of the interferons (Sugarman *et al.*, 1985). Negative interactions also occur, since IL-1 has been reported to inhibit TNFα cytotoxicity (Holtman and

Wallach, 1987). However, IL-1 also synergised with TNFα in the elimination of cultured melanoma cells (Ruggerio and Baglioni, 1987), demonstrating that the action of the same combination of cytokines can vary on different target cell lines.

Experimental Design

Seven tumour cell lines were chosen for this study. These included 4 melanoma cell lines. A375 - an amelanotic melanoma cell line derived from metastatic melanoma. DX3 - a second amelanotic metastatic melanoma for comparison with A375. LT5.1 - DX3 cells which had been passaged through a nude mouse to increase its metastatic activity. SK23 - a metastatic melanotic melanoma. Also included were the erythroblastoma cell line K562, which is known to be NK sensitive: Epstein-Barr virus transformed B-cell line, Daudi, which is resistant to NK cells in 4h cytotoxicity assays: and U937, a promonocytic cell line which is sensitive to killing by TNFα.

The aims of this phase of experimentation were firstly to determine the cytotoxic activity of several cytokines (IL-1, IL-2, TNFα, LT and IFNγ, as well as that of purified lymphocytes and monocytes on the seven cell lines chosen for study, and secondly to study the role of pre-incubation of tumour cells with cytokines and/or cytotoxic drugs on lymphocyte and monocyte tumouricidal activity.

3.1 Cytokines

3.1.1 Direct Cytotoxicity of Tumour Cells by Cytokines

A large number of cytokines which modulate the immune response to tumour cells *in vivo* and *in vitro* have been reported. Several cytokines (TNFα, LT, IL-2, IFNγ and IL-1β) were chosen for study, because they have been reported to exert a range of direct and modulatory effects, alone or in combination, on leucocytes and tumour cells. Additionally, most of them have been used clinical trials to investigate their role as anti-cancer agents alone or in combination with cytotoxic drugs and/or adoptive immunotherapy by leucocytes.

3.1.2 Tumour Necrosis Factor Alpha (TNFα)

Tumour necrosis factor-α has been reported to be directly cytotoxic for a large variety of tumour cell lines *in vitro* (Helson *et al.,* 1975), although many other lines have proved to be resistant to attack by this cytokine. The cytotoxic activity of TNFα for the 7 cell lines, and the effect of pre-treatment of target cells with Act-D on TNFα killing was investigated.

Figure 3.1

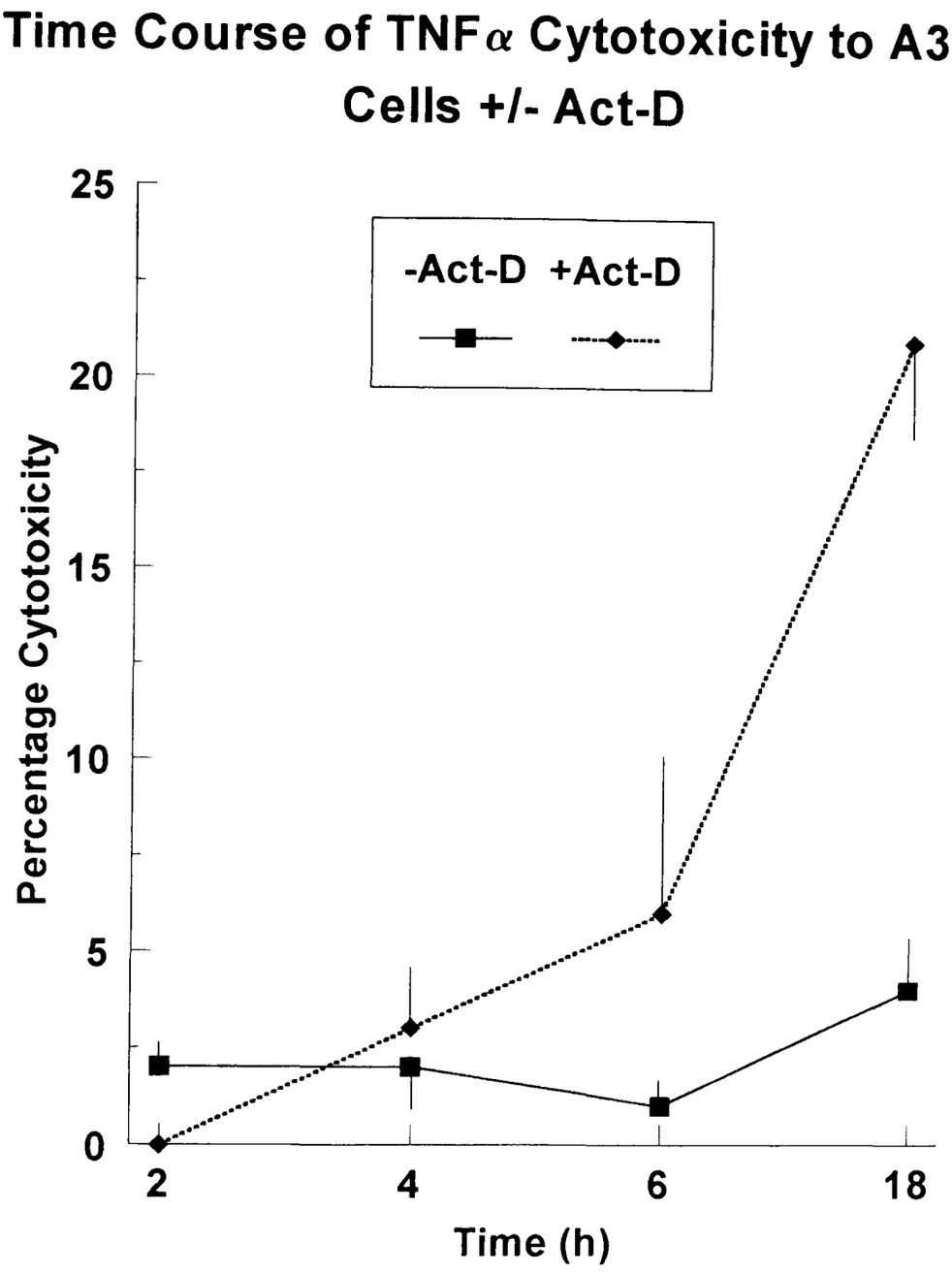

FIGURE 3.1. Time course of TNFα cytotoxicity against A375 cells +/- Act-D. Tumour necrosis factor-α (1000U/ml) was incubated in 4 parallel cultures with $Na^{51}CrO_4$ labelled A375 cells untreated or pre-incubated for 3h with Act-D (0.1μg/ml). Supernatants were harvested after 2, 4, 6 and 18h, their radioactivity measured, and specific cytotoxicity calculated. In 3 experiments (means and SEMs shown), Act-D pre-treatment of A375 cells resulted in significant ($p<0.05$ by MWU and PTT) TNFα cytotoxicity at 6 and 18h. At 18h (but not 2,4 or 6h). Killing of Act-D pre-treated A375 cells by TNFα was significantly greater ($p<0.05$ by MWU and PTT) than that of untreated A375 cells.

A375 cells were incubated with recombinant TNFα at a concentration of 1000U/ml for 2, 4, 6 and 18h, in the presence or absence of Act-D (0.1mg/ml) (Figure 3.1). Each experiment was performed in triplicate. In 3 such time course experiments no significant tumour cell killing was seen at incubation times less than 18h, irrespective of whether the target cells were untreated or pre-treated with Act-D for 3h, although some cytotoxicity (6%; compared with spontaneous $Na^{51}CrO_4$ release) was seen at 6h for Act-D pretreated A375 cells. Cytotoxicity mediated by TNFα against Act-D pretreated A375 cells was significantly greater than against untreated A375 cells after 18h, indicating that A375 cells were sensitised to the action of TNFα (at 1000U/ml) by pre-incubation with Act-D.

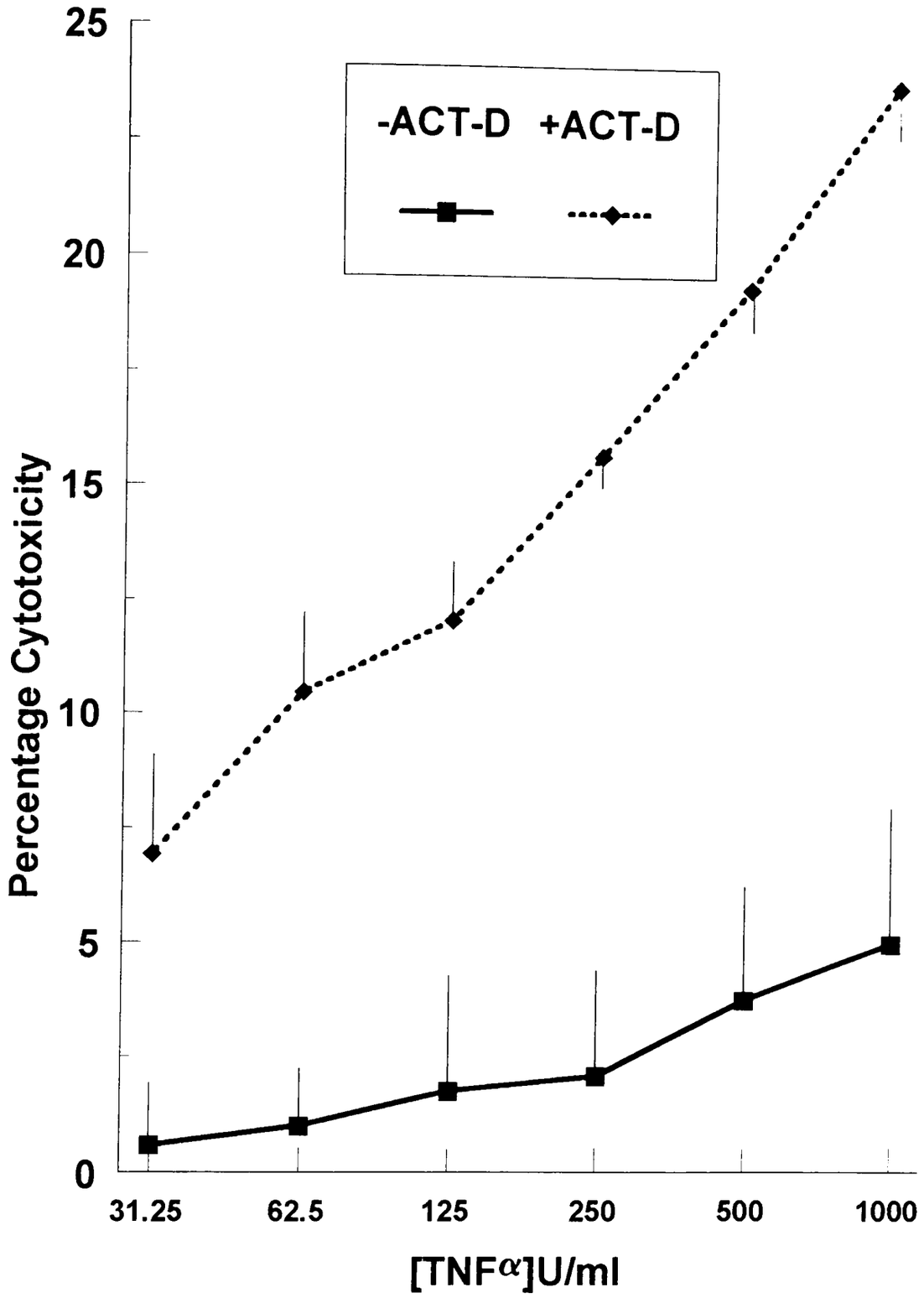

FIGURE 3.2. Dose response of TNFα cytotoxicity against A375 cells. Tumour necrosis factor-α (31.25-1000U/ml) was incubated with $Na^{51}CrO_4$ labelled A375 cells for 18h and cytotoxicity measured. The results shown are means and SEMs from 12 experiments. Significant ($p<0.05$ by PTT and MWU) TNFα cytotoxicity against untreated A375 cells was observed at [TNFα] above 62.5U/ml, and at all concentrations of TNFα tested against A375 cells pre-treated with Act-D (0.1μg/ml for 3h). Pre-incubation of the tumour cells with Act-D significantly ($p<0.05$ by PTT and MWU) increased TNFα cytotoxicity compared with untreated A375 cells at all concentrations of TNFα tested.

Figure 3.2 shows the mean dose response curves for TNFα cytotoxicity (at concentrations of 31.25, 62.5, 125, 250, 500 and 1000U/ml) for A375 cells at 18h from 12 experiments. Tumour necrosis factor-α alone induced relatively low levels of A375 cell killing at the highest concentration tested (*i.e.* 1000U/ml). Pre-treatment of the target cells with Act-D (at 0.1mg/ml) significantly enhanced TNFα cytotoxicity These results show that TNFα was not highly cytotoxic for fresh A375 cells at the concentrations used, and that pre-treatment with Act-D caused the target cells to become susceptible to killing by this cytokine, although the actual levels of cytotoxicity values were still relatively low at concentrations up to 1000U/ml.

In 4 further experiments, inclusion of a monoclonal antibody against TNFα significantly inhibited killing of Act-D pretreated A375 cells by TNFα at all concentrations tested. These experiments indicate that TNFα killing was specific and that the monoclonal antibody was effective in blocking the tumouricidal activity of TNFα (Figure 3.3).

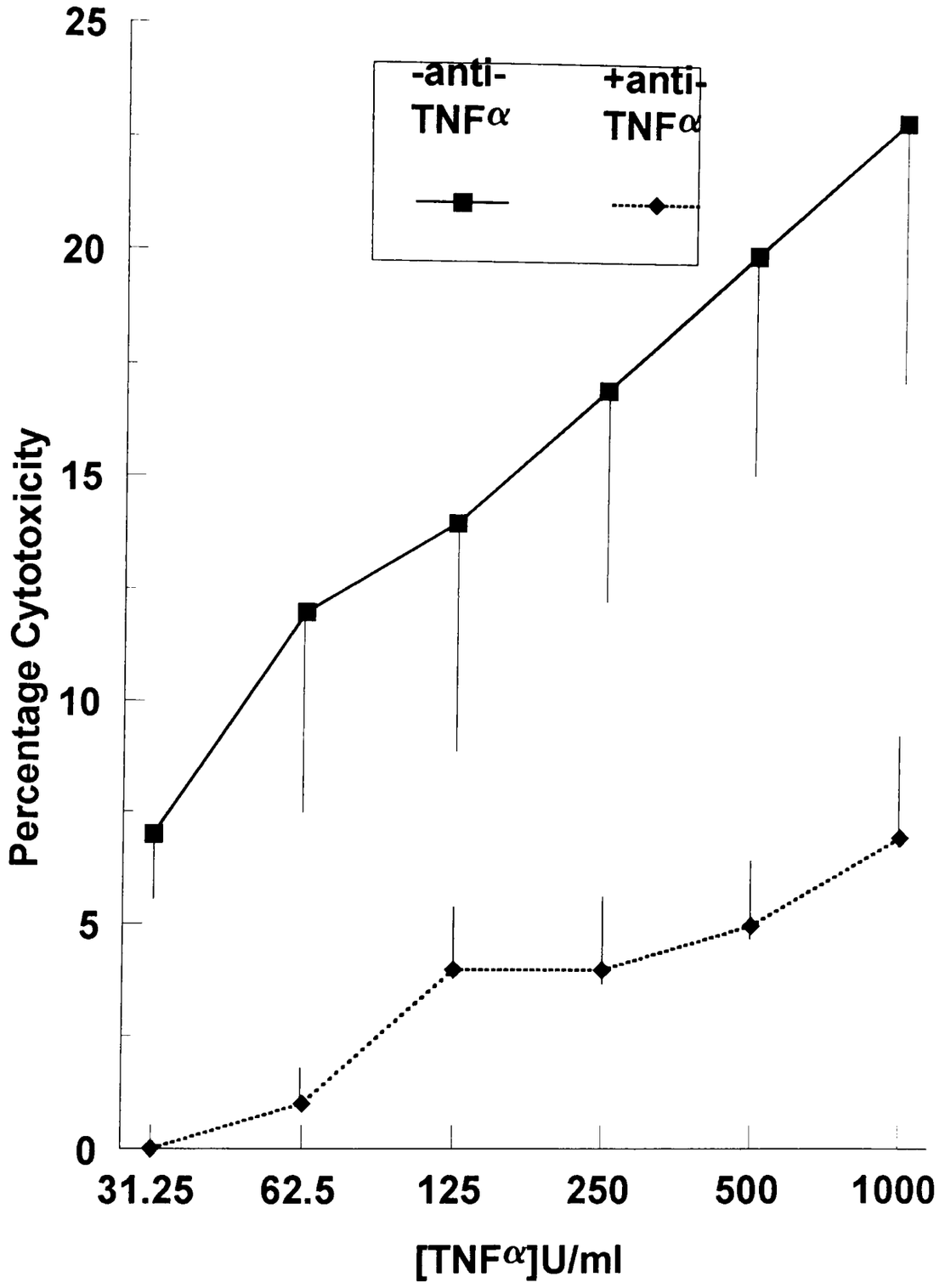

FIGURE 3.3. Effect of anti-TNFα antibody on TNFα killing of A375 cells. Tumour necrosis factor-α (31.25-1000U/ml) cytotoxicity for A375 cells (pre-treated with Act-D (0.1μg/ml)) was measured alone and in the presence of a neutralising antibody to TNFα (10μg/ml). In 4 experiment (means and SEMs are shown), cytotoxicity was significantly inhibited in the presence of the antibody at all concentrations of TNFα tested ($p<0.05$ by MWU and PTT; also by WSR and PTT for comparison of complete dose response curves).

Tumour necrosis factor-α cytotoxicity for three other melanoma cell lines, as well as K562, Daudi and U937 was also measured. Table 3.1 summarises the maximal TNFα killing (at 1000U/ml) of cell lines untreated and pre-incubated with Act-D (0.1μg/ml) for 3h prior to addition of cytokine.

Cell Line	Source	n	Cytotoxicity -ActD (%)	Cytotoxicity +ActD (%)	Significance
A375	**Melanoma**	12	**5** (1)	**24** (3)	**p<0.05**
DX3	**Melanoma**	3	**6** (1)	**26** (2)	**p<0.05**
SK23	**Melanoma**	3	**18** (2)	**18** (3)	p>0.05
LT5.1	**Melanoma**	3	**8** (3)	**21** (3)	**p<0.05**
K562	**Erythroblast**	6	**16** (2)	**15** (5)	p>0.05
Daudi	**EBV, B-Cell**	4	**17** (4)	**15** (4)	p>0.05
U937	**Promonocyte**	3	**23** (5)	**52** (7)	**p<0.05**

TABLE 3.1. TNFα killing of seven tumour cell lines. Tumour necrosis factor-α (1000U/ml) cytotoxicity (after 18h) was assessed against seven tumour cell lines untreated or pre-incubated with Act-D (0.1μg/ml for 3h). n = number of experiments, and numbers in brackets are SEMs. TNFα was cytotoxic for all cell lines (p<0.05 compared to spontaneous release, by MWU), but SK23, K562, Daudi and U937 cells were more sensitive (p<0.05) to the action of TNFα than the A375, DX3 or LT5.1 cell lines. Pre-treatment of the tumour cells with Act-D increased TNFα killing of some, but not other, cell lines (see significance column: MWU and PTT).

As with A375 cells, the DX3 and LT5.1 cell lines were relatively unresponsive to the action of TNFα alone, but pre-treatment with Actinomycin-D enhanced (p<0.05) their susceptibility to killing. While SK23, K562 and Daudi cells were more sensitive than A375, DX3 and LT5.1 cells to the action of TNFα, there was no significant increase in cytotoxicity following preincubation with Act-D. The promonocyte cell line U937 was sensitive to TNFα, untreated, at a comparable level to the melanoma lines (A375, DX3 and LT5.1) after pre-treatment with Act-D. In contrast to the 3 other TNFα sensitive cell lines (SK23, K562 and Daudi), killing of U937 cells by TNFα was more than doubled by pre-incubation with Act-D. These results show that there is considerable

heterogeneity in the susceptibility of the various cell lines to killing by TNFα, as well as in their ability to be sensitised by Act-D to the action of TNFα.

Further studies on the direct action of cytokines on tumour cells were performed using only the A375 melanoma cell line, because of its inducible sensitivity to killing by Act-D, although either DX3 or LT5.1 cells would have been equally suitable.

3.1.3 Cytotoxicity of A375 cells by IL-1β, IFNγ IL-2 and LT

Using the same experimental design described for TNFα in the previous section, several other cytokines were tested for their direct cytotoxic activity against A375 melanoma cells, in order to determine whether they are directly involved as leucocyte cytotoxic effector molecules or whether they have a secondary role in the induction of other leucocyte cytotoxic mechanisms. Incubation of A375 cells with IL-1β (0.001-100U/ml), IFNγ (0.001-100U/ml), IL-2 (15-500U/ml) or LT (39-1250U/ml) for 2, 4 or 6h did not result in any detectable cytotoxicity ($p>0.05$ compared to spontaneous $Na^{51}CrO_4$ release), but after 18h minimal killing was observed with LT at 1250U/ml. Table 3.2 summarises the maximal (at the highest dose in each case) killing of A375 cells untreated or pre-incubated with Act-D by each of the above cytokines after 18h. Pre-treatment of the tumour cells with Act-D resulted in increased killing by IL-1β, IFNγ and LT ($p<0.05$ compared to untreated target cells), but IL-2 demonstrated no significant ($p>0.05$ compared to spontaneous $Na^{51}CrO_4$ release) cytotoxic activity on A375 cells before or after pre-treatment with Act-D.

Cytokine	Concentration	n	Cytotoxicity -ActD (%)	Cytotoxicity +ActD (%)	Significance
IL-1β	100U/ml	3	**1** (1)	**17** (5)	**p<0.05**
IL-2	500U/ml	4	**0** (0)	**0** (0)	p>0.05
IFNγ	100U/ml	4	**0** (0)	**13** (5)	**p<0.05**
LT	1250U/ml	3	**6** (2)	**!9** (6)	**p<0.05**

TABLE 3.2. Cytokine killing of A375 melanoma cells. Five cytokines were assessed for their cytotoxicity against untreated or Act-D pre-treated A375 cells after 18h incubation. The number of experiments is shown in the column headed n, and numbers in brackets are SEMs. Only lymphotoxin was cytotoxic for untreated A375 cells (p<0.05 compared to spontaneous release by MWU and PTT). Actinomycin-D pre-treated A375 cells were sensitive to IL-1β and IFNγ killing, and LT cytotoxicity was augmented (p<0.05 by MWU and PTT) by this treatment.

The cytokines tested were not highly cytotoxic (*i.e.*, <20% specific cytotoxicity) for A375 cells, even after target cell pre-treatment with Act-D. However, a longer incubation time may have been required for maximal cytokine killing to occur. Alternatively, other effects such as cytostasis (which cannot be determined using $Na^{51}CrO_4$ release) could have been induced. Further experiments were therefore performed to examine the effect of incubation with the cytokines IFNγ and IL-2 (which representative of cytokines which are directly cytotoxic and which have secondary effects on tumour cells) on the number of A375 cells recovered from culture after several days in culture.

3.1.4 Effect of Cytokines on Growth of A375 Cells

Interferon-γ has been reported to be cytotoxic to many tumour cell lines *in vitro* after 24-48h, but the $Na^{51}CrO_4$ release assay is unsuitable for these longer term assays (section 2.7.1). Some melanoma cell lines have been reported to express high (p55/p75) and low (p75) affinity IL-2 receptors (Rimoldi, 1993). The effect of IFNγ and IL-2 on A375 cells was therefore determined by incubation of tumour cells alone or in the presence of various concentrations of IFNγ or IL-2 for 48 or 72h

In 3 experiments, A375 cells at a single cell density (1 in 5 dilution from a confluent 25cm^2 tissue culture flask), were incubated in RPMI/FCS alone or containing IFNγ (250, 500 and 1000U/ml), or IL-2 (250, 500, 1000 and 2000U/ml of A375 cells for 48h (IFNγ) or 72h (IL-2). The cells were removed from the flasks and the number of viable cells (trypan blue negative) counted.

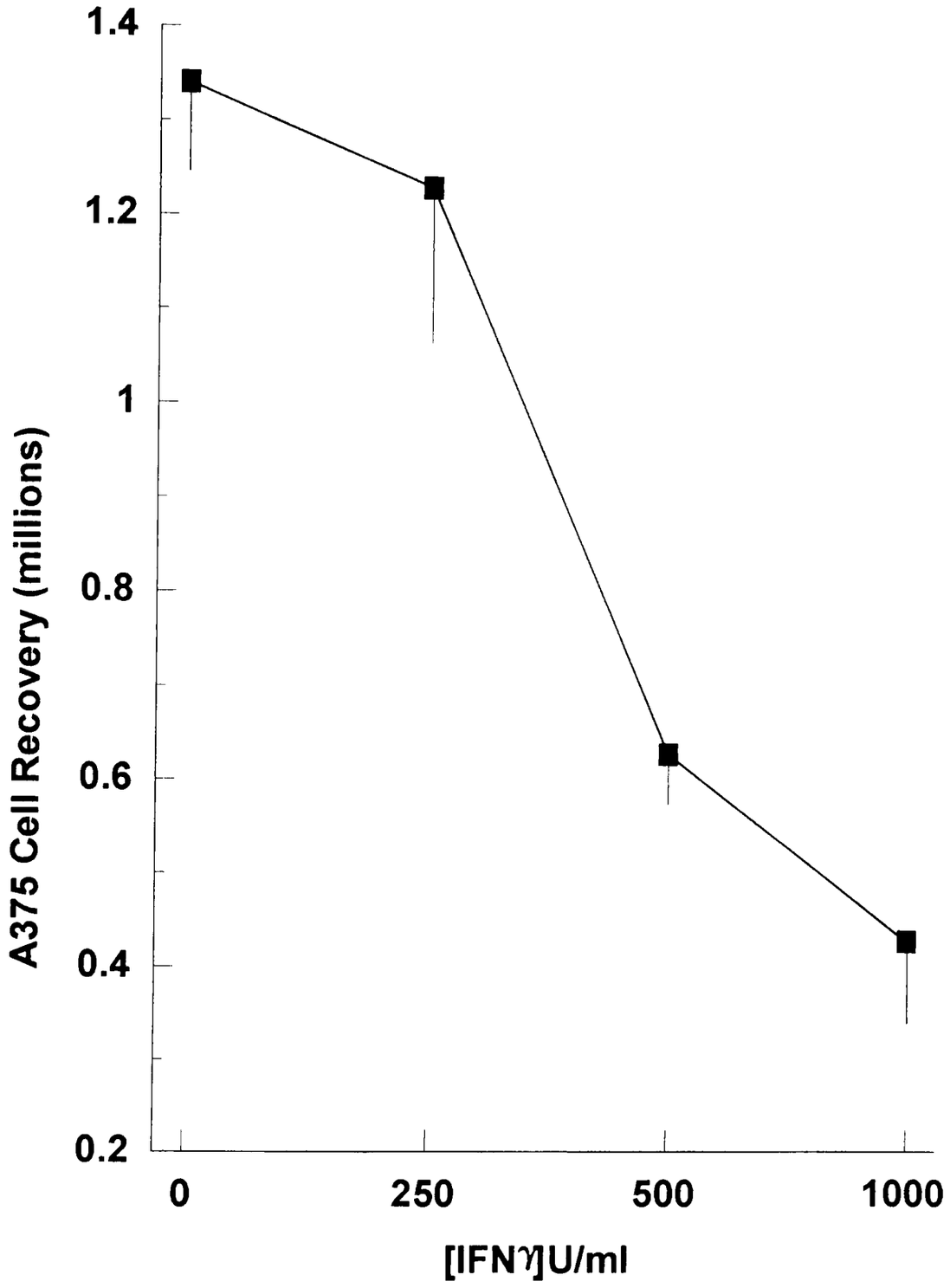

FIGURE 3.4. Effect of IFNγ on A375 cell growth. To each of 4 x 25cm^2 tissue culture flasks was added 7.5x10^5 A375 cells alone or containing IFNγ (250, 500 or 1000U/ml), and incubated for 48h at 37^{0}C. The cells were detached from plastic and a cell count and viability performed. Cell viability was >90% in all cases. The means (of viable cells recovered) and SEMs from 3 experiments are shown. In the absence if IFNγ, A375 cells expanded in number ($p<0.05$ by MWU and PTT), but a progressive decrease in cell yield was observed with increasing IFNγ concentration, which was significant ($p<0.05$ by MWU and PTT) at 500 and 1000U/ml.

A significant decrease in the number of cells recovered from culture was observed when target cells were incubated with IFNγ at concentrations of 500 and 1000U/ml, but not 250U/ml (Figure 3.4). These results indicated that high concentrations of IFNγ were cytostatic, or cytotoxic, for A375 cells in a dose-dependent manner after 48h. This reduction in cell yield could be due to the direct cytotoxic action of IFNγ or could result from natural cell death following IFNγ-induced cytostasis.

In contrast to IFNγ, IL-2 induced a dose-dependent increase in the number of A375 cells recovered from culture (Figure 3.5), reaching a maximum at 1000-2000U/ml. In the absence of IL-2, more cells were recovered from culture than in the IFNγ experiments. This was most likely due to the extra 24h incubation time in the IL-2 experiments which gave a greater yield of cells. These results suggest that IL-2 may be a growth promoting factor for A375 cells.

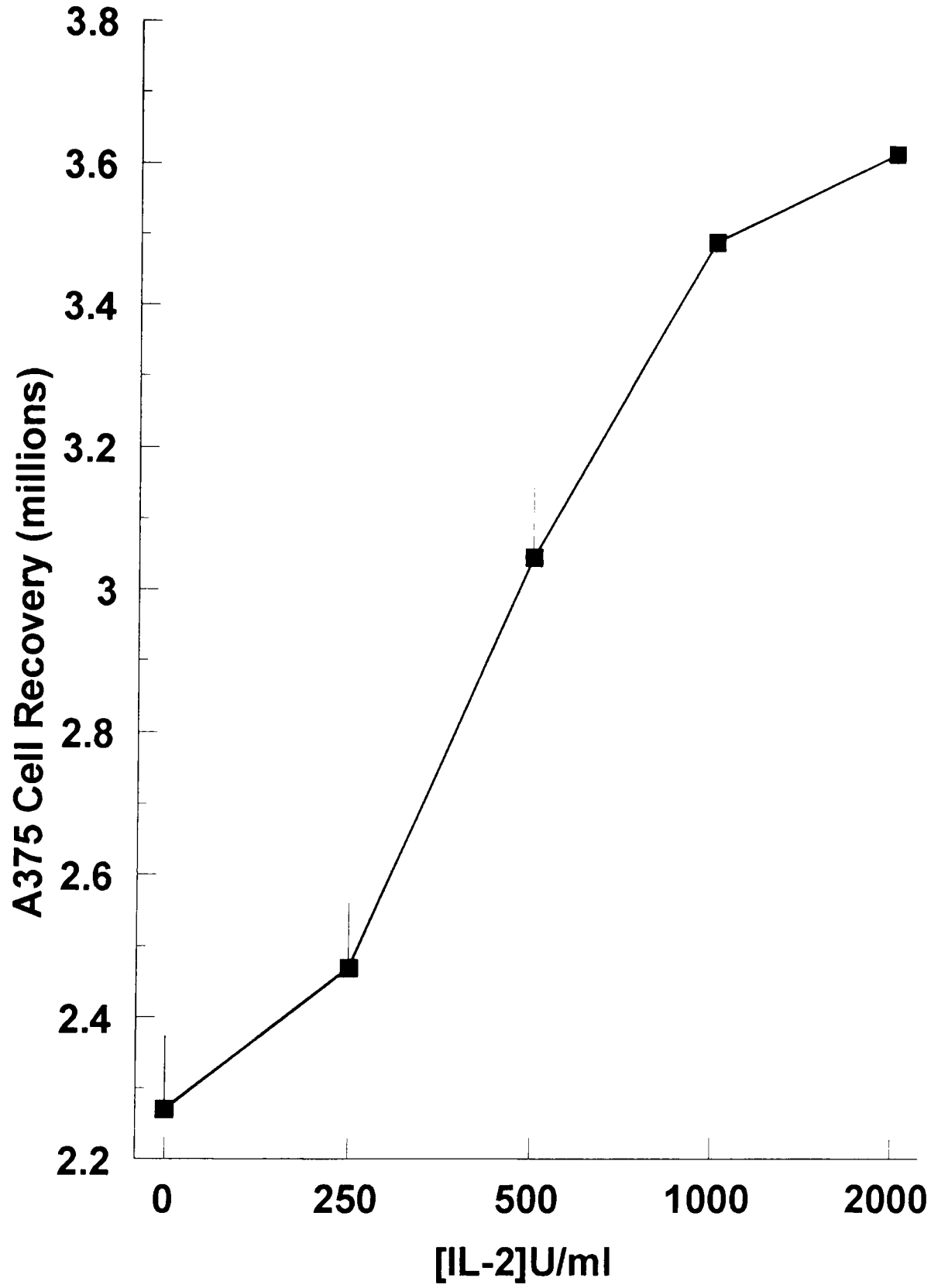

FIGURE 3.5. Effect of IL-2 on A375 cell growth. Tumour cells (A375; 7.5x105 in 10ml RPMI/FCS) were added to 5 x 25cm2 tissue culture flasks alone or in the presence of IL-2 (250-2000U/ml), and incubated for 72h at 37^0C. The cells were detached from plastic and a cell count and viability performed. Cell viability was >90% in all flasks. The means and SEMs from 3 experiments are shown. Addition of IL-2 to the cultures resulted in an increased yield of cells at all concentrations ($p<0.05$ at all points compared to no added IL-2, by MWU and PTT). In addition, a significant increase in yield was observed between 250 and 500U/ml ($p<0.05$ by MWU and PTT) as well as 500 and 1000U/ml ($p<0.05$ by MWU and PTT), but not between 1000-2000U/ml IL-2 ($p>0.05$).

For IL-2 to promote cell growth it must first bind to one of its receptors. Experiments were thus performed to determine whether A375 cells expressed p55 (CD25; IL-2 receptor-α) or p75 (IL-2 receptor-β) IL-2 receptors (section 2.5.0). Figure 3.6a is a representative histogram showing log fluorescence for direct FITC labelled anti-CD25 monoclonal antibody on the surface of A375 cells together with the corresponding isotype control.

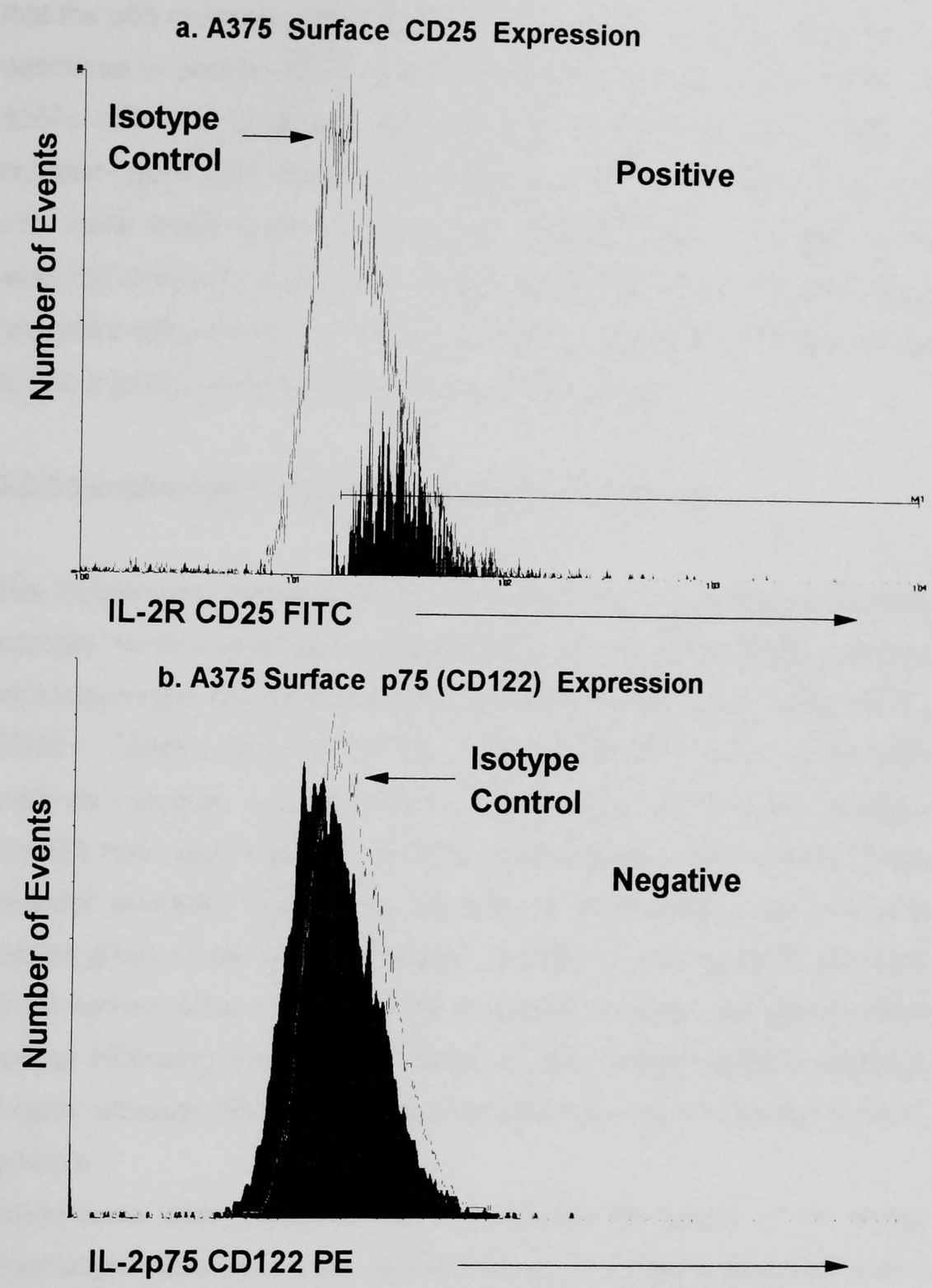

FIGURE 3.6. A375 cell surface CD25 and p75 IL-2 receptors. A375 cells were labelled with anti-CD25, anti-p75 or isotype control as described in section 2.4. Figure 3.6a shows that the histogram for anti-CD25 staining is right shifted compared to the isotype control, indicating that A375 cells possess the IL-2 receptor (α-chain). In contrast, no positive events were detected for the p75 antigen (figure 3.6b), indicating that A375 cells do not possess the IL-2 receptor β-chain.

The CD25 histogram is right shifted compared with the control, indicating that the p55 molecule was present on these cells. Using the calculations described in section 2.5.3, a mean (from 3 experiments) of 11456 (+/- 4267) p55 IL-2 receptors was detected on cultured A375 cells. In contrast figure 3.6b shows that no events from directly labelled PE anti-p75 were more fluorescent than the isotype control, indicating the absence of p75 on A375 cells. These results show that low affinity IL-2 receptors are present on A375 cells, and provides further evidence that IL-2 is a growth stimulatory molecule for this cell line.

3.2.0 Lymphocyte Cytotoxicity Against Tumour Cells

The lymphocyte population from normal donors is comprised of several subsets, some of which are spontaneously and non-specifically cytotoxic for tumour cells. Examples of these are the NK cells (CD4-, CD8-, CD3-, CD16+, CD56+) and the CD16+, CD8+ cytotoxic T-cells. Other cells such as cytotoxic T-cells (CD3+, CD8+, CD3+, CD16-) are antigen-specific and require antigen processing and presentation via the T-cell receptor and MHC before they are able to kill a specific cell line, or a related group of cell lines. A minority of CD8+ T-cells however, are able to kill tumour cells in the absence of specific antigen processing. Most tumour infiltrating lymphocytes comprise CD8+ antigen specific cytotoxic T-cells, although CD4+ T-cells and NK cells have been detected in some tumours.

Experiments were performed to (1.) evaluate the ability of the whole lymphocyte population from normal donors to kill spontaneously the 4 melanoma cell lines, K562 (NK-sensitive), Daudi (NK-resistant), and the promonocyte cell line U937, untreated or pre-incubated with the protein synthesis inhibitors Act-D or cycloheximide; (2.) to determine the relative contributions of NK cells and cytotoxic T-cells to lymphocyte killing of A375 cells by depletion of CD8+ cells from whole lymphocyte populations; (3.) to investigate further the mechanisms of lymphocyte

killing by incorporating monoclonal blocking antibodies against TNFα and LT in the cytotoxicity assay.

3.2.1 Lymphocyte Cytotoxicity of A375 Melanoma Cells

Direct lymphocyte cytotoxicity was assessed using non-adherent lymphocytes which were >89% pure (section 2.2.10).

Fresh non-adherent lymphocytes were incubated with A375 cells +/- Act-D at effector:target ratios of 50:1 to 1.6:1 in doubling dilution for 18h at 37^0C (section 2.3.1.3).

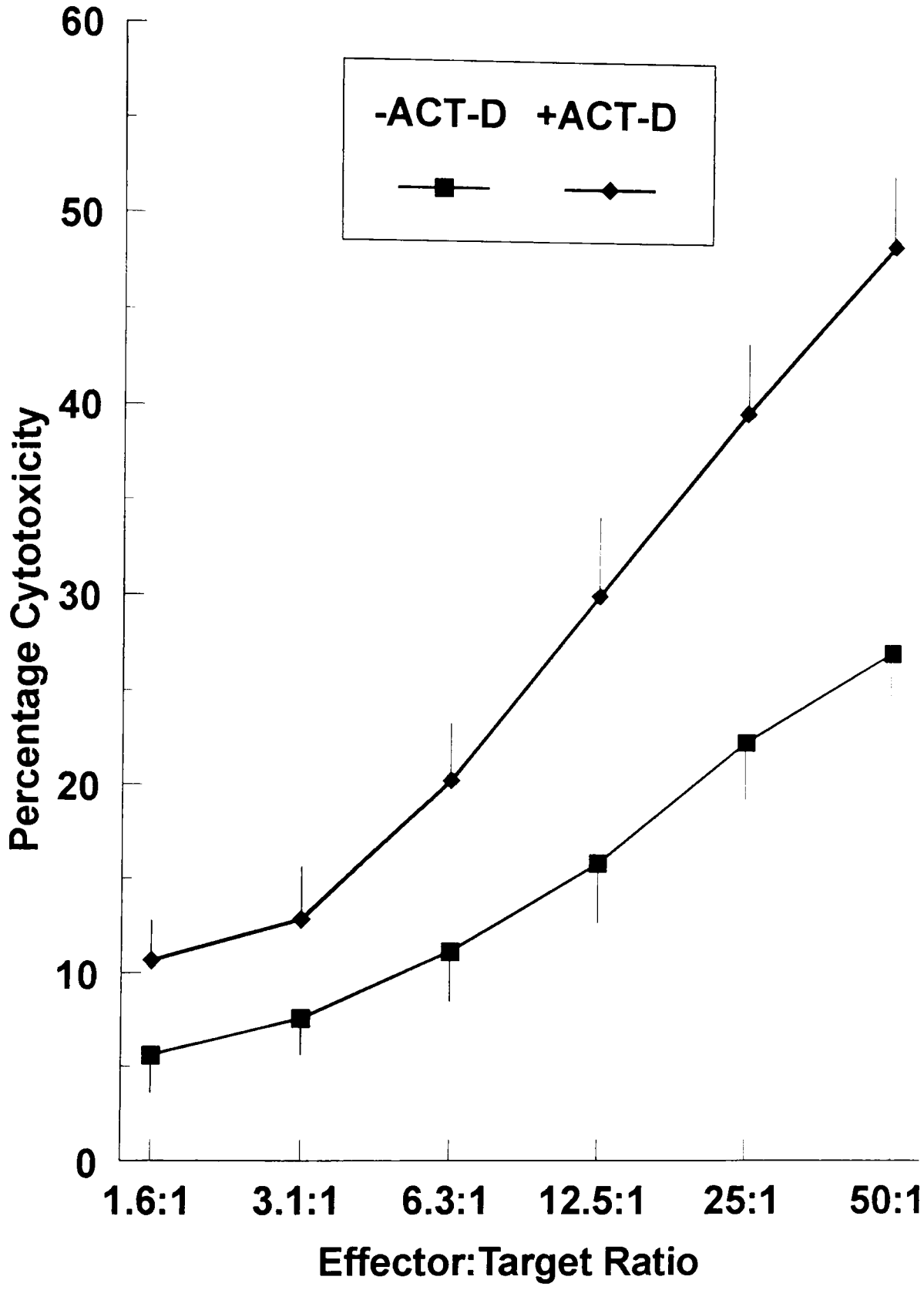

FIGURE 3.7. Lymphocyte killing of A375 cells. Lymphocytes were incubated for 18h with $Na^{51}CrO_4$ labelled A375 cells alone or pre-treated with Act-D (0.1μg/ml) for 3h, and the supernatants harvested and counted. The results shown are means and SEMs of specific lymphocyte cytotoxicity from 19 experiments. Lymphocytes were cytotoxic for untreated (6%) and Act-D pre-treated A375 cells at an effector target ratio (E:T) of 1.6:1 ($p<0.05$ compared to spontaneous release by WSR and PTT), which rose significantly to 15% and 50% ($p<0.05$ by WSR and PTT) respectively at an E:T of 50:1. Pre-treatment with Act-D significantly increased lymphocyte killing of A375 cells at all E:T ($p<0.05$ by WSR and PTT)

Figure 3.7 shows that lymphocyte cytotoxicity was effector:target cell ratio dependent giving maximal killing at an effector:target cell ratio of 50:1. Higher effector:target cell ratios were not used because there would be insufficient cells for experiments to be performed , and nutrient deprivation would have become a contributory factor to cytotoxicity in longer term assays. Actinomycin-D significantly ($p<0.05$) increased lymphocyte tumouricidal activity at all effector:target cell ratios tested. The slope of the cytotoxicity curve for untreated A375 cells was lower than that for Act-D pre-treated A375 cells, indicating that the mechanism of lymphocyte killing may not have been the same in each case. Maximal killing of untreated A375 cells by lymphocytes was greater than that seen with any of the cytokines alone or in combination, indicating either that there are other factors involved in the mechanism of lymphocyte killing of A375 cells or that the concentrations of effector cytokines at the cell-cell junctions reached levels in excess of those used in this study.

3.2.2 Time Course for Lymphocyte Killing of Tumour Cells

Tumour cell lines are extremely heterogeneous in their sensitivity to cytotoxic effectors, both in the degree of cytotoxicity generated and the incubation time required for maximal killing. For this reason, the time course for lymphocyte killing of K562 cells (already known to be relatively sensitive to lymphocyte killing after 4h, and A375 cells was investigated.

Figures 3.8 and 3.9 show the maximal lymphocyte cytotoxicity of K562 and A375 cells respectively (+/- Act-D), after 2, 4 6 and 18h incubation.

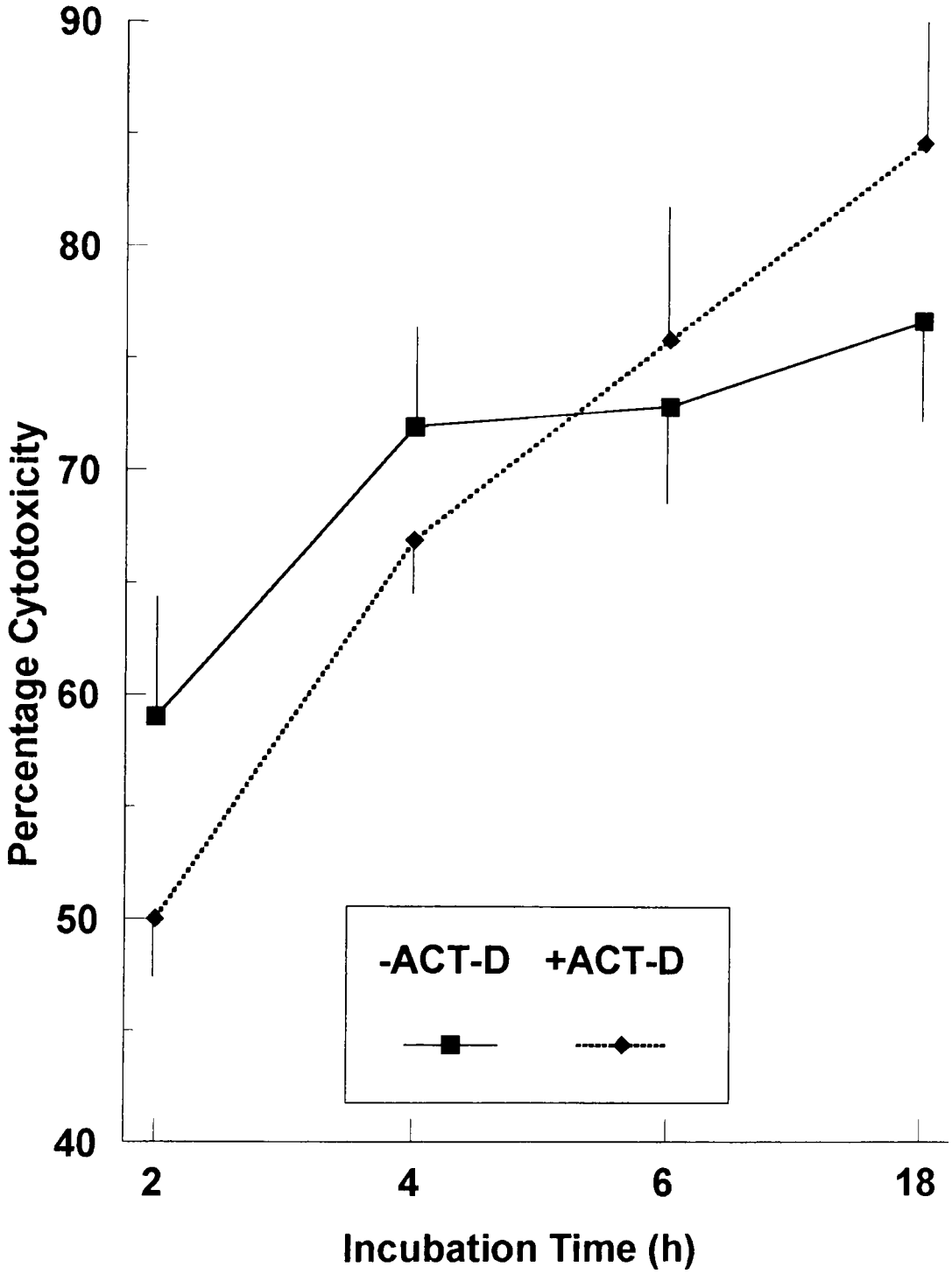

FIGURE 3.8 Lymphocytes were incubated with K562 cells at an effector:target cell ratio of 50:1. Four plates were set up in parallel, and harvested after 2, 4, 6 or 18h. The means and SEMS from 3 experiments are shown. Significant lymphocyte cytotoxicity of both untreated and Act-D pretreated target cells was observed after 2h (compared to spontaneous release; $p<0.05$ by MWU and PTT) which further increased after 4 (killing at 6h was not significantly different from that at 4h; $p>0.05$ by MWU and PTT) and 18h ($p<0.05$ compared to previous time point by MWU and PTT). Act-D pretreatment did not significantly affect lymphocyte killing of K562 cells at any time point (compared to untreated cells; $p>0.05$ by MWU and PTT).

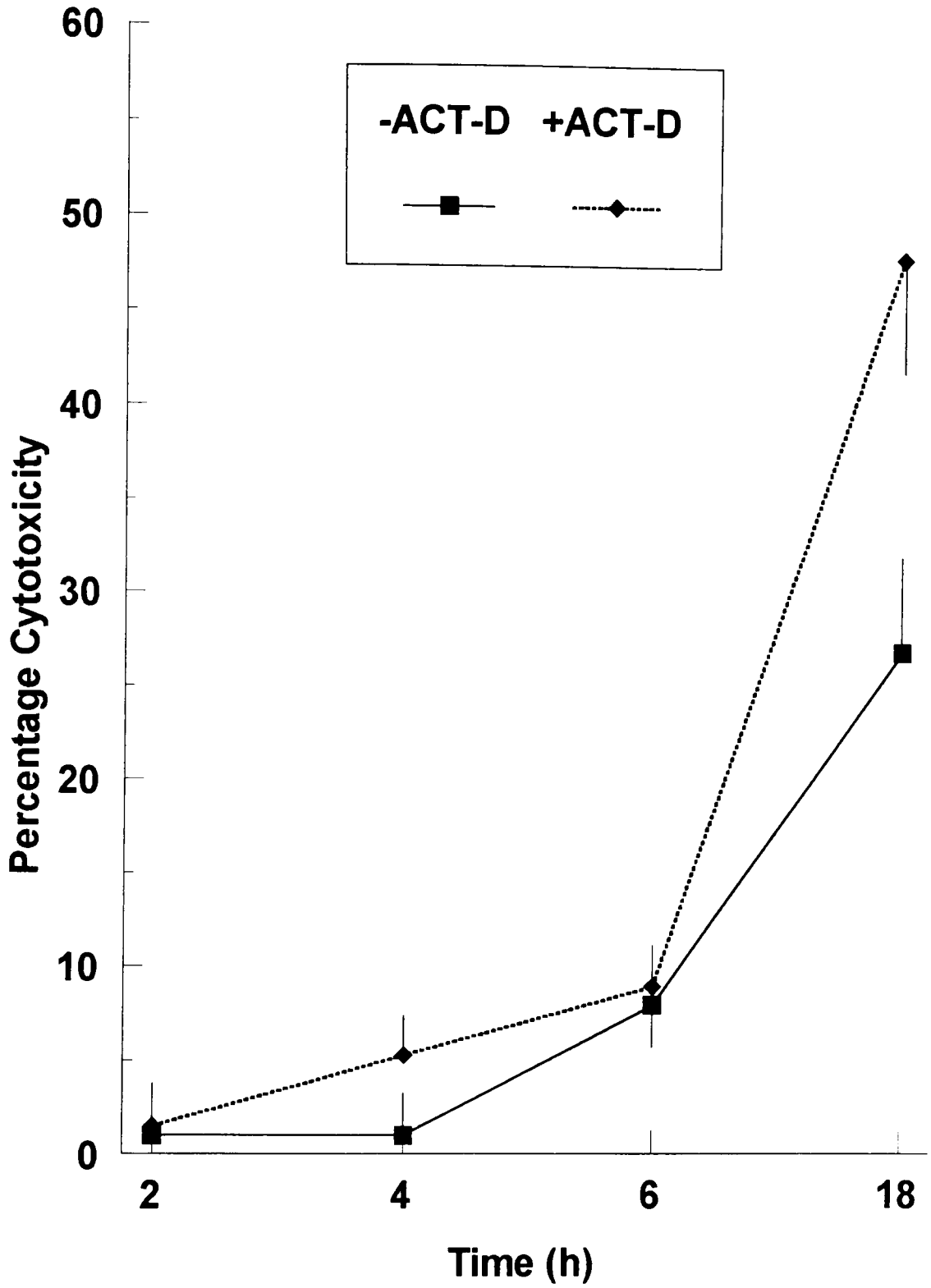

FIGURE 3.9. Time course of lymphocyte killing of A375 cells +/- Act-D. Lymphocytes were incubated with A375 cells at an effector:target cell ratio of 50:1. Four plates were set up in parallel, and harvested after 2, 4, 6 or 18h. The means and SEMS from 3 experiments are shown. Significant lymphocyte cytotoxicity ($p<0.05$, compared to spontaneous release by MWU and PTT) of untreated A375 cells (8%) was observed after 6h which increased to 25% after 18h ($p<0.05$ compared to 6h by MWU and PTT). Act-D treated A375 cells were sensitive to lymphocyte killing after 4h (5%; $p<0.05$ compared to spontaneous release by MWU and PTT), which rose to 36% after 18h (compared to 4 and 6h; $p<0.05$ by MWU and PTT). Act-D pretreatment significantly increased lymphocyte cytotoxicity of A375 cells after 18h ($p<0.05$ by MWU and PTT).

Lymphocytes were cytotoxic for K562 cells after 2h, and maximal killing was observed after 18h. Actinomycin-D pre-treatment of K562 cells did not result in significantly ($p>0.05$) increased lymphocyte cytotoxicity at any time point. In contrast, A375 cells were insensitive to lymphocyte killing at 2 and 4h, but cytotoxicity was observed at 6h ($p<0.05$ compared to spontaneous counts), which increased further after 18h incubation. In addition, Act-D significantly increased lymphocyte killing of A375 cells only after 18h incubation ($p<0.05$).

3.2.3 Effect of Cycloheximide on Lymphocyte Killing of A375 Cells

Increased lymphocyte cytotoxicity was not observed when A375 cells were pre-incubated for 3h with cycloheximide (a post-transcriptional protein synthesis inhibitor) at concentrations of 0.01, 0.1, 1 or 10mg/ml, (Figure 3.10), compared with untreated tumour cells. Pre-incubation of A375 cells with Act-D as a positive control resulted in increased cytotoxicity, as described in the previous sections.

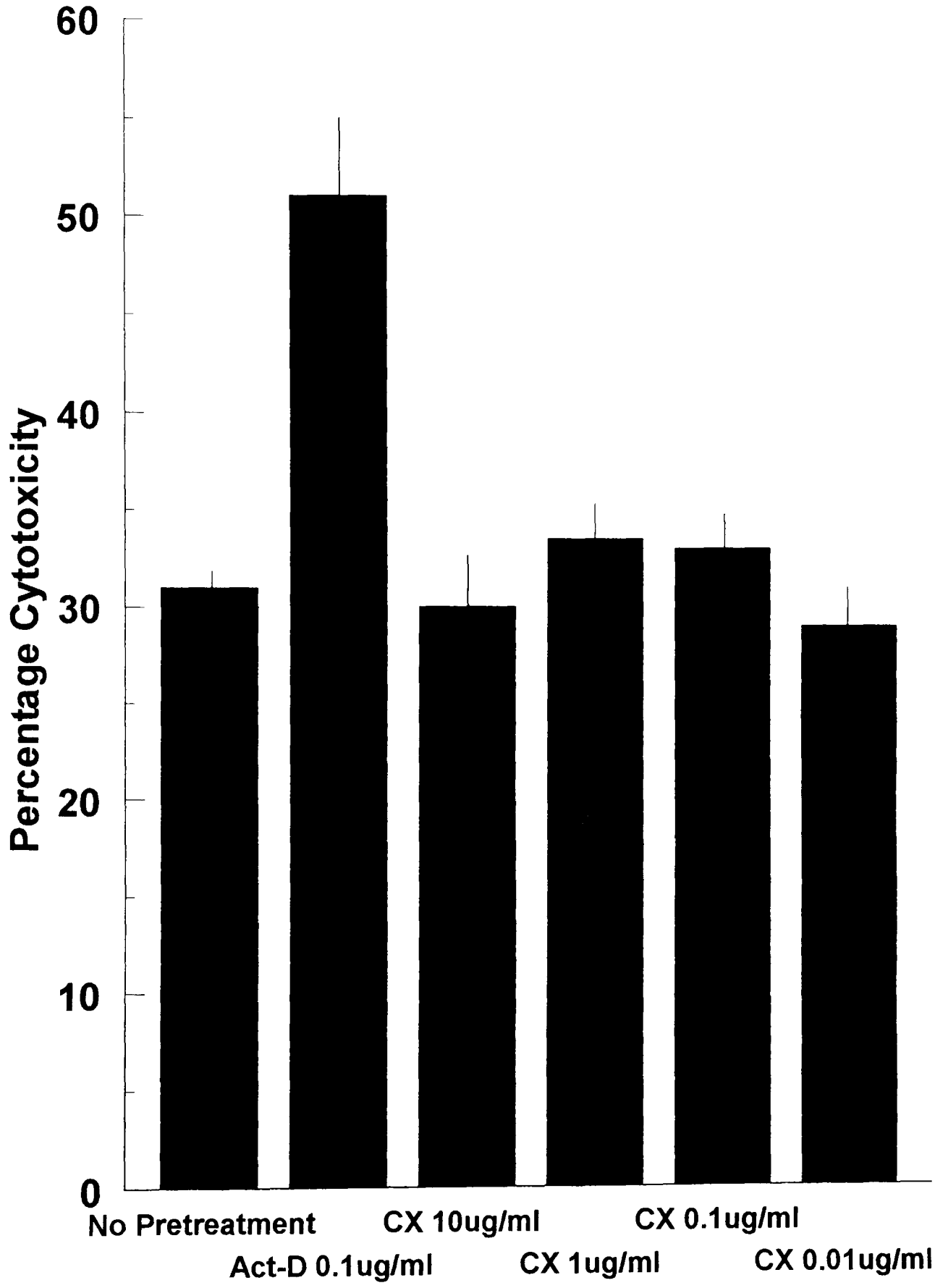

FIGURE 3.10. Effect of cycloheximide pre-treatment on lymphocyte killing of A375 cells. Lymphocyte cytotoxicity was assessed against A375 cells untreated or pre-incubated with Act-D (0.1μg/ml) or cycloheximide (CX, 0.01-10μg/ml), at an E:T of 50:1 after 18h incubation. Results are means and SEMs from 3 experiments. Although Act-D enhanced lymphocyte killing ($p<0.05$ by MWU and PTT), no augmentation was observed by target cell pre-treatment with CX at any concentration tested ($p>0.05$ by MWU and PTT).

3.2.4 Subset Depleted Lymphocyte Killing of A375 Cells

Highly purified subpopulations of lymphocytes for cytotoxicity experiments were prepared using a Becton Dickinson Facstar plus cell sorter. In order to separate the NK population from the T-cells, non-adherent lymphocytes (MNC incubated at 37^0C overnight, section 2.2.12) were divided into 2 aliquots, one of which was incubated with FITC labelled anti-CD8 monoclonal antibody, and passed through the cell sorter (section 2.2.12). The isolated population containing CD8+ cells was discarded and the remaining unlabelled cells, which were depleted of CD8+ cells was assessed for viability (>95% viable cells) and adjusted to 5 x 10^6 cells/ml for use in the cytotoxicity assay; after sorting <4% (+/- 2%, n=3) of CD8+ cells remained. The second aliquot was assessed for viability (section 2.2.16, >95% viable cells, n=3), adjusted to 5 x 10^6 cells/ml and included in the cytotoxicity assay.

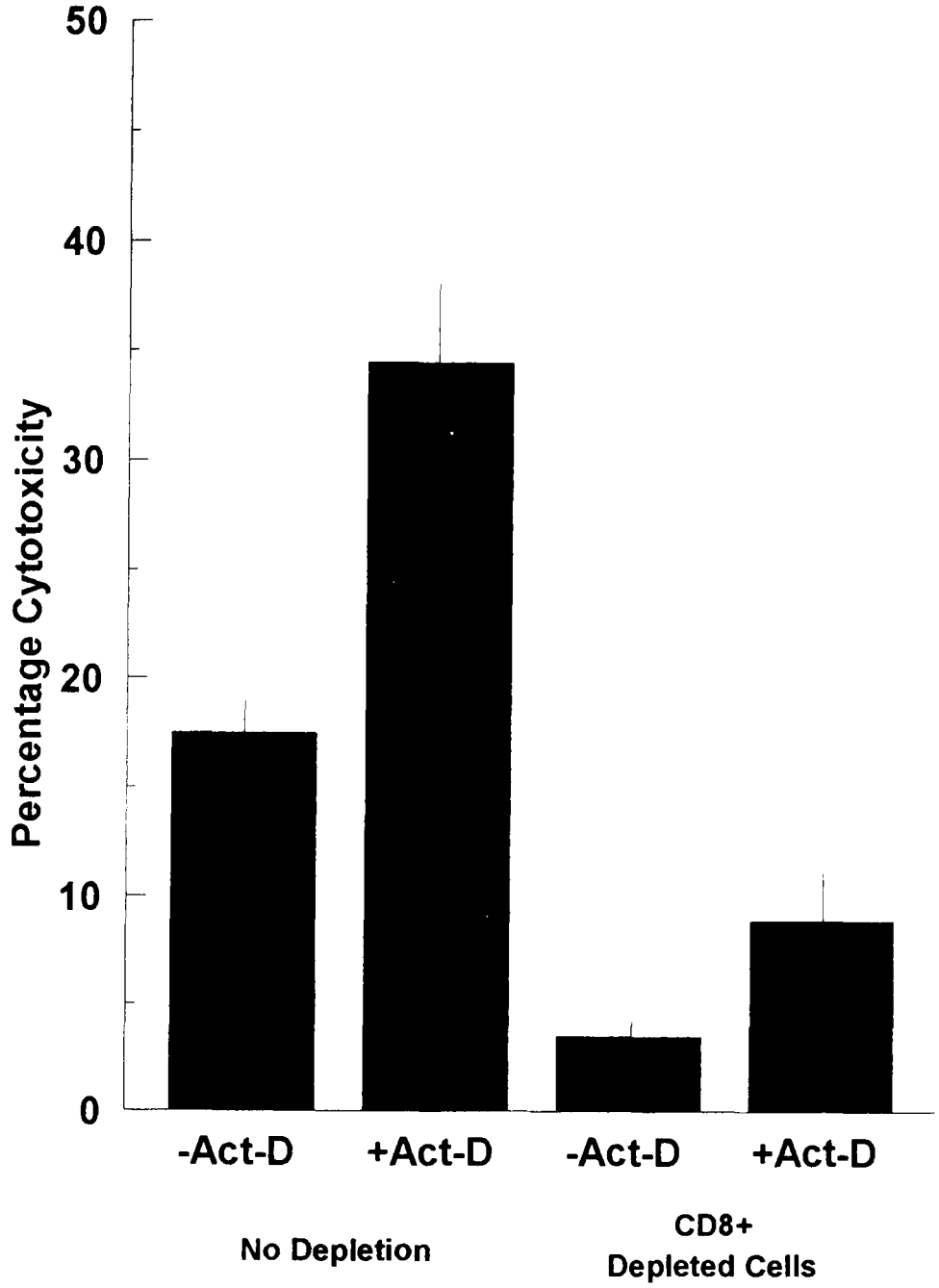

FIGURE 3.11. Effect of CD8+ cell depletion on lymphocyte killing of A375 cells. Lymphocytes were depleted of CD8+ cells as described in section 2.1.12. Cell viability was >95% for the whole and CD8+ cell depleted populations. The effector cells were incubated with untreated or Act-D pre-treated A375 cells for 18h at an E:T of 50:1 and specific cytotoxicity determined. In 3 experiments (means and SEMs are shown), removal of CD8+ cells from the lymphocyte population resulted in a 79% decrease in cytotoxicity ($p<0.05$ by MWU and PTT). Killing by fresh lymphocytes and CD8+ depleted cells was augmented by pretreatment of the target cells with Act-D ($p<0.05$ by MWU and PTT). However, cytotoxicity of Act-D pre-treated A375 cells by CD8+ depleted lymphocytes was significantly lower than that of whole lymphocytes against untreated tumour cells ($p<0.05$ by MWU and PTT).

Figure 3.11 shows that depletion of CD8+ cells almost completely abrogated the cytotoxic activity of lymphocytes against A375 cells. Pre-treatment of the target cells with Act-D enhanced the low level of killing (3%) by the CD8+ depleted cell population, as well as that of the whole lymphocyte population (as previously observed in section 3.2.2), but not to the same level of cytotoxicity observed in the whole lymphocyte population. It would therefore appear that the majority of lymphocyte cytotoxicity against A375 cells is due to cytotoxic CD8+ T-cells, rather than NK cells since this population was still present in the CD8+ depleted cell preparation. The residual cytotoxicity observed may have been due to some remaining CD8+ cells, monocytes or NK cells.

3.2.5 Lymphocyte Cytotoxicity to All Tumour Cell Lines

Non-adherent lymphocytes were tested for their cytotoxic activity against the 4 human melanoma cell lines A375, DX3, LT5.1 and SK23, as well as NK sensitive K562 cells (erythroblastoma) and NK resistant Daudi cells (EBV transformed B cells). Table 3.3 shows the mean maximal (Effector:Target Ratio 50:1) lymphocyte cytotoxicity for each cell line alone and following pre-incubation with Act-D.

Lymphocytes were directly cytotoxic for all lines tested and Act-D pre-incubation of the target cells resulted in increased tumouricidal activity only with the A375 and DX3 and SK23 melanoma lines. The melanotic melanoma cell line SK23 was the least susceptible to the action of lymphocytes. Daudi cells were moderately sensitive to lymphocyte killing, indicating either that an effector cell population other than NK cells was present or that Daudi cells become sensitive to lymphocyte killing after 18h The standard assay for the cytotoxicity of an NK resistant cell line is normally 4h.

	Source	n	Cytotoxicity -ActD (%)	Cytotoxicity +ActD (%)	Significance
A375	**Melanoma**	19	**27** (3)	**48** (3)	**p<0.05**
DX3	**Melanoma**	3	**31** (5)	**56** (2)	**p<0.05**
SK23	**Melanoma**	3	**18** (3)	**48** (5)	**p<0.05**
LT5.1	**Melanoma**	3	**32** (2)	**41** (4)	p>0.05
K562	**Erythroblast**	4	**42** (5)	**47** (5)	p>0.05
Daudi	**EBV, B-Cell**	3	**27** (7)	**22** (1)	p>0.05
U937	**Promonocyte**	3	**57** (5)	**53** (1)	p>0.05

TABLE 3.3. Summary of lymphocyte cytotoxicity against 7 cell lines. Untreated or Act-D pre-treated tumour cells were incubated with lymphocytes at an E:T ratio of 50:1 for 18h at 37^0C. Lymphocytes were cytotoxic for all cell lines (p<0.05 compared to spontaneous release by MWU and PTT). Pretreatment of the tumour cells with Act-D significantly increased (significance column) lymphocyte killing of A375, DX3 and SK23 cells compared to untreated target cells.

Cytotoxicity of tumour cell lines by lymphocytes was greater than that seen by any cytokine alone (sections 3.1.2 and 3.1.3) or in combination (pairs, section 3.1.3). There are several possible explanations for this, two of which have already been mentioned in the above sections (*i.e.* increased cytokine concentration at membrane junctions between effector and target cells, and effector cells may secrete a "cocktail" of effector molecules which are synergistic in their cytotoxic activity). Other explanations include 1. lymphocytes may not use these cytokines at all, but employ an entirely different mechanism of killing such as perforins or proteolytic enzymes. 2. Cytokines bound to the cell surface may behave differently to those secreted into the medium, as is the case with membrane bound TNFα. Further experiments on the mechanism of lymphocyte killing will be covered together with the possible mechanism of action of LAK cells in the next chapter.

Actinomycin-D enhanced the killing of some, but not other, cell lines to killing by cytokines and lymphocytes (table 3.3, significance column). Its mechanism of action is thought to work through protein synthesis inhibition at the level of mRNA synthesis.

3.2.6 Monocyte Cytotoxicity against Tumour Cell Lines

In addition to lymphocytes, monocytes are also present as infiltrating cells in many solid tumours, and have a large repertoire of direct cytotoxic effector mechanisms. Additionally, monocytes can present antigens and, thus, specifically activate T-cells to become tumouricidal. They also produce monokines which can modulate the behaviour of other monocytes, lymphocytes and tumour cells, as well as the extracellular matrix and the coagulation system. The direct cytotoxic activity of monocytes for A375 and K562 cells was therefore assessed.

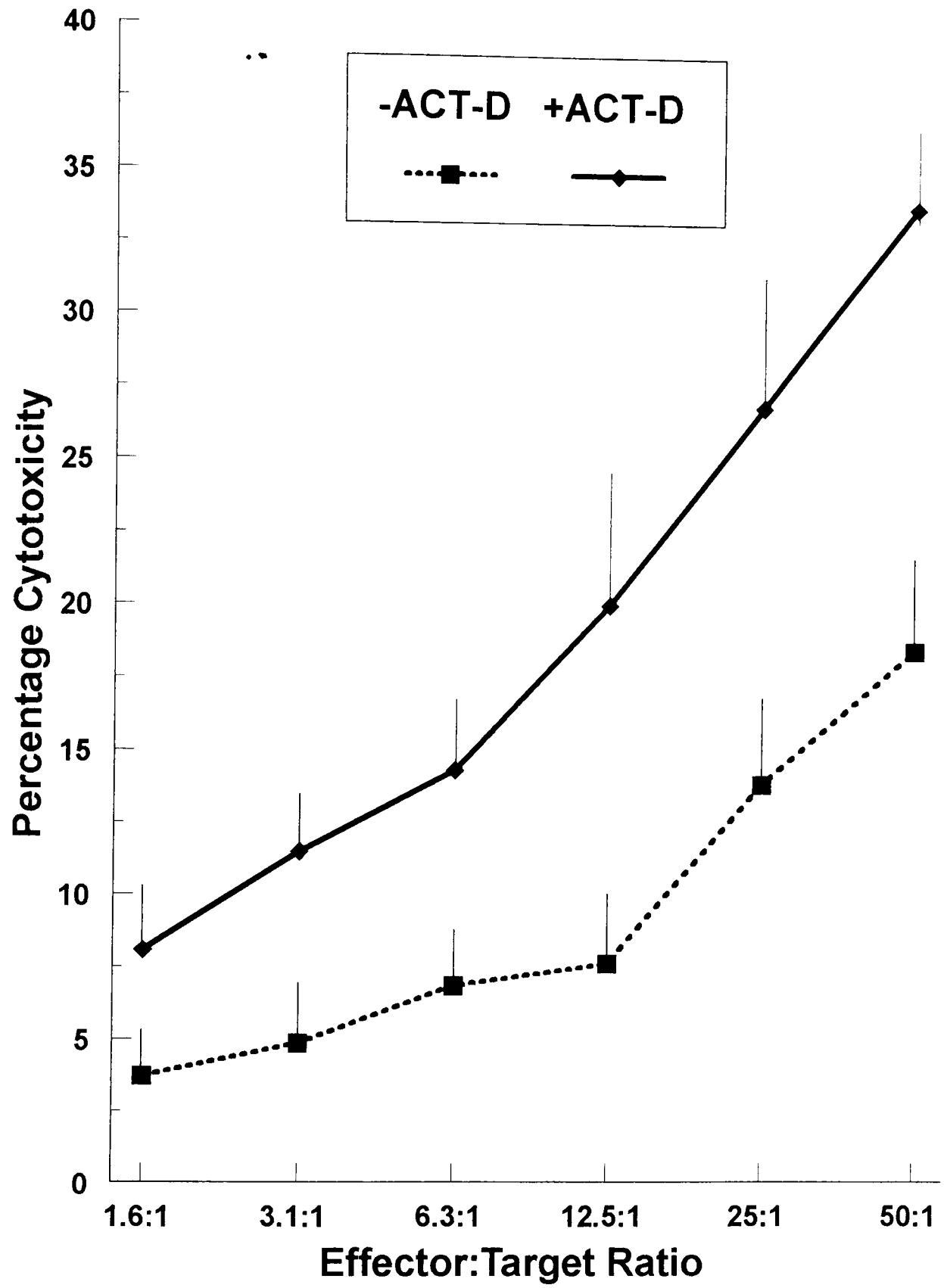

FIGURE 3.12. Monocyte cytotoxicity of A375 cells +/- Act-D. Monocyte cytotoxicity was assessed against untreated or Act-D pre-treated A375 cells at E:T ratios between 1.6:1 - 50:1 in an 18h $Na^{51}CrO_4$ releas eassay. The means and SEMs from 8 experiments are shown. On untreated A375 cells, monocytes were cytotoxic at an E:T of 3.1:1 (5%; compared to spontaneous release by WSR and PTT), which reached a maximum of 17% at an E:T of 50:1 ($p<0.05$ compared to 25:1 by WSR and PTT). Pre-treatment of A375 cells with Act-D significantly enhanced monocyte cytotoxicity at E:T of 3.1:1 and above, reaching a maximum of 35% at 50:1 ($p<0.05$ compared to 25:1 by WSR and PTT).

Freshly isolated monocytes were incubated for 18h with fresh A375 cells or A375 cells pre-treated with Act-D for 3h. Figure 3.12 shows the mean dose response curves from 8 experiments. Maximal monocyte killing occurred at an effector:target cell ratio of 50:1. Pre-treatment of A375 cells with Act-D significantly increased monocyte tumouricidal activity at all effector:target cell ratios.

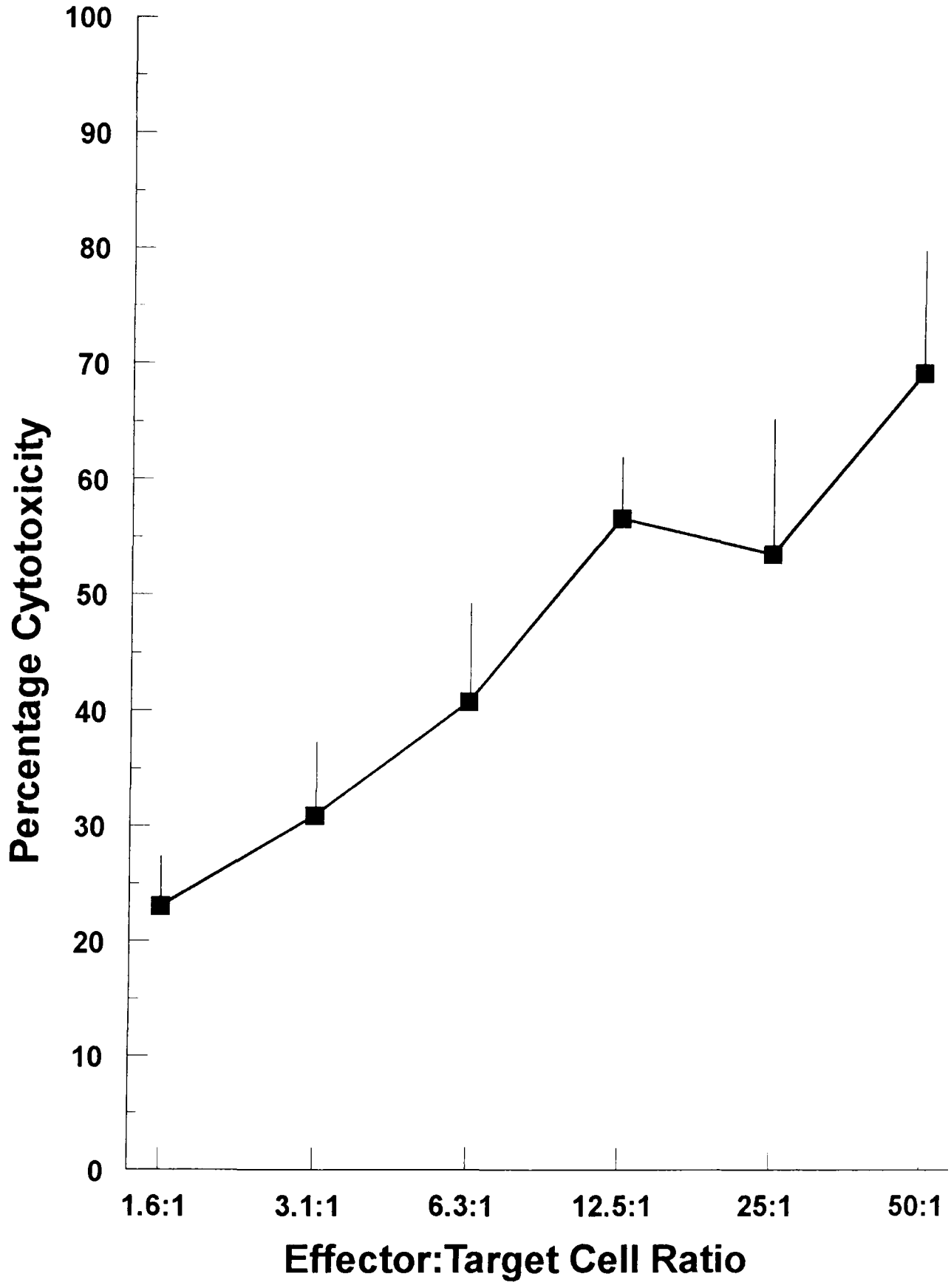

FIGURE 3.13. Monocyte cytotoxicity of K562 cells. Monocytes were incubated with $Na^{51}CrO_4$ labelled K562 cells at E:T ratios of 1.6:1 - 50:1. The means and SEMs from 3 experiments are shown. After 18h incubation, monocytes were cytotoxic against K562 cells ($p<0.05$ compared to spontaneous release by MWU and PTT), which increased in a dose dependent fashion to 73% at 50:1. Cytotoxicity at 50:1 was significantly greater ($p<0.05$ by MWU and PTT) than that at 6.3:1, but not 12.5:1 or 25:1.

Monocyte cytotoxicity against untreated K562 cells was also evaluated. Figure 3.13 shows the mean dose response curve from 3 experiments. Maximal cytotoxicity seen at an effector:target cell ratio of 50:1, was considerably higher than that observed at 50:1 against A375 cells, indicating that K562 cells were more sensitive to killing by monocytes. Pre-treatment of K562 cells with Act-D was not performed since no augmentation of killing was seen with TNFα or lymphocytes as effectors (sections 3.1.2 and 3.2.2 respectively).

3.2.7 Role of LPS in Monocyte Cytotoxicity of A375 Cells

Bacterial lipopolysaccharide activates resting monocytes causing production and secretion of cytokines including TNFα, IFNγ and IL-1. The presence of LPS in the monocyte separation medium could, therefore, activate monocytes to become non-specifically tumouricidal.

Lipopolysaccharide was incubated with A375 cells at concentrations of 0.001, 0.01, 0.1, 1.0, 10, 100 and 1000μg/ml for 18h. Cytotoxicity was only seen at the highest concentrations tested (*i.e.* 100 and 1000μg/ml, see Figure 3.14)

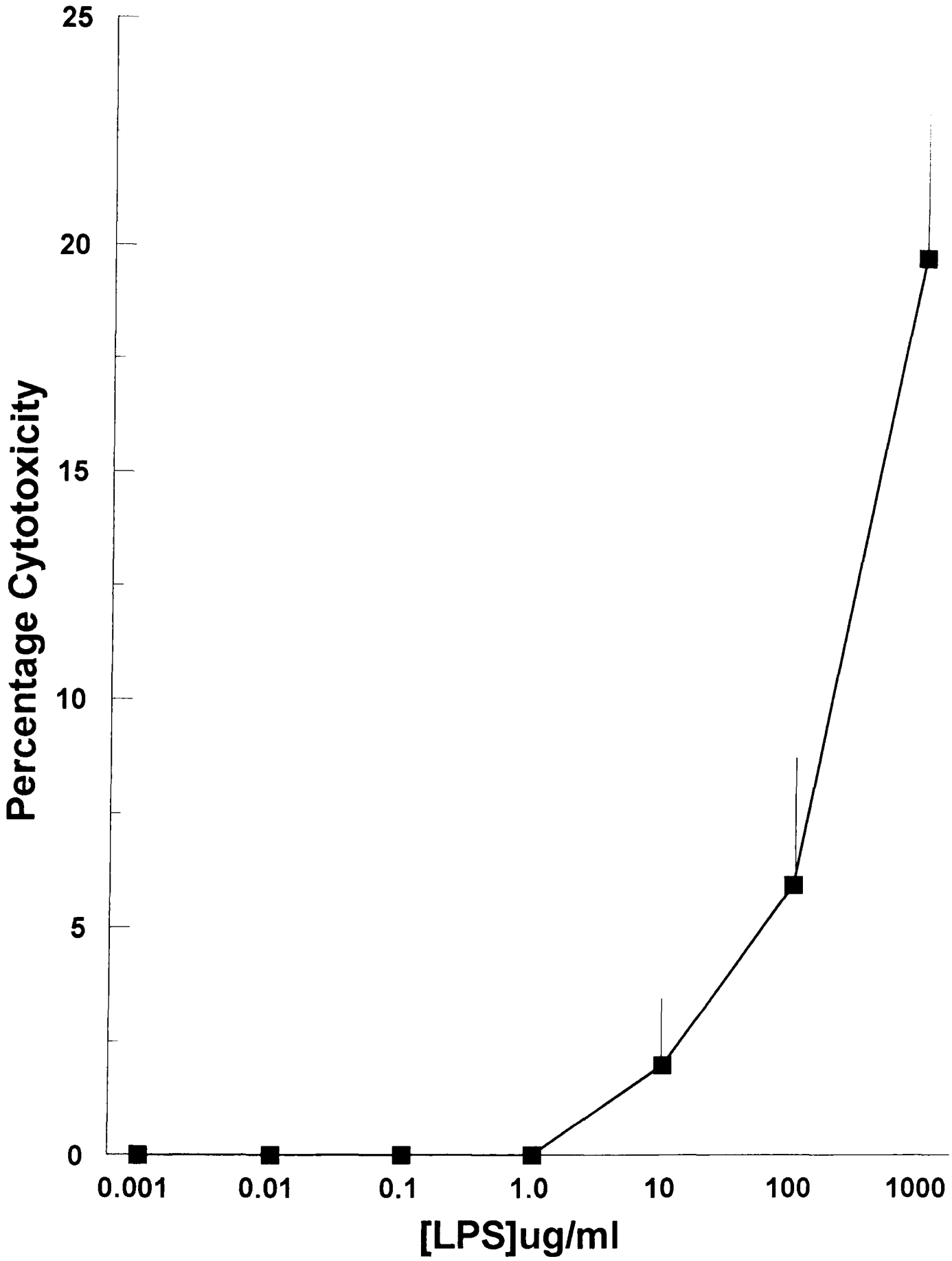

FIGURE 3.14. LPS cytotoxicity of A375 cells. Lipopolysaccharide (0.001-1000μg/ml) was incubated with $Na^{51}CrO_4$ labelled A375 cells for 18h at 37^0C. The supernatants were harvested and specific cytotoxicity calculated. The means and SEMs from 4 experiments are shown. Significant cytotoxicity (7%; $p<0.05$ by MWU compared to spontaneous release) was observed at 100μg/ml LPS, which rose to 19% at 1000μg/ml ($p<0.05$ compared to 100μg/ml by MWU).

These concentrations are far in excess of those which may be present in any tissue culture medium and thus it can be considered that any contaminating LPS would not be cytotoxic to the tumour cells. However, monocytes are activated by as little as 0.01μg/ml LPS (Chen *et al.*, 1986) which may be present in the tissue culture medium. To test whether monocytes could be activated (or further activated) by LPS to become cytotoxic, monocytes were incubated with A375 cells for 18h in the presence or absence of LPS (10μg/ml). In 2 experiments LPS had no effect on monocyte killing. However, the monocytes may have already been activated by contaminating LPS in the tissue culture medium. To circumvent this problem, monocyte cytotoxicity was performed in tissue culture media containing polymyxin-B (an antibiotic which inhibits LPS activity). No difference in monocyte killing of A375 cells (pre-treated with Act-D) was seen between experiments performed in 10% FCS or 10% FCS containing polymyxin-B. In addition, samples of the tissue culture media were sent to Wickham laboratories for a rabbit pyrogenicity test (British Pharmacopoeia 1993, page A172), which is a standard test used to judge the suitability of pharmaceuticals for injection into patients. RPMI and 10% FCS induced a temperature rise of 0.5^{0}C and 0.3^{0}C respectively, and were thus considered to have "passed" the test, and have been non-pyrogenic. These results suggest that any small amounts of LPS contaminating the tissue culture media did not contribute significantly to the monocyte cytotoxicity observed.

3.2.8 Comparison of A375 Cell Killing by TNFα, Lymphocytes and Monocytes.

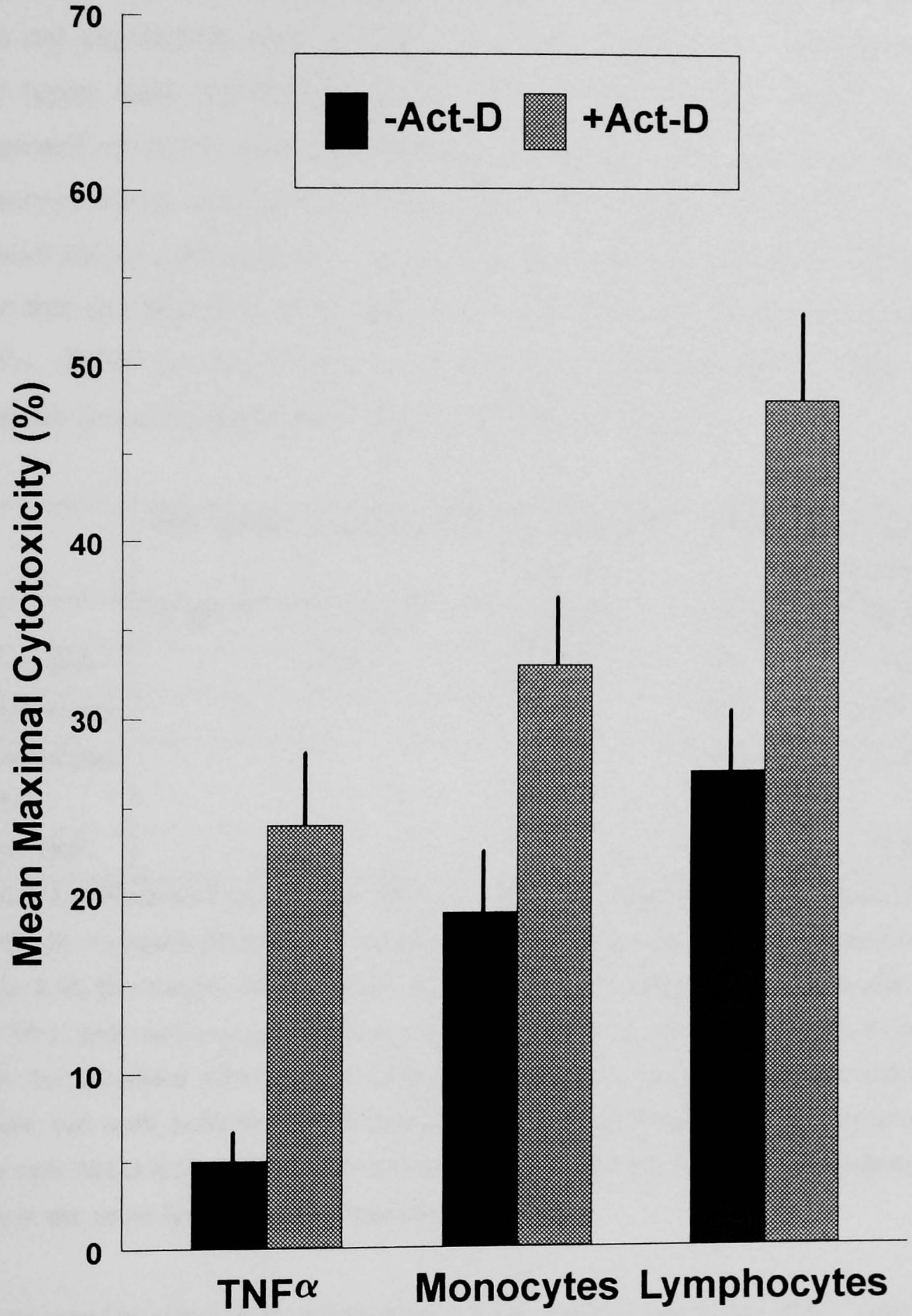

FIGURE 3.15. Comparison of TNFα, monocyte and lymphocyte killing of A375 cells. Cytotoxicity of untreated and Act-D pre-treated A375 cells by TNFα (n=12; section 3.1.2), lymphocytes (n=19; section 3.2.5) and monocytes (n=8; section 3.2.6) is shown. Statistical analysis comparing the levels of killing by each effector is presented in table 3.4.

Figure 3.15 shows the mean maximal cytotoxicity of A375 cells in the presence or absence of Act-D, induced by TNFα (1000U/ml; data from section 3.1.2), lymphocytes (data from section 3.2.5) or monocytes (data from section 3.2.6). Direct killing of untreated A375 cells by lymphocytes was not significantly greater than that of monocytes, but both effector cell types were significantly more cytotoxic than TNFα. Act-D pre-treatment of A375 cells significantly enhanced cytotoxicity over that observed using untreated A375 cells for each effector. On Act-D pre-treated target cells however, lymphocyte killing was significantly higher than that of monocytes which was in turn significantly higher than that of TNFα. Table 3.4 summarises the p-values obtained from a Mann-Whitney U-test between each effector-target combination.

	TNF +Act-D	**Lymphocytes**	**Lymphocytes +Act-D**	**Monocytes**	**Monocytes +Act-D**
TNF	**<0.0001**	**<0.0001**	**<0.0001**	**<0.001**	**<0.0001**
TNF +Act-D		NS	**<0.0001**	NS	**<0.01**
Lymphocytes			**<0.001**	NS	NS
Lymphocytes +Act-D				**0.0001**	**<0.05**
Monocytes					**<0.01**

TABLE 3.4. Statistical comparison of TNFα, monocyte and lymphocyte killing of A375 cells +/- Act-D. Statistical analysis was carried out on the data presented in figure 3.15, to compare the cytotoxic activities of TNFα (1000U/ml), lymphocytes (E:T 50:1) and monocytes (E:T 50:1) against untreated or Act-D pre-treated A375 cells. On untreated A375 cells, lymphocytes and monocytes killed to a similar degree, but were both more cytotoxic than TNFα. Pre-incubation of the tumour cells with Act-D increased killing by the 3 effectors, and the levels of cytotoxicity were in the order lymphocytes > monocytes > TNFα.

These results show that lymphocytes and monocytes killed A375 cells more effectively than did TNFα, either reaffirming that local concentrations of cytokine at effector-target cell junctions may be much higher than those used here, or that lymphocytes and monocytes do not exclusively employ TNFα as an effector molecule. Pre-treatment of A375

cells with Act-D enhanced lymphocyte and monocyte cytotoxicity to the same degree (54%), although the non-significantly higher value for lymphocyte killing of untreated target cells became significantly higher than that of monocytes after Act-D pretreatment. A possible explanation for this is that monocytes produce TNFα (among other effector molecules), whereas lymphocytes can also secrete LT, the cytotoxic action of which is augmented by target cell pre-treatment with Act-D.

3.3.0 Leucocyte Killing of Cytokine Pre-treated A375 Cells

Having established the direct cytotoxic activities of the selected cytokines and leucocytes, it was next important to evaluate the effect of combined cytokine and lymphocyte treatment on tumour cell cytotoxicity. Simple addition of cytokines and leucocytes together and observation of the resultant tumour cell killing would not produce meaningful results since many secondary interactions (such as the induction of other effector molecules) could occur between cytokines and cells, each of which might be responsible for any changes in cytotoxicity observed. Experiments were therefore performed to evaluate the effect of cytokine pre-treatment of tumour cells (with or without Act-D pre-treatment), on lymphocyte and monocyte killing of A375 cells.

3.3.1 Effect of Cytokine Pre-treatment of A375 Cells on Lymphocyte Cytotoxicity

A375 cells were pre-treated with IL-2 (500-2000U/ml), TNFα (100-500U/ml), or IFNγ (100-1000U/ml) for 3-72h. The cells were washed to remove any remaining cytokine and their sensitivity to lymphocyte cytotoxicity assessed. In some experiments the target cells were incubated with Act-D for 3h prior to inclusion in the cytotoxicity assay.

In 3 experiments, IL-2 and TNFα pre-treatment of A375 cells did not result in any increase in lymphocyte cytotoxicity compared to untreated A375 cells at any concentration tested, even after 72h, indicating that these cytokines are unable to augment lymphocyte cytotoxicity of A375

cells, even though IL-2 has some effect on the growth of these cells and TNFα will kill Act-D presensitised A375 cells (see section 3.1.2).

At 3h and 24h, IFNγ (concentration range 100-1000U/ml) pre-treatment had no effect on lymphocyte cytotoxicity of A375 cells, but in one experiment it induced a 57% increase in killing after 48h, and a 35% increase after 72h in a separate experiment. Maximal cytotoxicity was observed at an IFNγ concentration of 250U/ml (Figure 3.16)

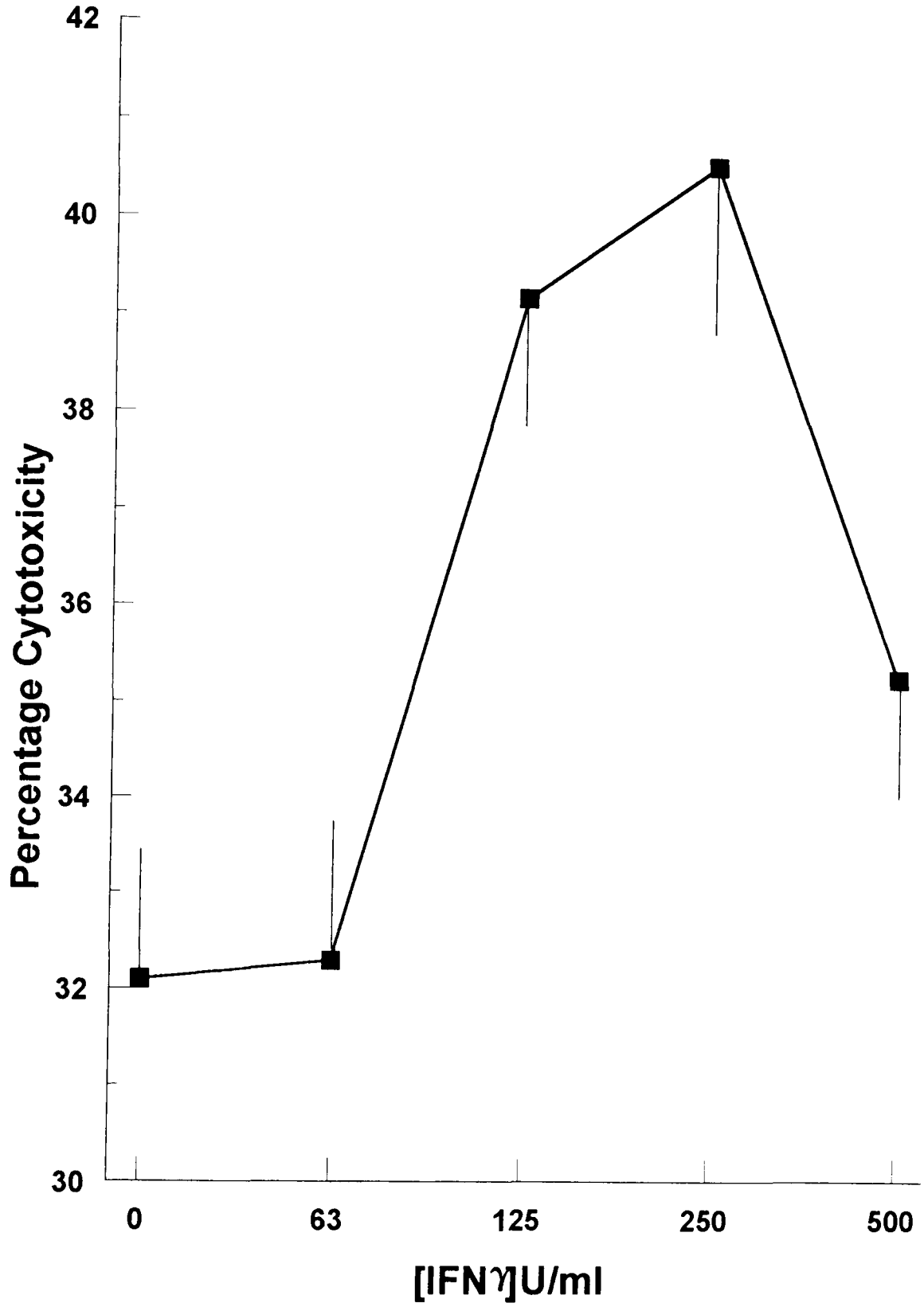

FIGURE 3.16. Lymphocyte killing of IFNγ pre-treated A375 cells. A375 cells were incubated with IFNγ (0-500U/ml) for 48h, $Na^{51}CrO_4$ labelled and their killing by lymphocytes assessed (E:T 50:1). In 3 experiments (means and SEMs shown), a significant increase in cytotoxicity was observed at an IFNγ concentration of 125 and 250U/ml ($p<0.05$ compared to untreated A375 cells by MWU and PTT), which subsequently decreased at 500U/ml IFNγ to a level which was similar to that of untreated A375 cells ($p>0.05$ compared to untreated A375 cells by MWU and PTT). Pre-incubation of A375 cells with IFNγ at 63U/ml had no significant effect on lymphocyte cytotoxicity ($p>0.05$ compared to untreated A375 cells by MWU and PTT).

At IFNγ concentrations above 250U/ml there was a decrease in lymphocyte cytotoxicity. This pattern of killing could be explained by the previous observation (section 3.1.4) that IFNγ significantly reduced the yield of A375 cells from culture at concentrations above 250U/ml. Concentrations of IFNγ up to 250U/ml may have been sufficient to sensitise (possibly by cytostasis) A375 cells for killing by lymphocytes, but concentrations greater than 250U/ml were directly cytotoxic to A375 cells. In the latter case, IFNγ, and possibly lymphocyte, sensitive target cells may have been selected out, leaving only resistant cells as targets in the cytotoxicity assay. Incubation of target cells with IFNγ for 48h followed by Act-D (0.1μg/ml) for 3h (Figure 3.17), augmented lymphocyte

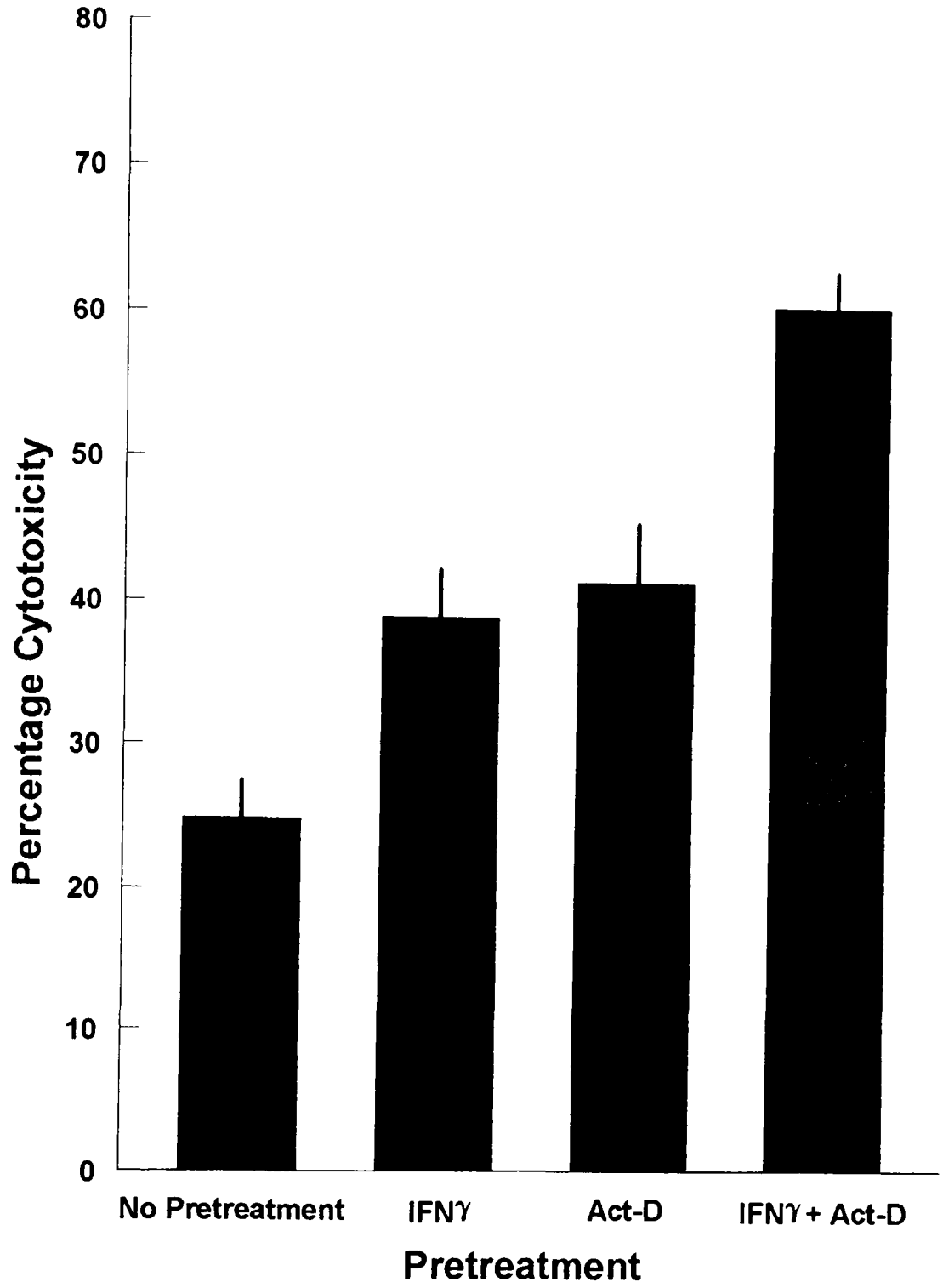

FIGURE 3.17. Effect of IFNγ and/or Act-D pretreatment on lymphocyte cytotoxicity of A375 cells. A375 cells were incubated for 48h in RPMI/FCS alone (2 x 25cm2 TC flasks) or containing IFNγ (250U/ml) (2 x 25cm2 TC flasks). One flask from each treatment was further incubated for 3h with Act-D (0.1μg/ml). After labelling with $Na^{51}CrO_4$, the cells were included as target cells in a lymphocyte cytotoxicity assay (E:T 50:1). In 3 experiments, killing was augmented by pretreatment of the target cells with either IFNγ or Act-D ($p<0.05$ by MWU and PTT). Combined pre-incubation with IFNγ and Act-D increased lymphocyte killing to a level which was only slightly greater than the sum of the increases observed with the individual agents ($p<0.05$ by MWU and PTT) This increase was not synergistic ($p>0.05$ compared to untreated cells plus the increases due to both IFNγ and Act-D individually; by MWU and PTT).

killing in 3 experiments. These results indicated that IFNγ and Act-D were synergistic in their sensitisation of A375 cells to killing by lymphocytes.

The results of pre-incubation of tumour cells with combinations of IFNγ and IL-2 or IFNγ and TNFα were not significantly different to those for IFNγ alone (data not shown), suggesting that there were no interactions between these cytokine combinations on A375 cells, as has been reported previously for IL-2 and IFNγ (Dealtry *et al.*, 1987).

3.3.2 Monocyte Killing of Cytokine Pretreated A375 Cells

Freshly isolated peripheral blood monocytes were assessed for their cytotoxic activity against untreated A375 cells, or A375 cells pre-treated with single cytokines, pairs of cytokines and/ or subsequent incubation with Act-D.

A375 cells were pre-incubated with IFNγ, and IL-2, with or without Act-D stimulation (section 2.2.4). In the absence of Act-D, none of the cytokines tested had any effect on monocyte killing when compared with untreated A375 cells (figure 3.18). Simultaneous pre-treatment of target cells with two cytokines again had no effect with the exception of the combination of IFNγ and IL-2 which induced an increase in monocyte cytotoxicity, thus demonstrating a synergistic interaction between these cytokines in the induction of monocyte cytotoxicity for A375 cells.

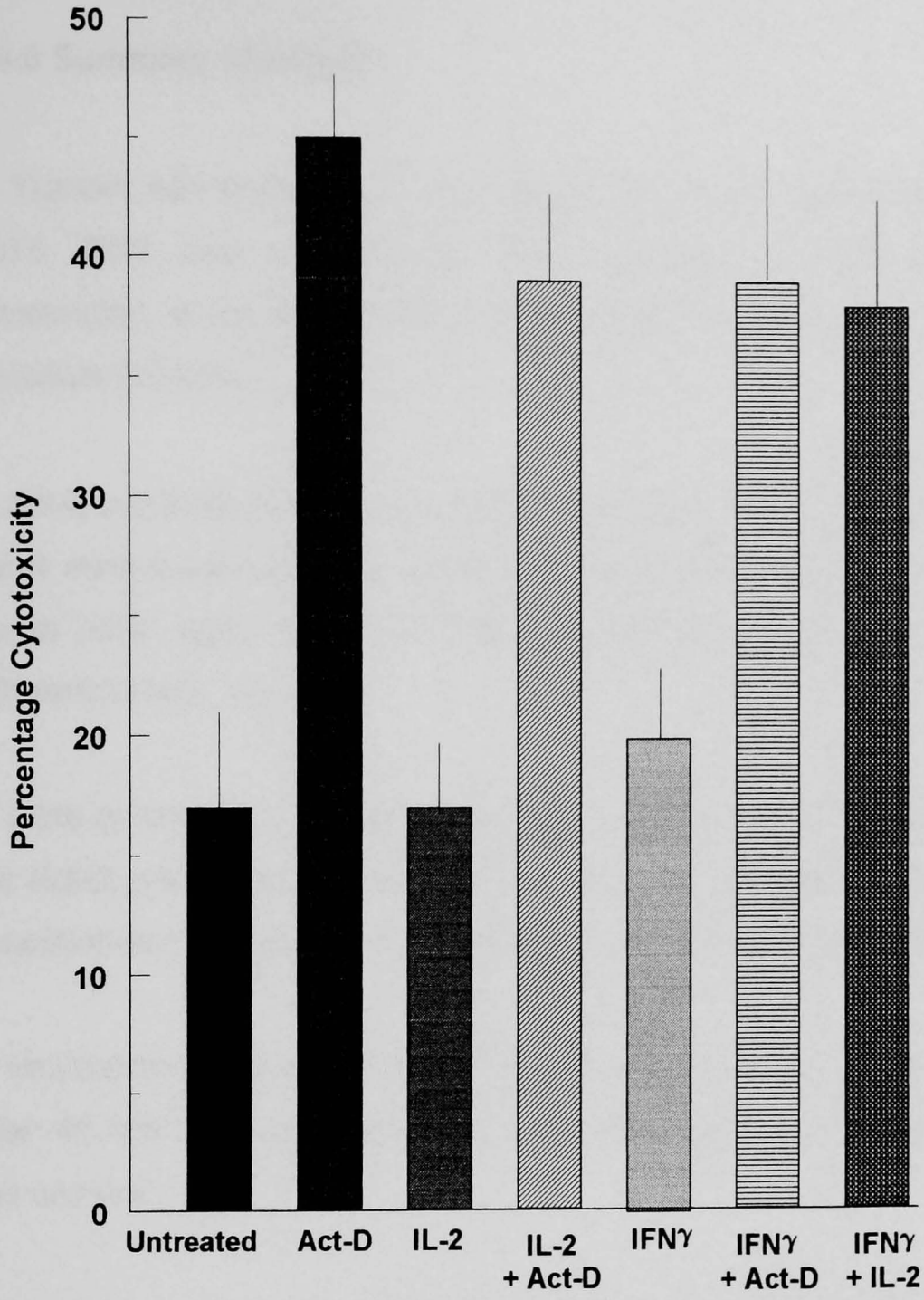

FIGURE 3.18. Tumour cells (A375) were incubated in duplicate 25cm2 TC flasks for 48h in RPMI/FCS alone or containing IFNγ (250U/ml), IL-2 (250U/ml) or IFNγ and IL-2. One flask from each group was subsequently treated with Act-D (0.1μg/ml) for 3h. After $Na^{51}CrO_4$ labelling, the A375 cells were cultured for 18h in the presence of purified monocytes (E:T 50:1) and specific cytotoxicity assessed. In 3 experiments, Act-D ($p<0.05$ compared to untreated cells by MWU and PTT), but not IFNγ or IL2 ($p>0.05$ by MWU and PTT), augmented monocyte cytotoxicity. Combined pre-treatment with either cytokine and Act-D resulted in increased cytotoxicity, but was not significantly different from that of Act-D alone ($p>0.05$ by MWU and PTT). However, increased monocyte killing was observed using a combination IFNγ and IL-2 pre-incubation ($p<0.05$ by MWU and PTT). Since IFNγ and IL-2 alone had no augmenting activity alone, the increase produced by their combination was synergistic.

3.4.0 Summary of Results

1. Tumour cell lines were heterogeneous in their response to TNFα. A375, DX3 and LT5.1 cells were relatively unresponsive (<10% cytotoxicity), whilst SK23, K562, Daudi (18h) and U937 cells were more sensitive (16-23%).

2. Act-D pre-treatment of tumour cells increased TNFα cytotoxicity in 3 of the 4 melanoma cell lines as well as U937 after 18h, but not K562 or Daudi cells, again demonstrating heterogeneity of response between different tumour cell lines.

3. Little or no killing of A375 cells was seen by IL-1β, IL-2, IFNγ or LT, but Act-D pre-treatment induced cytotoxicity by 3 of the 4 cytokines (the exception being IL-2), to levels between 13-19% specific cytotoxicity.

4. Incubation of A375 cells with IFNγ caused a decrease in the cell yield after 48-72h, indicating that IFNγ was either cytotoxic or cytostatic for this cell line.

5. Incubation of A375 cells with IL-2 resulted in an increased yield of cells after 72h. Flow cytometry demonstrated the presence of IL-2 receptors (α-chain) but not the β-chain on these cells.

6. Lymphocytes were cytotoxic for all cell lines tested, but there was heterogeneity in sensitivity between cell lines. The most sensitive lines were K562 (42%) and U937 (57%), and the least sensitive was SK23 (18%). Lymphocyte killing of the other cell lines tested ranged between 27-32% specific cytotoxicity.

7. Act-D pre-treatment augmented lymphocyte killing of 3 (A375, DX3 and SK23) of the melanoma cell lines, but not LT5.1, K562, Daudi or

U937. In contrast, pre-treatment of A375 cells with cycloheximide had no effect on lymphocyte killing.

8. Depletion of CD8+ cells from the lymphocyte population resulted in over 75% decrease in lymphocyte killing of untreated and Act-D pre-treated A375 cells. The effector lymphocytes in this system were, therefore, predominantly CD8+ cytotoxic T lymphocytes.

9. Monocytes were cytotoxic for A375 and K562 cells, and killing of A375 cells was augmented by pre-treatment with Act-D. Monocytes were probably not activated by low levels of contaminating LPS.

10. Fresh lymphocytes were significantly more cytotoxic for A375 cells than monocytes, which in turn were more cytotoxic than TNFα (at 1000U/ml).

11. Lymphocyte killing of A375 cells was only increased by incubation of target cells with IFNγ for 48-72h, and IFNγ treatment synergised with subsequent Act-D treatment of the target cells. No increase in killing was seen after A375 cells were pre-incubated with IL-2 or TNFα, even when the tumour cells were subsequently incubated for 3h in the presence of Act-D. No synergistic augmentation was observed between any pair of cytokines composed of IFNγ, IL-2 or TNFα.

12. Monocyte cytotoxicity of A375 cells was not augmented by pre-incubation of the target cells with IL-1β, IFNγ, TNFα or IL-2, but a synergistic increase in killing was observed with combined IFNγ and IL-2 pre-treatment. Incubation with IFNγ or IL-2 followed by Act-D pre-treatment of A375 cells also synergistically increased monocyte killing.

CHAPTER 4

LYMPHOKINE ACTIVATED KILLER (LAK) CELLS

4.0 Introduction

Interleukin-2 activates lymphocytes to become highly cytotoxic for a wide range of tumour cell lines (but not normal cells) *in vitro* (Grimm *et al.*, 1982). Over the past decade clinical trials have been undertaken to determine the therapeutic effects of injected recombinant IL-2 alone in patients with terminal cancer (Rosenberg, 1989; Mitchell, 1992). However, IL-2 at high concentrations (*i.e.*, sufficient to activate lymphocytes *in vivo*) proved to be highly toxic (Rosenberg *et al.*, 1987), and the therapeutic effect was marginal. In a total of 174 patients tested in six clinical trials with IL-2 (5×10^4 - $3 \times 10^7 U/m^2$) there was only 1 complete remission and 10 partial (>50% reduction in tumour mass) remissions (Lotze *et al.*, 1986; Creekmore *et al.*, 1987; Rosenberg *et al.*, 1987; Sano *et al.*, 1987; Thompson *et al.*, 1987; Whitehead *et al.*, 1987). Since the *in vivo* mechanism of action of IL-2 therapy was thought to be via the generation of LAK activity in the circulation and in the tumour infiltrating lymphocytes (Rosenberg *et al.*, 1985), further protocols were devised in which large numbers (typically 1-5 x 10^{10} cells) of peripheral blood mononuclear cells (lymphocytes and monocytes) were removed from patients, activated with high concentrations (between 500 - 2000U/10^6 cells) of IL-2, and re-introduced into the patient, together with lower doses of IL-2. In some trials this process was repeated on 2 or 3 occasions. Combined results from five such trials (Dutcher *et al.*, 1987; Fisher *et al.*, 1987; Paciucci *et al.*, 1987; Rosenberg *et al.*, 1987; West *et al.*, 1987) showed improved (but still relatively low) clinical response rates than was previously obtained using IL-2 alone. Of 228 patients treated there were 9 complete and 42 partial responses (*i.e.*, 4% complete and 18% partial compared to 0.6% complete and 6% partial responses using IL-2 alone). Although it was not possible to identify if patients were likely to respond to IL-2/LAK cell therapy, patients with renal cell carcinoma and malignant melanoma demonstrated an

increased responsiveness to this immunotherapy than other cancers (Rosenberg *et al.*, 1989).

The aims of the next series of experiments were: (1.) To compare the cytotoxic activity of LAK cells and IL-2 activated monocytes with untreated lymphocytes, monocytes and cytokines on the 7 tumour cell lines (with or without Act-D pre-treatment) *in vitro*; (2.) to determine the ability of LAK cells to kill autologous and heterologous normal cells; (3.) to assess the tumouricidal activity of LAK cell supernatants, and; (4.) to investigate the mechanism(s) by which unstimulated leucocytes and LAK cells adhere to and kill tumour cells.

4.1 LAK Cell Cytotoxicity Against Tumour Cells

4.1.1 IL-2 Activation of Lymphocytes

Various concentrations of IL-2 were used in previous studies to induce LAK activity. It was therefore necessary to determine the optimal concentration of IL-2 required for LAK cell generation in this study. In 3 experiments, peripheral blood lymphocytes at a concentration of 2 x 10^6/ml were incubated for three days alone or in the presence of rIL-2 (10, 50, 250 and 500U/ml), and their cytotoxicity against untreated or Act-D pre-treated A375 cells measured (figure 4.1).

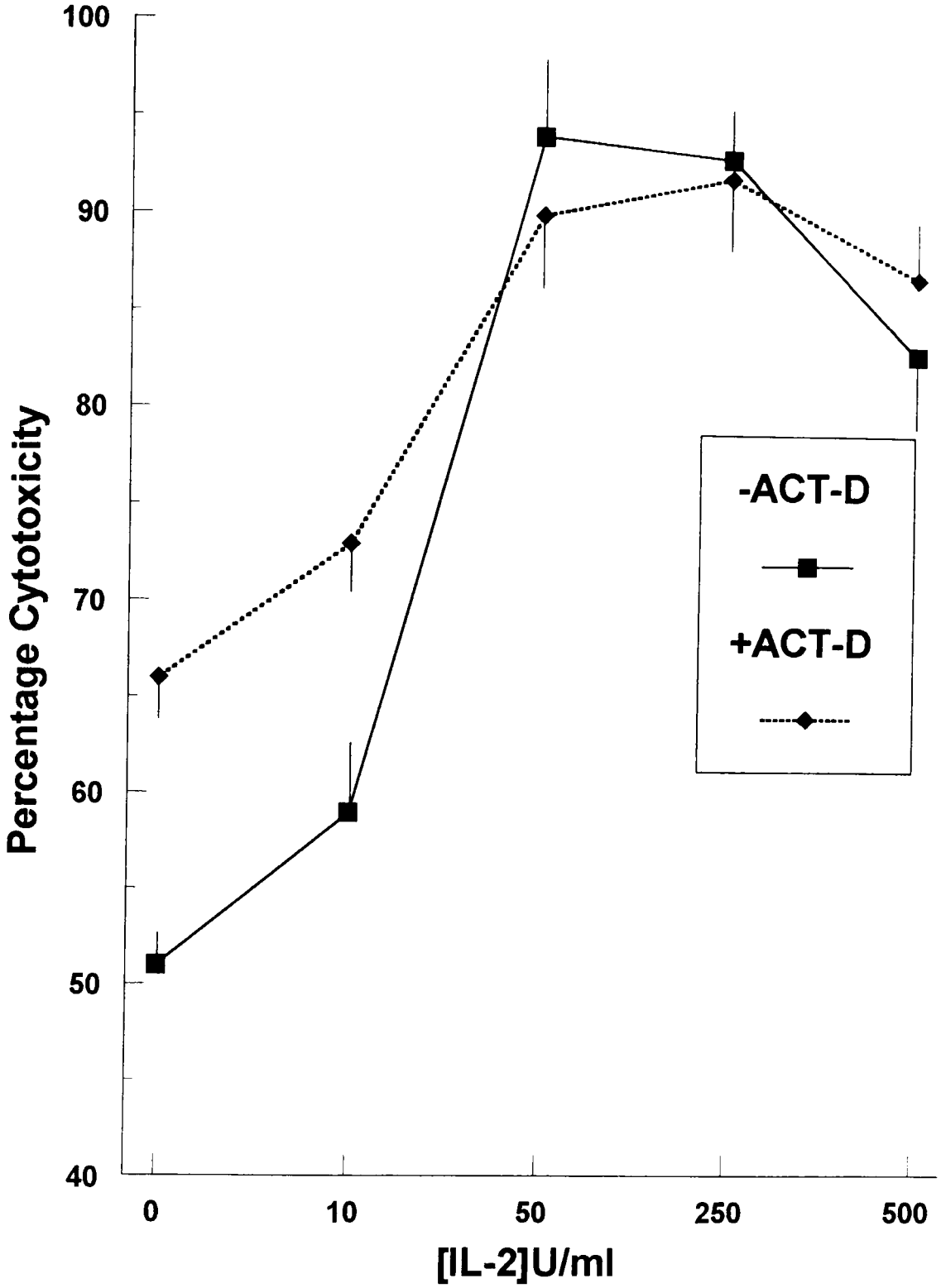

FIGURE 4.1. Effect of IL-2 on lymphocyte killing of A375 cells. Lymphocytes were incubated with RPMI/FCS alone (control cells) or containing IL-2 (10-500U/ml) for 72h, and their cytotoxicity against untreated or Act-D pre-treated A375 cells assessed (E:T 50:1). In 3 experiments (means and SEMs are shown), killing was augmented (compared to no IL-2; $p<0.05$ by PTT and MWU) by IL-2 at all concentrations tested. Maximal cytotoxicity was observed at 50U/ml IL-2 (compared to 10U/ml; $p<0.05$ by PTT and MWU), which did not increase further at higher IL-2 concentrations ($p> 0.05$ by PTT and MWU). Pre-incubation of A375 cells with Act-D resulted in increased lymphocyte cytotoxicity at 0 and 10U/ml IL-2 ($p<0.05$ by PTT and MWU), but not at the higher IL-2 concentrations ($p>0.05$ by PTT and MWU).

Control cells (lymphocytes cultured in the absence of IL-2 for 72h) were more cytotoxic (53%) than freshly isolated lymphocytes (mean 27% +/- %, see section 3.2) for untreated A375 cells. This may have been due to partial activation of the effector cells after 72h in culture. As with fresh lymphocytes, control cell killing was augmented by preincubation of the target cells with Act-D (Figure 4.1). Incubation of lymphocytes with IL-2 increased their killing of A375 cells in a dose-dependent manner, but no significant increase in killing was seen when A375 cells were pre-treated with Act-D, except at the lowest concentration of IL-2 tested (10U/ml). Since the cytotoxicity seen with 10U/ml IL-2 was not significantly different from that of the control (0U/ml IL-2) it is probable that this concentration of IL-2 was insufficient to induce LAK activity. Maximal cytotoxicity was seen at IL-2 concentrations of 50-250U/ml, and this higher concentration (250U/ml) was used to generate LAK cells in all further experiments. In contrast to lymphocytes (control cells), LAK cells were highly cytotoxic for A375 cells, generating over 90% specific cytotoxicity of these cells after 18h.

There are two possible explanations for the failure of Act-D pre-treatment of A375 cells to enhance LAK cell killing. Either LAK cells are extremely efficient at killing A375 cells, and at this level any increase induced by Act-D sensitisation would not be seen, or alternatively, LAK activity employs effector mechanisms distinct from those of unstimulated lymphocytes, which are independent of the action of Act-D.

4.1.2 Time Course of IL-2 Activation of Lymphocytes

Having determined the optimal concentration of IL-2 required for LAK cell production, the optimum incubation time for the culture of lymphocytes with IL-2 was assessed. Figure 4.2 shows the mean (3 experiments) cytotoxicity of untreated and Act-D pre-treated A375 cells by fresh lymphocytes and lymphocytes which had been incubated with IL-2 (250U/ml) for 4h, 1, 2, 3 and 7days.

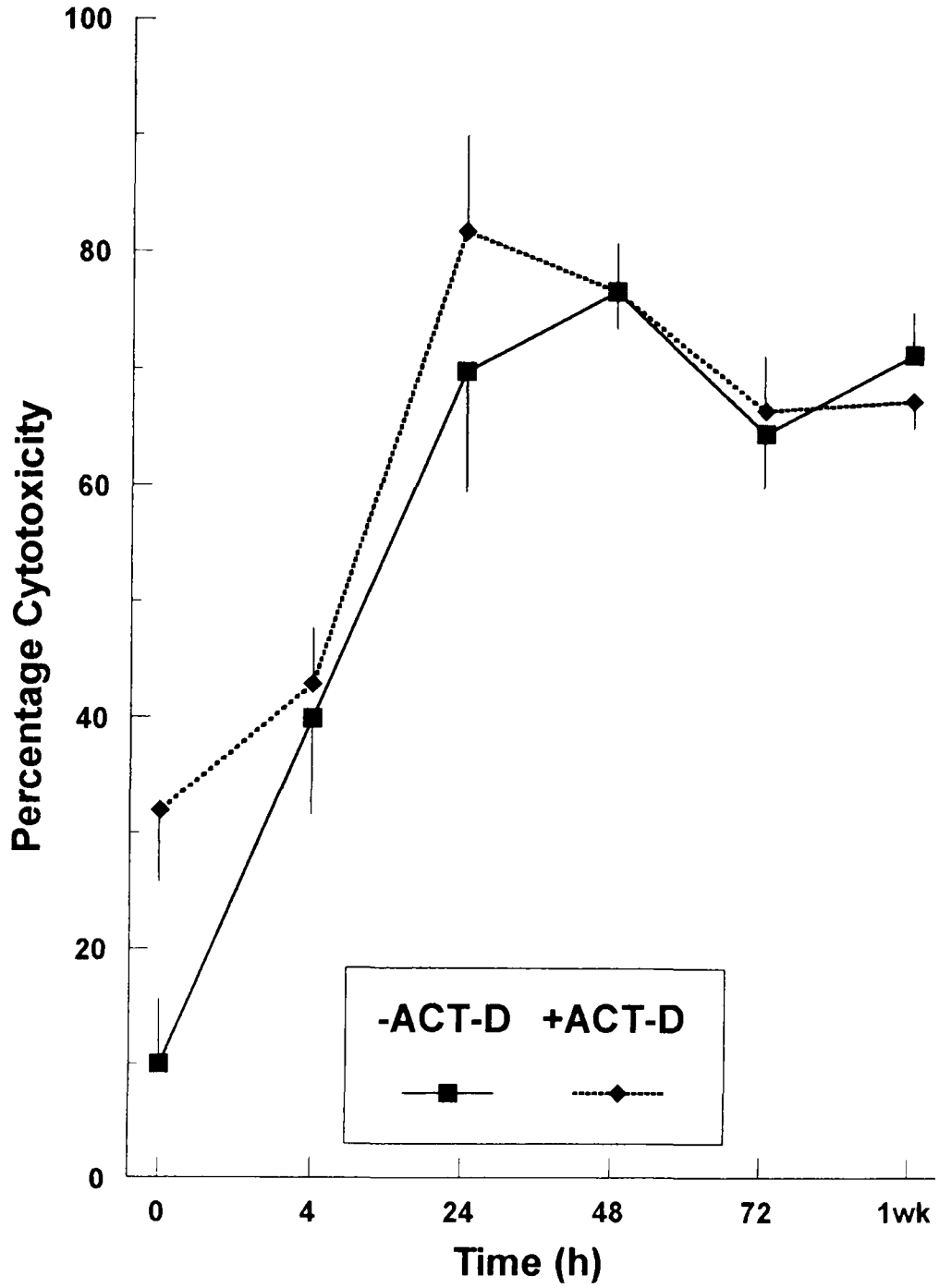

FIGURE 4.2. Time course for LAK cell generation. An aliquot of freshly isolated lymphocytes from a single donor were assessed for their cytotoxicity against untreated and Act-D pre-treated A375 cells (time 0). The remaining lymphocytes were divided between 5 culture flasks (at 2 x 10^6/ml), which were incubated in the presence of IL-2 (250U/ml) for 4h, 24h, 48h, 72h, or 1wk, and their killing of untreated or Act-D pre-treated A375 cells measured. In 3 experiments (means and SEMs are shown), significant killing of A375 cells was observed using fresh lymphocytes as effectors (compared to spontaneous release; $p<0.05$ by MWU and PTT), and cytotoxicity was increased by A375 cell treatment with Act-D ($p<0.05$ by MWU and PTT). Lymphocyte culture with IL-2 enhanced killing at all time points (compared to fresh lymphocytes; $p<0.05$ by MWU and PTT) to a maximum at 24h. Incubation with IL-2 for longer than 24h did not further increase cytotoxicity (compared to 24h; $p>0.05$ by MWU and PTT). Pre-incubation of A375 cells with Act-D did not increase cytotoxicity by IL-2 stimulated lymphocytes ($p>0.05$ by MWU and PTT).

As previously observed in section 3.2, a relatively low level of cytotoxicity to A375 cells by fresh lymphocytes was observed, but Act-D pre-treatment of the target cells resulted in increased killing. A significant increase in cytotoxicity was observed after as little as 4h incubation of lymphocytes with IL-2, and reached a maximum after 24h. Incubation of lymphocytes with IL-2 for longer than 24h did not result in a further increase in cytotoxicity. Act-D pre-treatment of A375 cells did not consistently enhance their killing by IL-2 activated lymphocytes. In further experiments LAK cells were prepared by incubation of lymphocytes with IL-2 for 24-72h.

4.1.3 Time Course for LAK Cell Cytotoxicity of A375 Cells

Since LAK cell killing of A375 cells was significantly greater than that of unstimulated lymphocytes, the next series of experiments compared the time course for LAK cell and lymphocyte (control cell) killing of A375 cells. LAK or control cells were incubated with untreated or Act-D pre-treated A375 cells for 1, 2, 4 or 18h, and the resulting cytotoxicity measured.

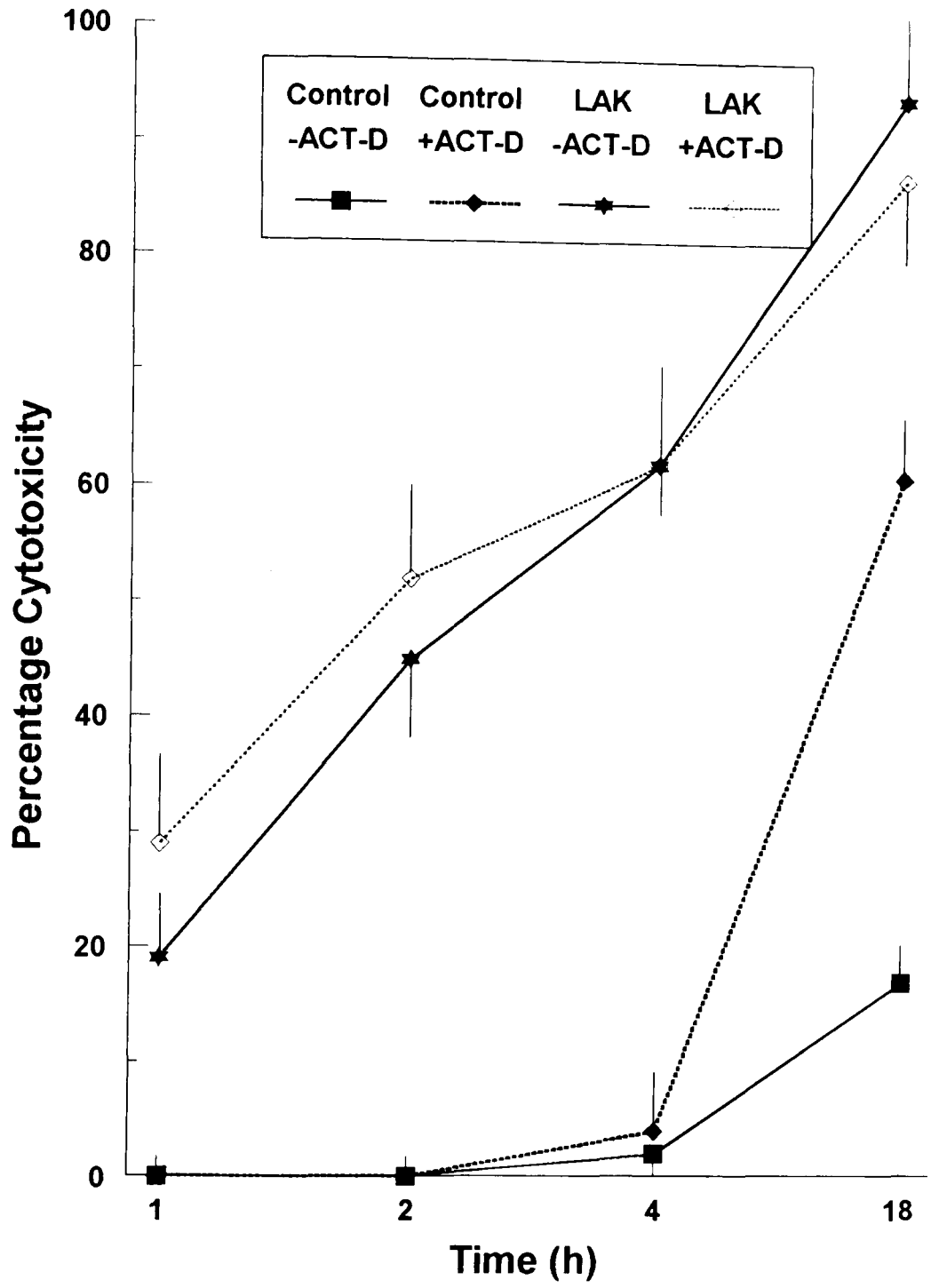

FIGURE 4.3. Time course of control and LAK cell cytotoxicity of A375 cells. Cytotoxicity of untreated or Act-D pretreated A375 cells by LAK cells (lymphocytes incubated with IL-2 (250U/ml), for 24h), or control cells (lymphocytes cultured for 24h in the absence of IL-2) was measured after 1, 2, 4 or 18h in co-culture. In 3 experiments (means and SEMs shown), significant LAK cell cytotoxicity against A375 cells was observed after 1h (compared to spontaneous release; $p<0.05$ by MWU and PTT), which further increased to a maximum at 18h ($p<0.05$ compared to previous time point for 2, 4 and 18h, by MWU and PTT). LAK cell cytotoxicity was not augmented by A375 cell pre-incubation with Act-D. Control cells were cytotoxic to A375 cells only after 18h incubation (compared to spontaneous release; $p<0.05$ by MWU and PTT), and killing was enhanced by target cell pre-treatment with Act-D ($p<0.05$ by MWU and PTT), but was still lower than the level of cytotoxicity observed for LAK cells ($p<0.05$ by MWU and PTT).

Figure 4.3 shows the mean maximal cytotoxicity (effector : target cell ratio 50:1) from three experiments. As with fresh lymphocytes, control cells did not show appreciable cytotoxicity until 18h, and this killing was augmented by Act-D pre-treatment of A375 cells. In contrast, LAK cells were cytotoxic for A375 cells after only one hour, and a time dependent increase in killing was seen up to a maximum of 93% specific cytotoxicity at 18h. Further time points were not measured because LAK cell cytotoxicity was already maximal (*i.e.*, approaching 100%) after 18h, and longer incubation times may result in altered cytotoxicity due to nutrient deprivation. In contrast to control cells, Act-D pre-treatment of target cells had no significant effect on LAK cell killing. LAK cell cytotoxicity was significantly greater than that of control cells ($p<0.05$; even control cell cytotoxicity of Act-D pre-treated A375 cells), at all time points.

4.1.6 TNFα Activation of Lymphocytes

IL-2 is the most important cytokine in the induction of LAK activity, but other cytokines, such as TNFα, have been reported to synergise with IL-2 in the modifying the number and activity of LAK cells generated in culture (Lee *et al.*, 1987), and to induce directly LAK activity. Freshly isolated lymphocytes were therefore incubated with TNFα for 72h, and their cytotoxicity against K562 and A375 (+/- Act-D) measured.

Figure 4.3 shows the mean maximal cytotoxicity (effector : target cell ratio 50:1) from three experiments. As with fresh lymphocytes, control cells did not show appreciable cytotoxicity until 18h, and this killing was augmented by Act-D pre-treatment of A375 cells. In contrast, LAK cells were cytotoxic for A375 cells after only one hour, and a time dependent increase in killing was seen up to a maximum of 93% specific cytotoxicity at 18h. Further time points were not measured because LAK cell cytotoxicity was already maximal (*i.e.*, approaching 100%) after 18h, and longer incubation times may result in altered cytotoxicity due to nutrient deprivation. In contrast to control cells, Act-D pre-treatment of target cells had no significant effect on LAK cell killing. LAK cell cytotoxicity was significantly greater than that of control cells ($p<0.05$; even control cell cytotoxicity of Act-D pre-treated A375 cells), at all time points.

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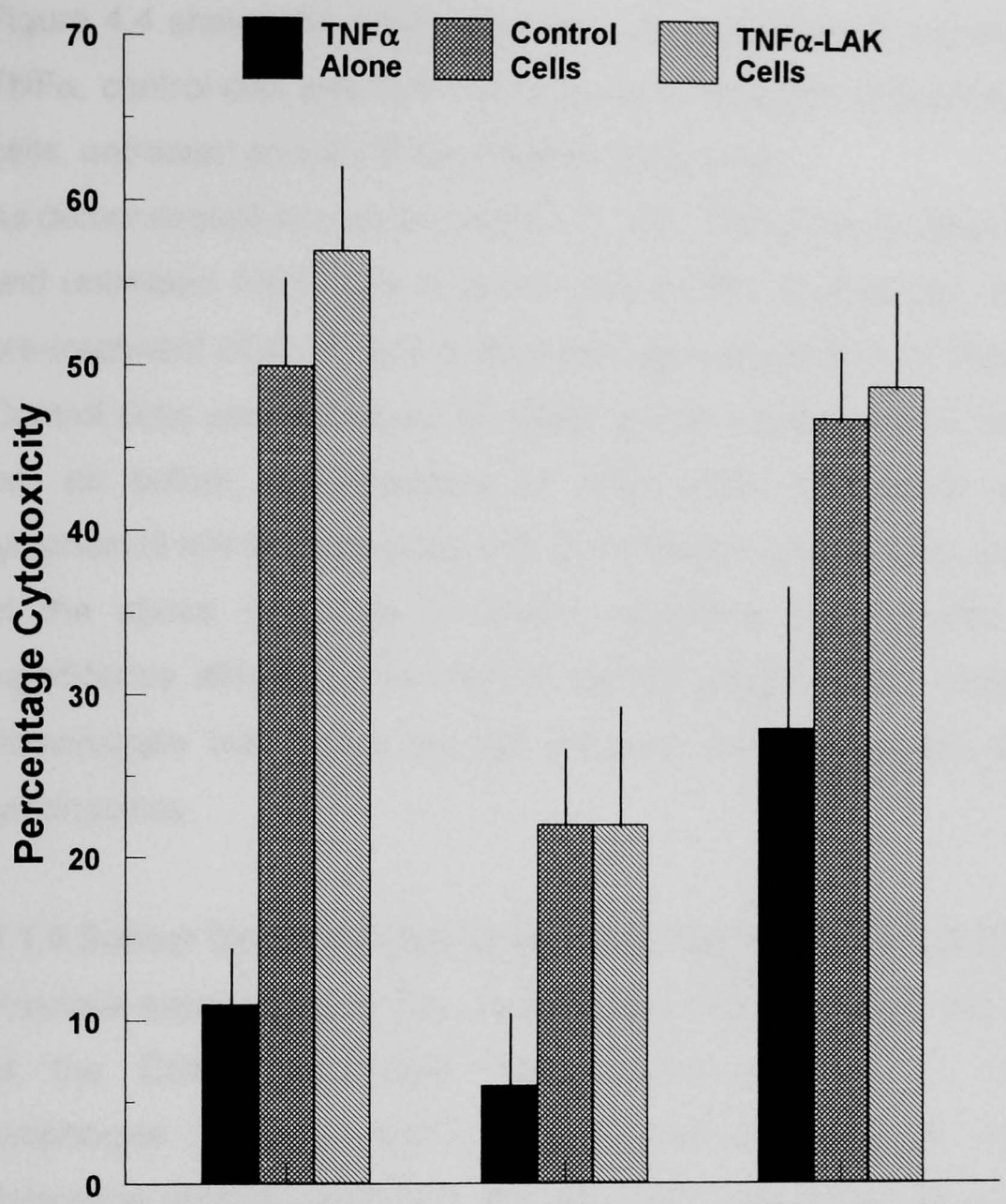


FIGURE 4.4. Effect of TNFα stimulation on lymphocyte killing of K562, A375 cells and A375 cells + Act-D. Lymphocytes were cultured in the presence of TNFα (1000U/ml) for 48h, and their cytotoxicity (at an E:T of 50:1) against K562 cells, A375 cells or Act-D pre-treated A375 cells compared to that of TNFα or unstimulated lymphocytes (cultured in RPMI/FCS for 48h). Three experiments were performed (means and SEMs shown). Unlike IL-2, pre-treatment of lymphocytes did not result in increased cytotoxicity against any cell line compared to unstimulated lymphocytes ($p>0.05$ by MWU and PTT), but increased killing was observed against Act-D pre-treated A375 cells (as previously shown in table 3.3). As a control, TNFα alone was incubated with the tumour cells. It was cytotoxic for untreated K562 and A375 cells (compared to spontaneous release; $p<0.05$ by MWU and PTT), and Act-D pre-treatment augmented TNFα killing of A375 cells (as previously shown in table 3.1; $p<0.05$ by MWU and PTT).

Figure 4.4 shows the mean maximal cytotoxicity from 3 experiments for TNFα, control cell, and TNFα stimulated lymphocyte cytotoxicity of K562 cells, untreated and Act-D pre-treated A375 cells.

As demonstrated previously (section 3.1.2), TNFα was cytotoxic for K562 and untreated A375 cells to a low level (<10% cytotoxicity), and Act-D pre-treatment of A375 cells enhanced their susceptibility to TNFα action. Control cells were cytotoxic for K562 and to a lesser extent A375 cells, but as before, pre-treatment of A375 cells with Act-D enhanced lymphocyte killing. In contrast to IL-2 stimulated lymphocytes, cytotoxicity of the above cell lines by TNFα stimulated lymphocytes was not significantly different from that of control lymphocytes. These results demonstrate that TNFα did not enhance the tumouricidal activity of lymphocytes.

4.1.5 Subset Depleted Control and LAK Cell Killing of A375 Cells

Previous experiments in this study (section 3.2.4) showed that depletion of the CD8+ lymphocyte subpopulation resulted in decreased lymphocyte killing of A375 cells. It was, therefore, of interest to determine whether depletion of CD8+ cells from LAK cell populations would produce a similar effect. Control (lymphocytes cultured for 24h in the absence of IL-2) and LAK cells were prepared as described in section 2.2.13, and each divided into 2 aliquots. One aliquot was adjusted to 5 x 10^6cells/ml and added directly to the A375 cells (section 2.3.1.3), and the other washed in Facsflow, labelled with antibody to CD8. Fluorescently labelled CD8+ cells were removed by fractionation in a cell sorter. The resulting cells were tested for viability (section 2.2.16), adjusted to 5 x 10^6 cells/ml and added to A375 cells in the cytotoxicity assay (section 2.3.1.3).

Figures 4.5 and 4.6 show the mean cytotoxicity dose response curves for killing of A375 cells by control and LAK cells respectively, as well as dose response curves for CD8+ depleted control and LAK cell killing.

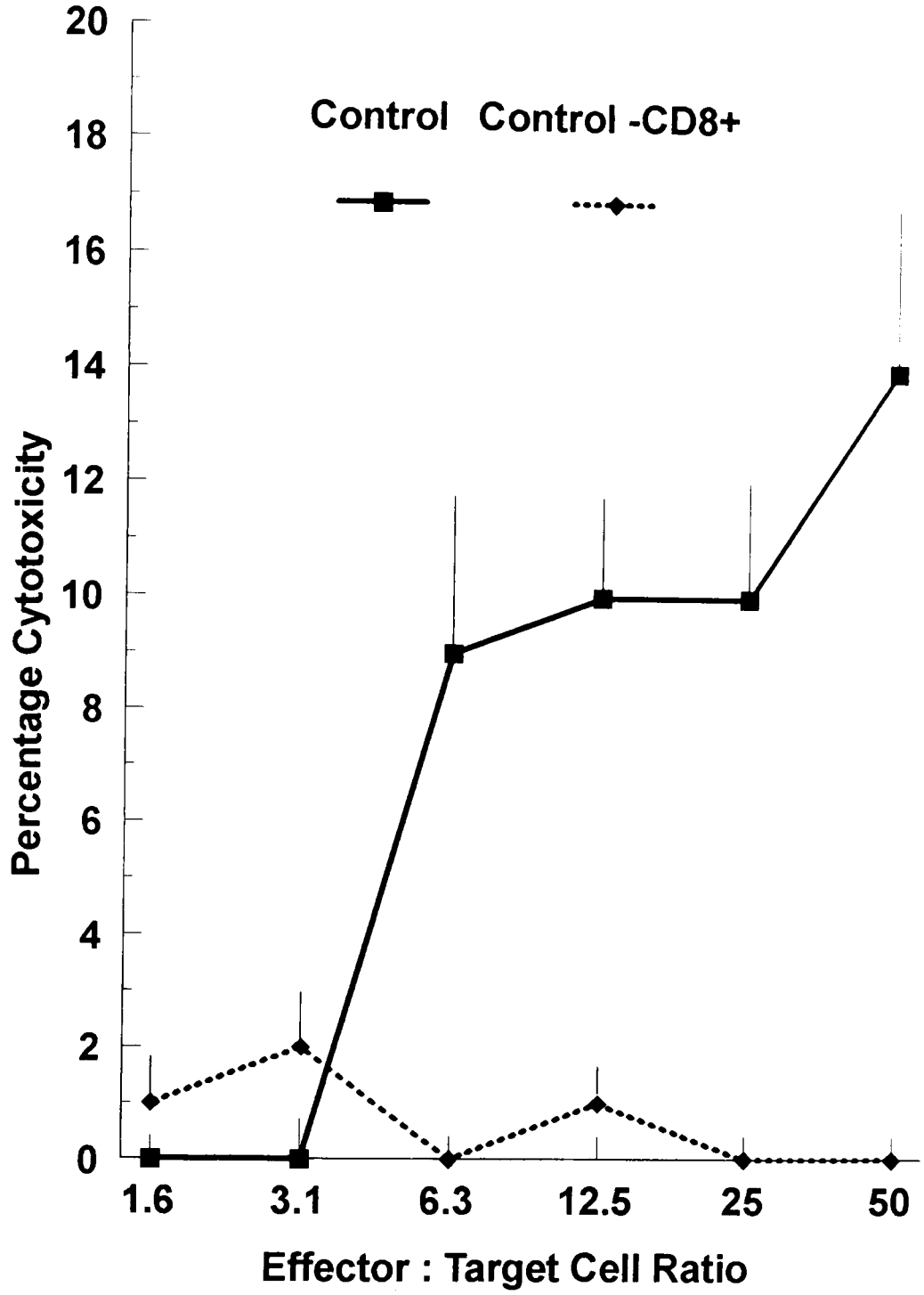


Figure 4.5. Cytotoxicity of A375 cells by control and CD8+ depleted control cells. Control cells (lymphocytes cultured in RPMI/FCS for 24h) were depleted of CD8+ cells (>96% depletion in 3 experiments) as described in section 2.1.12, and their cytotoxicity against untreated A375 cells compared with that of the whole lymphocyte population. In 3 experiments (>97% viability of both effector cell populations; means and SEMs shown), control cells were cytotoxic at an E:T of 6.3:1 (compared to spontaneous release; $p<0.05$ by MWU and PTT). reaching a maximum at 50:1 ($p<0.05$ compared to 25:1 by MWU and PTT). Depletion of CD8+ cells resulted in abrogation of control cell cytotoxicity (compared to the whole population; $p<0.05$ at E:T >=6.3:1 by MWU and PTT).

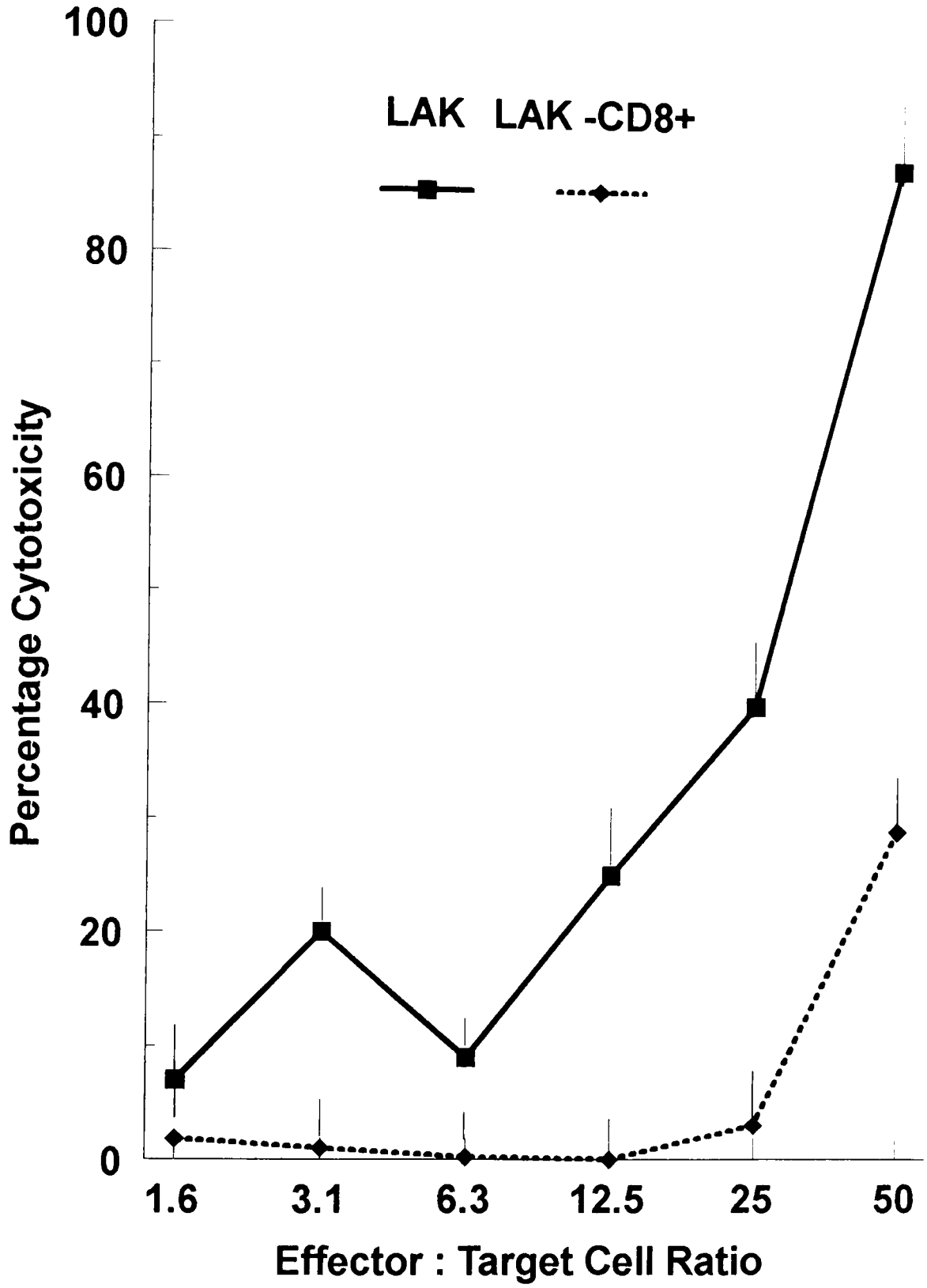


Figure 4.6. Cytotoxicity of A375 cells by LAK and CD8+ depleted LAK cells. Depletion of the CD8+ subset from 24h LAK cells (>94% depletion in 3 experiments) was performed as described in section 2.1.12. Cell viability was >97% for both populations. Cytotoxicity of LAK cells and CD8+ depleted LAK cells (E:T 1.6:1 to 50:1) against untreated A375 cells was determined in an 18h cytotoxicity assay. In 3 experiments (means and SEMs shown), significant cytotoxicity (compared to spontaneous release) was observed at all E:T for LAK cells, but only at 50:1 for CD8+ depleted cells ($p<0.05$ by MWU and PTT). Depletion of CD8+ cells resulted in significant inhibition of LAK cell cytotoxicity at all E:T above 1.6:1; $p<0.05$ by MWU and PTT).

Depletion of the CD8+ subpopulation from control cells (figure 4.5) resulted in complete abrogation of cytotoxicity in 2 experiments (in triplicate) at the highest effector:target cell ratio tested, whereas the same treatment of LAK cells in 3 experiments (in triplicate) resulted in a 75% inhibition of cytotoxicity (figure 4.6). In all experiments the CD8+ cell depletion was >94% and the viability of the sorted cells >97%. Since control cell cytotoxicity was initially fairly low (14%; but consistent with lymphocyte killing in section 3.2.2), the sensitivity of the assay may not have been sufficient to detect any residual cytotoxicity due to any remaining CD8+ or other contributing cell populations. In contrast, LAK cells were highly cytotoxic and a small (up to 6% CD8+ may have remained in the effector cell preparations) contamination of the depleted cell population with CD8+ cells was possibly responsible for a proportion of the residual cytotoxicity.

A contribution to the residual cytotoxicity by another cell population such as NK cells could not be discounted. Three further experiments were therefore performed to determine whether depletion of CD16+ (NK cells) would inhibit lymphocyte (control cell) cytotoxicity. No significant ($p>0.3$) reduction in control cell killing of A375 cells was observed (Figure 4.7). These results thus indicated that the cytotoxic activities of control (lymphocytes) and LAK cells against A375 cells were primarily due to the action of CD8+ MHC unrestricted cytotoxic lymphocytes.

Figure 4.7

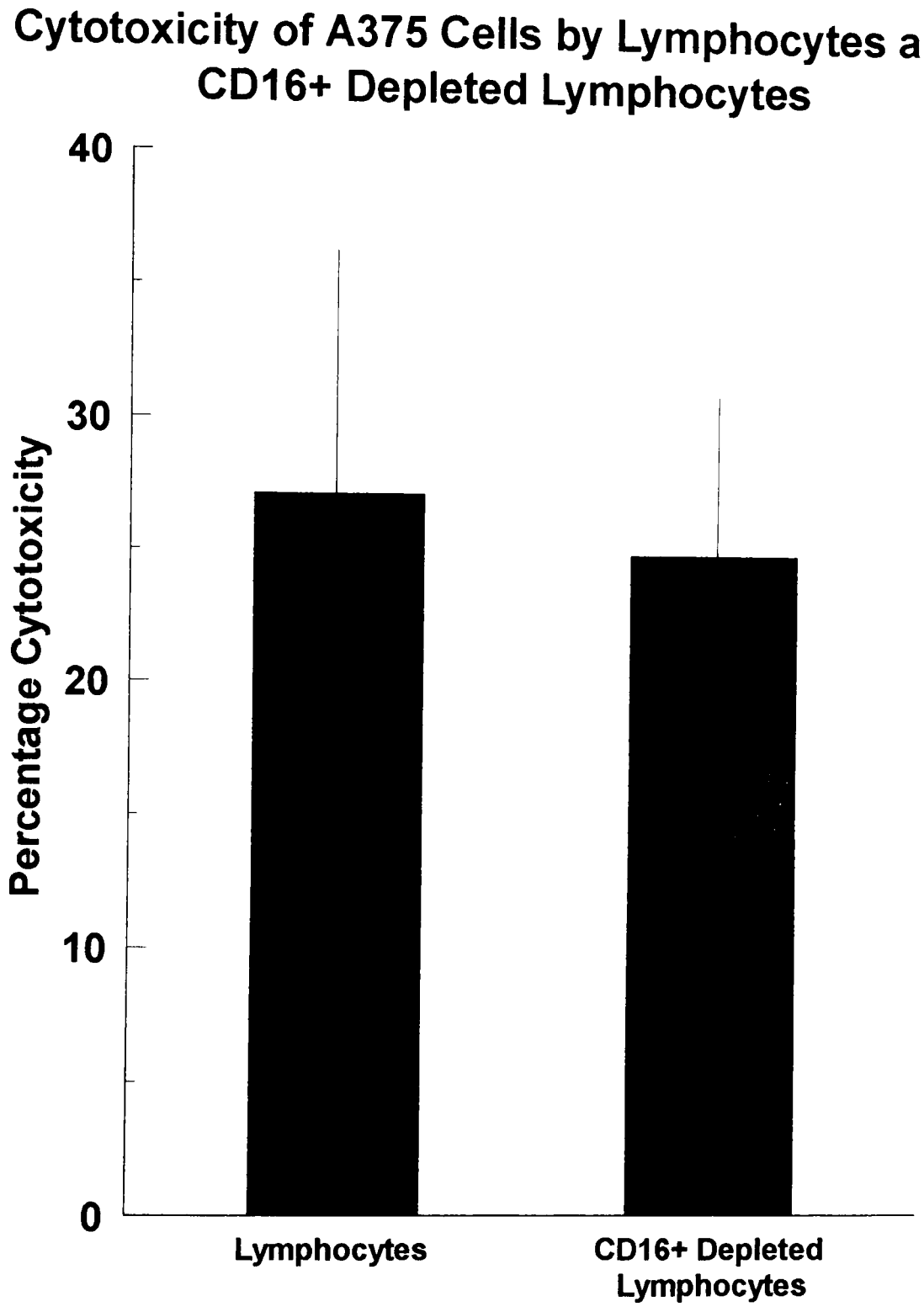


FIGURE 4.7. Cytotoxicity of A375 cells by lymphocytes and CD16+ depleted lymphocytes. Control cells (lymphocytes cultured in RPMI/FCS for 24h) were depleted of CD16+ cells (>95% depletion in 3 experiments) as described for CD8+ cell depletion in section 2.1.12, and their cytotoxicity against untreated A375 cells compared with that of the whole lymphocyte population. In 3 experiments (>97% viability of both effector cell populations; means and SEMs shown), control cells were maximally cytotoxic at an E:T of 50:1. Depletion of CD16+ cells did not result in any significant decrease in control cell cytotoxicity (compared to the whole lymphocyte population; $p>0.05$ by MWU and PTT).

4.1.6 LAK Cell Cytotoxicity of All Tumour Cell Lines

The tumouricidal activity of control and LAK cells was also tested against the other 6 tumour cell lines chosen for this study. Table 4.1 summarises the maximal cytotoxicity of control and LAK cells to the various target cells untreated or pre-treated with Act-D.

		Target Cells -Act-D		Target Cells + Act-D		Statistical Analysis	
Target	**n**	**Control**	**LAK**	**Control**	**LAK**	**Control**	**LAK**
A375	**13**	**38** (3)	**78** (4)	**50** (3)	**73** (2)	**p<0.05**	p>0.05
DX3	**3**	**34** (5)	**71** (6)	**59** (4)	**72** (5)	**p<0.05**	p>0.05
SK23	**3**	**30** (4)	**73** (6)	**63** (5)	**79** (7)	**p<0.05**	p>0.05
LT5.1	**3**	**44** (3)	**62** (5)	**51** (6)	**69** (5)	p>0.05	p>0.05
K562	**3**	**42** (6)	**76** (4)	**46** (4)	**79** (4)	p>0.05	p>0.05
DAUDI	**3**	**32** (3)	**97** (4)	**22** (5)	**92** (5))	p>0.05	p>0.05
U937	**3**	**64** (3)	**75** (4)	**51** (5)	**79** (4)	p>0.05	p>0.05

TABLE 4.1. Cytotoxicity of tumour cell lines +/- Act-D by control and LAK cells. The cytotoxic activity of control and LAK cells was assessed against seven untreated or Act-D pre-treated tumour cell lines in an 18h cytotoxicity assay. Results are expressed as the mean maximal cytotoxicity (at an E:T of 50:1) from "n" experiments, and numbers in brackets are SEMs. Statistical analysis by MWU and PTT of any increased cytotoxicity induced by Act-D pre-treatment of tumour cells is shown for control and LAK cells. Pre-incubation with Act-D only induced increased control cell cytotoxicity of A375, DX3 and SK23 cells. In addition, tumour cell killing by LAK cells was significantly greater than that of control cells against all untreated and Act-D pre-treated cell lines ($p<0.05$ by MWU and PTT).

Control cell (lymphocytes cultured in RPMI/FCS for 24h) killing of the various cell lines was very similar to that of fresh peripheral blood lymphocytes (section 3.2.5). Act-D pre-incubation of the target cells also augmented control cell cytotoxicity to a similar degree and in the same pattern as that previously observed for freshly isolated lymphocytes (section 3.2.2) These results indicate that unstimulated lymphocytes cultured for 24h at 37^0C (control cells) compared well with freshly

isolated lymphocytes with respect to tumour cell killing, and as such acted as a good autologous lymphocyte control for LAK activity.

In contrast, LAK cells were highly cytotoxic for all tumour cell lines tested; an effect that was not increased by pre-incubation of target cells with Act-D. Stimulation of lymphocytes with IL-2 would, therefore, seem to be an efficient way of generating highly tumouricidal cells prior to re-inoculation into the patient. Having determined the cytotoxic activity of LAK cells on a number of tumour cell lines, it was important to determine if normal cells such as autologous leucocytes, endothelial cells and fibroblasts were also susceptible to LAK cell activity.

4.2 LAK Cell Cytotoxicity Against Normal Cells

Most clinical protocols for LAK cell immunotherapy favour the re-introduction of LAK cells into cancer patients via the peripheral circulation. Here they encounter circulating immune cells and endothelial cells lining vessel walls. On crossing the vessel wall LAK cells may interact with fibroblasts prior to, and during, their infiltration into the tumour mass.

Experiments were performed to investigate the cytotoxicity of LAK and control cells to autologous lymphocytes and monocytes, and subsequently to heterologous human umbilical vein endothelial cells and fibroblasts.

4.2.1 Cytotoxicity of Autologous Lymphocytes and Monocytes by Control and LAK Cells

Control and LAK cells were prepared as previously described in section 2.2.13. A second sample of peripheral blood was the taken from the same donor who provided the control and LAK cells, lymphocytes and monocytes purified, $Na^{51}CrO_4$ labelled and used as target cells in the cytotoxicity assay. Act-D pretreated A375 cells were also tested as a positive control for cytotoxicity.

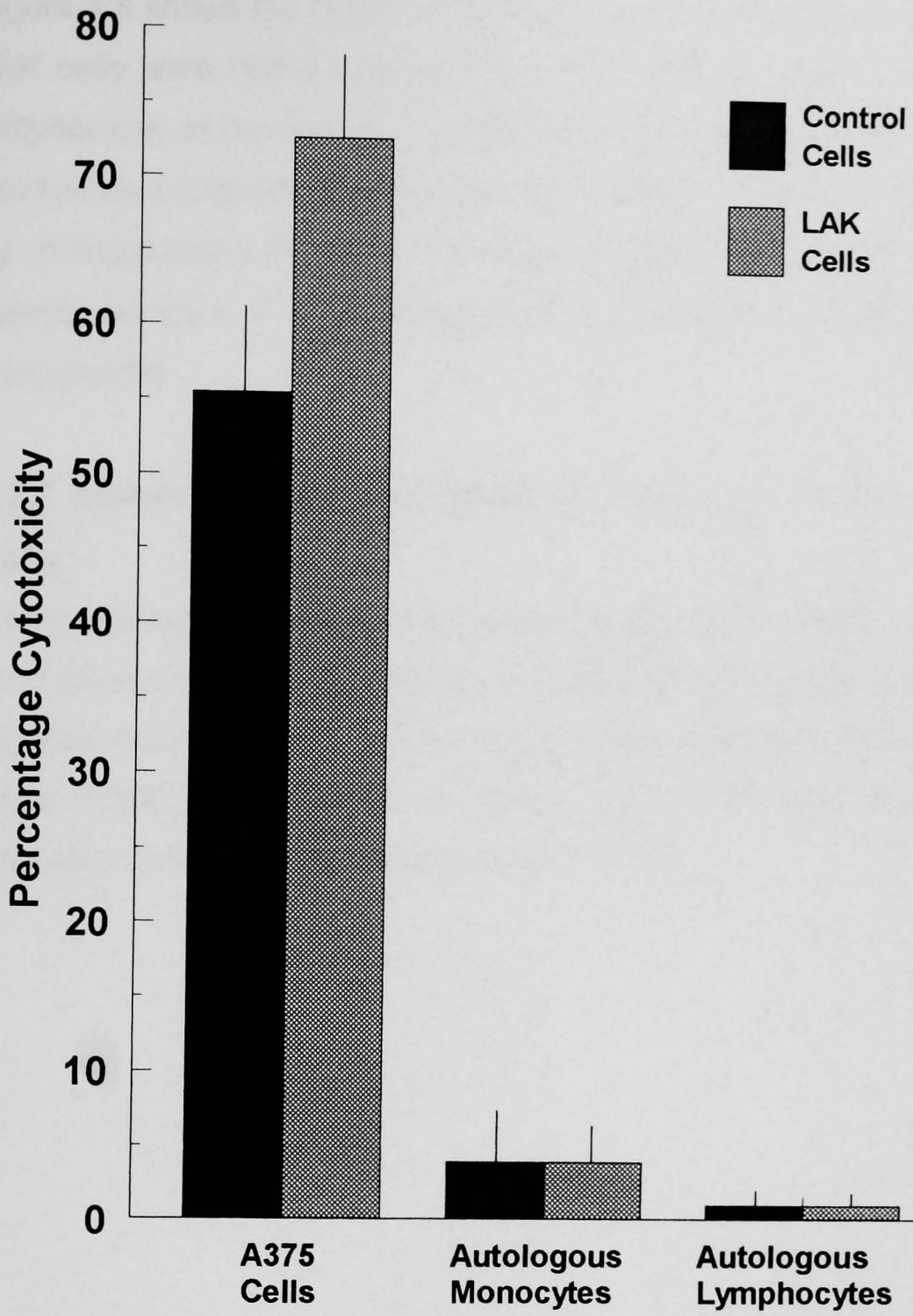


FIGURE 4.8. Control and LAK cell cytotoxicity of autologous monocytes and lymphocytes. Control and LAK cells (24h cultures) were prepared as described in section 2.1.13. After 24h, lymphocytes and monocytes were prepared from the peripheral blood of the same donor, $Na^{51}CrO_4$ labelled ($0.1\mu Ci/5x10^6$ cells for 30min), and their susceptibility to killing by autologous control and LAK cells (E:T 50:1) determined. Actinomycin-D pre-treated A375 cells were included in the assay as a positive control. In 3 experiments (means and SEMs are shown), control and LAK cells were not cytotoxic against autologous monocytes and lymphocytes (compared to spontaneous release; $p>0.05$ by MWU and PTT). In contrast, Act-D pre-treated A375 cells were efficiently killed by control cells (56%) and LAK cells (73%). LAK cell killing of A375 cells was significantly greater than that of control cells ($p<0.05$ by MWU and PTT).

Figure 4.8 shows the results of 3 experiments in which both control and LAK cells were highly cytotoxic for A375 cells, but not for autologous lymphocytes or monocytes. Extrapolation of these results to the *in vivo* environment suggests that circulating leucocytes would not be damaged by re-introduced LAK cells, and would therefore be available for further recruitment by IL-2, LAK cells and other released cytokines to become tumouricidal.

4.2.3 Endothelial Cell and Fibroblast Killing by Control and LAK Cells

Since capillary, venous or arterial endothelial cells cannot be obtained form normal donors, heterologous endothelial cells and fibroblasts were isolated from human umbilical vein (human umbilical vein endothelial cells, HUVEC) as described in section 2.2.14, and their cytotoxicity by LAK and control cells assessed (section 2.3.3).

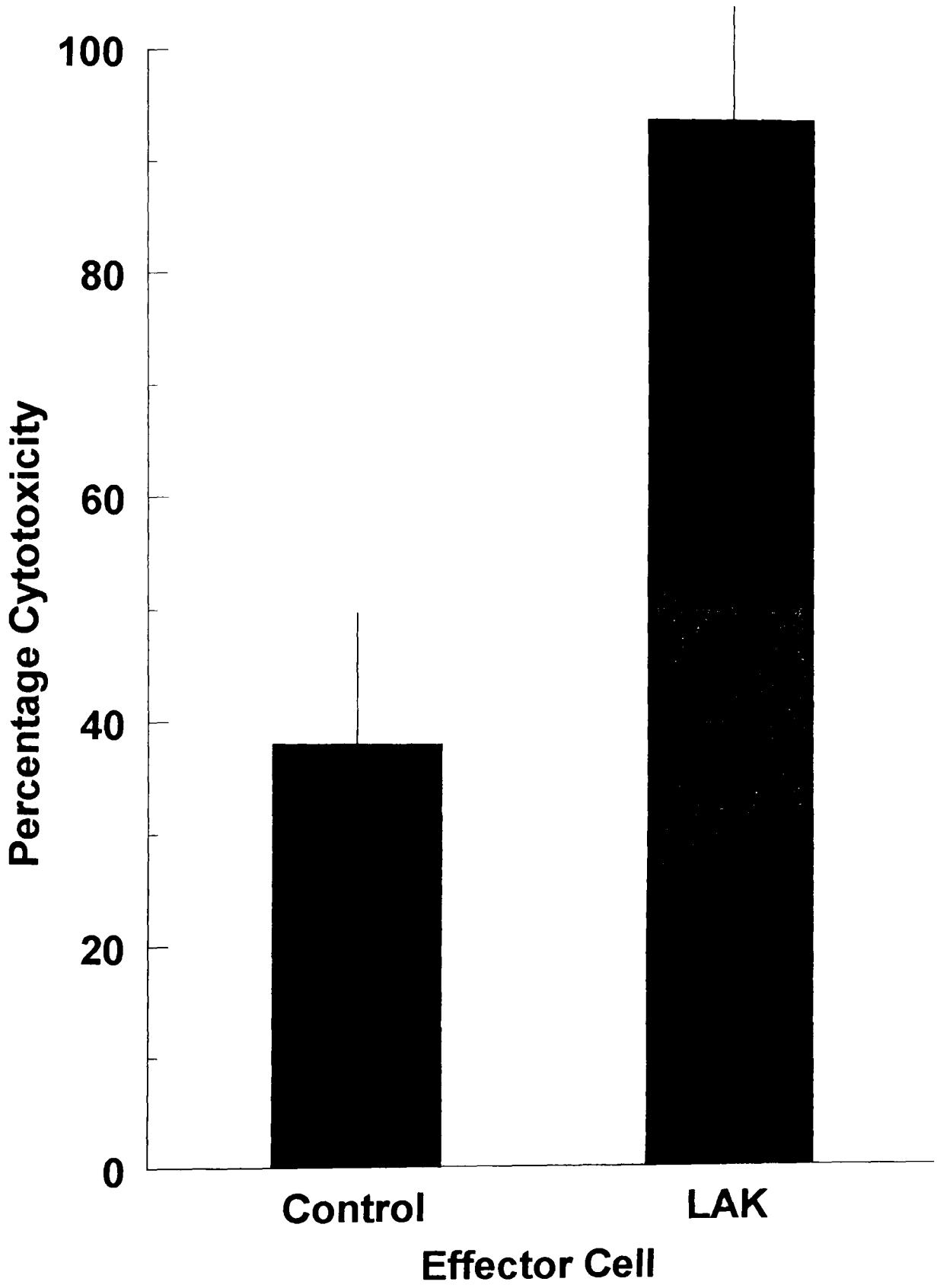


FIGURE 4.9. LAK cell cytotoxicity of human umbilical vein endothelial cells. Human umbilical vein endothelial cells (HUVEC) were isolated as described in section 2.1.14, $Na^{51}CrO_4$ labelled, and their cytotoxicity by control and LAK cells (24h culture: [IL-2] 250U/ml) assessed at an E:T of 50:1, after 18h incubation (see section 2.2.9). The results are expressed as the means and SEMs from 7 experiments. LAK cells were highly cytotoxic (93% specific cytotoxicity) against HUVEC, and killing was significantly greater than that observed for control cells (38%; $p<0.05$ by WSR, MWU and PTT).

Figure 4.9 shows the mean maximal cytotoxicity of endothelial cells by control and LAK cells. Control cells were moderately cytotoxic (38%) after 18h, but LAK cells were highly cytotoxic for endothelial cells, producing over 90% killing after 18h. In order to determine the time course for LAK cell killing of endothelial cells, experiments were performed in which LAK cell cytotoxicity was measured after 1, 2, 4 or 18h.

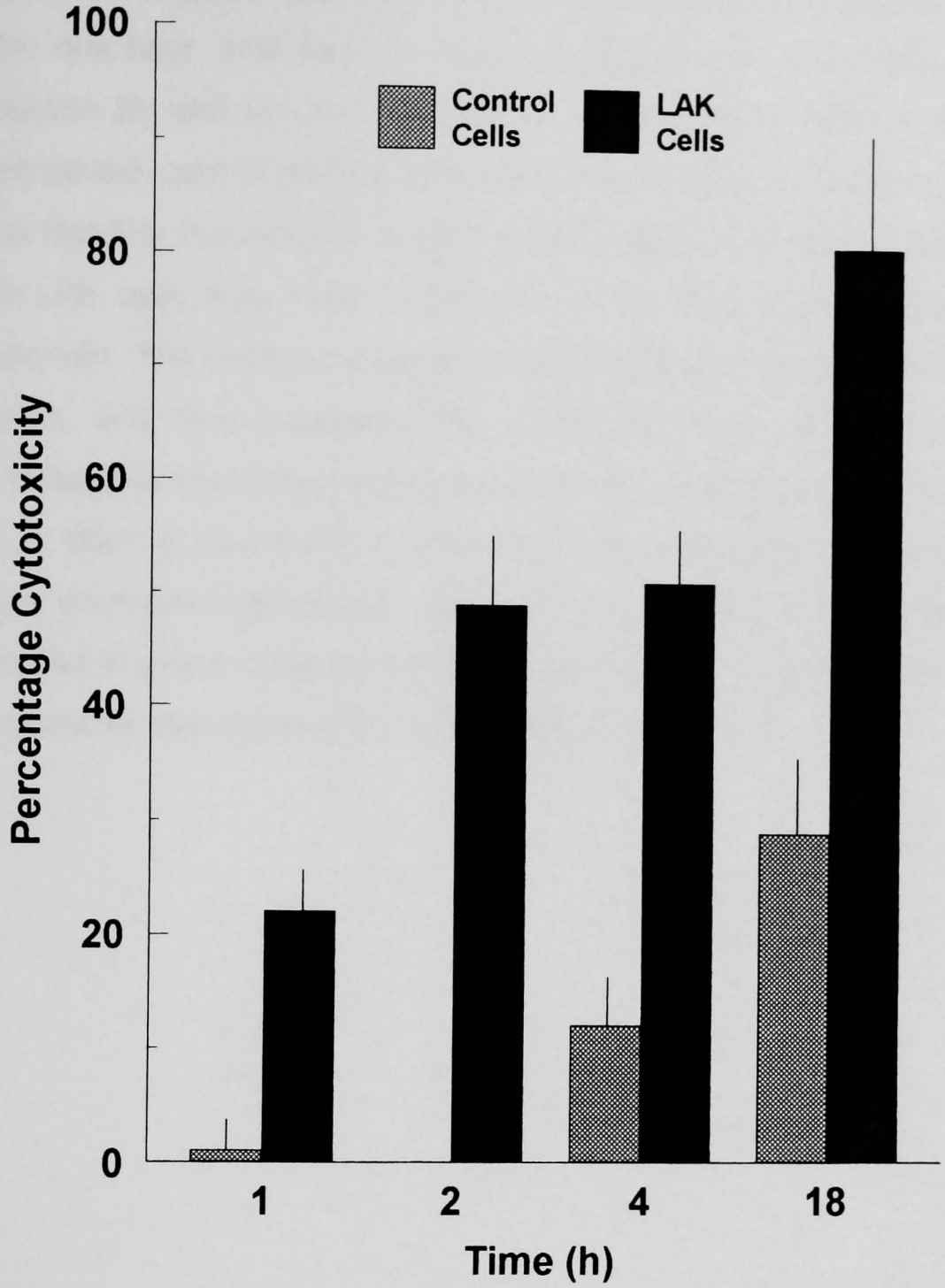


FIGURE 4.10. Time course of endothelial cell cytotoxicity by LAK cells. Control and LAK cells were incubated with $Na^{51}CrO_4$ labelled HUVEC at an E:T of 50:1 for 1, 2, 4 or 18h, and the level of cytotoxicity assessed as described in section 2.2.9. In 3 experiments (means and SEMs shown), endothelial cells were sensitive to killing by LAK cells after 1h incubation (compared to spontaneous release; $p<0.05$ by MWU and PTT). LAK cell cytotoxicity increased after 2h (compared to 1h; $p<0.05$ by MWU and PTT), and again at 18h (compared to 2h and 4h; $p<0.05$ by MWU and PTT). Control cells were only cytotoxic after 4h incubation (compared to spontaneous release; $p<0.05$ by MWU and PTT), and killing was further increased after 18h ($p<0.05$ by MWU and PTT). Cytotoxicity by control cells was significantly less than that of LAK cells at all corresponding time points ($p<0.05$ by MWU and PTT).

Figure 4.10 shows that LAK cells were cytotoxic for endothelial cells after one hour, and that this killing increased after 2h and 4h ($p>0.05$ between 2h and 4h) and again after 18h incubation. Within the same time period, control cells generated little or no cytotoxicity until 18h.

The fact that the HUVEC used in these experiments were allogeneic to the LAK cells may have contributed to the high levels of cytotoxicity observed. The umbilical cord is situated between the placenta and the foetus, and thus possesses the foetal genotype, which has a 50% homology to the maternal genotype. Since it was impractical to remove up to 60ml of peripheral blood from a newborn baby to produce LAK cells which were genetically identical to the HUVEC, an experiment was devised in which maternal LAK cells were tested for cytotoxicity against endothelial cells derived from the umbilical cord vein.

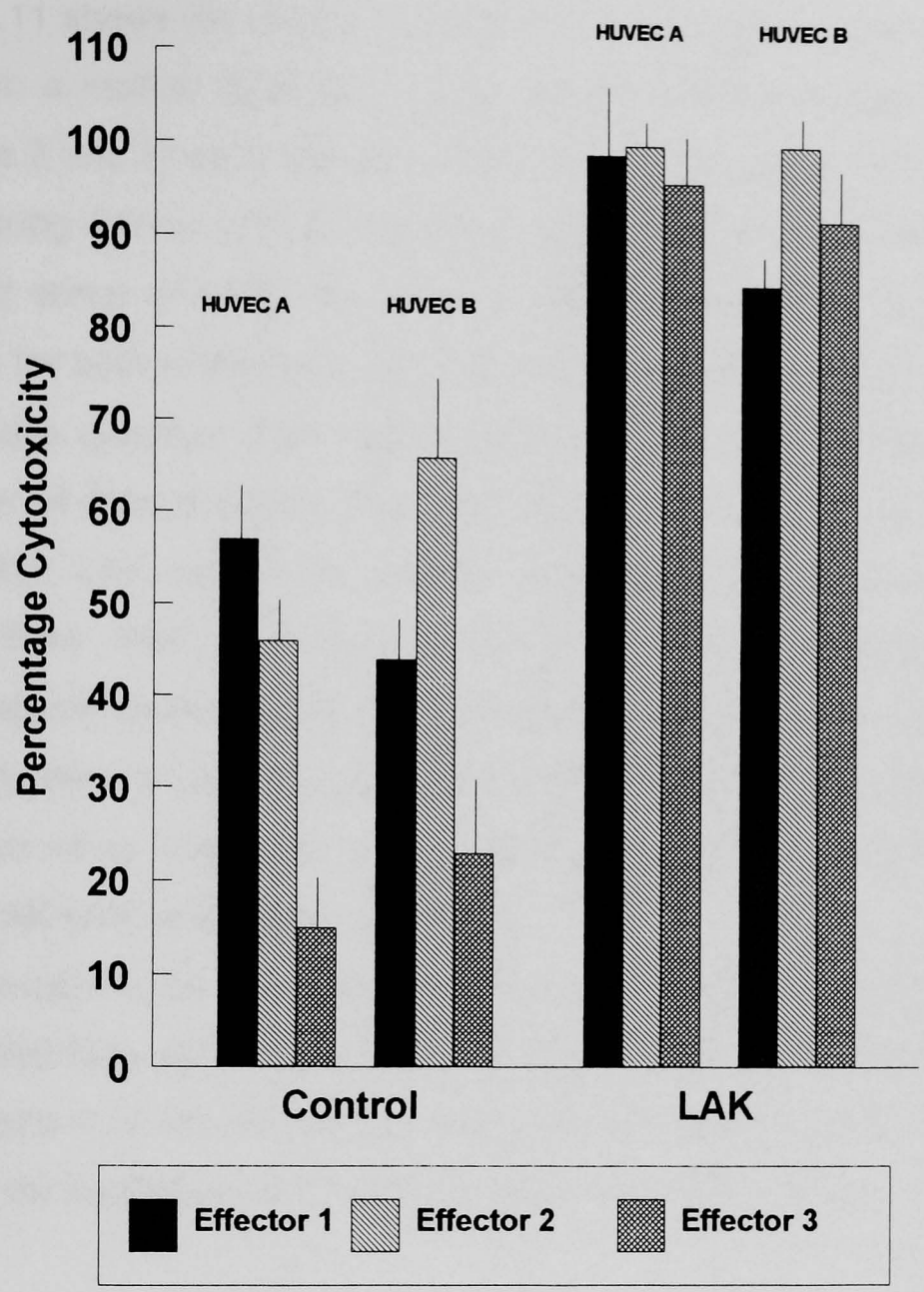


FIGURE 4.11. Cytotoxicity of autologous and heterologous HUVEC by control and LAK cells. Human umbilical vein endothelial cells from 2 donors were isolated as described in section 2.1.14. Lymphocytes from 3 donors were cultured in the presence (LAK cells), or absence (control cells) of IL-2 (250U/ml) for 24h, and their cytotoxicity against the cultured HUVEC assessed in an 18h $Na^{51}CrO_4$ release assay. The results are the means and SEMs of triplicate samples from a single assay. Effector 1 and HUVEC A were lymphocytes and endothelial cells from mother and offspring respectively. No other donors of lymphocytes or HUVEC were related. LAK cells from all 3 donors were highly cytotoxic for both HUVEC, and were not significantly different from each other ($p>0.05$ by MWU and PTT). Control cells from all donors were less cytotoxic than LAK cells ($p<0.05$ by MWU and PTT). Donor 3 cells were the least cytotoxic ($p<0.05$ by MWU and PTT) to both HUVEC 1 and HUVEC 2. Control cells from donor 1 and 2 were equally cytotoxic for HUVEC 1 ($p>0.05$ by MWU and PTT), but donor 1 cells were more cytotoxic against HUVEC 2 than were cells from donor 1. ($p<0.05$ by MWU and PTT).

Figure 4.11 shows the results of an experiment in which control and LAK cells from a mother (Effector 1) and 2 other normal unrelated donors (Effectors 2 and 3) were tested for their cytotoxicity against HUVEC from the offspring (of the mother mentioned above; HUVEC A) and another unrelated donor (HUVEC B). Control cells from all three donors were cytotoxic for both endothelial cell cultures, but control cells from effector 3 were less cytotoxic than control cells from the other 2 donors. LAK cells from all donors were highly cytotoxic for both HUVEC cultures. The finding that LAK cells from mother were highly cytotoxic to HUVEC derived from their offspring's umbilical cord suggests that genetic differences between the LAK cells and the HUVECs are not important in the mechanism of LAK cell killing of HUVEC. However, this result is not conclusive since there was only a 50% similarity in the genotypes of mother LAK cells and offspring HUVEC.

Further experiments were performed in which cultured fibroblasts from human umbilical veins were used as target cells for control and LAK cells. Figure 4.12 shows that LAK cells, but not control cells, were highly cytotoxic for fibroblasts at all effector:target cell ratios tested.

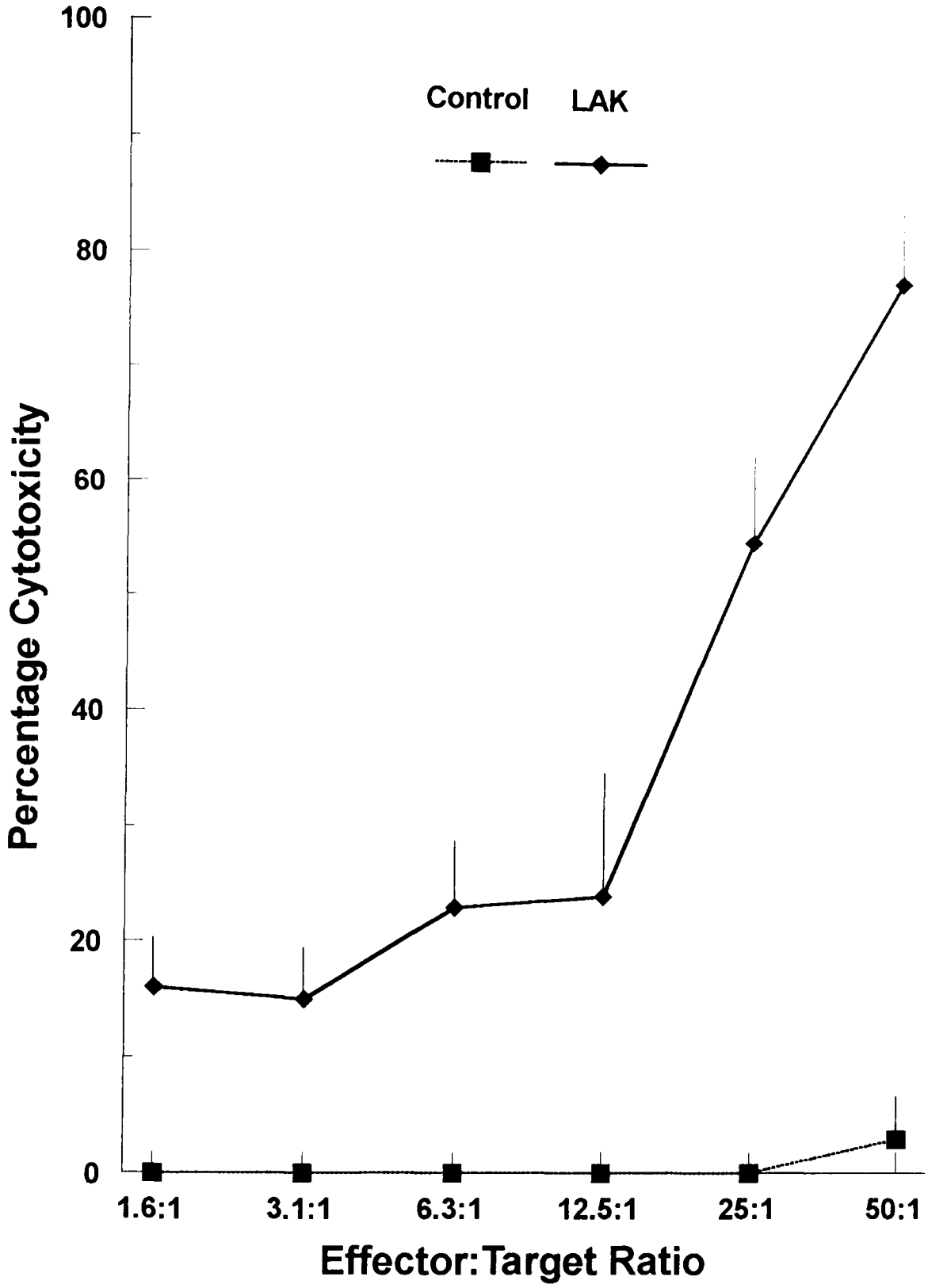


FIGURE 4.12. LAK cell cytotoxicity against cultured fibroblasts. Lymphocyte and LAK cell cytotoxicity against human umbilical vein fibroblasts was determined in an 18h $Na^{51}CrO_4$ release assay, as described in section 2.2.9. Means and SEMs from 3 experiments are shown. LAK cells were cytotoxic against fibroblasts at an E:T of 1.6:1 (compared to spontaneous release; $p<0.05$ by MWU and PTT), which increased significantly above 12.5:1 ($p<0.05$ by MWU and PTT) to a maximum at 50:1. Control cells were not significantly cytotoxic (compared to spontaneous release; $p>0.05$ by MWU and PTT) for fibroblasts at any effector : target cell ratio tested.

4.3 Cytotoxic Activity of LAK Cell Supernatants to Tumour Cells

Control and LAK cells may secrete cytotoxic effector molecules such as TNFα or LT into the surrounding medium, which are then able to kill tumour cells independent of their cell of origin. Alternatively, control and LAK cells may require direct adherence to their target cells in order to induce maximal cytotoxicity of tumour cells.

In order to determine whether lymphocytes and LAK cells secrete cytocidal molecules into their environment, thus causing tumour cell death, the cytotoxic activity of supernatants taken from control and LAK cell cultures was measured against the 7 tumour cell lines untreated or pre-incubated for 3h with Act-D.

		Cytotoxicity -Act-D (%)		Cytotoxicity +Act-D (%)		Statistical Analysis	
Target	n	Control	LAK	Control	LAK	Significance 1	Significance 2
A375	6	1 (1)	3 (2)	4 (1)	15 (4)	p>0.05	**p<0.05**
DX3	4	3 (1)	20 (2)	8 (2)	60 (6)	**p<0.05**	**p<0.05**
SK23	4	4 (2)	11 (2)	7 (1)	15 (3)	p>0.05	p>0.05
LT5.1	4	10 (2)	12 (4)	15 (2)	36 (3)	p>0.05	**p<0.05**
K562	3	15 (2)	36 (2)	18 (3)	32 (4)	p>0.05	p>0.05
DAUDI	3	0	10 (2)	0	13 (3)	p>0.05	p>0.05
U937	3	26 (2)	25 (2)	35 (2)	35 (4)	**p<0.05**	**p<0.05**

TABLE 4.2. Cytotoxicity of tumour cell lines +/- Act-D by control and LAK cell supernatants. Supernatants from cultures of control and LAK cells were collected after 24h incubation and stored at -70^{0}C prior to testing for cytotoxic activity against seven untreated and Act-D pre-treated tumour cell lines. The final dilution of supernatant was 1 in 4, and the incubation time with the tumour cells 18h. n = number of experiments, and numbers in brackets are SEMs. Statistical analysis by MWU and PTT of the effect (increased killing) of Act-D pretreatment on tumour cell killing by control (significance 1) and LAK cell (significance 2) supernatants is also shown. In addition, all Act-D pre-treated cell lines were more susceptible to killing by LAK cell supernatants than by those of control cells (p<0.05 by MWU and PTT). Of the untreated tumour cell lines, only DX3, SK23, K562 and Daudi cells were more efficiently killed by LAK cell supernatants (p<0.05 by MWU and PTT).

Table 4.2 shows that LAK cell supernatants were cytotoxic against all cell lines except A375 cells (compared to spontaneous counts; $p<0.05$). Pretreatment of target cells with Act-D significantly increased LAK supernatant killing of A375, DX3, LT5.1 and U937 cells. ACT-D pre-treatment of DX3 cells resulted in the largest increase in supernatant cytotoxicity observed (20% to 60%).

Control cell supernatants were cytotoxic against LT5.1, K562 and U937 cells ($p<0.05$ compared to spontaneous counts). Pre-incubation of the tumour cells with Act-D significantly enhanced control cell supernatant killing of DX3 and U937 cells.

The pattern and magnitude of TNFα killing of the cell lines untreated and pretreated with Act-D (section 3.1.2) was also very similar (except for SK23) to LAK supernatant cytotoxicity.

Killing of tumour cells by control and LAK cell supernatants was considerably lower than when the cells (section 3.2) were present in the assay. There are several possible explanations for this. First, the concentration of cytokine in the supernatants may be considerably less than that generated in the small volume within the cytotoxicity assay. Second, killing of tumour cell targets by control and LAK cells may require cell to cell contact. Third, control and LAK cells may only produce cytolytic effector molecules when in the presence of the target cells.

To address some of these points an experimental system was devised in which control and LAK cells were either in direct contact with A375 tumour cells, or separated from them by a porous polycarbonate membrane (section 2.3.2). This membrane ensured that the effector and target cells were relatively close to each other within the assay chamber, but no cell to cell contact could occur, although molecules were able to pass freely between the two cell layers.

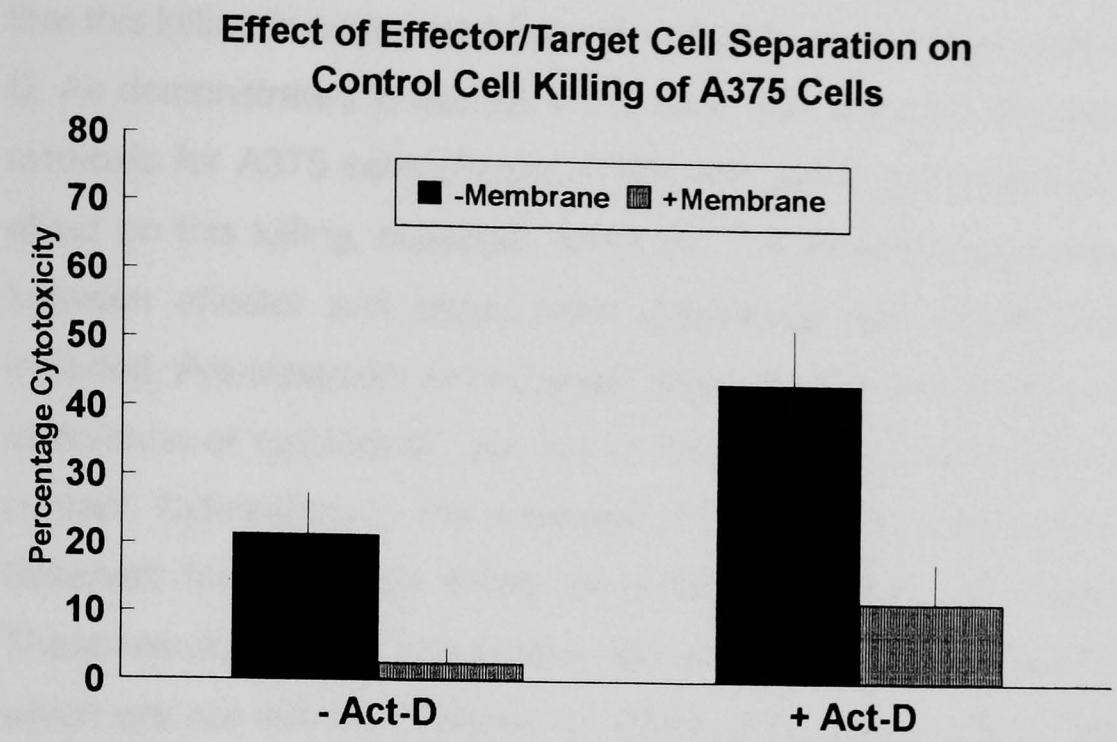
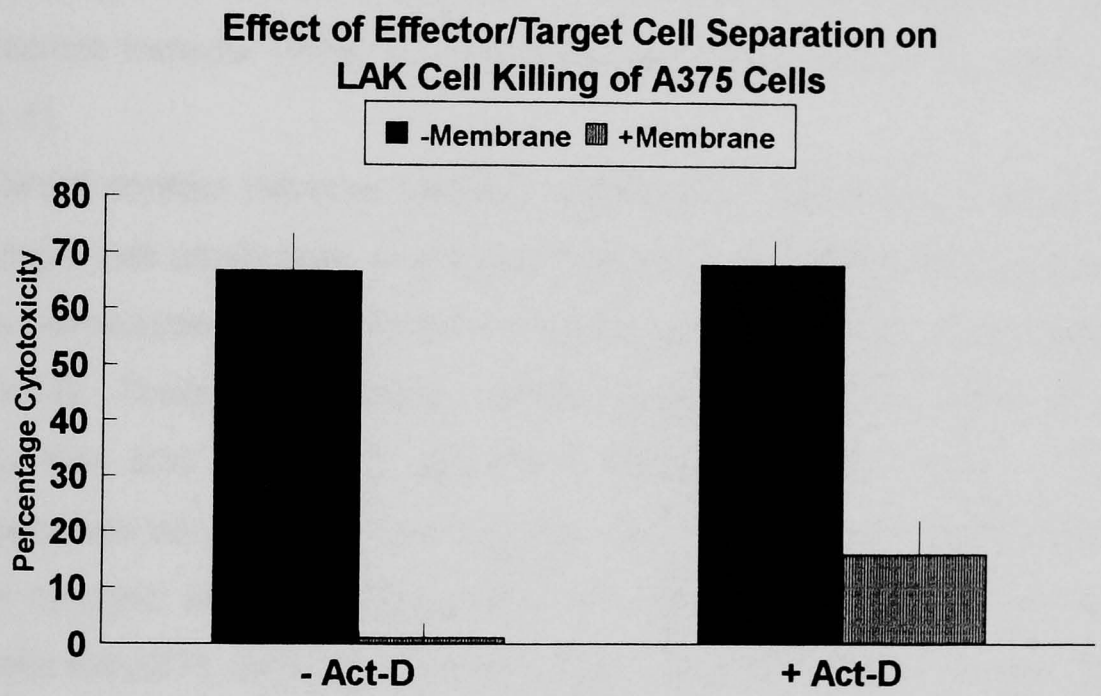


Figure 4.13



FIGURES 4.13 and 4.14. Control and LAK cell cytotoxicity against untreated and Act-D pre-treated A375 cells was assessed after 18h incubation in the presence or absence of 0.8mm pore size polycarbonate membranes, as described in section 2.2.7. In 3 experiments (means and SEMs are shown), control and LAK cell killing of untreated A375 cells was completely inhibited by the presence of membranes ($p < 0.05$ compared to no membrane: $p > 0.05$ compared to spontaneous release by MWU and PTT). As previously observed, control cell ($p < 0.05$ by MWU and PTT), but not LAK cell killing ($p > 0.05$ by MWU and PTT) was augmented by pre-treatment of the tumour cells with Act-D. Significant killing was also observed against Act-D pre-treated A375 cells in the presence of membranes ($p < 0.05$ by MWU and PTT) and control or LAK cells, but was less than when the membranes were absent ($p < 0.05$ by MWU and PTT).

Figure 4.13 again shows control cell cytotoxicity against A375 cells and that this killing is augmented by preincubation of the target cells with Act-D. As demonstrated in section 4.1.7, LAK cells (figure 4.14) were highly cytotoxic for A375 cells (Figure 4.14), and Act-D pre-treatment had no effect on this killing. However, when the 0.8mM membrane was placed between effector and target cells cytotoxicity was almost completely inhibited. Pre-treatment of the target cells with Act-D resulted in a partial restoration of cytotoxicity, but not to the levels seen with cells in direct contact. Cytotoxicity in the presence of membranes was similar to that observed for A375 cell killing by control and LAK cell supernatants. These results suggest that control and LAK cells produce soluble factors which are not directly cytotoxic for A375 cells, but become tumouricidal to target cells pretreated with Act-D. Possible candidates for these factors include TNFα, LT and IFNγ (or a combination of these: section 1.1)

Direct contact between effector and target cells results in much greater target cell death than with either high dose recombinant cytokines or cell supernatants in the presence or absence of target cell pretreatment with Act-D. There are several possible explanations for these results. 1. Control and LAK cells require to adhere to their targets in order to generate very high concentrations (*i.e.*, higher than tested in this study) of cytolytic effector molecules at the cell junctions. 2. Control and LAK cells kill A375 cells by a cytokine independent mechanism (*e.g.* perforins or proteolytic enzymes). 3. A combination of both 1 and 2. Some of these possible mechanisms of killing will be investigated further in the next sections

4.4 Inhibition of Control and LAK Cell Killing by Anti-TNFα and Anti-LT Antibodies

To investigate if TNFα and LT had a prominent role in the mechanism of control and LAK cell killing of A375 cells, monoclonal blocking antibodies against TNFα and LT were included in the cytotoxicity assay, together with a non-reactive monoclonal antibody (PDS1, an anti-retinal-S-antigen IgG1, (Banga *et al.*, 1989); appendix V).

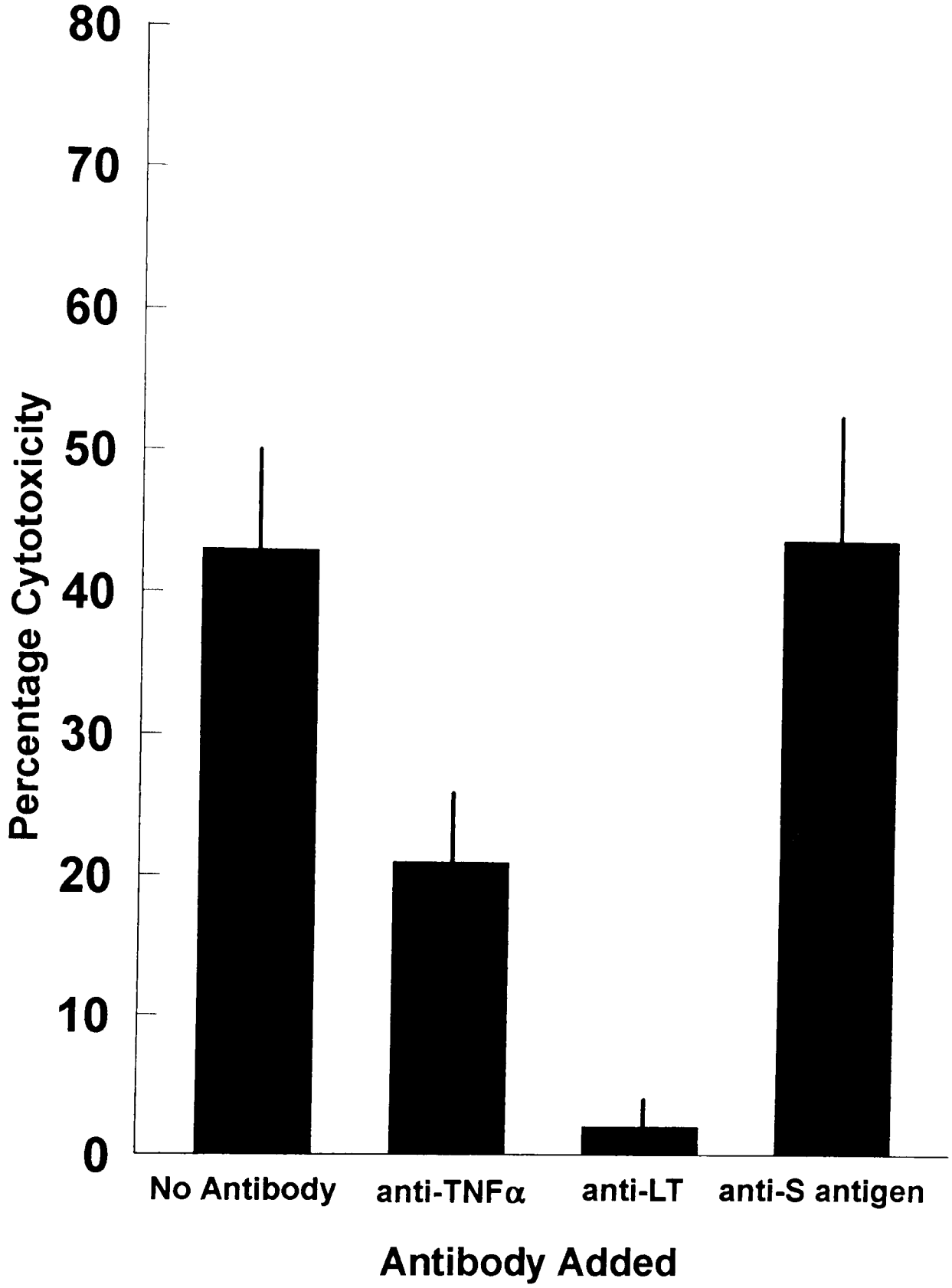


FIGURE 4.15. Effect of anti-TNFα and anti-LT on control cell killing of A375 cells. Control cell killing of Act-D pretreated A375 cells was assessed alone and in the presence of monoclonal anti-TNFα (100μg/ml), anti-LT (100μg/ml) or anti retinal S-antigen (100μg/ml) for 18h at an E:T of 50:1. The results are expressed as means and SEMs from 6 experiments. Cytotoxicity was partially inhibited (50%; $p<0.05$ by WSR and PTT) by anti-TNFα and almost completely inhibited by anti-LT (95%; $p<0.05$ by WSR and PTT compared to No antibody and anti-TNF; also $p>0.05$ for anti-LT compared to spontaneous release.). No inhibition was observed in the presence of anti-S-antigen ($p>0.05$ by WSR and PTT).

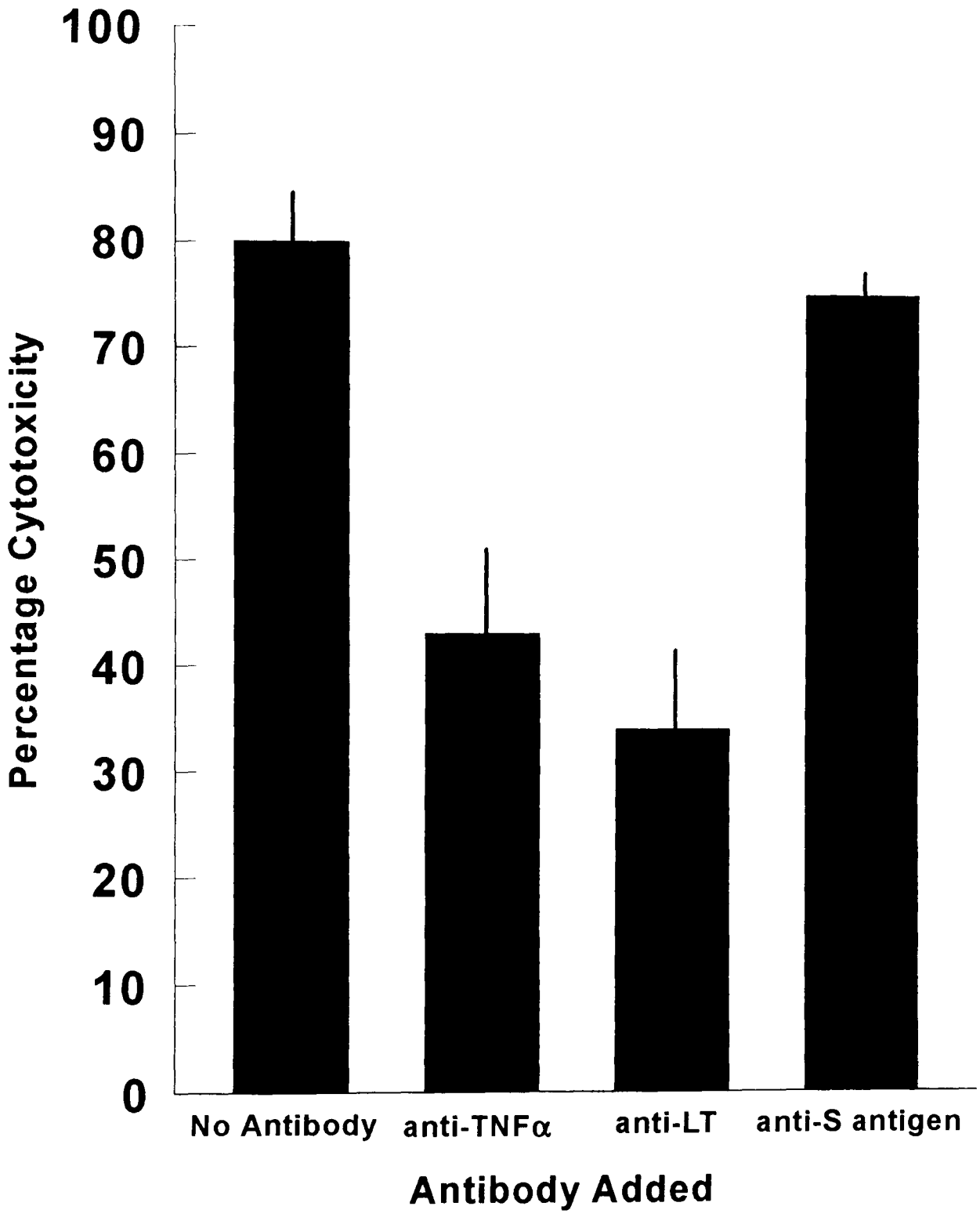


FIGURE 4.16. Effect of anti-TNFα and anti-LT on LAK cell killing of A375 cells. Killing of Act-D pretreated A375 cells by LAK cells was assessed alone and in the presence of monoclonal anti-TNFα (100μg/ml), anti-LT (100μg/ml) or anti-retinal S-antigen (100μg/ml) for 18h at an E:T of 50:1. The results are expressed as means and SEMs from 6 experiments. Cytotoxicity was partially inhibited (48%; $p<0.05$ by WSR and PTT) by anti-TNFα and by anti-LT (57%; $p<0.05$ by WSR and PTT; also $p>0.05$ for anti-LT compared to anti-TNFα.). No inhibition was observed in the presence of anti-S-antigen ($p>0.05$ by WSR and PTT).

Figures 4.15 and 4.16 show the mean maximal cytotoxicity (from six experiments) of control and LAK cells in the presence or absence of the various antibodies. Control cell killing was inhibited by anti-TNFα, and almost completely by anti-LT. LAK cell cytotoxicity was inhibited to a similar degree ($p>0.05$) by anti-TNFα and anti-LT. No inhibition of cytotoxicity by anti-S-antigen was observed.

4.5 Effect of IL-2 on Monocyte Killing of A375 Cells

IL-2 stimulation of monocytes has been reported to increase their cytotoxicity to tumour cells (Malkovsky *et al.*, 1987). Experiments were therefore performed to determine whether IL-2 augmented monocyte killing of A375 cells. Monocytes were incubated for 1-3 days with IL-2 (500U/ml) prior to their incubation with A375 cells. In 4 experiments no enhancement of monocyte cytotoxicity by IL-2 was seen.

Summary of Results

1. The optimal concentration of IL-2 for LAK cell production was between 50-250U/ml, and the optimal time of production was between 24-48h. Increased killing of A375 cells was seen after as little as 1h incubation of lymphocytes with IL-2 (250U/ml).

2. TNFα was unable to induce LAK activity by purified lymphocytes.

3. Depletion of CD8+ lymphocytes completely abrogated control cell killing of A375 cells (pretreated with Act-D), and caused LAK cell cytotoxicity to decrease by a mean of 69%, indicating that lymphocyte and LAK cell cytotoxicity was caused predominantly by CD8+ cells.

4. Control cell killing of the 7 tumour cell lines was very similar to lymphocyte killing for DX3, Daudi and U937 cells, but slightly greater for the other 4 cell lines. This may have been due to activation of control cells by a component of FCS. Control cells were cytotoxic to Act-D pretreated tumour cells in the same pattern as that observed using fresh lymphocytes.

5. LAK cell killing of the 7 tumour cell lines was considerably greater than that of control cells (62-97%), and killing was not augmented by pretreatment of target cells with Act-D.

6. LAK or control cells were not cytotoxic to autologous lymphocytes or monocytes, but were highly cytotoxic to heterologous HUVEC after as little as 1h incubation. Control cells were less cytotoxic than LAK cells to HUVEC, but did not kill heterologous fibroblasts, which were very sensitive to LAK cell killing.

7. Control cell supernatants were cytotoxic to LT5.1, K562 and U937 cells (10-26%), but not to the other cell lines (<5% cytotoxicity). Act-D

pretreatment of tumour cells caused increased killing by supernatants in the same pattern as control cells and fresh lymphocytes, but to a lesser degree.

8. LAK cell supernatants were cytotoxic for 5 of the cell lines (10-36%), whilst A375 and SK23 cells were relatively unresponsive (<8%). Act-D pretreatment of tumour cells augmented killing in the same pattern as fresh lymphocytes, control cells and control cell supernatants.

9. Control and LAK cell cytotoxicity to A375 cells was considerably reduced when cell-cell contact was inhibited by polycarbonate membranes. The remaining cytotoxicity in wells containing membranes was augmented by tumour cell pretreatment with Act-D (in a similar fashion to that observed with TNFα and control and LAK cell supernatants), as were control cells, but not LAK cells, in the absence of membranes.

10. Control and LAK cell killing of A375 cells was inhibited by blocking antibodies directed against TNFα and LT, thus implicating these cytokines in the mechanism of lymphocyte and LAK cell cytotoxicity.

11. IL-2 pretreatment of monocytes did not affect monocyte cytotoxicity of A375 cells.

CHAPTER 5

THE ROLE OF COAGULATION IN LAK CELL CYTOTOXICITY OF A375 CELLS

5.0 Introduction

It was demonstrated in the previous section that LAK cells were extremely cytotoxic for all tumour cell lines tested, as well as endothelial cells and fibroblasts *in vitro*. It was, therefore, of interest to investigate why LAK cells re-injected into cancer patients did not apparently kill tumour cells as effectively as they do *in vitro*. There are two possible reasons for this. Firstly, LAK cells may never reach the tumour mass, possibly because they lack the appropriate homing mechanism(s), or their function is inhibited in the circulation, either by inhibitory molecules or by non-specific adherence to the vessel wall. Secondly, inhibition of LAK cell activity may occur when the LAK cells infiltrate the tumour mass, either by the release of immunosuppressive molecules (*e.g.* TGFβ) from the tumour cells, or by some component of the tumour stroma (*e.g.* laminin, fibrin).

One important observation is that all measurements of LAK activity *in vitro* in this and other studies have been performed in tissue culture medium with or without human or bovine serum where all coagulation mechanisms have been activated and fibrin produced, whereas the *in vivo* circulation consists almost exclusively of plasma, containing the full complement of coagulation components. The experiments reported here were designed to compare the effects of plasma and serum on control and LAK cell killing of A375 cells *in vitro*.

Fibrinogen and other coagulation/fibrinolytic molecules are also present in the extracellular matrix in inflammation and cancer. Here the coagulation system can be activated by leucocytes (or LAK cells) and tumour cells to generate fibrin which becomes part of the tumour stroma. In order to metastasise, tumour cells must also be capable of degrading their extracellular matrix, including fibrin. Further experiments were therefore performed to investigate the procoagulant and fibrinolytic activities of control, LAK and A375 cells, and

the correlation between these cellular activities with possible LAK cell-tumour cell interactions *in vivo*.

5.1 Inhibition of Cellular Cytotoxicity by Plasma Components

5.1.1 Effect of Plasma on Control and LAK Cell Cytotoxicity of A375 Cells

To determine whether lymphocyte and LAK cell killing were inhibited by plasma, blood was taken from a single donor into either sodium citrate (citrated plasma) or glass (serum), and the resulting plasma compared with serum for it's ability to modify lymphocyte and LAK cytotoxicity.

Initial experiments investigated the effect on control and LAK cell cytotoxicity of RPMI containing serum or plasma at graded concentrations. Four experiments were performed to investigate the effect of different concentrations of serum on lymphocyte cytotoxicity of A375 cells (figure 5.1).

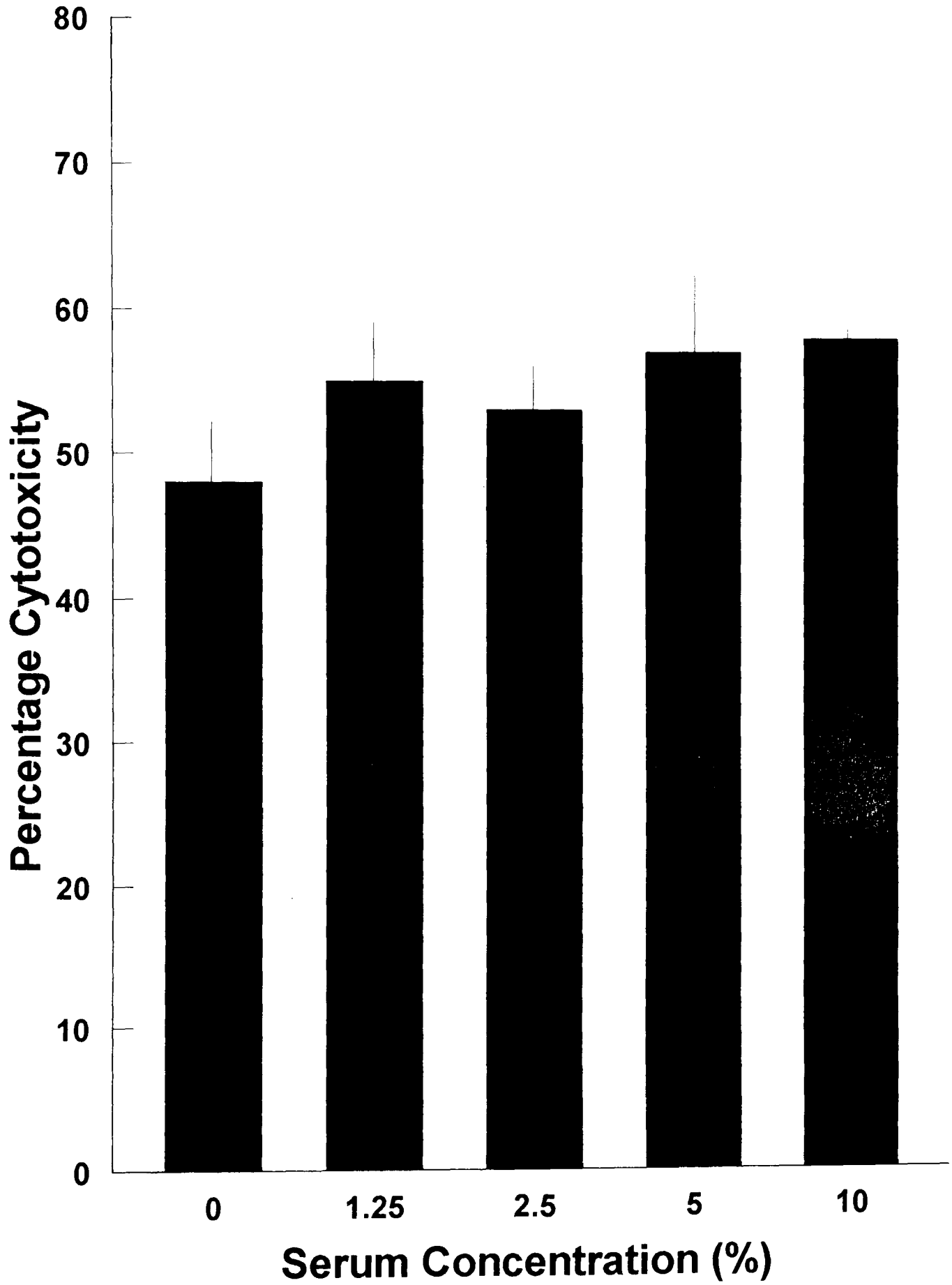


FIGURE 5.1. Effect of serum dilution on lymphocyte cytotoxicity of A375 cells. Freshly isolated lymphocytes ($5x10^6$ cells/ml) were incubated with Act-D pre-treated A375 cells ($1x10^5$ cells /ml) for 18h, in RPMI alone or containing heat inactivated serum at concentrations of 1.25, 2.5, 5 and 10%. The results are means and SEMs from 4 experiments. No significant difference was observed between RPMI or any serum dilution ($p>0.05$ by MWU and PTT).

There was little or no change in cytotoxicity at any concentration of serum tested, although a slight, but not significant ($p>0.05$) decrease was observed in RPMI alone compared with any of the serum dilutions. This indicated that reduction of the serum concentration to 1.25% in the assay did not result in any change in the level of cytotoxicity due to nutrient deprivation, nor were any components of the serum (such as PF4 or TGFβ) responsible for increasing cellular cytotoxicity in serum compared to tissue culture medium alone. The cytotoxicity values for 5% serum will be shown in subsequent experiments, except where stated. In addition, sodium citrate was not directly cytotoxic for A375 cells at the same concentrations that were present when plasma was added to the assay plate, nor did it directly inhibit control and LAK cell killing of A375 cells. Any inhibition of cytotoxicity observed was therefore due to the presence of plasma.

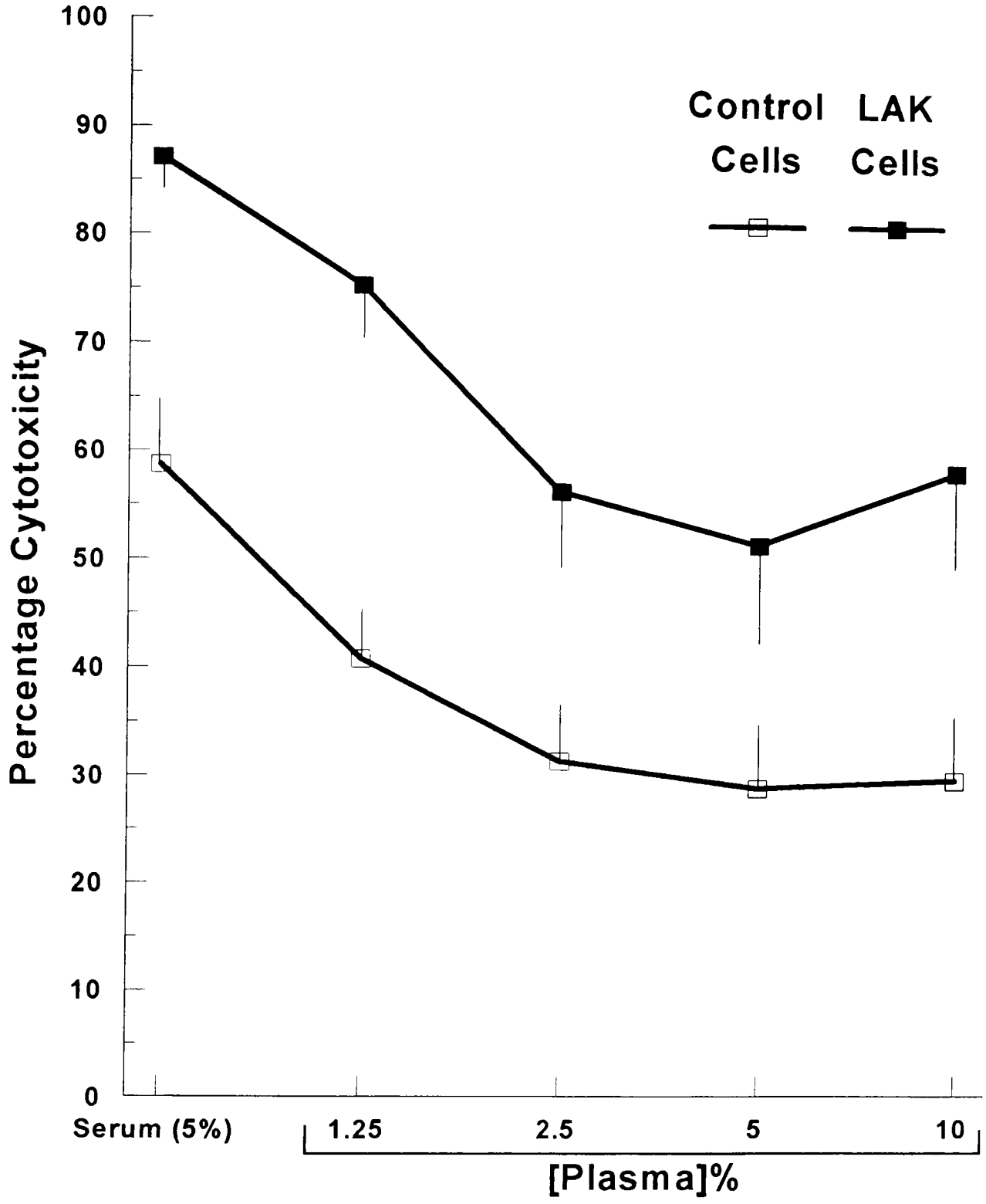


FIGURE 5.2. Effect of plasma on control and LAK cell killing of tumour cells (A375). Lymphocyte (control) and LAK cell cytotoxicity (at an E:T of 50:1) against Act-D pre-treated A375 cells was assessed in the presence of 5% serum or plasma at 1.25, 2.5, 5 and 10%. Means and SEMs from 6 experiments are shown. Cytotoxicity by LAK cells was significantly inhibited at plasma concentrations of 2.5%, 5% and 10% compared to serum ($p<0.05$ by WSR, PTT and MWU. Control cell killing was inhibited at all plasma concentrations tested ($p<0.05$ by WSR, PTT and MWU). No significant difference in inhibition was observed at plasma concentrations >2.5% for LAK cells and 1.25% for control cells. LAK cell cytotoxicity was significantly greater than that of control cells in serum, and at all plasma concentrations ($p<0.05$ by WSR, PTT and MWU).

Figure 5.2 is a summary of 6 experiments comparing plasma with donor matched serum. LAK cell killing was significantly inhibited by plasma at 2.5%, 5% and 10%, and control cell killing was significantly inhibited by all concentrations of plasma tested.

Following the observation that the addition of plasma rather than serum to the cytotoxicity assay resulted in significant inhibition of killing, further experiments were carried out to investigate the molecular component(s) responsible for this inhibition.

5.1.2 Effect of Heat Inactivation on Plasma Inhibition of Killing

In contrast to fresh plasma no significant ($p>0.05$) inhibition of killing was seen when plasma was incubated for 30min at $56^{\circ}C$ prior to inclusion in the cytotoxicity assay (figure 5.3). These results indicate that a heat labile molecule, or molecules, are either directly responsible for inhibition, or contribute to inhibition by another factor.

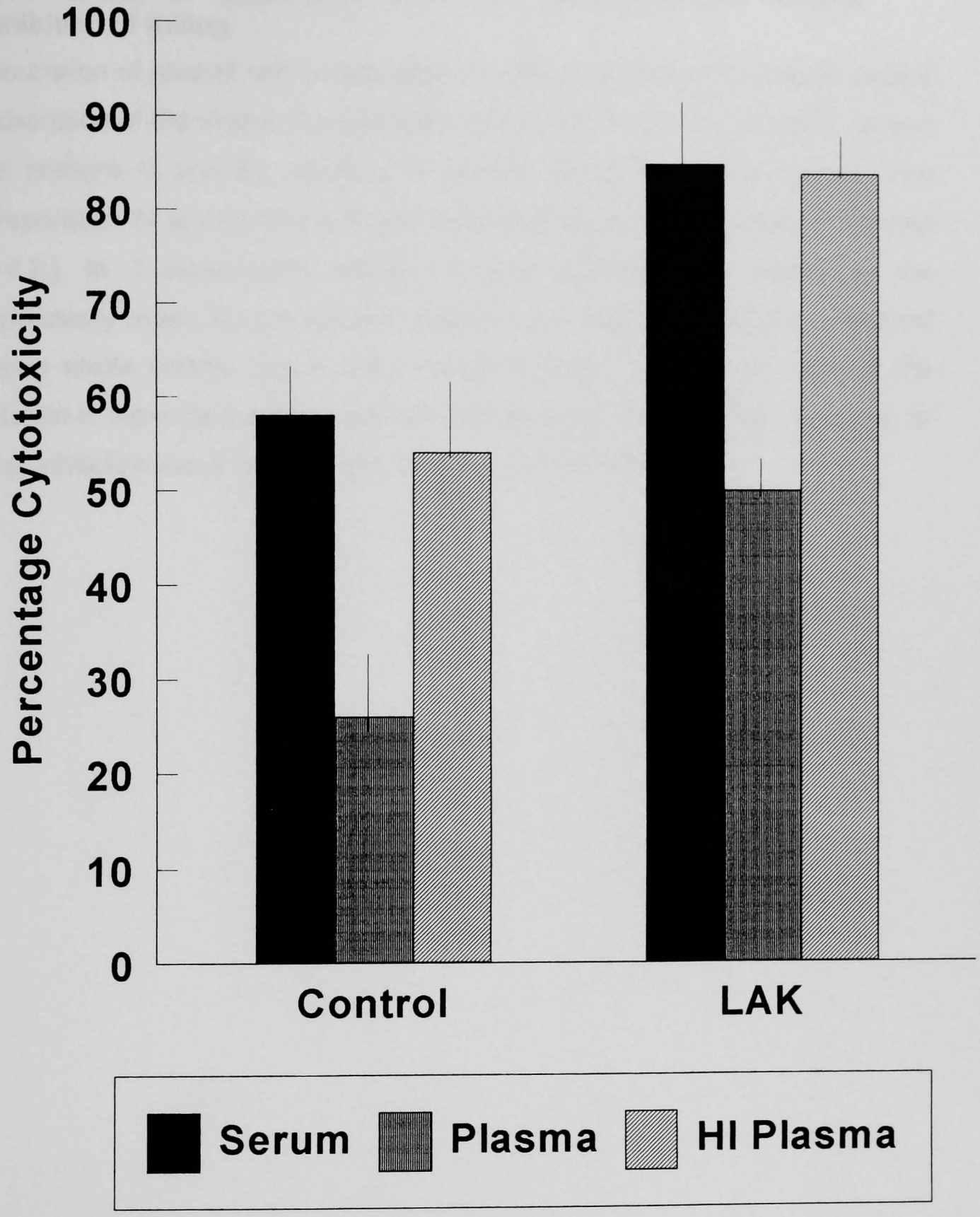


FIGURE 5.3. Comparison of serum, plasma and heat inactivated plasma on control and LAK cell cytotoxicity of A375 cells. Matched serum and plasma was obtained as described in section 2.3.1. The serum and an aliquot of plasma were heat inactivated (HI) at 56^0C for 30min. Lymphocyte and LAK cell cytotoxicity (at an E:T of 50:1) against Act-D (0.1μg/ml) pretreated A375 cells was assessed in the presence of 5% HI-serum, plasma, or HI-plasma after 18h. In 3 experiments (means and SEMs are shown), plasma significantly inhibited control and LAK cell killing (compared to HI-serum; $p<0.05$ by MWU and PTT), but heat inactivation of plasma resulted in restoration of cytotoxicity to the level of HI-serum ($p>0.05$ compared to serum by MWU and PTT).

5.1.3 Effect of Aluminium Hydroxide Adsorption on Plasma Inhibition of Killing

Incubation of plasma with a saturated solution of aluminium hydroxide causes adsorption of the vitamin-K dependent clotting factors (II, VII, IX and X as well as proteins C and S), resulting in plasma deficient in these factors (The preparation of aluminium hydroxide adsorbed plasma is described in section 2.4.2.). In 3 experiments addition of this factor-deficient plasma to the cytotoxicity assay did not result in inhibition of cytotoxicity ($p>0.05$), observed using whole plasma (figure 5.4), indicating either that one or more of the vitamin-K dependent clotting factors contributed to the inhibition of killing, or that inhibition was a result of their action in clot formation.

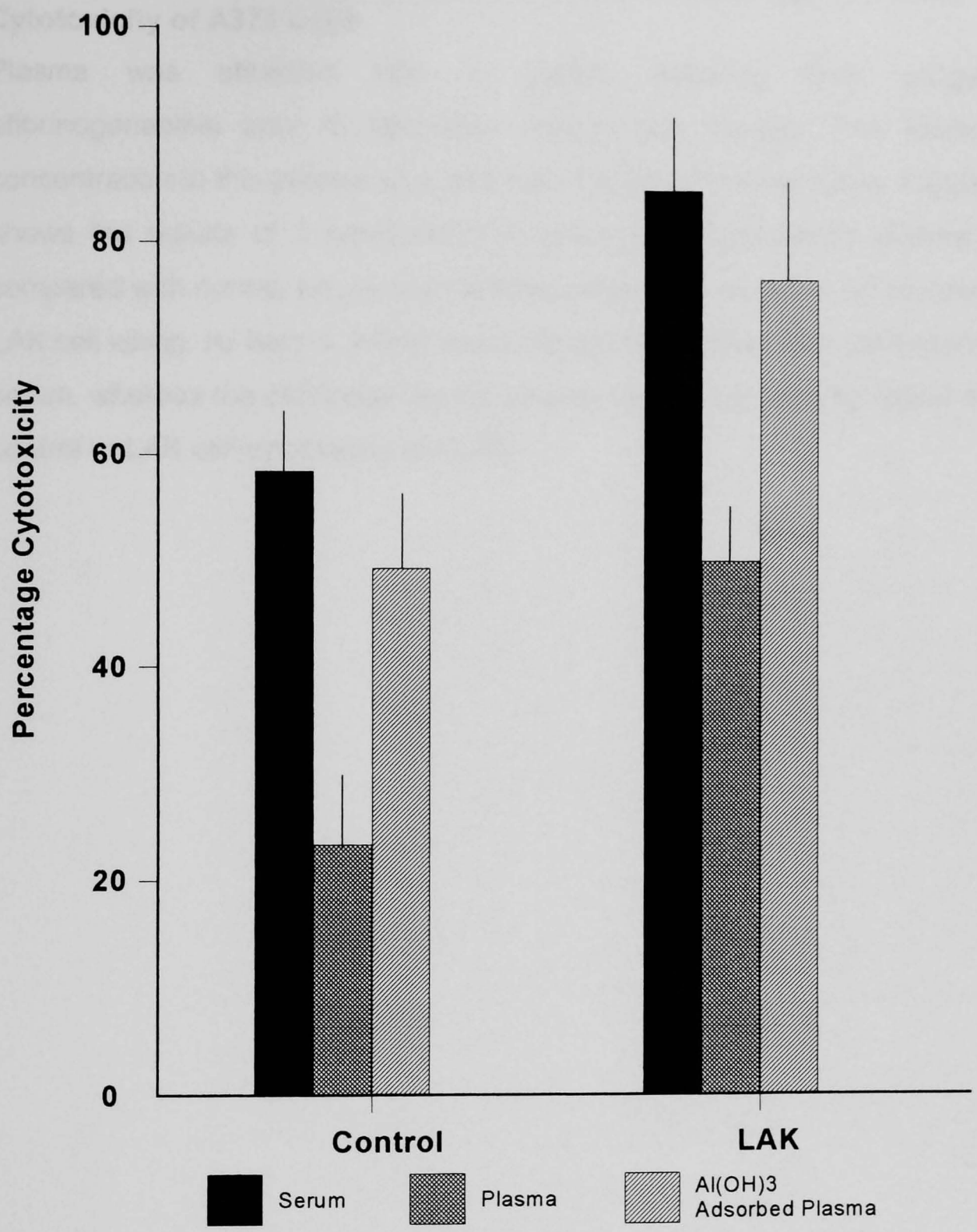


FIGURE 5.4. Control and LAK cell cytotoxicity against A375 cells in the presence of plasma or aluminium hydroxide adsorbed plasma. Aluminium hydroxide adsorbed plasma was prepared (section 2.3.2) and compared with matched HI-serum and plasma for its effect on lymphocyte (control cell) and LAK cell cytotoxicity (at an E:T of 50:1) against Act-D pre-treated A375 cells after 18h. In 3 experiments (means and SEMs are shown), removal of the vitamin-K dependent clotting factors rendered plasma unable to inhibit control and LAK cell cytotoxicity ($p>0.05$ compared to serum by MWU and PTT), which was observed in whole fresh plasma ($p<0.05$ compared to plasma by MWU and PTT).

5.1.4 Effect of Afibrinogenaemic Plasma on Control and LAK Cell Cytotoxicity of A375 Cells

Plasma was obtained from a patient suffering from congenital afibrinogenaemia prior to fibrinogen replacement therapy. The fibrinogen concentration in this plasma was less than 1% of the normal value. Figure 5.5 shows the results of 3 experiments in which afibrinogenaemic plasma was compared with normal serum and matched plasma for it's effect on control and LAK cell killing. As before, killing was inhibited by plasma when compared with serum, whereas the afibrinogenaemic plasma did not significantly inhibit either control or LAK cell cytotoxicity ($p>0.05$).

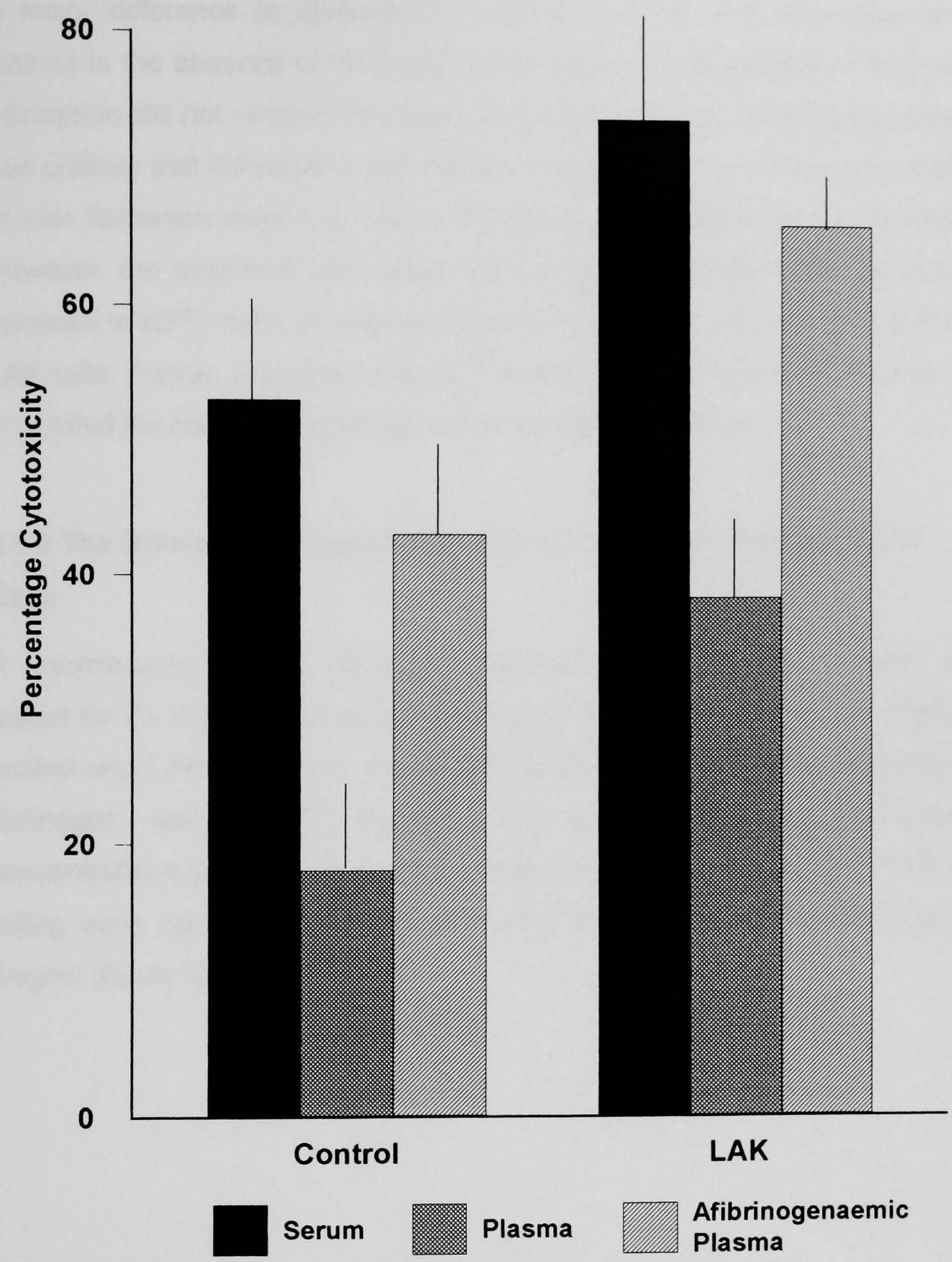


FIGURE 5.5. Effect of afibrinogenaemic plasma on control and LAK cell cytotoxicity of A375 cells. Lymphocyte and LAK cell cytotoxicity (at an E:T of 50:1) against Act-D (0.1μg/ml) pre-treated A375 cells was assessed in the presence of 5% HI-serum, matched plasma or plasma obtained from a patient with congenital afibrinogenaemia. In 3 experiments (means and SEMs shown), control and LAK cell cytotoxicity was not significantly inhibited in the presence of afibrinogenaemic plasma. In contrast, whole plasma inhibited control cell killing by 59% ($p<0.05$ compared to serum by MWU and PTT), and LAK cell killing by 49% ($p<0.05$ by MWU and PTT).

A major difference in composition between plasma and afibrinogenaemic plasma is the absence of fibrinogen in the latter. Since aluminium hydroxide adsorption did not remove fibrinogen, but was unable to inhibit cytotoxicity, it was unlikely that fibrinogen itself was the inhibitory factor, and that the process of clot formation itself (*i.e.* fibrin formation) was responsible for inhibition. However, the possibility also remained that fibrinogen may either be directly cytotoxic to A375 cells, or may upregulate the cytotoxic activity of control and LAK cells. Further experiments were, therefore, performed to examine the role of purified fibrinogen in lymphocyte and LAK cell cytotoxicity.

5.1.5 The Role of Fibrinogen in Control and LAK Cell Killing of A375 Cells

A commercially purified fibrinogen preparation (CRTS, Lille, France) was tested for it's direct cytotoxic activity against A375 cells and for it's effect on control and LAK cell killing. Figure 5.6 (inset) shows that this preparation of fibrinogen was directly cytotoxic for tumour cells at physiological concentrations (3mg/ml), in a dose dependent manner. Control and LAK cell killing were not significantly affected ($p>0.05$) by addition of fibrinogen at 3mg/ml (figure 5.6).

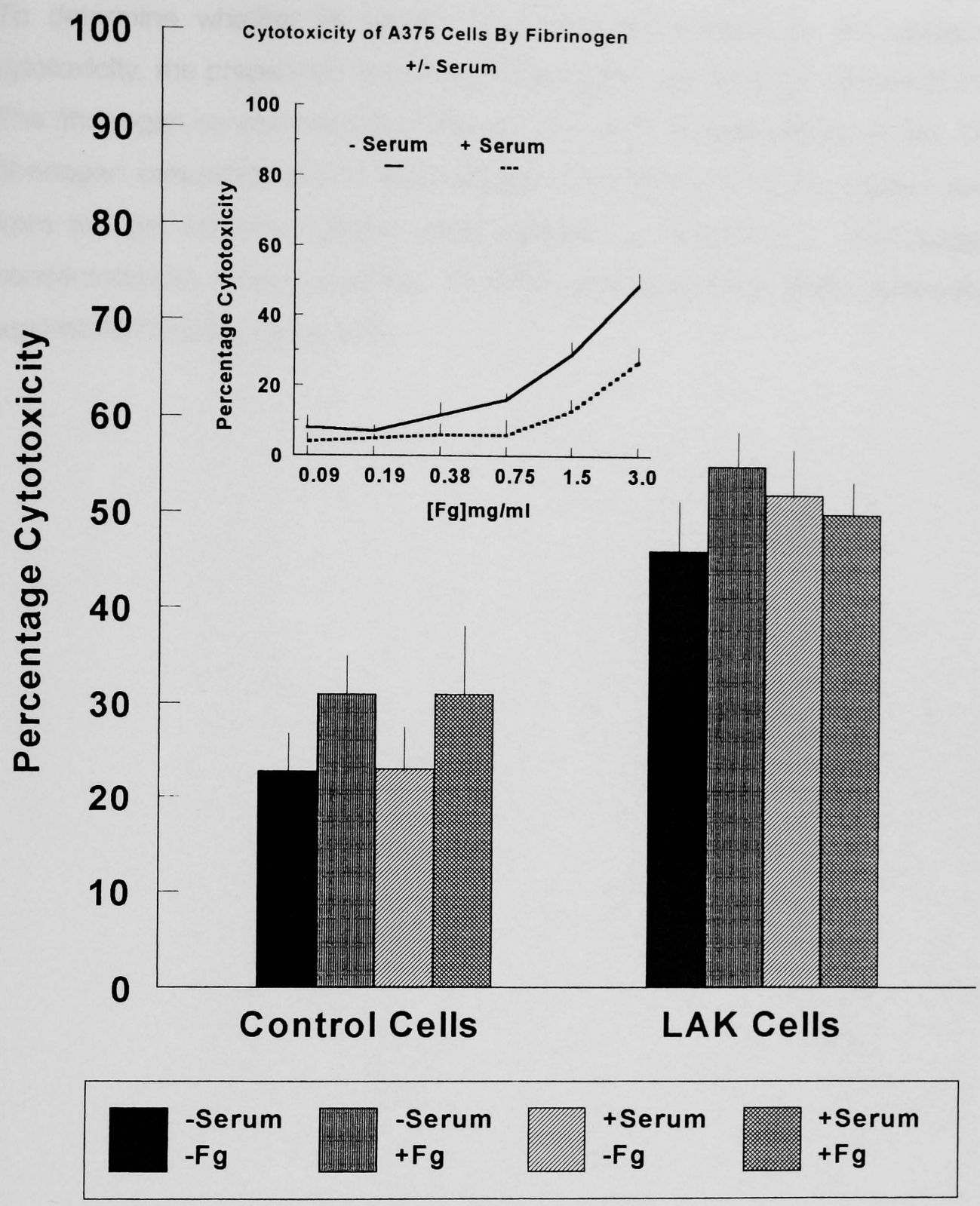


FIGURE 5.6. Effect of fibrinogen on control and LAK cell cytotoxicity of A375 cells. A commercial fibrinogen (Fg) preparation was tested (at 0.09 - 3.0mg/ml: 3mg/ml is the physiological concentration of Fg in the circulation) for its cytotoxicity against A375 cells in the presence or absence of 5% serum (inset graph). In 3 experiments (means and SEMs shown), a dose dependent increase in cytotoxicity was observed (compared to spontaneous release; $p<0.05$ by MWU and PTT), but the level of killing was lower in the presence of serum at Fg concentrations of 0.75, 1.5 and 3.0mg/ml ($p<0.05$ by MWU and PTT). The main figure shows that Fg (at 3mg/ml) did not significantly affect control or LAK cell cytotoxicity (at an E:T of 50:1) against A375 cells (in 3 experiments; means and SEMs shown), either in the presence, or absence, of 5% serum ($p>0.05$ by MWU and PTT).

To determine whether fibrinogen itself was responsible for the observed cytotoxicity, the preparation was further purified by gel filtration (section 2.4.9). The fibrinogen concentrations of the fractions were determined by ELISA. The fibrinogen concentrations of the fractions corresponding to the protein peak from the gel filtration column were adjusted to 3mg/ml (*i.e.,* physiological concentrations), where possible, in RPMI and tested for direct cytotoxicity against A375 cells (figure 5.7).

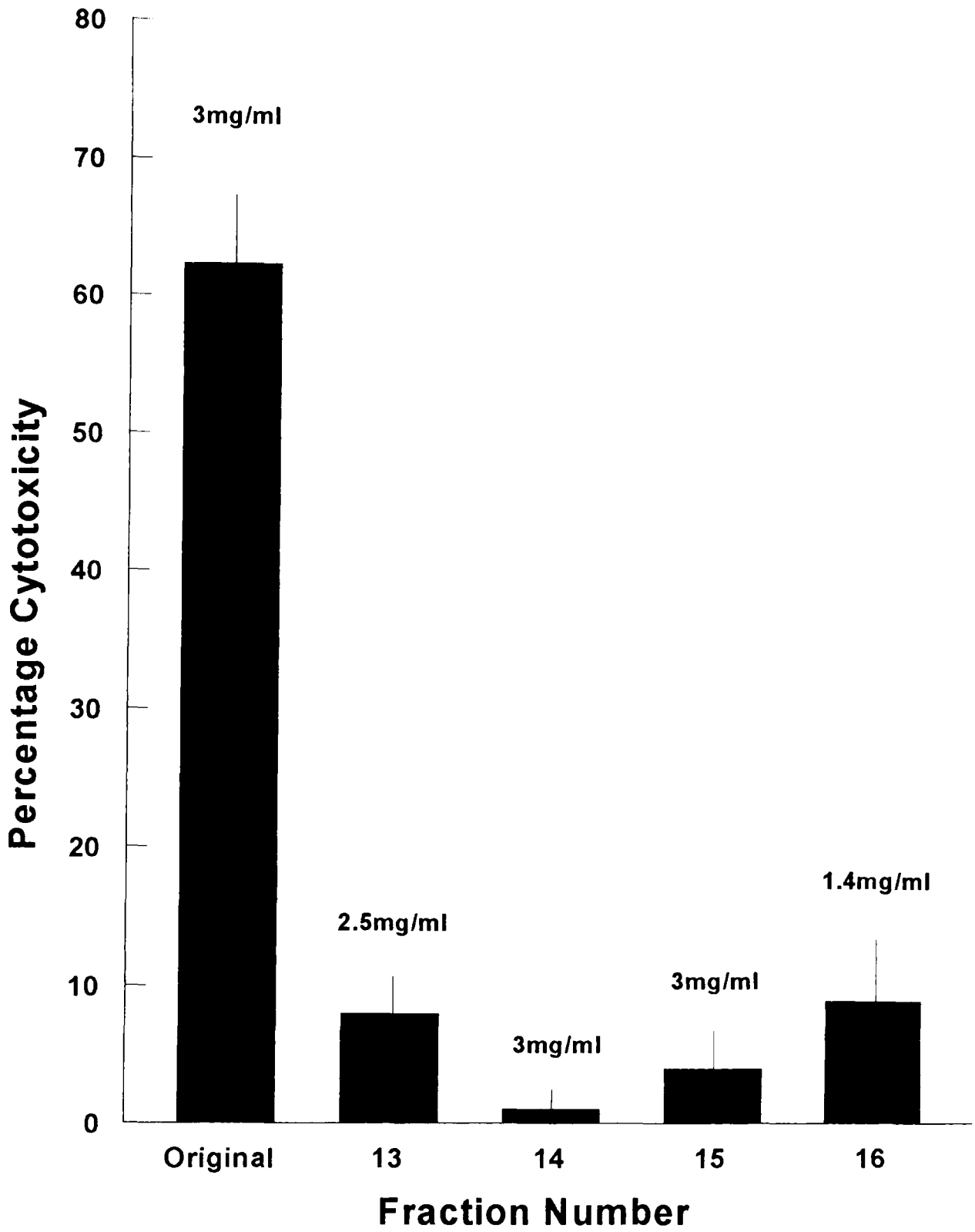


FIGURE 5.7. Effect of fibrinogen and gel filtered fractions on A375 cells. After gel filtration, the fibrinogen containing fractions (see section 2.3.9-11) were assessed for their cytotoxic activity against A375 cells. The concentration of Fg in the assay was physiological (*i.e.* 3.0mg/ml) for fractions 14 and 15, but the initial concentration of Fg in fractions 13 (5.0mg/ml) and 16 (2.8mg/ml), combined with the 1 in 2 dilution in the cytotoxicity assay, required these fractions to be tested at concentrations of 2.5 and 1.4mg/ml. In 3 experiments (means and SEMs are shown), fractions 13 and 16 were cytotoxic against A375 cells (compared to spontaneous release; $p<0.05$ by MWU and PTT), but killing was considerably less than that of the original product ($p<0.001$ by MWU and PTT). No significant killing was observed in the presence of fractions 14 and 15 (compared to spontaneous counts; $p>0.05$ by MWU and PTT).

Fractions 13, 14, 15 and 16, which contained fibrinogen (figure 5.7; see also phast gels, section 2.4.11) showed little cytotoxicity for A375 cells, compared with the unfractionated product. One of the low molecular weight fractions (fraction 23) was more acidic than other fractions (as determined by colour change of phenol red in the RPMI) and was also assessed for its cytotoxic activity, and found to be highly cytotoxic (figure 5.8).

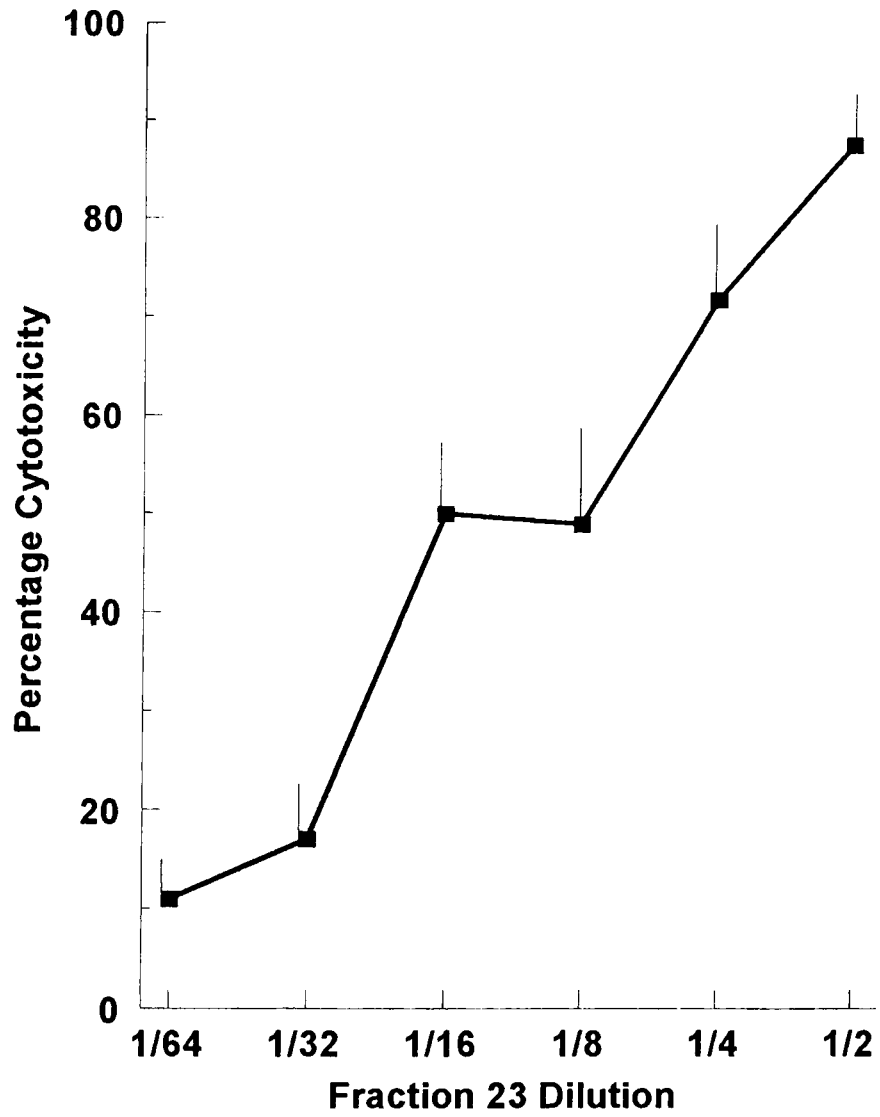


FIGURE 5.8. Cytotoxicity of A375 cells by fraction 23. Fraction 23 from the gel filtration of fibrinogen, which constituted the low molecular weight fraction, was examined for its cytotoxic activity (at a dilution range of 1/64 - 1/2 final) against A375 cells in an 18h $Na^{51}CrO_4$ release assay. In 3 experiments (means and SEMs shown, significant cytotoxicity (compared to spontaneous release; $p<0.05$ by MWU and PTT) was observed at a dilution of 1/64, and rose to a maximum of 88% at 1/2 ($p<0.05$ compared to 1/4 by MWU and PTT).

The dose-dependent cytotoxic activity of fraction 23 was probably not caused by low molecular weight peptides since silver staining (which is a more

sensitive stain than coomassie blue for the detection of proteinacious material) of the PHAST gels (performed by Andrew Lawrie from the Research and Development coagulation laboratory) failed to resolve any proteinacious material. The osmolality of fraction 23 was then measured and found to be 367mOsm/Kg which was considerably above the normal range (280-300mOsm/Kg). This raised osmolality may have accounted for the observed cytotoxicity.

Since fraction 14 was the least cytotoxic of the fractions in the fibrinogen peak, it was retested in place of the unpurified original commercial Fg preparation for its influence on LAK and control cell killing of A375 cells. Figure 5.9 shows that after gel filtration, fibrinogen (fraction 14) was not directly cytotoxic for A375 cells, and had no significant ($p>0.05$) effect on LAK and control cell cytotoxicity.

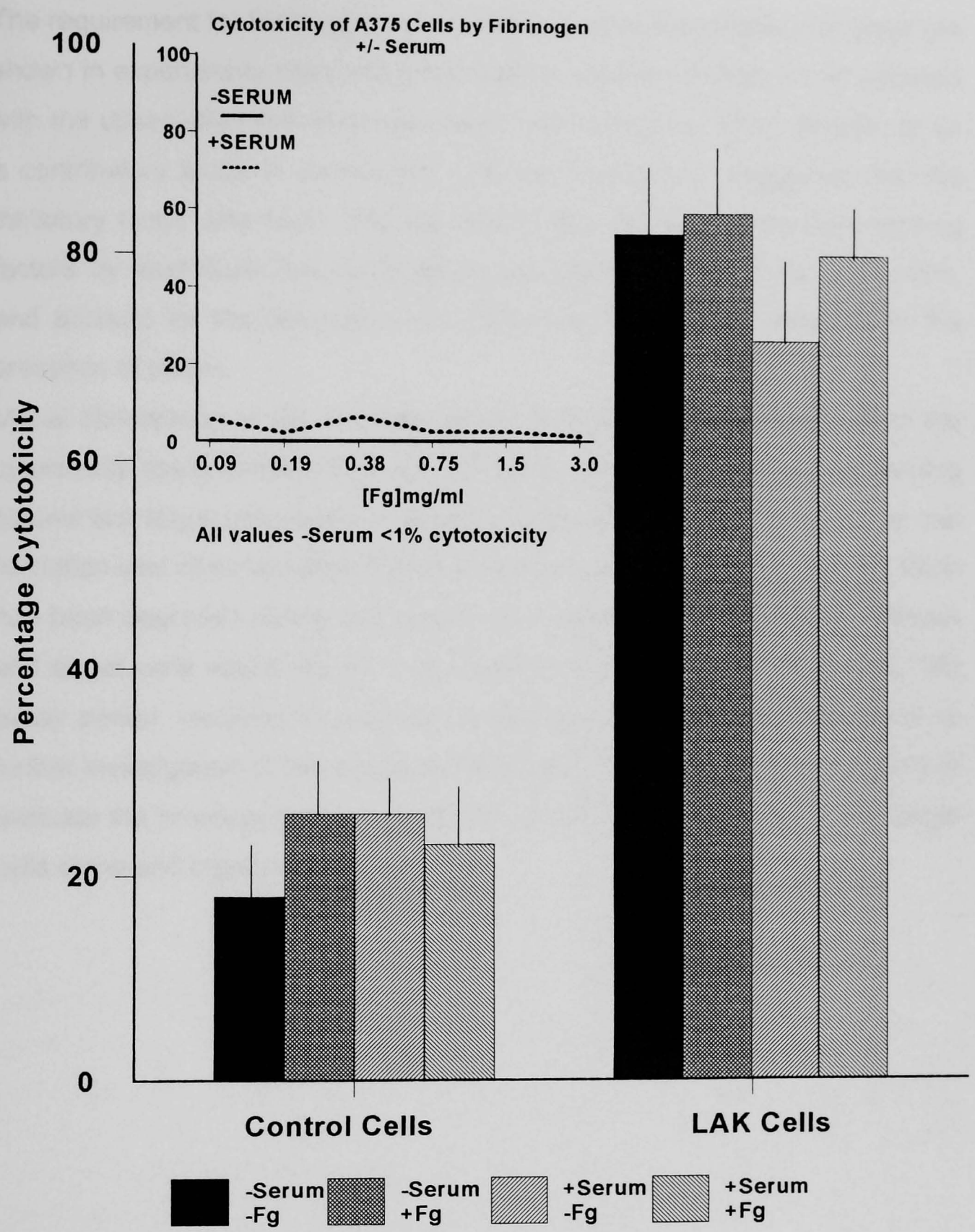


FIGURE 5.9. Effect of purified (fraction 14) fibrinogen on control and LAK cell killing of A375 cells. Gel filtered fibrinogen (fraction 14) was tested for cytotoxicity against A375 cells in the presence or absence of serum (inset graph) at concentrations of 0.09 - 3.0mg/ml after 18h. No significant killing was observed by this fraction at any concentration tested. The main figure shows that lymphocyte (control) and LAK cell cytotoxicity (at an E:T of 50:1) of A375 cells was not affected by the presence of fibrinogen or serum, alone or in combination ($p>0.05$ by MWU and PTT).

The requirement for fibrinogen to be present in order for inhibition to occur (as shown in experiments using afibrinogenaemic plasma; section 5.1.4), coupled with the observation that fibrinogen itself was ineffective, either directly, or as a contributory factor in control and LAK cell cytotoxicity, suggested that the inhibitory factor was fibrin. The removal of the vitamin-K dependent clotting factors by aluminium hydroxide would significantly reduce fibrin production, and account for the restoration of cytotoxicity to the level observed in the presence of serum.

Visual observation of the microtitre plates during the incubation period of the cytotoxicity assay showed that clot formation occurred in the wells containing plasma and target cells (with or without effector cells) in 2-4h. However, no clot formation was observed after the full incubation time, suggesting that the fibrin had been degraded during this period. As a result of this fibrinolysis, effector and target cells would not be in the presence of solid fibrin for the full 18h assay period, resulting in possible restoration of cytotoxicity. Thus, prior to further investigation of the causative factor(s) of inhibition, it was necessary to evaluate the procoagulant and fibrinolytic properties of the effector and target cells alone and together.

5.2 Procoagulant and Fibrinolytic Activities of LAK and A375 Cells

5.2.1 Observation of Clot Formation and Dissolution in the Cytotoxicity Assay

In order to observe clot formation more clearly during the 18h cytotoxicity assay period the volumes of all constituents were scaled up from a total of 200µl in 96 well microtitre plates to 2ml total volume in 24 well plates. Control, LAK or A375 cells were added to the plates alone or in combination. Cell concentrations and thus effector:target cell ratios were those which produced maximum killing in the cytotoxicity assay. After 0, 1, 2, 4, 6 or 18h one plate was removed from the incubator and each well inspected for clot formation or dissolution. 0.5ml aliquots were taken for analysis of thrombin and D-dimer.

Time (h)	Control	LAK	A375	A375 +Control	A375 +LAK	RPMI
0	-	-	-	-	-	-
1	+++	+++	+	++	++	-
2	+++	+++	++	++	+	+
4	+++	+++	-	-	-	+
18	+++	+++	-	-	-	+

TABLE 5.1. Visual assessment of clot formation by A375, control and LAK cells. Control lymphocytes, LAK cells, A375 cells, RPMI and mixtures of control and LAK cells with A375 cells (at an E:T of 50:1) were incubated in the presence of normal plasma (5%) for 0, 1, 2, 4 or 18h, as described in section 2.3.4, and a visual assessment of clot formation made. Supernatants were taken from relevant wells after each time point and stored for analysis of thrombin and D-dimer generation. The results shown were repeated in 2 further experiments. A score of: (-) indicated no observable clot formation; (+) - partial clotting, resulting in increased viscosity of the cell suspension; (++) - a solid clot which disintegrated on agitation; (+++) - a solid clot which was not dispersed by agitation. Solid clots were observed after 1h when control or LAK cells were incubated alone, but in all cultures containing A375 cells, clot formation was less strong than control or LAK cells alone and no clots were present after 4h incubation.

Table 5.1 shows the visual assessment of clot integrity over 18h for control, LAK, A375 cells and tissue culture medium alone. In addition, clot visualisation is shown for mixtures of control and LAK cells with target cells at an

effector:target cell ratio of 50:1. The plasma concentration was 5% throughout. After 1-2h control and LAK cells induced the formation of a solid clot which was still apparent after 18h. A375 cells, alone or in combination with control or LAK cells induced a sticky, but not solid, clot after 1-2h, which had disappeared after 4h. Plasma (5%) added to RPMI alone generated a very stringy, friable clot after 2-4h which remained after 18h. These results demonstrated that control, LAK and A375 cells expressed procoagulant activity. Furthermore, A375 cells possessed fibrinolytic activity, demonstrated by clot dissolution in all wells in which these cells were present. The following two sections further evaluate the procoagulant and fibrinolytic properties of control, LAK and A375 cells.

5.2.2 Evaluation of the Procoagulant Activities of Lymphocytes, LAK and A375 Cells

In order to quantify the above observations (see table 5.1), thrombin activity (using the chromogenic substrate S2238) was measured in supernatants collected from each well and time point (see figure 5.10).

In 3 experiments, RPMI induced minimal thrombin activity throughout the 18h assay period. A375 cells induced a sharp peak in activity after 1h which subsequently decreased over the assay period reaching a level slightly above the starting value after 18h. Control and LAK cells alone induced a sharp rise in thrombin activity after 1h which decreased slightly over 18h ($p>0.05$ compared to 1h), but still remained considerably higher than the initial observation. Combination of control or LAK cells with A375 cells induced the greatest increase in thrombin activity after 1-2h which then fell to levels similar to those of control and LAK cells alone after 18h. The decrease in thrombin activity after 18h was probably due to partial breakdown of the thrombin. Experiments were carried out to further elucidate the mechanism(s) of the procoagulant activities of LAK and A375 cells.

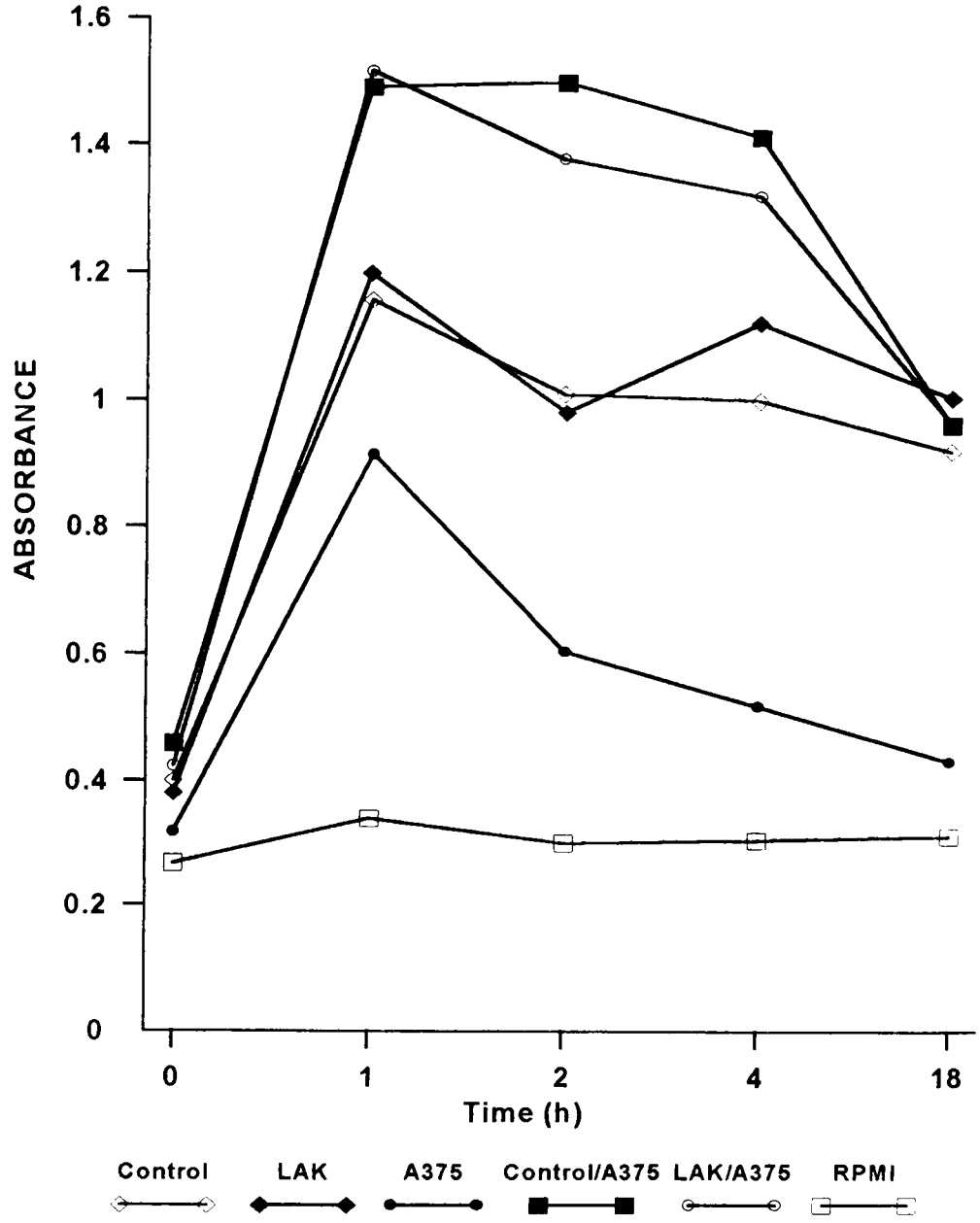
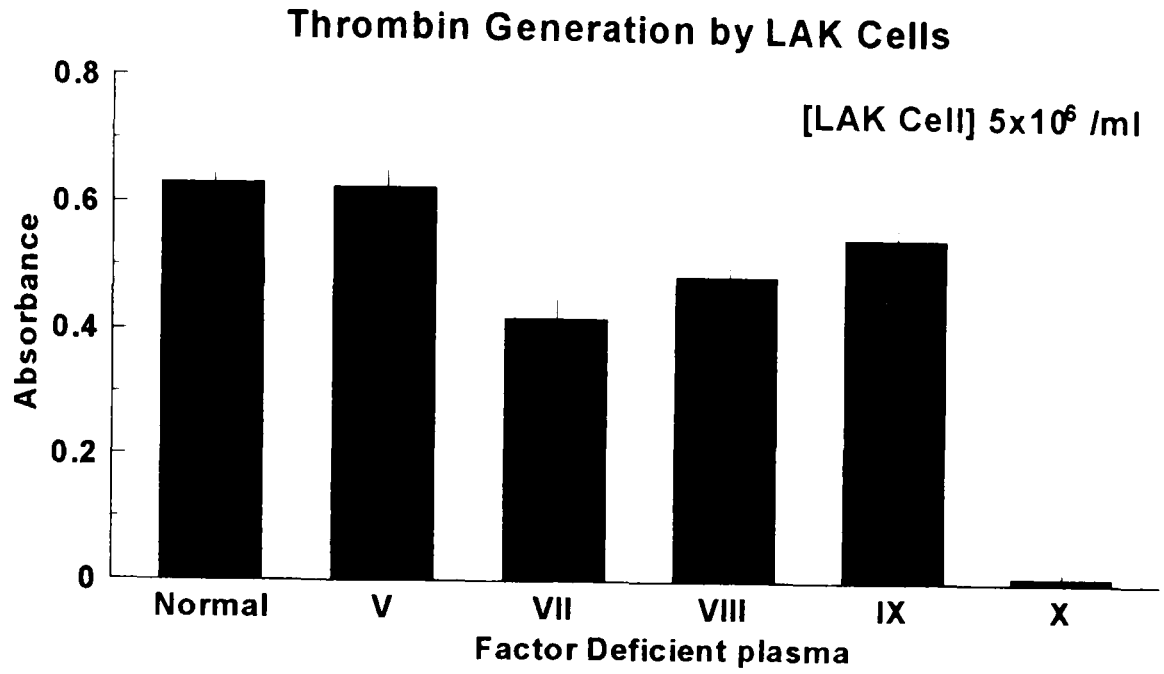


FIGURE 5.10. Time course of thrombin generation during LAK cell cytotoxicity of A375 cells. Supernatants collected from cultures of RPMI, control ($5x10^6$ cells/ml), LAK ($5x10^6$ cells/ml), or A375 cells ($1x10^5$ cells/ml), alone, and control or LAK cells together with A375 cells in the presence of 5% normal plasma, were assessed for thrombin activity using the chromogenic substrate S2238 (see section 2.3.5). Three experiments were performed, of which the data shown are means from a representative experiment All cell types alone or in combination induced a sharp increase in absorbance after 1h (compared to values at time 0; $p<0.05$ by MWU and PTT), with the absorbance for A375 cells < control or LAK cells ($p<0.05$ by MWU and PTT) < control or LAK + A375 cells ($p<0.05$ by MWU and PTT). Absorbances for control or LAK cells alone decreased slightly (but not significantly) after 18h, but a marked drop in absorbance was observed after 18h (2h for A375 cells alone) in supernatants from cultures containing A375 cells ($p<0.05$ by MWU and PTT).

Tumour cell lines have been reported to possess direct factor X activating activity, also known as cancer procoagulant activity (Gordon *et al.,* 1975). Experiments were therefore performed to assess the ability of A375 cells to directly activate factor-X. A375 cells were incubated with factor X (0.16, 0.31, 0.63, 1.25, 2.5 and 5U/ml) for 0.25, 1, 4 and 18h, after which the supernatants were removed and stored at -70^{0}C. In addition the cells were washed and the plates stored at -70^{0}C. Supernatants and cell lysates were subsequently thawed, and incubated in triplicate with the chromogenic substrate S2222. As a positive control factor Xa was also assayed. No change in absorbance was measured in any of the test wells, but the positive control reacted with the substrate within minutes, indicating that A375 cells did not secrete cancer procoagulant into the supernatant. The absence of colour change in the cell lysates indicated that factor X had not been adsorbed or internalised and subsequently activated to factor Xa by the cells.

To investigate the mechanism of procoagulant activity further, LAK and A375 cells were incubated separately with plasma, factor V, VII, VIII, IX and X deficient plasmas (5% final dilution), as well as RPMI alone, for 4h, the supernatants removed and tested for thrombin activity. The absorbance values for the plasmas in tissue culture medium alone (RPMI) were subtracted from those of the plasmas mixed with cells. Figure 5.11 shows the results of a representative experiment (of 3 performed). After 4h, plasma in RPMI generated a tenuous clot which was reflected in the generation of thrombin (absorbance of 0.166). No clots or thrombin activity were seen in any wells containing factor deficient plasmas in the absence of cells (*i.e.,* cultured in RPMI). LAK cells induced greater thrombin activity in whole plasma than the control (whole plasma in RPMI). In addition thrombin activity (and also clotting) was also observed with LAK cells in factor V, VIII and IX and to a lesser extent factor VII, but not factor X deficient plasma. A375 cells induced thrombin activity in plasma (above that of the control) factor VII, VIII and IX, but not factor X deficient plasma. The absorbance values for A375 cells were lower than those for LAK cells because the A375 cell density was lower. The cell concentrations reflected those in the cytotoxicity assay (*i.e.* LAK 50:1 A375).



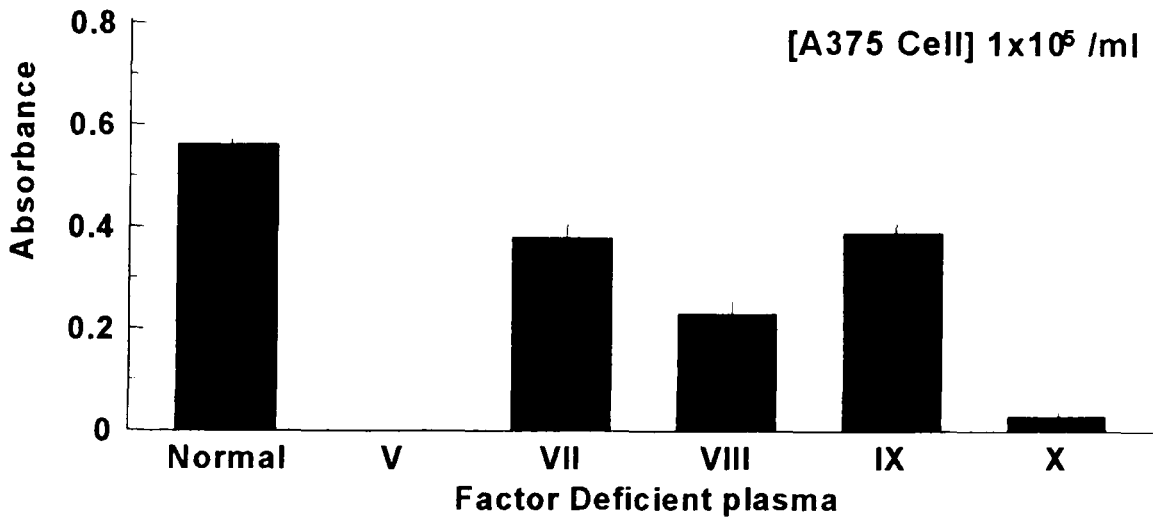


FIGURE 5.11. Thrombin generation by LAK cells and A375 cells. LAK cells ($5x10^6$ cells/ml), or A375 cells ($1x10^5$ cells/ml), were incubated with normal plasma (5%) or plasmas deficient in coagulation factors V, VII, VIII, IX or X for 4h, supernatants collected and thrombin activity determined using the chromogenic substrate S2238 (see section 2.3.5). RPMI was also incubated with each plasma and the absorbances subtracted from those of LAK or A375 cells. The results are means and SEMs from 3 experiments. Thrombin activity was induced by LAK cells to a similar degree ($p>0.05$ by MWU and PTT) in the presence of normal and factor V, VIII and IX deficient plasmas, but the absorbance was significantly decreased in factor VII deficient plasma. LAK cells were unable to induce thrombin activity in factor X deficient plasma. Significant (compared to background (RPMI); $p<0.05$ by MWU and PTT), but reduced ($p<0.05$ compared to normal plasma) thrombin activity was induced in factor VII, VIII and IX deficient plasmas by A375 cells, but not in factor V and X deficient plasmas.

Factor VIII and factor IX deficient plasmas supported thrombin generation in the presence of LAK or A375 cells, suggesting that the procoagulant activities of these cells are primarily via the extrinsic, or tissue factor, coagulation pathway. Both cell types were unable to induce clotting of factor X deficient plasma, indicating that they were unable to directly activate prothrombin to thrombin. The inability of A375 cells to clot factor X deficient plasma confirmed the earlier observation in this section that A375 cells were unable to directly activate factor X (*i.e.,* did not produce cancer procoagulant), or prothrombin (some cancers lines have been reported to release enzymes which were able to activate prothrombin directly (Semeraro and Donati, 1981b)).

The ability of LAK cells to induce thrombin activity in factor V deficient plasma suggested that these cells possessed factor Va activity. However, a small number of contaminating monocytes in the LAK cell preparations may have been responsible for the clotting of factor V deficient plasma. In contrast, A375 cells were unable to clot factor V deficient plasma. Both LAK cells and A375 cells supported thrombin generation in the presence of factor VII deficient plasma. LAK cells may have induced clot formation in factor VII deficient plasma as a combined result of cell surface factor Va and activation of the intrinsic pathway to supply factor Xa for the conversion of prothrombin to thrombin. Alternatively, both cell types may have produced tissue factor (a cell derived cofactor required for factor VIIa activity) which was bound to residual amounts of factor VIIa derived from the serum in which the cells were cultured prior to assay. However, lymphocytes are not known to produce tissue factor, and it is more likely that LAK cells adsorbed tissue factor/factor VIIa complexes from the serum in which they were cultured. From these results, further experiments were performed to determine if LAK cells, monocytes and A375 cells possessed factor V, tissue factor or factor VII on their surface, using flow cytometry (section 2.5). Figures 5.12 - 5.14 are representative histograms of the test fluorescence intensity (FL1) versus the fluorescence intensity of the FITC labelled isotype control on the X axis versus the number of positive events on the Y axis, for factor V, tissue factor and factor VII respectively, on the surface of A375 cells (a.), LAK cells (b.) and monocytes (c.).

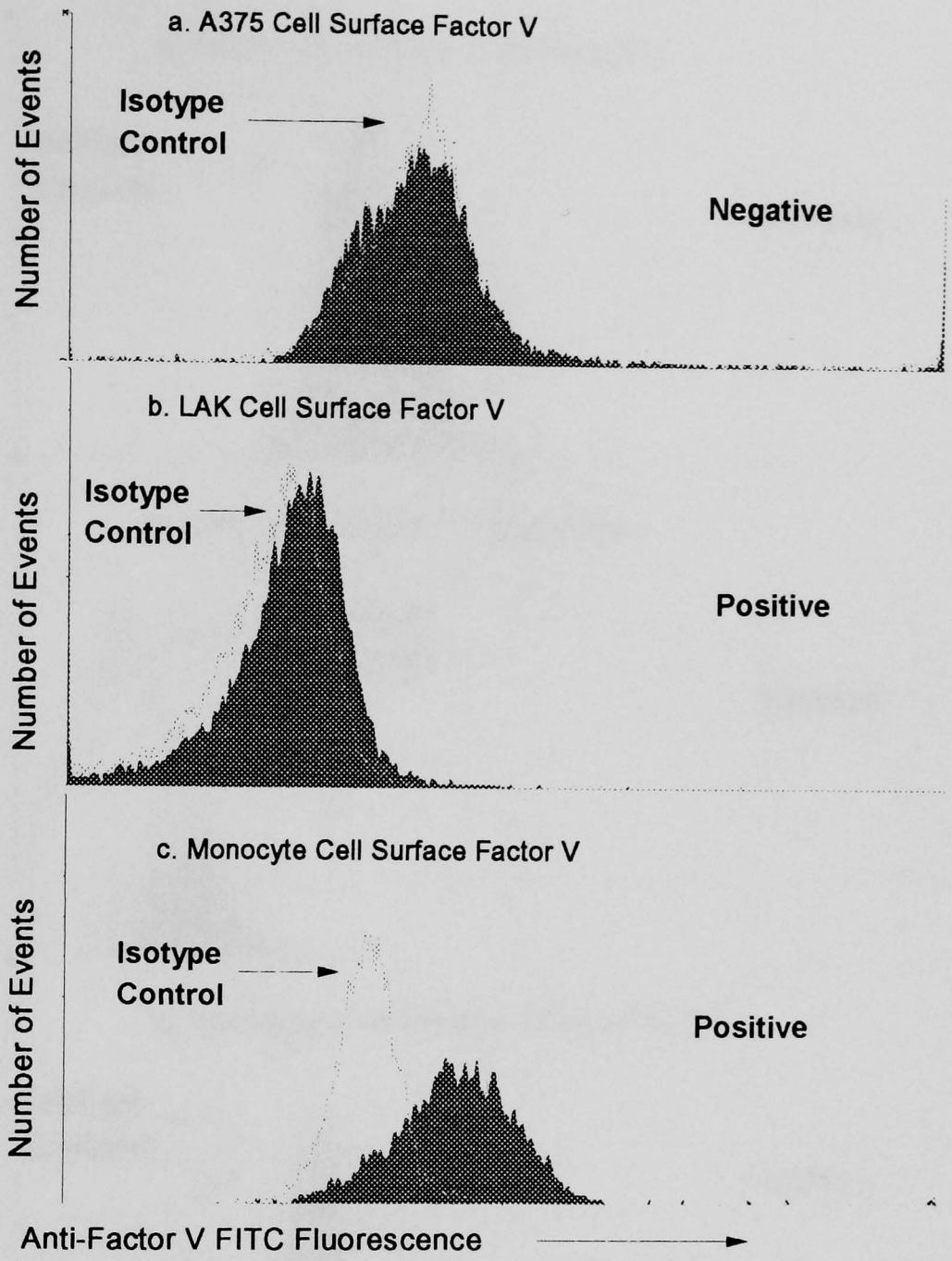


FIGURE 5.12. A375 cell, LAK cell and monocyte surface factor V expression. A375 cells, LAK cells or monocytes were stained with anti-factor-V or isotype control, and flow cytometry performed as described in section 2.4. Ten thousand events were counted in each experiment. Three (A375 and monocytes) or 5 experiments (LAK) were performed, of which this result is representative. Both LAK cells and monocytes were positive for cell surface factor V (compared to the isotype control), but A375 cells showed no positive events with greater fluorescence than the isotype control.

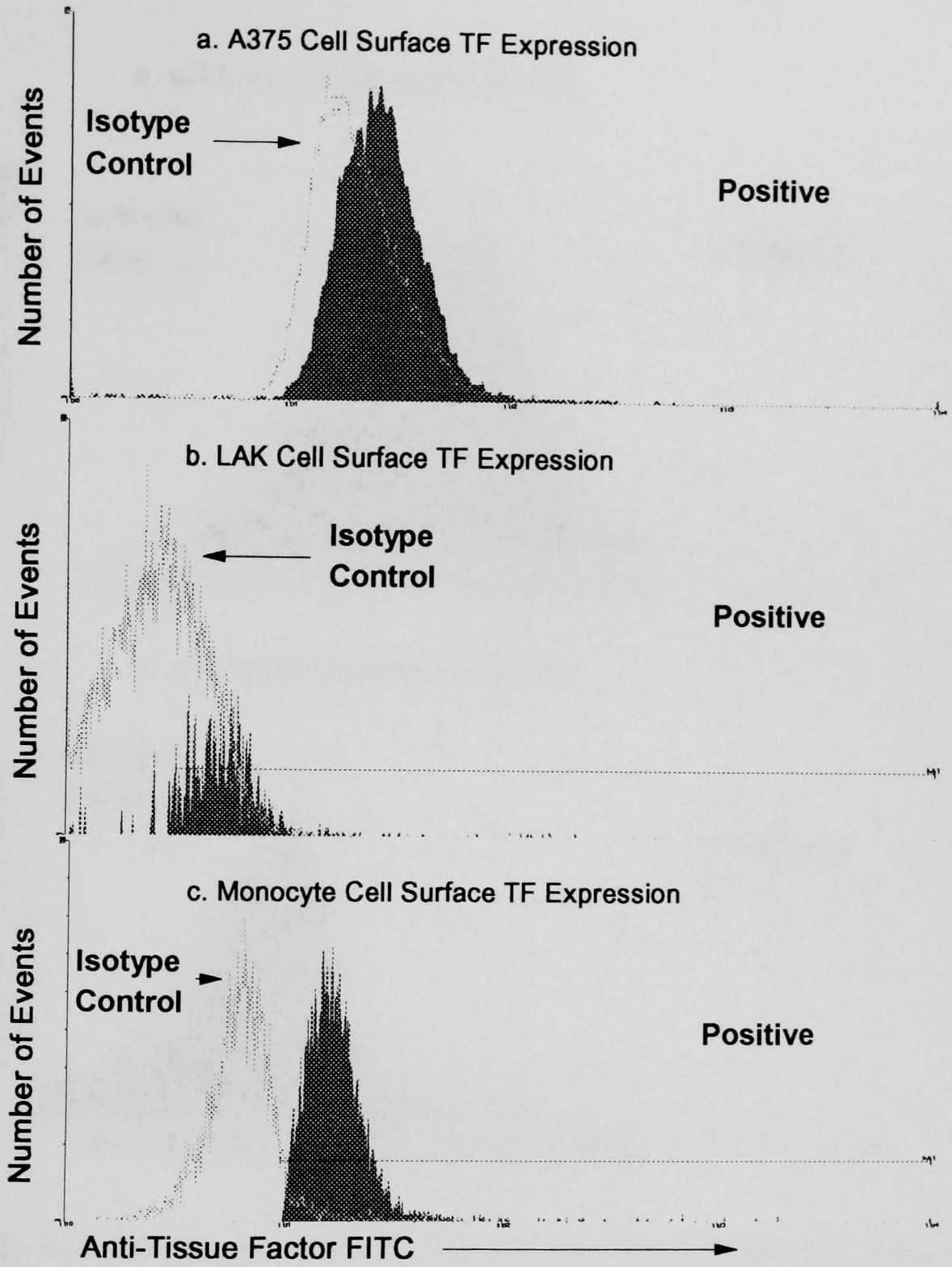


FIGURE 5.13. A375 cell, LAK cell and monocyte cell surface tissue factor expression. A375 cells, LAK cells or monocytes were stained with anti-TF or isotype control, and flow cytometry performed as described in section 2.4. Ten thousand events were counted in each experiment. Three (A375), 7 (LAK cells) or 6 experiments (LAK) were performed, of which this result is representative. All cell types were positive for tissue factor expression compared to the isotype control.

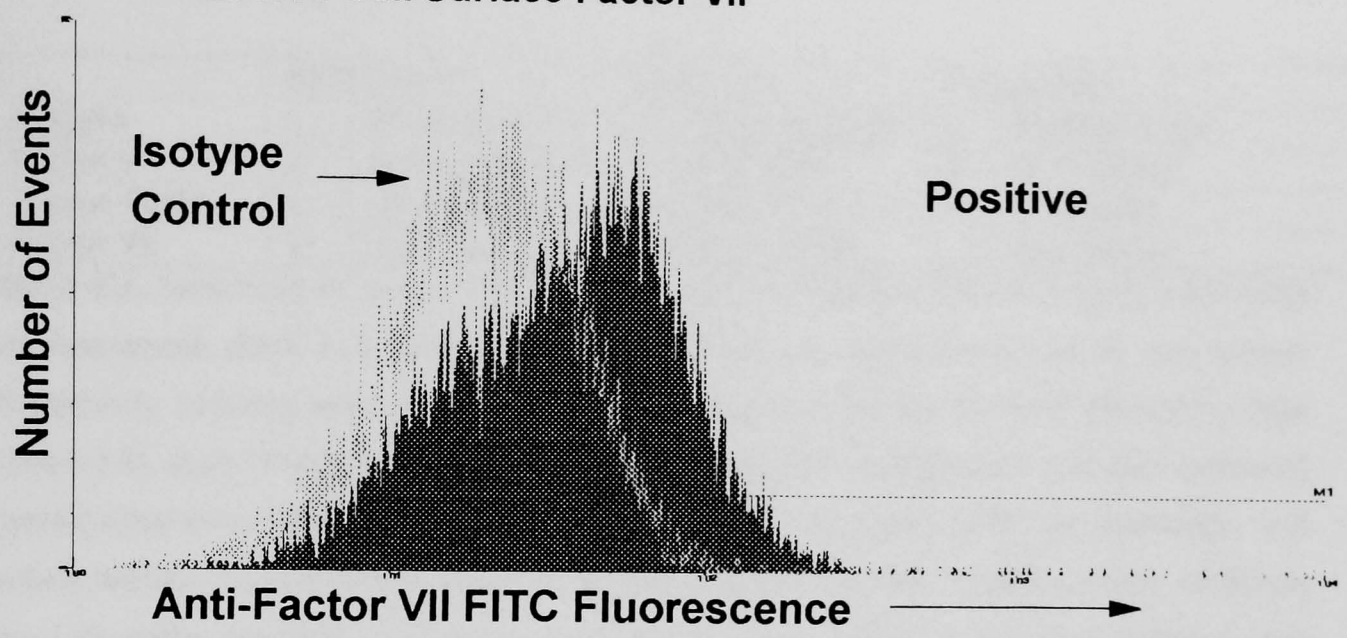


FIGURE 5.14. A375 cell and LAK cell surface factor VII expression. A375 cells or LAK cells were stained with anti-factor VII or isotype control, and flow cytometry performed as described in section 2.4. Ten thousand events were counted in each experiment. Three experiments were performed, of which this result is representative. Both cell types were positive for factor VII expression compared to the isotype control.

Table 5.2 shows the mean number of factor V, tissue factor and factor VII antibody binding sites present on the surface of A375 cells, LAK cells and monocytes (calculations are described in section 2.5).

	A375 Cells		LAK Cells		Monocytes	
Antigen	**n**	**Binding Sites**	**n**	**Binding Sites**	**n**	**Binding Sites**
Factor V	**3**	**None Detected**	**5**	**650** (109)	**3**	**4422** (1032)
Tissue Factor	**3**	**16453** (2812)	**7**	**692** (153)	**6**	**2576** (553)
Factor VII	**3**	**2267** (146)	**3**	**1300** (134)		**Not Tested**

TABLE 5.2. Detection of factor-V, factor VII and tissue factor on A375 cells, LAK cells and Monocytes. A375 cell, LAK cell and monocyte cell surface factor-V, TF and factor-VII antibody binding sites were measured (in n experiments) by flow cytometry (see section 2.4). Anti-TF was a directly labelled antibody and anti-factorV and anti-factor-VII binding sites were assessed indirectly. Results are means and SEMs (in brackets). Cell surface factor-V was present on monocytes in approximately 7 fold greater numbers than LAK cells, and was not detected on the surface of A375 cells. Tissue factor was expressed by A375 cells in approximately 7 fold greater numbers than monocytes and 23 times greater than LAK cells. Factor-VII was present on A375 cells in approximately 50% greater amounts than on LAK cells.

A375 cells possessed tissue factor and factor VII, but not factor V on their surface, which is in agreement with their ability to induce thrombin activity in factor VII, but not factor V deficient plasma. The most likely mechanism of A375 cell procoagulant activity is the expression of tissue factor on the cell surface, which then binds activated factor VII from the culture environment, enabling the cells to initiate the coagulation system.

LAK cells also expressed cell surface tissue factor and factor VII (although to a lesser extent than tumour cells), indicating that the mechanism of procoagulant activity was similar to that of A375 cells. However, in addition to TF and factor VIIa expression, LAK cells also expressed cell surface factor V, which acts at a later stage of coagulation than factor VIIa. Thus, in the presence of factor Xa (activated by factor VIIa), and calcium ions (the phospholipid component is provided by the cell membrane), lymphocyte cell surface factor Va can activate prothrombin to thrombin, and hence fibrinogen to fibrin. It is probable that LAK cells synthesised the cell surface factor V, since recent reports have also shown that lymphocytes express mRNA for factor V (Shen *et al.*, 1993).

Monocytes were also positive for both factor-V and tissue factor which has been previously reported (Kappelmayer *et al*, 1993; Shen *et al.,* 1993), and are thus able to activate coagulation in a similar fashion to lymphocytes or LAK cells.

These results are thus consistent with the patterns of thrombin generation in factor deficient plasmas shown previously in this section.

Since the presence of A375 cells in cultures containing plasma resulted in dissolution of the fibrin formed (as shown in table 5.1 above), experiments were performed to characterise the fibrinolytic activity of these cells.

5.2.3 Evaluation of the Fibrinolytic Activities of Lymphocyte, LAK and A375 Cells

Supernatants taken from experiments described in section 5.2.1 (observation of cell induced clot formation in normal plasma) were assessed for D-dimer production as an indicator of the fibrinolytic activity of the cells (figure 5.15).

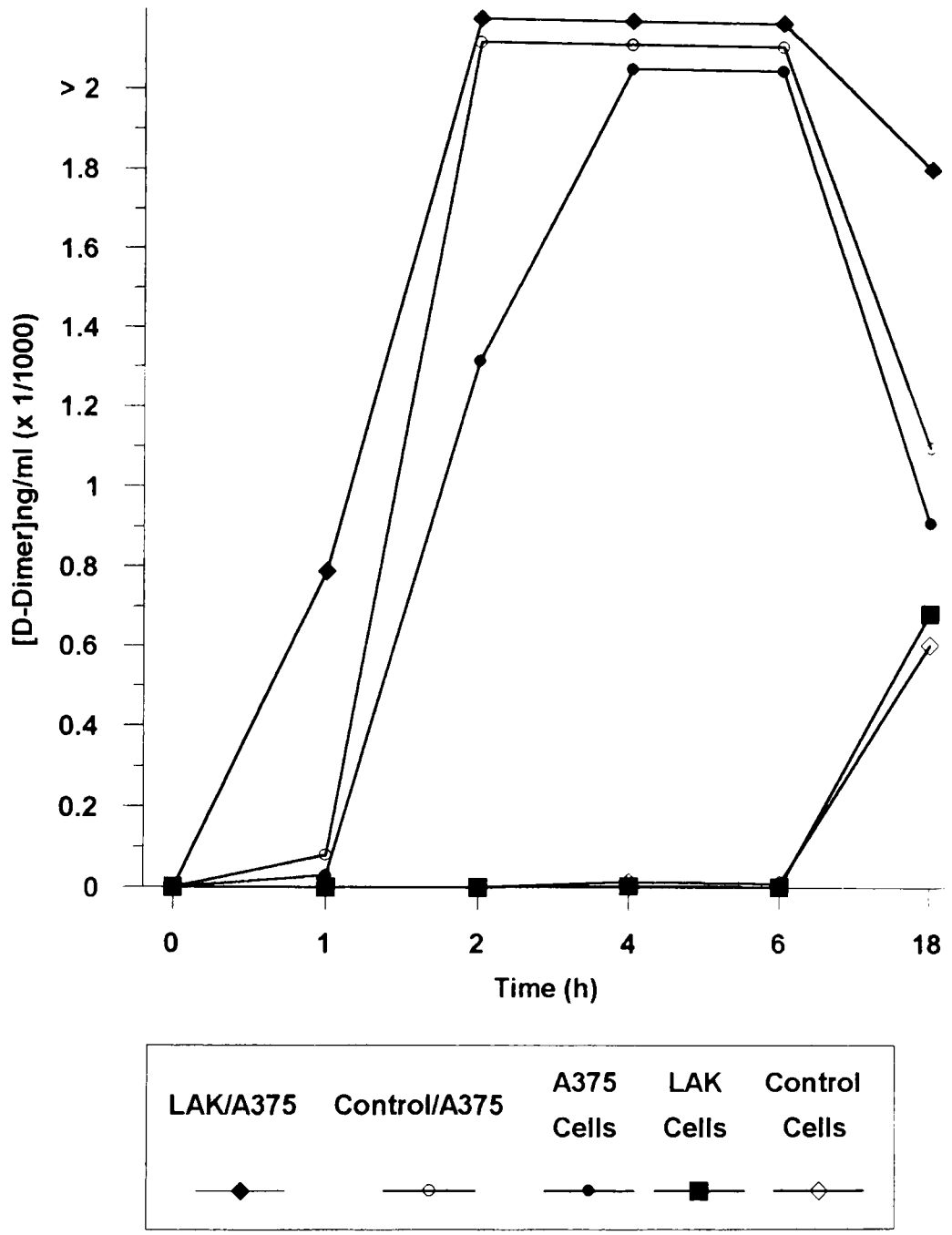


FIGURE 5.15. D-dimer production during LAK cell cytotoxicity of A375 cells. Supernatants collected from cultures (from 1 experiment, assayed x3; means shown; see section 2.3.4) of RPMI, control ($5x10^6$ cells/ml), LAK ($5x10^6$ cells/ml), or A375 cells ($1x10^5$ cells/ml), alone, and control or LAK cells together with A375 cells in the presence of 5% normal plasma, were assessed for the presence of D-dimer as described in see section 2.3.8. Cultures containing lymphocytes (control cells) or LAK cells alone induced significant D-dimer production only after 18h incubation (compared to RPMI; $p<0.05$ by MWU and PTT). A375 cells alone or a combination of A375 and control cells induced significant (A375 cells alone < A375/control; $p<0.05$ by MWU and PTT) D-dimer production after 2h (compared to RPMI; $p<0.05$ by MWU and PTT). Supernatants from cultures of A375 and LAK cells induced D-dimer after 1h ($p<0.05$ by MWU and PTT). The supernatants from cultures containing A375 cells induced levels of D-dimer of >2000ng/ml after 2h (A375/control, A375/LAK) or 4h (A375 cells alone), which dropped significantly after 6h incubation (compared to 4h; $p<0.05$ by MWU and PTT).

In wells containing RPMI and plasma no D-dimer production was seen over the assay period. A375 cells alone or in combination with control and LAK cells induced very high levels (plateau shown where results were above the standard curve, *i.e.,*. >2000ng/ml) of D-dimer after 1-2h which subsequently decreased (except LAK/A375) between 6-18h. In the wells containing control or LAK cells alone some D-dimer production was seen after 6-18h ($p<0.05$ compared to RPMI). These results show that control and LAK cells possessed very little fibrinolytic activity as measured by D-dimer production. This confirmed the previous observation that clots were still present after 18h when these cells were incubated with whole plasma. A375 cells however were highly fibrinolytic, and were thus likely to be responsible for the observed clot dissolution when cultured with lymphocytes or LAK cells.

As previously observed (table 5.1), cocultures of A375 cells with either lymphocytes (control cells) or LAK cells in the presence of plasma resulted in clot dissolution after 2-4h. However, in the microplate cytotoxicity assay (section 5.1.1), significant inhibition of cytotoxicity was observed after 18h. Further experiments were therefore performed to identify the mechanism of A375 cell fibrinolytic activity, and to determine the effect of antifibrinolytic agents on plasma inhibition of cellular cytotoxicity.

5.2.4 Fibrinolytic Activity of A375 Cells

As mentioned above, fibrin is a major constituent of the extracellular matrix in most, if not all, solid tumours. In order for tumour cells to grow and metastasise, they must be able to break down their extracellular matrix. There are two main ways in which this can be achieved. Firstly, production and secretion of proteolytic enzymes such as cathepsins, collagenases and elastase, all of which have specificity for fibrin in addition to their major substrates. Secondly, adsorption of plasminogen/fibrin complexes onto their surface, and secretion of plasminogen activators (u-PA and t-PA) to activate the plasminogen to plasmin, which subsequently degrades fibrin. Experiments were performed to identify the mechanism of A375 cell fibrinolytic activity.

A375 cells were grown to confluence in 96 well microtitre plates. The tissue culture medium was replaced with medium containing plasma, matched serum or plasma which had been passed through a lysine-sepharose column (to remove plasminogen; plasminogen-depleted plasma) at concentrations of 0.63, 1.25, 2.5, 5, 10 and 20% for 4h (the peak time of D-dimer production). Supernatants were washed from the cells and the chromogenic substrate S-2251 (specific for plasmin) added overnight at 37^0C, and the absorbance at 405nm measured.

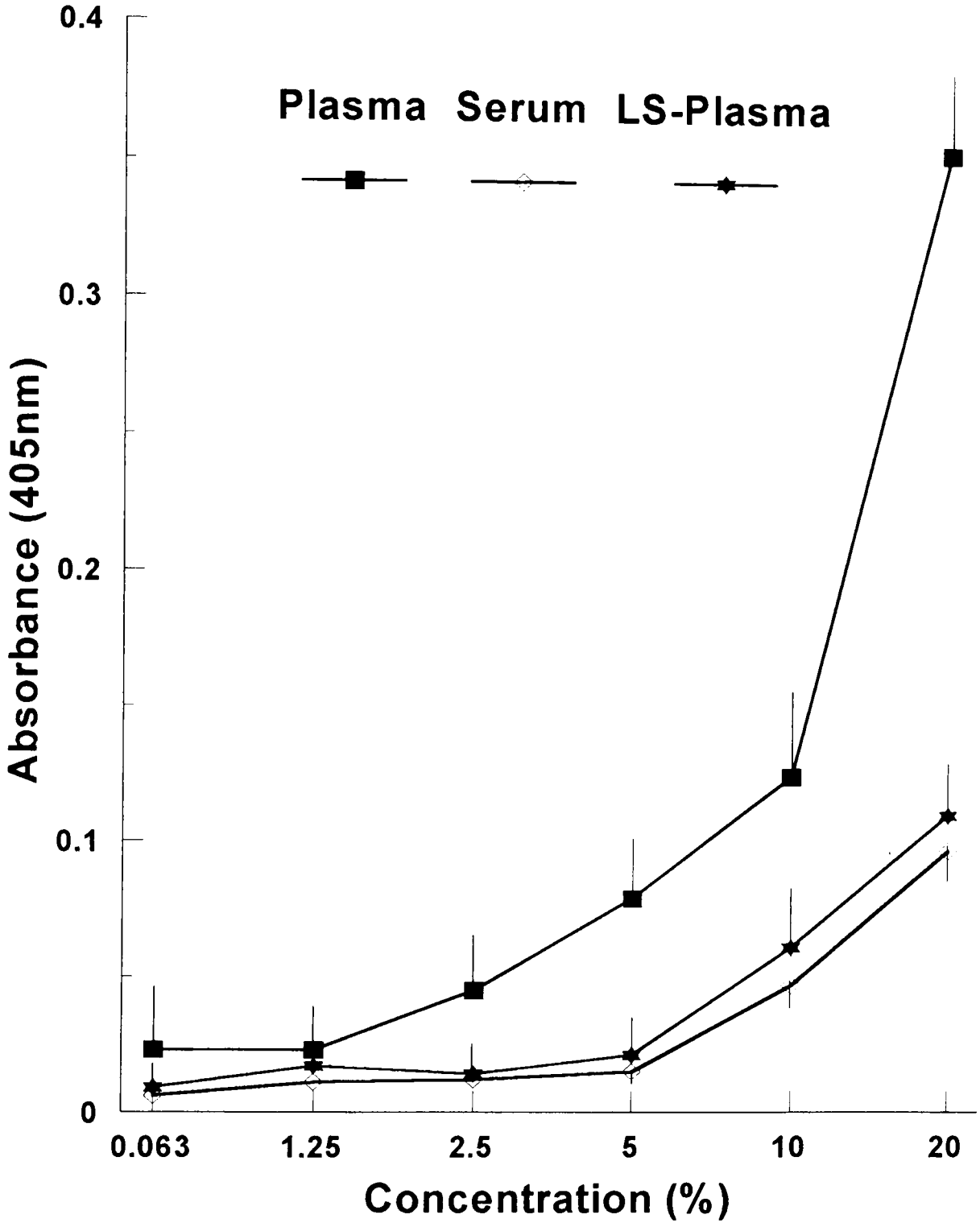


FIGURE 5.16. Plasmin activity on A375 cells treated with plasma, serum or plasminogen depleted plasma. Adherent A375 cells were incubated with HI serum, matched plasma, plasminogen depleted plasma or RPMI for 4h at 37^{0}C. The supernatants were removed and the cells further incubated with the chromogenic substrate S2251 (see section 2.3.7), and the absorbance at 405nm measured after colour development. The absorbance from RPMI and S2251 (blank; 0.147) was subtracted from test absorbances, and The means and SEMs from triplicate results are shown are shown. Plasmin activity from whole plasma was significantly greater than either serum or PDP at concentrations of 2.5% and above ($p<0.05$ by MWU and PTT), but some activity was observed in serum and PDP at 10% and 20%.

A375 cells incubated with serum and plasminogen depleted-plasma showed some plasmin activity at 10% and 20% (figure 5.16). The serum probably still contained some plasmin(ogen) after clotting had occurred, and the plasminogen depleted plasma may have contained residual amounts of plasminogen which was able to bind to the fibrin, and hence become activated by the A375 cells. Plasma however, showed higher absorbance values than either of the other preparations at concentrations of 2.5% and above ($p<0.05$ compared to serum). Maximal absorbance occurred in 20% plasma, which was considerably higher than that of serum and plasminogen depleted plasma ($p<0.05$), reflecting an increase in plasmin activity.

Clot formation was observed during the course of these experiments. Table 5.3 shows that no clot formation was observed in the serum control.

Time (h)	Serum (5%)	Normal Plasma (%)				Plasminogen Depleted Plasma				RPMI
		2.5	5	10	20	2.5	5	10	20	
0	-	-	-	-	-	-	-	-	-	-
0.5	-	-	-	-	-	-	-	-	-	-
1	-	++	++	-	-	-	-	-	-	+
2	-	++	++	++	-	+	+	-	-	+
4	-	-	-	-	--	++	++	+	-	+
18	-	-	-	-	--	+++	++	+	+	+

TABLE 5.3. Visual assessment of clot formation in serum, plasma and plasminogen depleted plasma. A375 cells ($1x10^5$/ml) were incubated in the presence of serum (5%), matched plasma (2.5-20%) or plasminogen-depleted plasma (PDP; 2.5-20%) at 37^0C in 24 well microtitre plates, and clot formation observed visually for 0-18h. This experiment was repeated twice and these results are representative. A score of: (-) indicated no observable clot formation; (+) - partial clotting, resulting in increased viscosity of the cell suspension; (++) - a solid clot which disintegrated on agitation; (+++) - a solid clot which was not dispersed by agitation. Clot formation was observed in 2.5% and 5% plasma after 1h, and in 2.5%, 5% and 10% plasma after 4h, but no clotting was seen in 20% plasma. No clots were present at any concentration of plasma after 4h. Clotting was observed in 2.5% PDP and solid clots were still present after 18h. The strength of clot observed decreased with increasing PDP concentration.

Clotting was observed with plasma and A375 cells at 1 and 2h, but as before (table 5.1, section 5.2.1), the clots were no longer present after 4h.

plasminogen depleted plasma took longer to clot than whole plasma, but clots were still present after 18h, indicating that fibrinolysis had been inhibited. Since solid clots were still present after 18h in cultures of A375 cells and PDP, experiments were performed to compare LAK cell killing of A375 cells in the presence of either plasma or PDP.

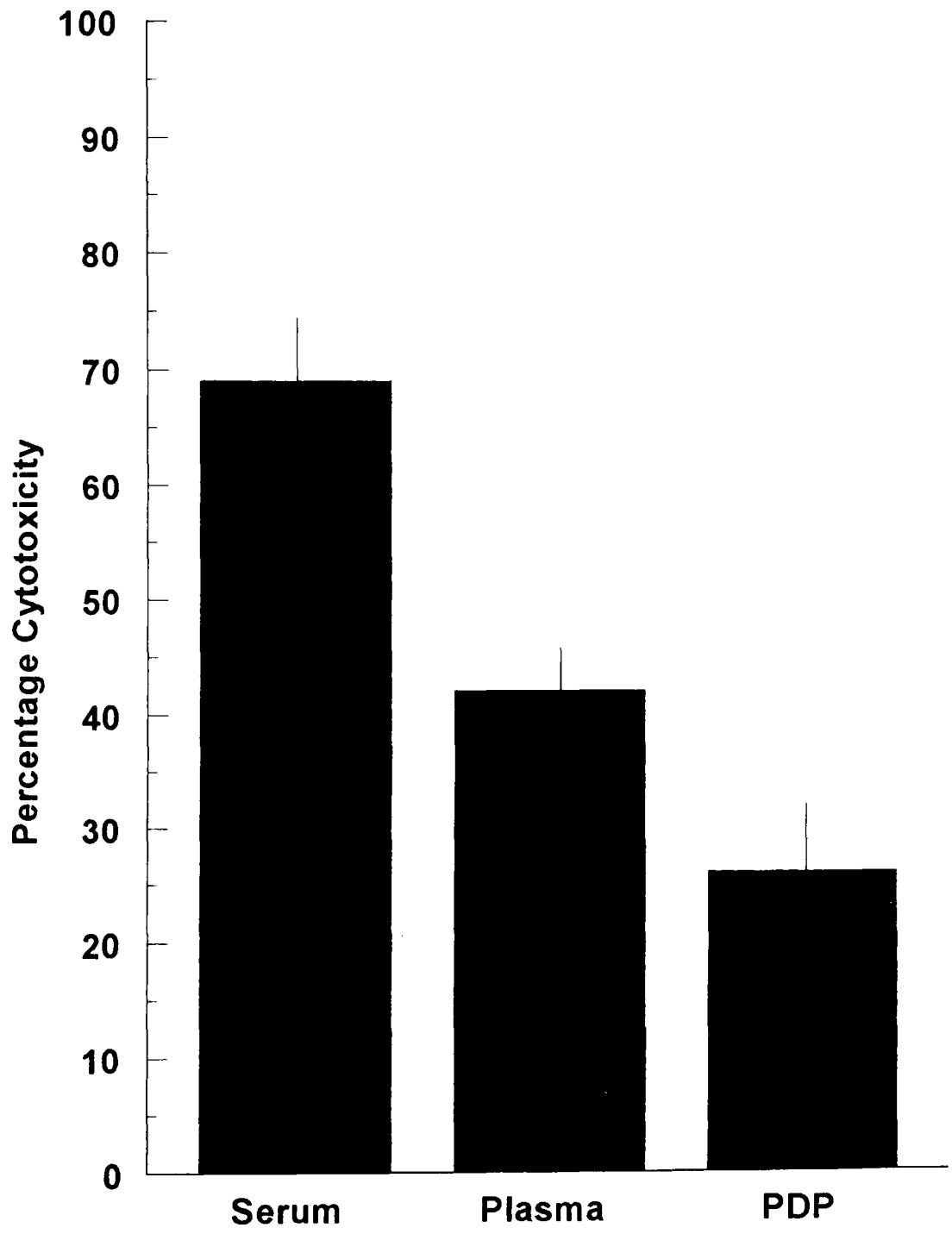


FIGURE 5.17. Inhibition of LAK cell killing of A375 cells by plasma and plasminogen depleted plasma (PDP). Cytotoxicity of $Na^{51}Cro_4$ labelled A375 cells by LAK cells was performed in the presence of HI serum (5%), plasma (5%; matched to the serum) and plasminogen depleted plasma (PDP; 5%; see section 2.3.3) after 18h. The means and SEMs from 3 experiments are shown. As previously observed LAK cell cytotoxicity was inhibited by plasma (compared to serum; $p<0.05$ by MWU and PTT), and inhibition was increased in the presence of PDP (compared to plasma; $p<0.05$ by MWU and PTT).

Figure 5.17 shows the mean maximal LAK cell cytotoxicity obtained from 3 experiments. As previously observed (section 5.1.1, figure 5.2) killing was inhibited when plasma was included in the assay, but inclusion of plasminogen depleted plasma resulted in increased inhibition compared with plasma ($p<0.05$). In addition, clots were still visible in wells containing plasminogen depleted plasma after 18h, whereas no solid clots were observed in wells containing whole plasma after 4h (see also table 5.3). The presence of a fibrin clot in the assay wells was probably responsible for the increased inhibition of cytotoxicity observed.

These results suggested that plasmin was probably the major fibrinolytic agent when A375 cells were combined with plasma, since removal of plasminogen resulted in increased inhibition of killing. Plasmin activity in fresh plasma can also be inhibited by the addition of synthetic lysine analogues such as epsilon-amino-caproic-acid (6-amino-hexanoic acid (eACA)). The following experiments examined the effect of eACA addition to plasma on inhibition of LAK cell cytotoxicity against A375 cells.

5.3 Fibrin Structure and LAK Cell Cytotoxicity of A375 Cells

5.3.1 Effect of eACA on Plasma Inhibition of Lymphocyte and LAK Cell Cytotoxicity

The synthetic lysine analogue eACA, functions by binding to lysine binding sites on plasminogen, resulting in a conformational change in the plasminogen molecule (Brockway and Castellino, 1971). For plasmin to cleave fibrin(ogen), it must bind via its lysine binding sites to lysine residues on fibrin(ogen). Blocking these lysine binding sites on plasminogen prevents the formed plasmin from binding to and enzymatically degrading fibrin(ogen).

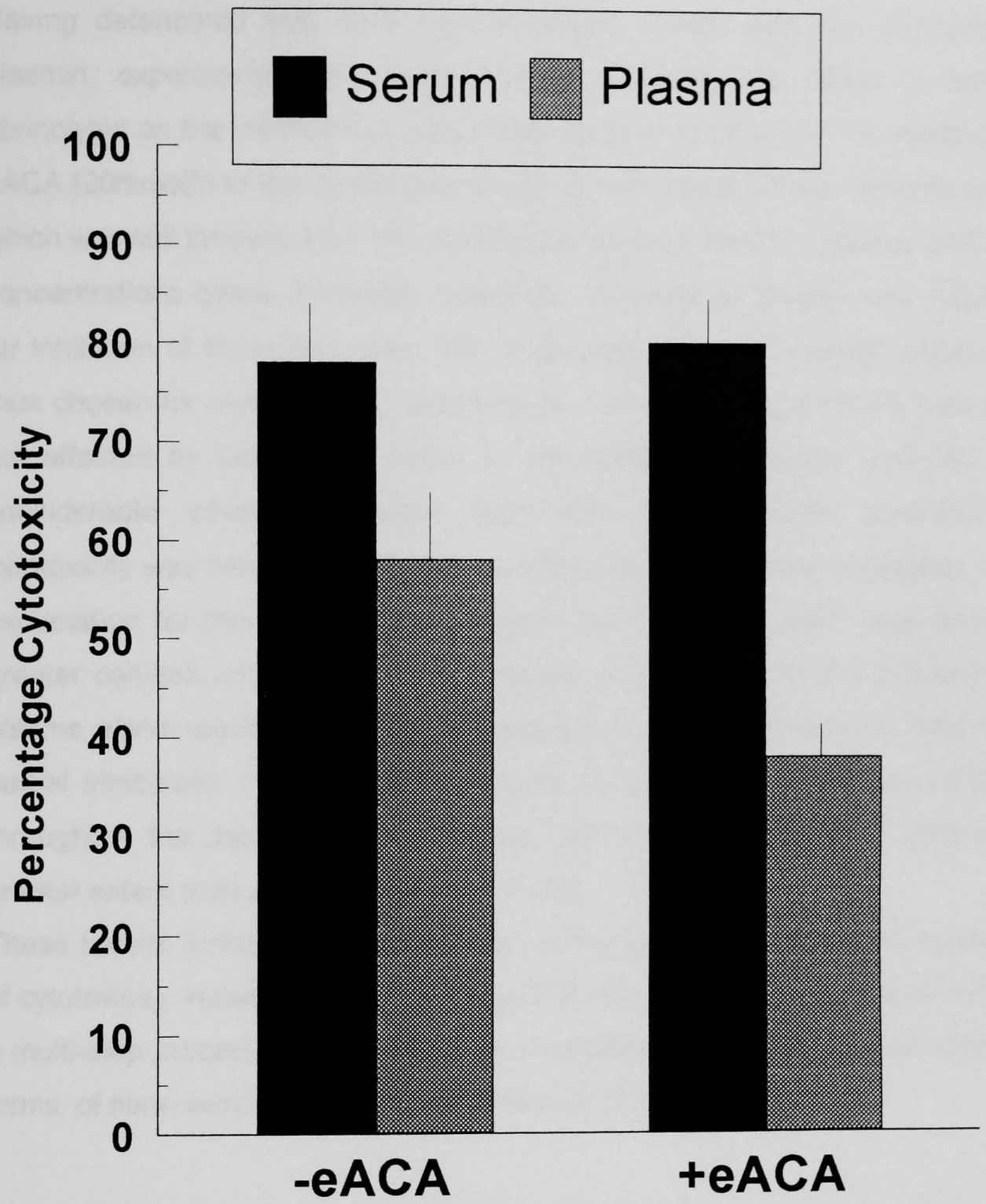


FIGURE 5.18. Killing of A375 cells by LAK cells in plasma or serum: effect of eACA. LAK cell cytotoxicity (E:T 50:1) against $Na^{51}CrO_4$ labelled A375 cells was determined after 18h in the presence of HI serum (5%) or matched plasma (5%)./ Parallel cultures also included the synthetic lysine analogue eACA (20mmol/l). The means and SEMs from 5 experiments are shown. Solid clots were observed in all wells containing eACA after 18h, but not in wells in which eACA was absent. Killing of A375 cells by LAK cells was inhibited in plasma (compared to serum; $p<0.05$ by WSR and PTT), and inhibition was further increased in the presence of eACA (compared to plasma; $p<0.05$ by WSR and PTT). Addition of eACA to cultures containing serum had no effect on LAK cell cytotoxicity ($p>0.05$ by WSR and PTT).

Having determined that A375 cell fibrinolytic activity was due primarily to plasmin, experiments were performed to determine the effect of halting fibrinolysis on the inhibition of cytotoxicity by plasma (figure 5.18). Addition of eACA (20mmol/l) to the cytotoxicity assay allowed solid clot formation to occur which was still present after 18h. A375 cells were not directly killed by eACA at concentrations below 50mmol/L. However, 20mmol/l or greater was required for inhibition of fibrinolysis after 18h. A concentration of 20mmol/l eACA was thus chosen for use in further experiments. LAK cell killing of A375 cells was not affected by addition of eACA in the presence of serum ($p>0.05$), but considerable inhibition (greater than with plasma alone ($p<0.05$)) of cytotoxicity was seen when eACA was added in the presence of plasma. One explanation for this is that the fibrinolytic activity of the A375 cells allowed greater cell-cell contact in the later stages of the assay in the presence of plasma alone resulting in a partial restoration of cytotoxic activity (and thus partial inhibition). Inhibition of fibrinolysis by eACA (*i.e.,*. retention of fibrin throughout the assay period) inhibited LAK cell killing of A375 cells to a greater extent than in plasma alone ($p<0.05$).

These results further suggest that fibrin is the factor responsible for inhibition of cytotoxicity. However, fibrin formation from fibrinogen to cross-linked fibrin is a multi-step process. It was, therefore, necessary to determine which form, or forms, of fibrin were responsible for inhibition of cytotoxicity.

5.3.2 Effect of Fibrin Crosslinking on Inhibition of LAK Cell Cytotoxicity

Fibrinogen is converted into fibrin by the thrombin-induced release of two small peptides, fibrinopeptides A and B. The resulting fibrin monomers spontaneously bind to each other to form a non-covalently linked polymer. Thrombin also activates another enzyme, factor XIII, which covalently cross-links the formed fibrin. Experiments were performed to determine whether cross-linking of fibrin was necessary for inhibition of cytotoxicity to occur.

Plasma was obtained from a patient with factor XIII deficiency (kindly provided by Dr Osier, Bournmouth General Hospital). Factor XIII was measured and the

α-subunit was not present in detectable amounts, indicating that no fibrin crosslinking was possible in the cytotoxicity assay (the factor XIII assay was kindly performed by Dr Ian Mackie, University College Hospital, London). Factor XIII-deficient plasma was compared with whole plasma and serum for its effect on LAK cell cytotoxicity against A375 cells (figure 5.19)..

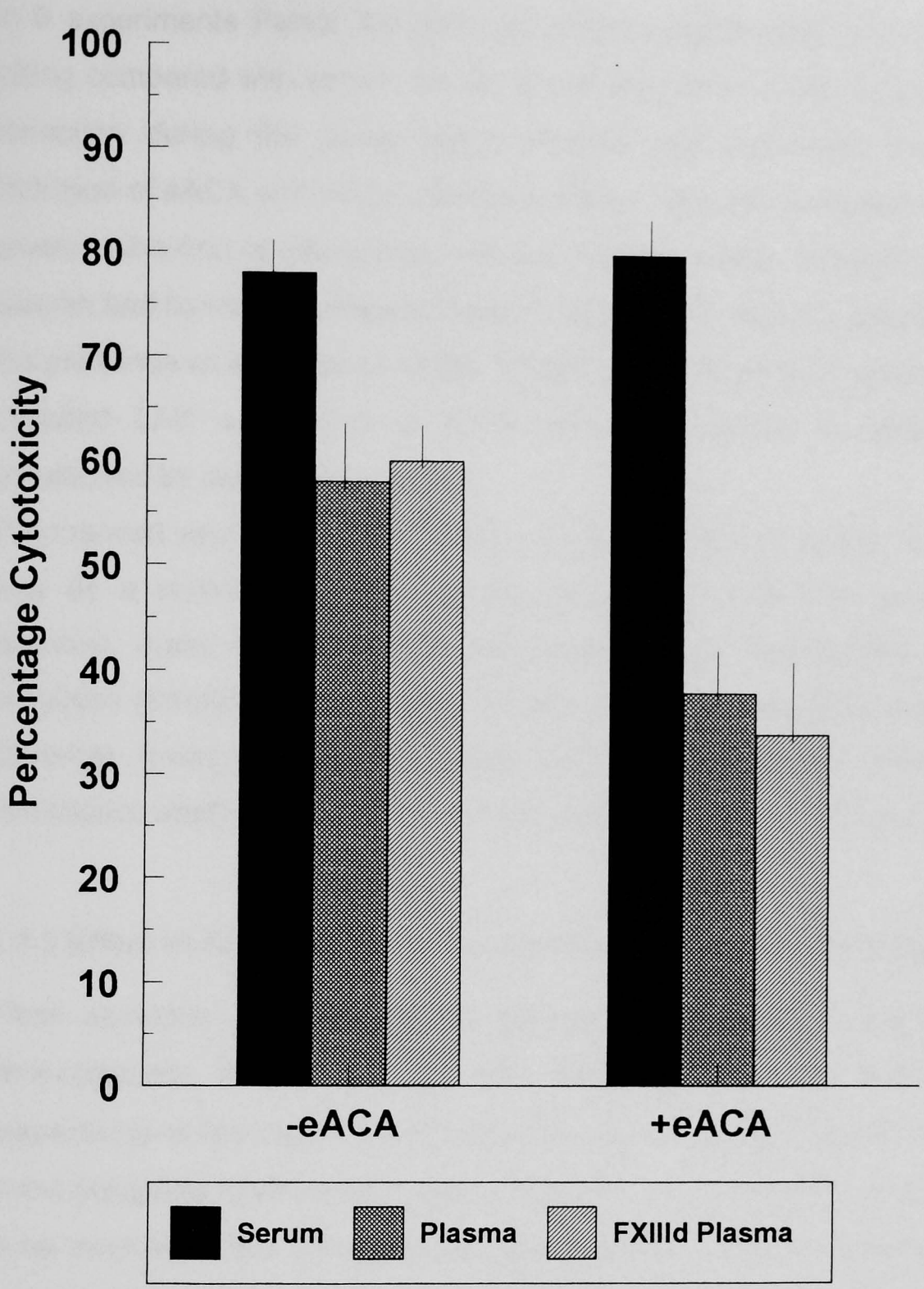


FIGURE 5.19. LAK cell cytotoxicity of A375 cells in plasma, FXIII deficient plasma or serum: effect of eACA. LAK cells were incubated with $Na^{51}CrO_4$ labelled A375 cells (+/- eACA 20mmol/l) for 18h in the presence of HI serum (5%), matched plasma (5%) or plasma deficient in factor XIII (5%). Results are means and SEMs from 6 experiments. Solid clots were present in wells containing eACA after 18h, but not in those in which eACA was absent. Cytotoxicity was inhibited by plasma and FXIII deficient plasma (compared to serum; $p<0.05$ by WSR and PTT) to a similar degree ($p>0.05$ by WSR and PTT compared to each other). Inhibition by both plasmas was increased when eACA was present in the assay (compared to plasmas -eACA; $p<0.05$ by WSR and PTT), but no difference in inhibition was observed between whole plasma and factor XIII deficient plasma ($p>0.05$ by WSR and PTT). Killing in serum was not affected by the presence of eACA ($p>0.05$ by WSR and PTT).

In 6 experiments Factor XIII deficient plasma significantly inhibited LAK cell killing compared with serum, as did whole plasma ($p<0.05$). Inspection of clot formation during the assay again showed that fibrinolysis had occurred. Inclusion of eACA with whole plasma or Factor XIII-deficient plasma resulted in greater inhibition of killing than with the plasmas alone. Inhibition of killing by plasma and factor XIII deficient plasma were not significantly different, either in the presence or absence of eACA. These results show that polymerised fibrin inhibited LAK cell killing of A375 cells, irrespective of whether it was crosslinked by activated factor XIII.

Polymerised and cross-linked fibrin in the extracellular matrix of the tumour may be a contributory factor to the inhibition of LAK cell activity *in vivo.* However, many cancer patients also have various intravascular coagulation problems (Lindahl *et al.,* 1990), among which are elevated levels of fibrin monomer. It was, therefore, of interest to continue this series of experiments to investigate whether fibrin monomer also inhibited LAK cell killing of A375 cells.

5.3.3 Effect of Fibrin Monomer on LAK Cell Killing of A375 Cells

Fibrin monomer is formed by the sequential thrombin induced cleavage of fibrinopeptides A and B (FPA and FPB) from the $A\alpha$ and $B\beta$ chains respectively of fibrinogen. The N-terminal regions of the α chains of fibrin end in the tripeptide Gly-Pro-Arg in many species, including man, and are thought to be involved in the spontaneous polymerisation of fibrin monomer. In 1978, Laudano and Doolittle prepared Gly-Pro-Arg and found that it inhibited fibrin polymerisation. The addition of a fourth residue such as sarcosine or proline significantly increased the activity of the peptide, but addition of proline had an additional advantage of making the peptide more resistant to proteolytic attack because of the presence of the imino acid. Gly-Pro-Arg-Pro was, therefore, of use in this study to distinguish between the effects of fibrin monomer and polymerised fibrin on LAK cell killing of A375 cells.

Initial experiments showed that GPRP was not directly cytotoxic to A375 cells or LAK cells at concentrations between 1-20mmol/l. In 3 experiments GPRP (20mmol/l final concentration) was added to plasma, and their combined effect on LAK cell cytotoxicity was measured. However, if GPRP was added to

plasma alone, the fibrin monomer formed could be degraded by plasmin. For this reason, a combination of GPRP and eACA was also included in the assay. Since fibrin monomer can also be cross-linked by factor XIII, a combination of GPRP and eACA were added to factor XIII-deficient plasma and the effect of all these combinations on LAK cell cytotoxicity was determined. In one experiment supernatants were collected from all wells after the 18h incubation period and stored at -70^{0}C for subsequent measurement of thrombin and D-dimer activity.

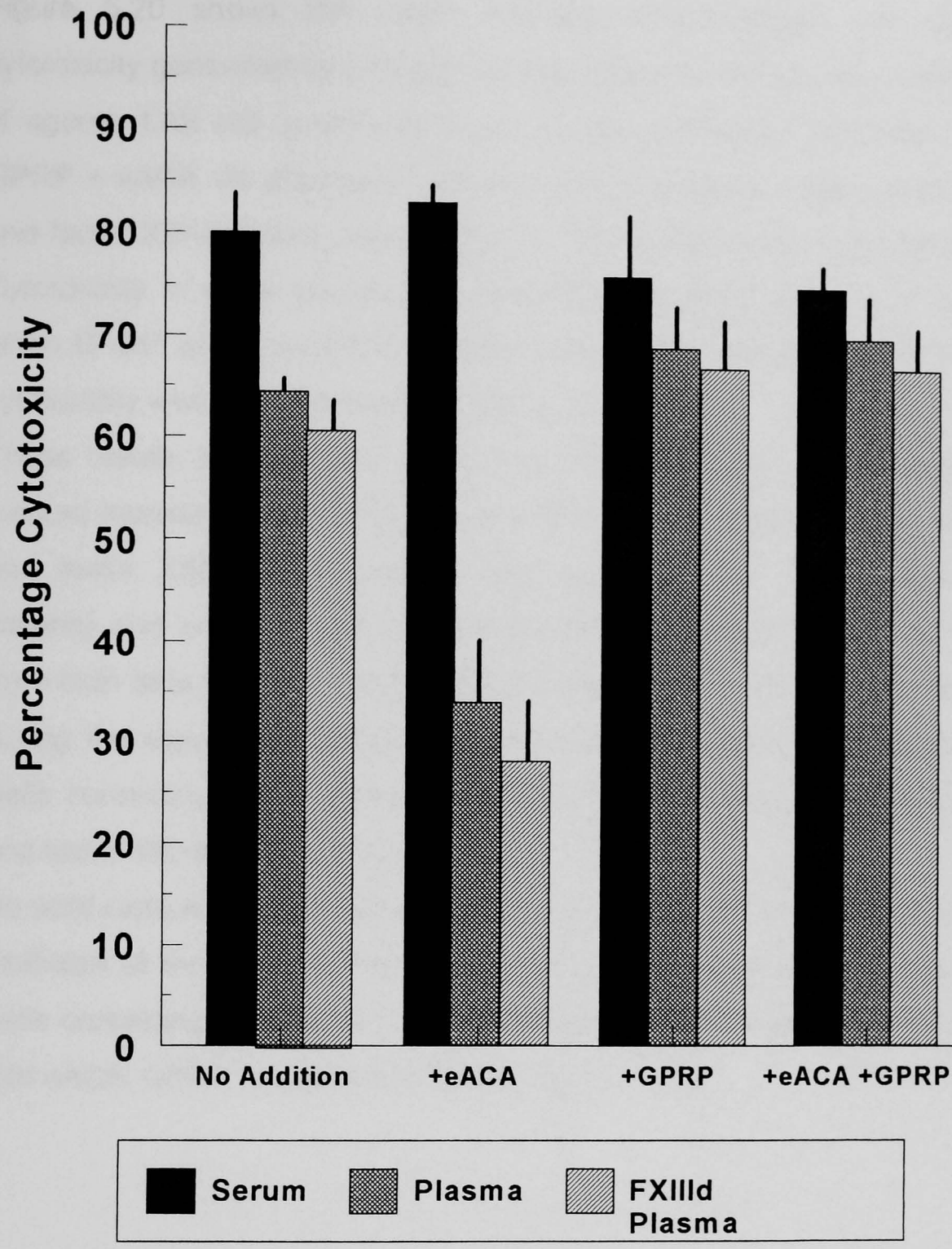


FIGURE 5.20. Killing of A375 cells by LAK cells in plasma, FXIII deficient plasma or serum: effect of eACA and GPRP. LAK cell cytotoxicity (at an E:T of 50:1) of A375 cells was performed in the presence of HI serum (5%), matched plasma (5%) or factor XIII deficient plasma after 18h. In addition the effect of eACA (20mmol/l), GPRP (20mmol/l) or eACA and GPRP was investigated. Supernatants were taken from each well for subsequent thrombin and D-dimer estimation. Solid clots were observed after 18h only in wells containing plasma or FXIII deficient plasma and eACA alone. In 3 experiments (means and SEMs shown), plasma and factor XIII deficient plasma inhibited cytotoxicity (compared to serum; $p<0.05$ by WSR and PTT), to a similar degree ($p>0.05$ by MWU and PTT compared to each other). As before, eACA increased inhibition, but cytotoxicity in all cultures containing GPRP was not significantly different to that in serum ($p>0.05$ by MWU and PTT). LAK cell cytotoxicity in serum was not affected by addition of eACA, GPRP or eACA and GPRP ($p>0.05$ by MWU and PTT).

Figure 5.20 shows the mean maximal (effector:target cell ratio 50:1) cytotoxicity generated by LAK cells in the presence of the above combinations of agents. LAK cell cytotoxicity in serum was unaffected by GPRP, eACA or GPRP + eACA. As previously observed, cytotoxicity in plasma (section 5.1.1) and factor XIII-deficient plasma (section 5.2.6) was lower than that in serum. Cytotoxicity in either plasma was greatly reduced by addition of eACA, but when GPRP alone or GPRP together with eACA were present in the assay cytotoxicity was restored almost to the serum level.

These results showed that inhibition of fibrinolysis (by eACA) resulted in a marked increase in the inhibition of LAK cell cytotoxicity induced by plasma and factor XIII-deficient plasma, and suggests that cross-linked (normal plasma) and uncross-linked (factor XIII-deficient plasma) polymerised fibrin were both able to inhibit killing. Analysis of D-Dimer in the supernatants taken during the experiment showed that <30ng/ml D-Dimer was produced in any wells containing eACA, compared with >2000ng/ml for normal plasma alone and factor XIII-deficient plasma alone .

No solid clots were observed in wells containing GPRP, but this was not due to inhibition of thrombin formation, since thrombin activity was measured in all wells containing plasma, factor XIII-deficient plasma or serum in combination with eACA, GPRP or eACA and GPRP (figure 5.21).

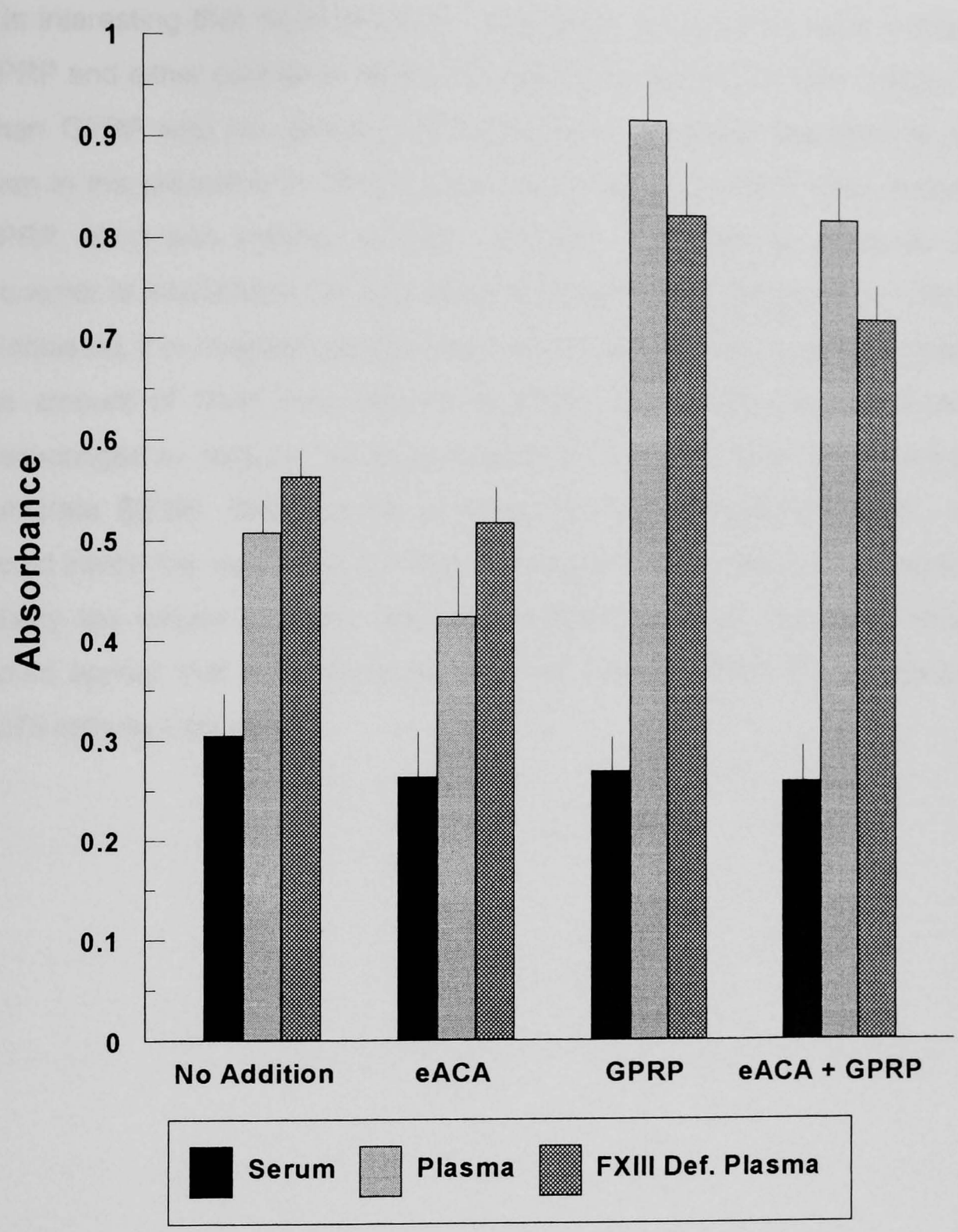


FIGURE 5.21. Thrombin activity from wells containing serum, plasma or FXIII deficient plasma +/- eACA, GPRP or eACA+GPRP. Supernatants collected from 18h cocultures of LAK cells ($5x10^6$/ml) with A375 cells ($1x10^5$/ml) in the presence of HI serum, matched plasma or factor XIII deficient plasma (+/- eACA (20mmol/l), GPRP (20mmol/l) or eACA + GPRP) were assessed for thrombin activity using the chromogenic substrate S2238 (see section 2.3.5). The results shown are means and SEMs of absorbances from 3 experiments. Cell cultures in whole plasma and factor XIII deficient plasma exhibited greater thrombin activity than in serum ($p<0.05$ by MWU and PTT), but not to each other ($p>0.05$ by MWU and PTT). In addition, thrombin activity in plasma and factor XIII deficient plasma was greater in cultures also containing GPRP (compared to plasmas alone; $p<0.05$ by MWU and PTT).

It is interesting that more thrombin generation was seen in wells containing GPRP and either plasma or factor XIII-deficient plasma (but not in serum) than when GPRP was not present. Since thrombin converts fibrinogen to fibrin, even in the presence of GPRP, it can be considered that in wells containing GPRP, fibrin was present as fibrin monomer. Although an assay for fibrin monomer is available (COA-SET fibrin monomer, KABI Diagnostica, Uxbridge, Middlesex), it is inappropriate for use in the present study because it measures the amount of fibrin monomer by its ability to increase the conversion of plasminogen to plasmin, which subsequently interacts with the chromogenic substrate S2390. Many of the samples of interest contained eACA, which would inhibit the reaction in the fibrin monomer assay, resulting in negative or falsely low values for fibrin monomer production. From the these results it would appear that fibrin monomer was not able to inhibit the cytotoxicity of A375 cells by LAK cells.

5.4 Summary of Results

1. Control and LAK cell cytotoxicity of A375 cells was inhibited in the presence of whole plasma at concentrations of 1.25%, 2.5%, 5%, and 10%.

2. Heat-inactivated plasma was not inhibitory to control and LAK cell killing of A375 cells, demonstrating that the inhibitory factor(s) were heat labile.

3. Plasma which had been adsorbed with aluminium hydroxide did not inhibit control or LAK cell killing of A375 cells, suggesting that one, or more, of the vitamin-K dependent clotting factors (FII, FVII, FIX, FX, Proteins S and C), were involved in the inhibitory process.

4. Afibrinogenaemic plasma did not inhibit control or LAK cell cytotoxicity of A375 cells, suggesting that either fibrinogen or one of its products was responsible for the inhibition of cytotoxicity observed in whole plasma.

5. Purified fibrinogen did not influence control or LAK cell killing of A375 cells, and these results suggested that fibrin formation (or a later stage of coagulation or fibrinolysis) was required for inhibition of cytotoxicity to occur.

6. Control cells, LAK cells and A375 cells all demonstrated procoagulant activity in recalcified whole plasma, as measured by observation and thrombin generation.

7. LAK cells induced clot formation in factor V, VII, VIII and IX, but not factor X deficient plasmas, suggesting that the procoagulant activity of these cells was predominantly via the extrinsic (tissue factor) coagulation pathway, and that they possessed FVa activity. Subsequent flow cytometry demonstrated the presence of factor V, factor VII and low levels of TF on the surface of these cells. Since lymphocytes do not synthesise tissue factor, it was probably absorbed onto the cell surface bound to factor VIIa.

8. A375 cells induced clot formation in factor VII, VIII and IX, but not factor V or X deficient plasmas. These results showed that A375 cell procoagulant activity was probably via the extrinsic (tissue factor) coagulation pathway, and that these cells did not possess factor V activity. As with LAK cells, clotting of factor VII deficient plasma by A375 cells may have been due to tissue factor bound to contaminating factor VIIa derived from serum in the medium used to culture the cells prior to assay. Flow cytometry confirmed the presence of TF, but not factor V on the surface of these cells.

9. A375 cells were highly fibrinolytic, and clots formed in whole plasma were lysed within 4-6h in coculture. Elevated levels of D-dimer were observed after as little as 1h in culture. Cultures of control and LAK cells induced relatively little (compared with A375 cells) D-dimer production after 18h, but solid clots were still observed in these cultures after 18h incubation.

10. A375 cells had the ability to activate plasminogen on their surface, as measured by reaction with the chromogenic substrate S2251. Incubation of these cells with plasminogen depleted plasma resulted in the presence of solid clots in these cultures after 18h, suggesting that plasminogen activation was the predominant fibrinolytic activity of these cells. Plasminogen depleted plasma inhibited LAK cell killing of A375 cells to a slightly greater extent than whole plasma. This may have been due to the presence of solid clots in the assay wells for the whole 18h incubation period.

11. Addition of eACA (an antifibrinolytic agent) to the cytotoxicity assay also increased inhibition of LAK cell cytotoxicity of A375 cells over that observed in whole plasma alone.

12. Inhibition of LAK cell cytotoxicity to A375 cells was also observed in the presence of factor XIII-deficient plasma, suggesting that fibrin cross-linking was not important in the inhibitory mechanism. As with whole plasma, addition of eACA caused increased inhibition of LAK cell killing of A375 cells in factor XIII deficient plasma.

13. Addition of Gly-Pro-Arg-Pro (a fibrin polymerisation inhibitor) to plasma or factor XIII-deficient plasma, in the presence or absence of eACA, almost completely restored LAK cell cytotoxicity of A375 cells to the level seen in serum, suggesting that fibrin monomer was unable to inhibit killing.

Chapter 6

DISCUSSION

The main objective of this study was to investigate why poor clinical responses are obtained with LAK cell immunotherapy, when LAK cells are highly cytotoxic to tumour cells *in vitro*. To this end, experiments were performed firstly to evaluate the cytotoxic activities of lymphocytes, monocytes and LAK cells against a variety of unstimulated and cytokine pretreated tumour cell lines *in vitro*; secondly, to establish *in vitro* the efficacy of chemotherapeutic drugs (Act-D and cycloheximide) on the enhancement of leucocyte, cytokine and LAK cell cytotoxicity to tumour cells; thirdly, to investigate the mechanism(s) and predominant leucocyte subtype(s) involved in lymphocyte cytotoxicity of tumour cells: and lastly to determine whether any factor(s) present in the circulation or the tumour environment inhibit the cytotoxic activity of infiltrating leucocytes and LAK cells.

The interactions between cytokines, cytotoxic drugs, immune cells, cancer cells and the vasculature during immunotherapy is highly complex. The main purpose of the experiments in chapter 3 was to isolate and investigate *in vitro,* the interactions between cytokines, tumour cells and unstimulated peripheral blood mononuclear cells, in order to better understand the contributions made by these components to tumour destruction during immuno/chemo-therapy.

Unstimulated normal lymphocytes were cytotoxic to K562 and U937 cells after 4h, but a longer incubation time (18h) was required for killing of the melanoma cell lines. Daudi cells were also insensitive to killing by lymphocytes after 4h. but moderate cytotoxicity (27%) was observed after 18h. In many previous studies (*e.g.,* clinical trials undertaken by Amgen Corp. in the Rayne Institute at St Thomas' Hospital London (unpublished data)) the K562 and Daudi (or Raji, another EBV transformed cell line) cell lines have been used as standard *in vitro* indicators of NK and LAK cell activity respectively in 4h cytotoxicity assays. However, these cell lines may not be appropriate markers of the

in vivo effect of immunotherapy, since the patient's tumour cells may be more or less responsive to treatment. Inclusion of a tumour cell line of the same type as that of the patient (or cells grown directly from a biopsy of the specific tumour to be treated) may therefore yield more information about the potential efficacy of treatment in the individual patient.

Lymphocytes were cytotoxic against the seven cell lines after 18h, but there was a heterogeneous response between the tumour cell lines. The most sensitive cell lines were K562 and U937. Lymphocyte killing of A375, DX3 and SK23 cells was augmented by target cell pre-treatment with Act-D, but cycloheximide (another protein synthesis inhibitor), was unable to augment lymphocyte cytotoxicity against any of the cell lines tested. In 2 further experiments (data not shown), no enhancement of lymphocyte cytotoxicity was observed after pre-treatment of the tumour cell lines with the cytotoxic drugs melphalan and vinblastine.

It may be inferred from these results that the mechanism of action of these cytotoxic drugs on sensitive tumours *in vivo* may not be wholly due to their direct cytotoxic activity, but may be due in part to their sensitisation of tumour cells to the cytotoxic action of lymphocytes and monocytes already present in the tumour. This may be especially important in the areas of tumour which are distant from blood capillaries, where drug concentrations can be considerably lower than those in areas closer to capillaries. It may be useful prognostically therefore, to culture cells from tumour biopsies for short periods of time in order to determine their sensitivity to killing by leucocytes in combination with various chemotherapeutic agents prior to treatment.

Freshly isolated monocytes were also cytotoxic against the melanoma cell line A375 and to K562 cells after 18h. Monocyte killing was maximal at an effector:target cell ratio of 50:1 on both cell lines. There is some controversy as to whether unstimulated monocytes are able to kill tumour cells at all (Chen *et al.,* 1986), since monocytes in culture may be inadvertently activated by contaminating LPS. To address this point, the antibiotic polymyxin-B (which bind to and inactivates LPS) was added to the tissue culture media and monocyte cytotoxicity reassessed. No difference in monocyte killing of A375 and K562 cells between untreated

and polymyxin-B treated media was seen, indicating that LPS was not responsible for the monocyte cytotoxicity observed. In addition, the media used in these experiments was determined to be non-pyrogenic in a rabbit pyrogenicity test, again indicating low levels or the absence of contaminating LPS. A more direct (and thus conclusive) test for the presence of LPS would have been the limulus amoebocyte lysate (LAL) assay, but this was not available in the department at the time these experiments were carried out.

Depletion of CD8+ cells from freshly isolated lymphocytes resulted in over 75% inhibition of A375 cell cytotoxicity, suggesting that CD8+ lymphocytes were primarily responsible for killing in this system.

Cytokines are important in the modulation of immune reactivity to tumours in three main ways. (1) They can activate immune cells and thus increase their cytotoxic activity towards tumour cells (*e.g.* IL-2, IFNγ,). 2. Some cytokines can sensitise tumour cells to the cytotoxic action of leucocytes (IFNγ, IL-1), and (3), they can act directly as cytotoxic effector molecules (*e.g.* TNFα; IFNγ, LT). Experiments were therefore carried out to examine the direct cytotoxic activity of several cytokines (section 3.1) which had been reported to have direct or modulatory activities on leucocyte killing of tumour cells.

TNFα cytotoxicity was assessed against the seven cell lines untreated or preincubated with Act-D and as with lymphocyte killing a heterogeneous response was observed. Three of the four melanoma cell lines (A375, DX3 and LT5.1) were relatively unresponsive to the action of TNFα, but increased sensitivity was seen when these cells were pretreated with Act-D. In contrast, the melanoma cell line SK23, as well as K562 and Daudi cells were more sensitive (untreated) to TNFα killing than the three melanoma lines mentioned above, but Act-D pretreatment failed to enhance TNFα cytotoxicity of these cells. Not surprisingly, U937 was the most TNFα sensitive cell line and killing was greatly augmented by pretreatment with Act-D. With the exception of SK23 and U937, the pattern of augmentation of TNFα cytotoxicity by Act-D was similar to that seen using lymphocytes as effector cells, implying that TNFα may be an

effector molecule in lymphocyte killing of these tumour cell lines. The percentage cytotoxicity values for TNFα (1000U/ml) killing were consistently lower than those observed for maximal lymphocyte (effector:target cell ratio 50:1) killing of the corresponding cell lines, whether untreated or preincubated with Act-D (section 3.2.8). This may be because TNFα was only one of a number of effector molecules in lymphocyte killing. Alternatively, the local concentration of cytokine generated when lymphocytes adhere to tumour cells may greatly exceed that used in these experiments.

The cytotoxic activities of several other cytokines were also measured on the A375 melanoma cell line. (section 3.1.3). A375 cells were resistant to killing by IL-1β, but cytotoxicity was seen when the target cells were preincubated with Act-D. IL-1β has been reported to be cytostatic for A375 cells (Onozaki *et al.,* 1985), but cytostasis could not be measured in the $Na^{51}CrO_4$ release assay because the target cells would still remain intact and thus retain chromium. Since neither IL-1β nor Act-D were cytotoxic in their own right at the concentrations used, their combination was therefore synergistic in the induction of A375 cell cytotoxicity. In these experiments IL-1β behaved similarly to TNFα, which is consistent with the considerable overlap in their reported biological activities (Balkwill, 1989).

IFNγ is known to exert it's cytotoxic activity by the *de novo* synthesis of protein (Branca and Baglioni, 1982) in the target cell, and thus requires up to 72h incubation with target cells (Stuart *et al.,* 1988) to induce maximal killing. The observed lack of direct cytotoxicity by IFNγ on A375 cells after 18h was therefore consistent with these reports. As with IL-1β and TNFα, IFNγ and LT were cytotoxic to Act-D pretreated but not untreated A375 cells. These results suggested that combined cytokine and cytotoxic drug therapy may increase the response in cytokine or drug resistant tumours. IL-2 was not cytotoxic alone or when the target cells had been pretreated with Act-D. IL-2 has not been reported to be cytotoxic to tumour cell lines, but was included in this series of experiments to confirm that any residual IL-2 remaining in LAK cell

preparations was not directly responsible for any increased cytotoxicity observed (section 3.1.3). It is clear from these results that only relatively low levels of cytotoxicity could be obtained using cytokines alone or in various combinations, even when the target cells were preincubated with Act-D. With the exceptions of IFNα treatment of hairy cell leukaemia and some lymphomas, clinical trials using one or more cytokines in combination have also shown significant therapeutic value in the minority of cases (Wong and Goeddel, 1986; Pfizenmaier *et al.,* 1987; Fransen *et al.,* 1986)). However, in the present study, the target cells remained in direct contact with cytokine for only 18h, whereas tumour cells *in vivo* may be subjected to infiltrating leucocyte derived cytokines for much longer periods of time. Further experiments were thus devised in which A375 cells were cocultured with IFNγ, IL-2 or TNFα at various concentrations for up to 72h prior to their use as target cells (+/- Act-D) for lymphocyte or monocyte cytotoxicity (section 3.3). In the course of these experiments the initial number of cells placed in each flask was the same. After the incubation with cytokine the number of viable cells was assessed. When A375 cells were incubated with IFNγ for 72h, a significant dose dependent decrease in cell yield was observed compared to that of untreated cells This could either have been due to the direct cytotoxic action of IFNγ on A375 cells after 72h, whereas 18h (see above) was insufficient to demonstrate IFNγ cytotoxicity, or IFNγ was cytostatic for A375 cells after 72h, and the observed decrease in cell yield was the result of natural cell wastage over the incubation period.

Under the same experimental conditions, a significant increase in cell yield was observed when A375 cells were incubated with IL-2 for 48h, suggesting that IL-2 was a growth stimulatory factor for these cells. In order for IL-2 to stimulate cell growth it must first bind to its receptor. Further experiments using flow cytometry were therefore carried out to determine if these receptors were present on A375 cells (3.1.4). In 3 experiments no p75 receptors were found on A375 cells, but a mean of 11456 +/- 4267 p55 (CD25) receptors were detected. The presence of

IL-2 receptors on A375 cells provided further evidence for IL-2 as a growth stimulatory molecule for A375 cells. Other tumour cell lines (Rimoldi *et al.,* 1993) have recently been reported to express low affinity (p75) and (p55) as well as high affinity IL-2 receptors (both p75 and p55). The presence of IL-2 receptors on the tumour cells of patients undergoing IL-2 immunotherapy may be a negative prognostic indicator for this form of treatment. However, in the case of IL-2/LAK cell therapy this growth stimulatory effect of IL-2 on tumour cells may be outweighed by IL-2 stimulation of tumouricidal activity by LAK cells and tumour infiltrating lymphocytes. As with tumour sensitivity to cytotoxic drugs, the determination of IL-2 receptor numbers on tumour biopsies prior to treatment may be clinically beneficial. Incubation of A375 cells with TNFα had no significant effect on the yield of viable cells after 72h compared with the untreated cultures.

Pretreatment of A375 cells with IFNγ for 48h or 72h resulted in increased killing of these cells by lymphocytes and monocytes. IFNγ pretreatment may upregulate certain molecules (*e.g.* MHC class I and II) on the target cell surface which enhance lymphocyte recognition of or adherence to tumour cells, resulting in increased cytotoxicity. IFNγ is known to upregulate the expression of HLA class II molecules on tumour cells (Pfizenmaier *et al.,* 1987). This increase in class II molecule expression may have been responsible for the observed increase in lymphocyte killing of A375 cells. Alternatively, the target cells may have been "weakened" by incubation with IFNγ, resulting in increased sensitivity of the tumour cells to killing by leucocytes. Incubation of A375 cells with IFNγ for 48h, followed by Act-D for 3h induced a synergistic increase in lymphocyte cytotoxicity. This may be the result of target cell attack via both of the above mechanisms. Act-D may have weakened the target cells by inhibition of protein synthesis, and hence inhibition of the target cell repair systems, whereas IFNγ may have induced the target cells to express molecules recognised by lymphocytes. These results thus provide further evidence to support the use of combined immunotherapy

and chemotherapy, although the sensitivity of each tumour to these cytotoxic agents should be determined prior to treatment.

Preincubation of A375 cells with a combination of IFNγ and IL-2 (section 3.3.1) did not result in any increase in lymphocyte cytotoxicity over that induced by IFNγ alone, which is in agreement with other published data (Dealtry *et al* 1987). In contrast, monocyte cytotoxicity was synergistically augmented by IFNγ and IL-2 presensitisation of tumour cells. This combination of cytokines has been reported to produce an additive therapeutic effect when administered to mice bearing pulmonary metastases or intraperitoneal growths of weakly immunogenic tumours (Kedar *et al*, 1992). Tumour cell pretreatment with IFNγ and TNFα has been reported to result in a synergistic increase in lymphocyte cytotoxicity (Balkwill and Smyth, 1987). However, in this study, no further increase in cytotoxicity was observed when TNFα was added in combination with IFNγ. Act-D pretreatment did not induce any further increase in lymphocyte or monocyte cytotoxicity using combinations of cytokines than with IFNγ alone.

During cytokine/cytotoxic drug treatment of cancer, the therapeutic agents (such as TNFα, LT and IFNγ) may interact, not only with tumour cells directly, but with host lymphocytes and monocytes, both in the peripheral blood and in the tumour mass, to further augment the anti-tumour response. Since IL-2 was not cytotoxic for the tumour cell lines used in this study (IL-2 has not been reported to be cytotoxic for any tumour cell lines), it is likely that its main therapeutic effect is activation of lymphocytes *in vivo* to the tumouricidal state (LAK cell production). In order to overcome the toxic side effects of high dose IL-2 infusion into patients undergoing immunotherapy clinical trials, leucocytes were removed from the peripheral blood of cancer patients, and activated with high concentrations of IL-2 prior to their reinfusion into the cancer patients. The next phase of experimentation in this study was thus to investigate the tumouricidal properties of LAK cells, and to assess their interactions with other blood components and normal cells (*in vitro*),

which they encounter on their passage through the peripheral blood and extravascular environment towards the tumour (Chapter 4).

IL-2 stimulates lymphocytes to become highly cytotoxic to a wider variety of cell lines than unstimulated lymphocytes (LAK activity). The optimum concentration of IL-2 required to generate LAK activity against A375 cells was found to be between 50-250U/ml, and maximal cytotoxicity was demonstrated by lymphocytes which had been cultured in the presence of IL-2 (250U/ml) for as little as 24h (section 4.1.3). The mean cytotoxicity of control (lymphocytes cultured for the same period of time as LAK cells, but excluding IL-2) cells for the melanoma and Daudi cell lines was slightly higher than that seen using fresh lymphocytes as effector cells (section 4.1.7, Table 1). This slight increase may be due to activation of the lymphocytes by a component of FCS (Lamph *et al.,* 1988). Cytotoxicity of some tumour cell lines (A375, DX3, SK23 LT5.1) by control cells was augmented when they were pretreated with Act-D. This occurred in the same general pattern, and to a similar degree, as that observed for fresh lymphocytes (section 4.1.7). In contrast, LAK cells were considerably more cytotoxic to all cell lines than either fresh lymphocytes or control cells, and this cytotoxic activity was not further increased by preincubation of the tumour cells with Act-D. The very high levels of LAK cell cytotoxicity to the tumour cells may mask any effect due to Act-D. Another explanation of these results is that LAK cell killing may employ a different mechanism of cytotoxicity than lymphocytes or control cells, which is independent of tumour cell repair systems involving protein synthesis. Whereas lymphocytes required 18h incubation with target cells, LAK cells were cytotoxic to tumour (A375) cells after as little as 1-2h in coculture (section 4.1.3).

Fresh lymphocyte, control and LAK cell cytotoxicity against A375 cells was almost completely depleted when the CD8+ lymphocyte population was removed by cell sorting (section 4.1.6), indicating that these cells were primarily responsible for cytotoxicity against this cell line. This hypothesis was supported by the observation that CD16+ cell depletion did not significantly alter lymphocyte cytotoxicity against A375 cells. Reports in the 1980s (Herberman *et al.,* 1987) suggested that LAK

activity was induced primarily in the CD16+, CD3- large granular lymphocyte population (NK cells). CD8+ cytotoxic T-cells were subsequently found to possess LAK activity after stimulation with IL-2 (ref). The predominant lymphocyte subtype present in many solid tumours are of this latter type (Lotzova, 1992). It is now considered that activated NK cells may act within the circulation and have a role in the prevention of metastatic tumour spread, while CD8+ LAK cell tumouricidal activity is directed more towards extravasation, infiltration and cytotoxicity of neoplastic cells within the tumour mass.

IL-2 treated monocytes (>95% purity) did not generate any increased cytotoxicity of A375 cells compared to unstimulated monocytes (E:T ratio 50:1). In 1987 Malkovsky and co-workers reported that recombinant IL-2 enhanced monocyte cytotoxicity against tumour cells. However, of the two target cell lines tested only one (T24, a human bladder cancer cell line) demonstrated increased sensitivity to IL-2 treated monocytes (E:T ratio 40:1), whereas McSa-1 (derived from a murine fibrosarcoma), which is a known LAK cell sensitive cell line, was not sensitive to IL-2 stimulated monocytes. These data suggest that although increased monocyte cytotoxicity may be stimulated against some cell lines by IL-2, LAK cells are able to kill a much wider spectrum of tumour cell types. LAK cells would therefore be a better choice of effector cell for adoptive immunotherapy than would monocytes. In addition, monocytes are adherent to their culture vessels, and would require additional treatment in order to remove them from the flasks. This treatment (usually EDTA) may block the function of the cell adhesion molecules on the monocyte surface, which could be involved in monocyte homing to the tumour site. Lastly, monocytes are present in peripheral blood in much lower numbers than lymphocytes. It would therefore be much more difficult to obtain monocytes in sufficient numbers for immunotherapy.

There are several mechanisms by which lymphocytes and LAK cells are able unrestrictedly to kill tumour cells. These include the release of soluble molecules such as TNFα, LT and IFNγ, or the release of the calcium dependent pore forming proteins, perforins. Monoclonal blocking

antibodies against TNFα and LT were available in the department, and were included in the cytotoxicity assay to determine whether they would inhibit control or LAK cell killing of A375 cells (section 4.4). In addition an "irrelevant" monoclonal antibody of the same IgG subtype (anti retinal antigen-S) was included as an antibody control. Control (lymphocyte) killing was partially inhibited by anti-TNFα and almost completely inhibited by anti-LT. Anti-antigen-S had no effect on control cell killing. These results suggest that lymphotoxin and tumour necrosis factor were the primary effector molecules used by lymphocytes in this system. LAK cell killing was again partially inhibited by anti-TNFα, and in contrast to lymphocyte killing, was only partially inhibited by anti-LT. This may have been because the percentage specific cytotoxicity was much higher using LAK effectors than control cells. The percentage inhibition of cytotoxicity was very similar for control (41%) and LAK (43%) cells. Thus the amount of antibody added to the LAK cell cultures was insufficient to completely inhibit killing, even though the amount used was the highest which could be obtained (1 in 2 final dilution of stock antibody) in this assay system. Alternatively, LAK cells may employ another effector mechanism such as perforin mediated cell lysis, in addition to secretion of TNFα and LT. It can be concluded from these results however, that TNFα and LT play a highly significant (if not exclusive) role in lymphocyte and LAK cell mediated killing of A375 cells.

TNFα and LT alone were relatively ineffective as direct cytotoxic agents on tumour cells, even at over 1000U/ml, but were directly implicated (see antibody inhibition of killing above) in lymphocyte and LAK cell killing of A375 cells. This apparent contradiction could be explained by the previously stated hypothesis that cell adhesion is required for cytotoxicity to occur, and that the concentration of cytokine secreted into the intercellular junction may far exceed that used in these experiments. The sensitivity of the tumour cell lines to control and LAK cell supernatants was therefore examined (section 4.3.1). Cytotoxicity of the tumour cell lines by control and LAK cell supernatants was consistently considerably lower than the killing observed with control and LAK cells. The maximum

level of tumour cell killing by the control cell supernatants was very similar to TNFα cytotoxicity in five of the seven cell lines (SK23 and Daudi cells were more responsive to TNFα than to supernatant). Slight (but not statistically significant) increases in killing were observed on some cell lines when the target cells were preincubated with Act-D, which generally matched the augmentation seen with TNFα or control cells as effectors. LAK cell supernatants were more cytotoxic to all cell lines than control cell supernatants, and this killing was augmented on five of the tumour cell lines in the same pattern as TNFα. As with control cell supernatants, the maximum killing seen with LAK cell supernatants was considerably lower than that observed using intact cells as effectors. It was interesting to observe such a general similarity between the cytotoxicity of the cell supernatants with that of TNFα (and LT), considering that the supernatants comprise a heterogeneous mixture of effector molecules (Limb *et al.,* 1989). Since control and LAK cell killing was inhibited by monoclonal antibodies to TNFα and LT, this data would suggest that these cytokines behave in a similar fashion to the recombinant forms when contained in complex mixtures. TNFα can also be expressed as a 23kDa membrane bound cytokine on activated lymphocytes (Ichinose *et al.,* 1988; Bakouche *et al.,* 1988), and this may account in part for the increased LAK cell cytotoxicity compared with that of the supernatants, as well as the inhibition of LAK cell killing by anti-TNFα. In addition, another member of the TNF family, fas or APO1, is implicated in cytotoxic lymphocyte mediated killing of target cells (Tartaglia *et al*, 1993). T-cell mediated crosslinking of the fas ligand on the target cell surface can result in the initiation of apoptosis, and this mechanism may also account for the increased cytotoxicity observed when effector and target cells were in direct contact.

To test whether cell-cell contact was necessary for cytotoxicity, control or LAK cells were incubated with A375 cells (+/- Act-D) in the presence or absence of 0.8u pore size polycarbonate membranes (section 4.3.1). The membranes allowed the diffusion of soluble molecules from effector cells to target cells (and vice-versa), while preventing the cells from

coming into direct contact. Cytotoxicity of A375 cells by control and LAK cells was almost completely inhibited by the presence of the membranes. However, some killing was observed when the target cells were pretreated with Act-D, indicating that a soluble effector molecule, or molecules, had come into contact with the tumour cells, and that sensitisation of the tumour cells was required for killing to occur. This pattern of cytotoxicity has already been observed using recombinant soluble cytokines (TNFα, LT, IL-1β and IFNγ) (section 4.1.7), and it is likely that these molecules were responsible for cytotoxicity in the presence of the membranes. In summary, cell-cell contact between effector and target cells resulted in more efficient killing of the target cells than with cytokines alone, and this increased cytotoxicity may have been caused by an increased cytokine concentration at the intercellular junction, or by exposure of the target cells to membrane bound effector molecules or by a combination of these mechanisms.

Prior to their infiltration into the tumour mass, LAK cells first come into contact with autologous unstimulated peripheral blood leucocytes and endothelial cells and, on passing out of the blood stream, fibroblasts. In this study LAK cells were not cytotoxic to autologous lymphocytes or monocytes (section 4.2.2). There are conflicting reports as to whether LAK cells kill autologous lymphocytes and monocytes *in vitro* (Balkwill, 1989). LAK cells were highly cytotoxic for allogeneic human umbilical vein endothelial cells and fibroblasts. Control cells showed some cytotoxic activity to endothelial cells after 4-18h, but this may have been due to partial activation of the lymphocytes by components of the FCS, as discussed previously. However, control cells did not kill fibroblasts after 18h in culture. This may be because fibroblasts are more robust cells than HUVEC and would thus be more resistant to killing by partially activated lymphocytes. Extrapolation of these results to the *in vivo* environment would suggest that reinjected LAK cells adhere to and non-specifically kill endothelial cells and underlying fibroblasts. These interactions would result in severe damage to the blood vessel lining, allowing the extravasation of both blood cells and plasma. This vascular

leakage syndrome has been reported as a common side effect of IL-2/LAK cell immunotherapy (Gemlo *et al.*, 1988). The non-specific adherence of LAK cells to endothelium in the peripheral circulation may inhibit LAK cell homing to the tumour site (assuming that LAK cells are able to home), resulting in the observed low numbers of effector cells arriving at the tumour, compared with the number of cells inoculated.

Having determined that IL-2 activated leucocytes efficiently killed a wide range of tumour cell types *in vitro*, the following chapter of results investigates the difference between the high levels of cytotoxicity observed *in vitro*, and the relatively poor clinical responses obtained in clinical trials using adoptive immunotherapy with LAK cells (chapter 5).

Blood samples taken from patients who have undergone LAK cell therapy show considerable LAK cell activity against allogeneic and autologous tumour cells for several days after the initial infusion, although little LAK cell homing or tumour cytotoxicity has been observed *in vivo*. Reintroduction of IL-2 activated lymphocytes into the bloodstream of cancer patients results not only in their interaction with cellular components of the blood and extravascular tissue, but also with various molecules in the circulation and extracellular matrix. One major environmental difference between LAK cell therapy and the cytotoxicity assay is that LAK cells are introduced into an *in vivo* environment consisting predominantly of plasma, and *in vitro* assays are generally performed in serum. Experiments were therefore performed to determine if LAK cell cytotoxicity *in vitro* was altered by the presence of plasma instead of serum in the assay. A375 cell killing by control and LAK cells was significantly, but not completely, inhibited in the presence of plasma compared with serum. Using purified fibrinogen and thrombin Gunji and Gorelik in 1988 reported that 5-10% plasma or serum could be toxic to effector or target cells in a murine system. No data was shown to substantiate this claim, and the only reference quoted (Fogel *et al.*, 1978) did not include plasma in the study. Thereafter, in the murine report and a later paper using human effector and target cells (Gunji *et al.*, 1990), they only reported data using a 5% or lower concentration of plasma in the cytotoxicity assays. A similar unsubstantiated claim for the

toxicity of serum or plasma at concentrations greater than 5% was made in a paper by Shinji Atagi in 1992, although in this study, cytotoxicity in 10% serum was not different from that in 5% or lower.

Having determined that control and LAK cell cytotoxicity of A375 cells was inhibited in the presence of plasma it was next necessary to determine which component(s) of plasma was responsible for the inhibition. There were two main ways in which this could be approached. First, the use of purified molecules such as fibrinogen and thrombin to generate a clot in the assay plate, and to examine the effect of this and various anticoagulants and inhibitors of fibrin polymerisation on the inhibition of cytotoxicity. This was the main approach taken by Gorelik and others. Although the use of such purified systems gives more definitive results than the use of whole plasma, the results do not accurately reflect the physiological environment to which LAK cells are exposed on reinjection into the patient. In addition, the integrity and pore size of the fibrin gels generated using purified molecules is different to those produced using plasma, and thus are not representative of the structure and composition of fibrin formed within and around solid tumours. The second approach was to continue using plasma derived clots in the assays, but to modify the plasma in such a way that the role of the individual components of the coagulation system in the inhibition of cytotoxicity could be identified. The advantage of this is that all the components of the coagulation system are present together, so the interactions between them are more representative of those which occur in the tumour environment. It was this second approach which was chosen for further experiments in this study.

No inhibition of A375 cell killing by control or LAK cells was seen in plasma which had been heat inactivated at 56 degrees for 45 minutes, indicating that a heat labile molecule was involved in inhibition of cytotoxicity. (section 5.1.2) A similar (though not complete) restoration of cytotoxicity was seen when plasma had been adsorbed with aluminium hydroxide. This showed either that one of the vitamin-K dependent clotting factors was responsible for inhibition or that the process of clot formation itself (*i.e.* fibrin formation) was the causative agent. To test

this, the cytotoxicity assay was performed in the presence of afibrinogenaemic plasma from one of the patients attending the haemophilia centre. Again, no inhibition of killing was observed, indicating firstly that fibrinogen or some later component of the clotting process was the inhibitory factor, and secondly that cytotoxicity was not inhibited by any of the other components of the coagulation system present in normal plasma.

The role of fibrinogen in the inhibition of cytotoxicity was examined by addition of a commercial fibrinogen preparation to the cytotoxicity assay, either alone or in the presence of serum. Serum was present as a control for potential nutrient deprivation during the course of the experiments. Unfortunately, this preparation of fibrinogen proved to be directly cytotoxic for the tumour cells. It was therefore decided to further purify the fibrinogen by gel filtration (section 2.4.9). After gel filtration the cytotoxic activity was present in the low molecular weight fraction, whereas the fractions containing clottable fibrinogen were no longer cytotoxic to A375 cells (section 5.1.5). The presence of fibrinogen in the assay neither enhanced nor inhibited control or LAK cell cytotoxicity of A375 cells, thus confirming that the process of clot formation (fibrin generation) was required for inhibition of cytotoxicity to occur. These results are in general agreement with those published by Gorelik and others using purified fibrinogen and thrombin, and would suggest that fibrin deposition within the stroma of solid tumours prevents the adherence of infiltrating lymphocytes (and monocytes) to the tumour cells, resulting in a suppression of immune reactivity towards the tumour mass.

Many tumour cell types exhibit procoagulant activity, which may be specific or non-specific. Specific mechanisms include the production of factors (such as tissue factor, factor X, thrombin) by the tumour cells which result in the activation the of prothrombin, or the secretion of cytokines which activate mononuclear leucocytes and/or endothelial cells. Non-specific mechanisms include activation of the coagulation system by products released from necrosing tumour cells or from normal cells which have been disrupted by tumour invasion, or by the

stimulation of a host inflammatory response. Experiments were therefore performed in which RPMI, control cells, LAK cells and A375 cells alone or in combination with control or LAK cells at an effector:target cell ratio of 50:1 were incubated with fresh citrated plasma for 0, 1, 2, 4 or 18h.(Results Chapter 3) Observation of clot formation showed that both control and LAK cells induced the formation of solid clots within 1h, which were still present after 18h. In contrast, A375 cells induced clot formation within 1-2h, but no solid clot was evident after 4h even when A375 cells were mixed with either effector cell type. Thrombin activity against the chromogenic substrate S-2238 was measured on supernatants from these cultures and confirmed the visual observation of clot formation. These results demonstrated that lymphocytes, LAK cells and A375 cells possessed procoagulant activity, and that A375 cells were also fibrinolytic.

In order to determine the nature of the procoagulant activity by each cell type, LAK cells or A375 cells were incubated with fresh citrated plasma or plasmas deficient in various coagulation factors (section 5.2) for 4h (After this time the fibrinolytic activity of A375 cells would result in fibrin degradation) and, as before, thrombin generation was measured in the supernatants (section 5.2.2). A375 cells were unable to activate factor X directly (section 5.2.2) or to clot factor X deficient plasma, indicating that these cells do not possess cancer procoagulant activity (Gordon *et al.*, 1975). Both A375 cells and LAK cells induced clotting of factor VII deficient plasma, suggesting that they both expressed tissue factor activity. Subsequent flow cytometric analysis for tissue factor antigen on the surface of these cells (section 5.2.2) confirmed these observations. The presence of tissue factor on A375 and LAK cells enabled them to bypass the intrinsic pathway of coagulation, and thus induce clots in factor VIII and factor IX deficient plasma. Although A375 cells produce tissue factor, lymphocytes (and thus LAK cells) have not been reported to secrete this factor. It is likely therefore that LAK cell surface tissue factor was complexed with factor VIIa derived from the serum in which the cells were previously cultured. The predominant procoagulant activities of these cells were therefore via the extrinsic pathway. LAK

cells (but not A375 cells) were also able to clot factor V deficient plasma, implying that they possessed direct factor V activating activity, or had factor V present on their surface. Again, flow cytometric analysis confirmed the presence of factor V antigen on the surface of LAK cells, and its absence on A375 cells. Synthesis of factor V by lymphocytes has recently been reported (Shen *et al.,* 1993). Whether fibrin deposition within and around solid tumours is beneficial to the patient or to tumour growth is not fully understood. In many inflammatory reactions fibrin deposition acts to isolate the inflammatory lesion from the host so that it can be easily dealt with by the host immune response. Cancer has been likened to an inflammatory lesion, and like other inflammatory conditions are characterised by the infiltration of leucocytes. The procoagulant activity of LAK cells is therefore consistent with an attempt by the immune system to isolate the tumour from the host. On the other hand, the procoagulant and fibrinolytic properties of tumour cells would suggest that they are able to lay down their own extracellular matrix when required and to degrade it in order to grow or metastasise.

The blood vessels within tumours are more permeable to plasma proteins than those of normal tissues,. This is due firstly to a decrease in the organisation of the endothelium, with frequent gaps between endothelial cells and often incomplete or absent basement membrane; and secondly to the release of vascular permeability factors from the tumour cells. Extravasated fibrinogen is thus clotted in the tumour environment, and often forms the initial matrix for the growth of tumour cells prior to the deposition of collagen. In order to grow and metastasise, tumour cells must have the capacity to degrade their extracellular matrix (*i.e.* collagen and fibrin). Collagen is degraded by collagenases secreted by the tumour cells. There are two ways in which fibrinolysis may occur within the tumour stroma. 1. fibrin is susceptible to the action of proteolytic enzymes secreted by the tumour cells such as collagenases, cathepsins and elastase. 2. the tumour cells may adsorb or specifically bind to the plasminogen-fibrin complex, and subsequently secrete plasminogen activators (uPA or tPA) to activate plasminogen to plasmin, resulting in fibrinolysis. Within the tumour mass there is a

continuous process of fibrin formation, and fibrinolysis at the growing edges of the tumour. The fact that there is always solid fibrin within the tumour is a result of the continuous supply of fibrinogen from the circulation. This is not the case in the *in vitro* assay plate, where the supply of fibrinogen is fixed. In this case, after the fibrinogen has been clotted, the fibrinolytic activity of the tumour cells will completely degrade the clot, leaving no solid fibrin within the plate. Consequently, the effector and target cells can again come into close contact, and cytotoxicity is re-established. This is the probable reason why the inhibition of cytotoxicity observed in this study was less (around 20% maximum) than that observed by Gorelik and others using pure clotting factors, where fibrinolysis was not observed. The use of plasma in the assays, and hence the observation of both clotting and fibrinolysis, is therefore more representative of the role of coagulation within tumours *in vivo* than is the use of purified clotting factors. In this study experiments were performed to determine the nature of A375 cell (as well as control and LAK cell) fibrinolytic activity. The supernatants taken previously for analysis of thrombin generation were also tested for D-dimer, a marker of fibrinolytic activity (section 5.2.4). After 18h a small amount of D-dimer was measured in the cultures containing control or LAK cells alone, indicating that these effector cells probably express a low level of fibrinolytic activity, although it was insufficient to eliminate all of the solid fibrin from the assay well. This low level of fibrinolytic activity by lymphocytes and LAK cells may be of use in the process of tumour infiltration by these cells, although the process would be relatively slow when compared to the fibrinolytic activity of the tumour cells. In all cultures containing A375 cells, very high levels (> 2μg/ml) of D-dimer were measured, indicating that A375 cells were highly fibrinolytic. This correlated well with the observed pattern of clot dissolution in the assay plates. To determine if the observed fibrinolytic activity was due to plasmin, A375 cells were incubated for 4h with plasma, serum, or plasma which had been passed down a lysine-sepharose column (section 2.4.3) in order to remove plasminogen. Serum and plasma (and plasminogen

depleted plasma; PDP) were obtained from the same donor. After removal of the supernatant (plasmin requires a cell surface (phospholipid) for it's activity) the cells were incubated with the plasmin specific chromogenic substrate S-2251 (section 5.2.4). Some plasmin activity was seen with PDP and serum at 10 -20% (probably reflecting residual plasminogen), but whole plasma showed a considerable increase in plasmin activity compared with either serum or plasminogen depleted plasma at a concentration of 2.5% and above. In addition, solid clots were still present after 4h incubation of PDP with A375 cells, whereas those generated from whole plasma were not. These results indicated that the fibrinolytic activity of A375 cells *in vitro* was primarily due to the action of membrane bound plasmin. Addition of PDP to the cytotoxicity assay resulted in greater inhibition of LAK cell killing than plasma, indicating that the presence of a solid clot throughout the assay period was responsible for this increased inhibition. Since PDP is not present in cancer patients, and is therefore not representative of the physiological state, further inhibition experiments were therefore carried out in the presence of eACA, a specific plasmin inhibitor (section 5.2.5) which has been used as a therapeutic antifibrinolytic agent (Brockway and Castellino, 1971). In the presence of eACA and whole plasma, solid clots were still present in the assay wells after 18h, confirming that fibrinolysis had been inhibited. Inhibition of A375 cell killing by LAK cells in the presence of plasma was significantly increased in the presence of eACA, confirming that the presence of solid fibrin throughout the assay period resulted in a greater inhibition of cytotoxicity than with plasma alone. These results also indicated that inhibition of cytotoxicity was not caused by the production of fibrinopeptides since no fibrinolysis had occurred (as determined by observation and D-dimer production in supernatants.

In normal systems, fibrin is covalently crosslinked by activated factor XIII, with up to six crosslinked bonds per fibrin monomer subunit. This results in a polymer which is mechanically much stronger than non-crosslinked fibrin polymer. It was therefore of interest to investigate if fibrin crosslinking was important in the inhibition of cytotoxicity observed

using whole plasma. Plasma was obtained from a patient with congenital factor XIII deficiency and included in the cytotoxicity assay together with normal plasma and serum. A significant reduction in LAK cell cytotoxicity of A375 cells was seen by both plasma and factor XIII deficient plasma compared with normal serum. Addition of eACA to the assay further increased the inhibition of both plasmas to similar levels, demonstrating that fibrin crosslinking by factor XIII was not important in the inhibition of LAK cell cytotoxicity.

In addition to the generation of fibrin within and around the tumour mass, the majority of cancer patients exhibit some form of systemic activation of the coagulation system (Zacharski *et al.,* 1992). As a result activated coagulation products such as fibrin monomer (complexed to fibrinogen) may be present in the peripheral blood. LAK cells reintroduced into the blood stream of cancer patients may therefore bind to fibrin monomer, prior to extravasation into the tumour microenvironment. It was therefore important to determine whether fibrin monomer also inhibited LAK cell killing of tumour cells.

Fibrin monomer is formed by the thrombin mediated cleavage of the Aα and Bβ chains of fibrinogen. The resultant fibrin monomer then spontaneously polymerises to form non-crosslinked fibrin. Fibrin polymerisation in the cytotoxicity assay was inhibited by the addition of the tetrapeptide Gly-Pro-Arg-Pro (section 5.3.3). In addition, the experiments were also performed in factor XIII deficient plasma to prevent factor XIII mediated crosslinking of the fibrin monomer subunits. In order to prevent fibrinolysis the experiment was also carried out in the presence of eACA. (section 5.3.1). As before some inhibition of LAK cell killing of A375 cells was seen in the presence of whole plasma and factor XIII deficient plasma, but inhibition was greatly increased in the presence of eACA. Addition of Gly-Pro-Arg-Pro to the system (section 5.3.3) caused cytotoxicity levels in both plasma and factor XIII deficient plasma to return to just below that observed in serum. Under the experimental conditions of these experiments, it would therefore appear that fibrin monomer was unable to inhibit LAK cell cytotoxicity against

A375 cells *in vitro*. Extrapolation of these results to the *in vivo* environment would suggest that in LAK cell immunotherapy, inhibition of immune cytotoxic activity occurs within the tumour, and is caused by polymerised fibrin, irrespective of whether it is crosslinked by factor XIII. This hypothesis is further supported by the previous observation that LAK cells are highly cytotoxic to endothelial cells, and that this cell damage may account for the vascular leakage syndrome observed during therapy. In addition, leucocytes removed from patients undergoing LAK cell therapy show considerable LAK cell activity *in vitro*, although the effect of the cell separation procedure on the removal of any inhibitory agent cannot be excluded.

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Appendix I

LIST OF ABBREVIATIONS

A23187	Calcium Ionophore
Act-D	Actinomycin-D
ADCC	Antibody dependent cellular cytotoxicity
ADP	Adenine nucleotide diphosphate
APTT	Activated partial thromboplastin time
BBS	Borate buffered saline
BCG	*Bacillus Calmette Guerin*
CD	Cluster determination
Con A	Concanavalin A
CP	Cancer procoagulant
cpm	Counts per minute
CX	Cycloheximide
DIC	Disseminated intravascular coagulation
eACA	epsilon-amino-caproic-acid
EBV	Epstein Barr Virus
EDTA	Ethylene-diamine-tetra-acetic acid
ELISA	Enzyme linked immunosorbant assay
ELT	Euglobulin lysis time
F/P	Fluorophore to protein ratio
FBP	Fibrinopeptide B
FCS	Foetal calf serum
FDP	Fibrin degradation products
FITC	Fluorescein isothiocyanate
FL-1	Fluorescent channel 1
FPA	Fibrinopeptide A
FSC	Forward light scatter
GPRP	Gly-pro-arg-pro
GPRP	Glycine-Proline-Arginine-Proline
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule-1

ICF	Intravascular coagulation and fibrinolysis
IFNα	Interferon alpha
IFNβ	Interferon beta
IFNγ	Interferon gamma
Ig	Immunoglobulin
IgG	Immunoglobulin G
IL-1	Interleukin-1
IL-1RA	Interleukin-1 receptor antagonist
IL-1RI	Interleukin-1 receptor type I
IL-1RII	Interleukin-1 receptor type II
	Interleukin-1 binding protein
IL-2	Interleukin-2
IL-6	Interleukin-6
i.v.	intra-venous
LAK	Lymphokine activated killer
LAL	Limulus amoebocyte lysate assay
LFA-1	Leukocyte function antigen-1
LGL	Large granular lymphocyte
LPS	Lipopolysaccharide
LT, TNFb	Lymphotoxin
MAC	Membrane attack complex
MBSN	Mean binding site number
mcab	Monoclonal antibody
MDP	Muramyldipeptide
MESF	molecules of equivalent soluble fluorophore
MHC	Major histocompatability complex
MNC	Mononuclear cells
MPIF	Monocyte procoagulant inducing factor
MWU	Mann-Whitney U-test
NF-κB	Nuclear factor-κB
NIBSC	National Institute for Biological Standardisation and Control
NIH	National institute of Health
NK	Natural killer

NS	Not significant
PA	Plasminogen activators
PCA	Procoagulant activity
PE	Phycoerythrin
Per CP	Peridinin chlorophyll protein
PF4	Platelet factor 4
PG12	Prostacyclin
PGE_2	Prostaglandin E_2
PHA	Phytohaemagglutinin
Poly (I,C)	Polyionic:polycytodylic acid
PT	Prothrombin
SCCL	Small cell carcinoma of the lung
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SSC	Side scatter
Tc	Cytotoxic T cell
TC flask	Tissue culture flask
TCGF	T cell growth factor
TCR	T-cell receptor
TF	Tissue factor
TIL	Tumour infiltrating lymphocytes
TNFα	Tumour necrosis factor alpha
tPA	Tissue type plasminogen activator
uPA	Urokinase type plasminogen activator
UPTT	Unpaired students T-test
VPF	Vascular permeability factor
WSR	Wilcoxon Signed Rank test

Appendix II

Addresses of Companies Cited

Alpha Laboratories
40 Parham Drive
Eastleigh
Hants
SO5 4NU

American Diagnostica
222 Railroad Avenue
PO BOX 1165
Greenwich
CT 06836-1165
USA

Amersham International PLC
UK Sales Office
Lincoln Place
Green End
Aylesbury
Bucks
HP20 2TP

BDH (Merck Ltd)
Hunter Boulevard
Magna Park
Lutterworth
Leic
LE17 4XN

Becton Dickinson Immunocytometry Systems
Between Towns Road
Cowley
Oxford
OX4 3LY

Dako
16 Manor Courtyard
Hughenden Avenue
High Wycombe
Bucks
HP13 5RE

Flow Cytometry Standards Corporation
Academic Business Centre
Neils Bohruieg 11-13 K2
2333 CA Leiden
The Netherlands

Genzyme Diagnostics
50 Gibson Drive
Kings Hill
West Malling
Kent
ME19 6HG

Immuno Ltd
Arctic Hiuse
Rye Lane
Dunton Green
Near Seven Oaks
Kent
TN14 5HB

Immunotech SA
130 av de Lattre de Tassigny
BP 177
13276Marseille Cedex 9
France

Imperial Cancer Research Fund
Lincoln Inns Field
London
NW

KabiVitrum Ltd Diagnostica
Riverside Way
Uxbridge
Middlesex
UB8 2YF

Life Technologies
PO Box 35
Trident House
Renfrew Road
Paisley
Renfrewshire
Scotland
PA3 4EF

Luckham Ltd
Victoria Gardens
Burgess Hill
Sussex
RH15 9GN

Marathon Laboratory Supplies
Unit 6
55-57 Park Royal Road
London
NW10 1JP

National Blood Transfusion Service
South Thames Regional Centre
75 Cranmer Terrace
London
SW17 0RB

Nycomed (UK) Ltd
2111 Coventry Road
Sheldon
Birmingham
B26 3EA

Nyegaard Diagnostica
PO BOX 4220
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Mylen House
11 Wagon Lane
Sheldon
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Pharmacia Biosystems Ltd
Pharmacia LKB Biotechnology Div
Dory Avenue
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Quadratech
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Appendix III

Tissue Culture Media

RPMI - RPMI 1640 tissue culture medium (Life Technologies, Renfrewshire, Scotland) supplemented with 10mmol/L glutamine (Life Technologies, Renfrewshire, Scotland), penicillin sodium 50 units/ml and streptomycin 50 units/ml (Life Technologies, Renfrewshire, Scotland).

RPMI/FCS - RPMI supplemented with 10% v/v Australian foetal calf serum (Life Technologies, Renfrewshire, Scotland) which had been heat inactivated for 30min at 56^0C.

RPMI/AB - RPMI supplemented with 10% v/v human AB serum (South London Blood Transfusion Service, Cranmer Terrace, Tooting, London) which had been heat inactivated for 30min at 56^0C.

HBSS - Hanks balanced salt solution (without phenol red; Life Technologies, Renfrewshire, Scotland)

HBSS-Ca^{++}/-Mg^{++} - Hanks balanced salt solution without calcium, magnesium or phenol red (Life Technologies, Renfrewshire, Scotland)

Appendix IV

Cytokine Preparations

Recombinant human tumour necrosis factor-α (TNFα) was obtained from Genzyme Diagnostics, Kent.) as a solution containing 2 x 10^5 U/ml (1 unit being defined as the amount of TNFα required to generate half maximal cytotoxicity of Actinomycin-D stimulated L929 cells). The stock solution was aliquotted (10μl) into plastic cryotubes (Costar, U.K. Ltd.) and stored at -70^{0}C prior to use. For use, 10μl of TNFα was diluted to 2ml with RPMI to give 1000U/ml, and serial dilutions prepared from this solution.

Recombinant human tumour necrosis factor β (lymphotoxin, LT) was obtained from Genzyme Diagnostics, Kent., as 25μl of solution containing 5 x 10^4U/ml (1 unit being the amount of TNFα required to generate half maximal cytotoxicity of cycloheximide stimulated L929 cells). The solution was diluted to 200μl with RPMI and 10μl aliquots were stored at -70^{0}C prior to use. For use, 10μl LT was diluted to 2.5ml (1000U/ml) with RPMI to give 1000U/ml which represented the highest LT concentration used in the cytotoxicity assay.

Recombinant human interferon-γ (IFNγ) was obtained from Genzyme Diagnostics, Kent. (Code HG-IFN) as 1ml of solution containing 40mg protein with a specific activity of 2.5 x 10^7U/mg. One unit is defined as the amount of IFNγ required to provide equivalent anti-viral activity to that expressed by one unit (*i.e.* 50% inhibition of viral replication) of the NIH human IFNγ reference standard (Gg23-901-530). The stock preparation was aliquotted into plastic cryotubes (5μl) which were stored at -70^{0}C prior to use. For use, the stock solution was diluted with RPMI to 200U/ml, which constituted the highest initial concentration (representing a final concentration of 100U/ml) used in the cytotoxicity assay. In experiments investigating cytokine pre-treatment of tumour

cells (section 3.3), IFNγ was included in the cell cultures at final concentrations up to 1000U/ml.

Recombinant human interleukin-1β (IL-1β) was obtained from Genzyme Diagnostics, Kent. (Code BIL-1-C) as a sterile solution containing 0.5mg protein (specific activity 2 x 10^8 units/mg). One unit of IL-1β activity is defined as the amount of IL-1β which induces a 50% decrease in the growth of A375 melanoma cells. The stock preparation was aliquotted into 5μl aliquots and stored at -70^{0}C prior to use. For use, the stock solution was diluted with RPMI to 200U/ml, which constituted the highest initial concentration (representing a final concentration of 100U/ml) used in the cytotoxicity assay.

Recombinant human interleukin-2 (derived from *E.coli*) was obtained from Genzyme Diagnostics, Kent. (Code 2180-01) as lyophilised powder containing 4 x 10^6 BRMP units/mg IL-2. One BRMP unit of IL-2 activity is defined as the amount of IL-2 which is required to support half maximal incorporation of tritiated thymidine by CTLL-2 cells (an IL-2 dependent T-cell line). The powder was reconstituted with 1ml RPMI and 0.5ml was then diluted to 5ml with RPMI/FCS for the preparation of LAK cells. Appropriate serial dilutions were prepared as required.

Appendix V

Monoclonal Antibodies

Fluorescene isothiocyanate (FITC) labelled mouse anti-human immunoglobulin type 1 (IgG1) CD25, FITC labelled mouse anti-human (IgG1) CD8, and phycoerythrin (PE) labelled mouse anti- human (IgG1) p75 were obtained from Becton-Dickinson Immunocytometry Systems, Oxford, U.K..

FITC labelled mouse anti-human (IgG1) tissue factor was obtained from American Diagnostica Inc, Greenwich, U.S.A. and unlabelled mouse anti-human (IgG1) factor V was obtained from Sigma Chemical Co. Ltd., Poole, Dorset, U.K.

The isotype control (mouse IgG1 FITC and PE) directly labelled antibodies were obtained from Becton-Dickinson Immunocytometry Systems, Oxford, U.K.

The indirect isotype control (mouse IgG1), and Fab" rabbit anti-mouse FITC second layer were obtained from DAKO (High Wycombe, Bucks.).

Recombinant goat anti-human TNFα and TNFβ neutralising antibodies were obtained from R & D Systems, Minneapolis, USA.

Mouse anti-bovine retinal antigen-S was obtained from the Immunology Department at St Thomas' Hospital. Retinal S-antigen is highly conserved between species and reacts well against human retinal S-antigen (Banga *et al.*, 1989).

Appendix VI

Reagents for Fibrinogen ELISA

1. Carbonate-Bicarbonate Buffer, 0.05mol/L, pH 9.6

Distilled water

~950ml

Sodium carbonate, anhydrous 1.59g

Sodium hydrogen carbonate 2.93g

Sodium azide 0.2g

adjust pH with 4M Sodium hydroxide to pH 9.6

make to 1l with distilled water and store at 4^0C

2. Borate Buffered Saline (BBS), 0.02mol/L, pH 7.2

Distilled water ~9.5l

Boric acid

12.363g

Sodium chloride

87.740g

Tween 20

10.00ml

adjust pH with 4M sodium hydroxide (pH 7.2)

make up to 10l with distilled water

3. Citrate Phosphate Buffer,).1mol/L, (pH 5.0)

Distilled water

~950ml

di-sodium hydrogen orthophosphate dodecahydrate

23.87g

Citric acid 7.30g

adjust pH with 4M sodium hydroxide (pH 5.0), make up to 1l with distilled water and store at 4^0C

4. Peroxidase Substrate Solution

1,2 ortho-phenylenediamine dihydrochloride	75mg
Citrate phosphate buffer	15ml
6% Hydrogen peroxide	15ml

Appendix VII

Reagents for Phast Gel Electrophoresis

1. Coomassie Blue-R Stain (0.2%)

PORT 1

Five tablets of stain were added to 400ml distilled water and stirred for 10min. Methanol (600ml) was added and the solution filtered through a Whatman number 1 filter. For use, 50ml of stock stain solution was mixed with 50ml 10% acetic acid.

2. Destain

PORT 2

methanol	300ml
acetic acid	100ml
distilled water	600ml

3. Preserving Solutions (for 20% gels)

PORT 3

acetic acid	100ml
glycerol	100ml
distilled water	800ml