The Effect of Intraportal Mannitol on the Short-term *in Vivo* Distribution of Radiolabelled A-LAK Cells in Rats

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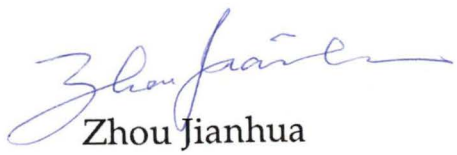

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Abstract

The effectiveness of adoptive immunotherapy of cancer using LAK cells and IL-2 depends on the accumulation of transferred effector cells at the tumour sites. *In vitro* LAK cell have been demonstrated to have broad cytotoxic activity to a wide variety of tumour cells in a non-major-histocompatibility complex-restricted manner, and independent of the presence of tumour specific antigens. LAK cells have now been used effectively in a small number of human trials. The effective delivery of these cells to the tumour site *in vivo* is one of the main aspects of this type of immunotherapy that requires further investigation. Conventional systemic infusion has shown a limited migration pattern of LAK cells. In this study the degree of entrapment of LAK cells in organs following local infusion has been determined. The large size and rigidity of LAK cells may be one mechanism which restricts the distribution of these cells. In addition, in this study the effect of mannitol, a hyperosmotic agent which increases the space between vascular endothelial cells, on the uptake of intraportal LAK cells into liver has been determined.

A-LAK cells are obtained by culture of N.W.P. lymphocytes with IL-2. In this study A-LAK cells were characterised by typical morphologic appearance, cell surface phenotype, and cytotoxic specificity. Purified A-LAK cells were morphologically large granular lymphocytes, 67%-90% of which showed the surface marker phenotype of NK/LGL cells (OX8). The population contained few pan-T cells (only 4.0-6.5% of cells expressing OX19). No B cell surface marker Ig was detectable in the A-LAK cell population. These cells showed high ability to lyse YAC-1 and P-815 cultured tumour target cells in 4h ^{51}Cr-release cytotoxic assays. At an

Effector:Target ratio of 40:1 A-LAK cells lysed 70% P815 and 100% YAC-1 cells.

After labelling A-LAK cells with ^{51}Cr, the effect of intraportal infussion of 30% mannitol on the distribution of intraportally infused A-LAK cells in liver was studied. The trafficking studies were carried out in three groups. In Group 1, ^{51}Cr labelled A-LAK cells were systemically infused through the tail vein of rats as a control group. In Group 2 and Group 3, A-LAK cells were infused into syngeneic rats through the portal vein without or following prior portal infusion of 30% mannitol. Two hours after LAK cell administration the rats were sacrificed and the radioactivity in liver, lung, spleen, blood, MLN, kidney and brain were measured to determine the distribution of A-LAK cells to these organs.

The results showed that intraportal mannitol was associated with an increased percentage of LAK cells in the liver compared with regionally infused LAK cells without mannitol (54% vs 24%; $P<0.0005$). The administration of intraportal mannitol was also associated with increased distribution of A-LAK cells into the brain (0.26% vs 0.08%; $P<0.05$) and MLN (0.05% vs 0.02%; $P<0.05$). There was no significant increase in uptake of A-LAK cells in lung (8.39% vs 6.01%), spleen (1.00% vs 0.98%), or kidney (1.44% vs 1.78%) following intraportal mannitol.

There was no significant increase of the A-LAK cell distribution to the liver by regional infusion without mannitol (24% vs 16%). Systemic injection gave greater A-LAK uptake into lungs (15.65%) than portal injection (6.05%, $P=0.05$). There was no significant differences of cell distribution in spleen, MLN, kidney and brain by these two routes of infusion.

These results showed that mannitol has the effect of increasing the distribution of LAK cells into the liver. Augmentation of LAK cell accumulation in the liver may help to enhance the tumouricidal activity of these cells to hepatic metastases. The results of this thesis suggest areas for further investigation into the effect of mannitol and other agents on A-LAK cell uptake into the liver following regional infusion.

CONTENTS

ABBREVIATIONS

A-LAK	adherent lymphokine-activated killer
BBB	blood brain barrier
BM	bone marrow
BSA	bovine serum albumin
Ci	curie
CLN	clavicle lymphonodus
CM	complete culture medium
cpm	count per minute
CR	complete regression
CTL	cytotoxic T lymphocyte
CY	cyclophosphamide
dl	deciliter
EDTA	ethylenediaminetetra-acetic acid
E/T	effector to target ratio
FCS	fetal calf serium
FITC	fluorescein isothiocyanate
g	gram
h	hour
HEV	high endothelial venules
IFN	interferon
Ig	immunoglobulin
IL-2	interleukin-2
ip.	intraperitoneal
iv.	intravenous
kg	kilogram
L	liter
LAK	lymphokine-activated killer
LAL	large agranular lymphocyte
LGL	large granular lymphocyte
LME	lysosomotropic amine
M	mole
Mab	monoclonal antibodies
mg	milligram
min	minute
ml	milliliter
MHC	major-histocompatibility complex
MLC	mixed lymphocyte culture

MLN	mesenteric lymphonode
MMC	mitomycin C
MNC	mononuclear cell
MLTC	mixed lymphocyte-tumour cell culture
MW	molecular weight
NH&MRC	national health and medical research council
NK	natural killer
NW	nylon wool
NWP	nylon wool passed (or non-adherent) lymphocyte
NT	non-tested.
PBL	peripheral blood lymphocyte
PBS	phosphate buffered saline
PMN	peripheral mononuclear cell
PR	partial regression
P-815	murine mastocytoma
rIL	recombinant interleukin-2
rpm	round per minute
SI	small intestinal
SR	spontaneous release
TCGF	T cell growth factor
TCM	tissue culture medium
T_h	T-helper
TIL	tumour infiltrate lymphocyte
TNF	tumour necrosis factor
T_s	T-suppressor
U	units
UV	ultraviolet
YAC-1	mouse T cell lymphoma
°C	degree centigrade
%	per cent
µg	microgram
µl	microliter
2-ME	2-Mercaptoethanol
5FU	5 fluorouracil
^{51}Cr	^{51}chromium
^{3}H	tritium
^{125}I	^{125}Iodine
^{111}In	^{111}Indium
^{99}mTc	technetium

^{75}Se $^{75}Selenium$

Chapter 1 Literature Review

1.1 Introduction

LAK cells, produced by culture of lymphocytes with a high concentration of IL-2 have been demonstrated to have broad cytotoxic activity against a wide variety of tumour cells (Lotze *et al* 1981; Grimm *et al* 1982; Rosenstein *et al* 1984; Lafreniere *et al* 1985; Mule *et al* 1985; Shu *et al* 1985; Papa *et al* 1986; Itoh *et al* 1986; Salup *et al* 1986; Vujanovic *et al* 1988b). LAK cells lyse tumour cells *in vivo* after gaining access to the target cells by extravasation through the microcirculation (Harcel *et al* 1991; Basse *et al* 1991a). Clinical studies using LAK cells with IL-2 have shown therapeutic value in cancer treatment (Rosenberg *et al* 1986a; 1986b; Benyunes *et al* 1993; Schoof *et al* 1993). However, systemic LAK/IL-2 therapy is associated with significant toxicity which is associated with the very large number of cells and high doses of rIL-2 infused (Rosenberg *et al* 1987; Kuebler *et al* 1993; Villani *et al* 1993). One solution to this problem may be the regional administration of LAK cells, possibly together with factors which enhance the distribution of LAK cells to the target organ.

Studies have shown that regional infusion of LAK cells could result in more effector cells accumulating at the target sites, thus decreasing the dosage of LAK cells and IL-2 required and minimising some side effects. Regional IL-2/LAK cell infusion has also been shown to be associated with anti-tumour effectiveness and lower toxicity (Lafreniere *et al* 1985b; Ettinghausen *et al* 1985; Basse *et al* 1991b; Kuppen *et al* 1992; Keilholz *et al* 1992; Scudeletti *et al* 1993; Yamamoto *et al* 1993). Local injection of LAK cells shows accumulation of injected cells at the tumour sites and tumour regression (Yoshida *et al* 1988; Basse *et al* 1992b; Hayakawa *et al* 1992; Belli *et al* 1992; Ibayashi *et al*

1993) suggesting that the accumulation of effector cells in the tumour is an important factor.

LAK cells have a limited pattern of migration after systemic administration with 47% accumulation in lungs 2h after infusion and then redistribution into liver 24h after infusion (Maghazachi *et al* 1988c, 1988b; Felgar *et al* 1990; Takai *et al* 1988). The limited distribution of LAK cells inhibits their optimal tumouricidal effect *in vivo*. This pattern may be the result of decreased extravasation of LAK cells because of their large size and high rigidity (Sasaki *et al* 1989). Mannitol has been shown to open the Blood-Brain Barrier by a mechanism involving increase of intercellular spaces between capillary endothelial cells (Franceschini *et al* 1988). In this study we investigated the effect of intraportal mannitol on the distribution of LAK cells infused into the portal vein.

During the course of these experiments there were not many reports concerning infusion of LAK cells regionally and no report of using reagents to increase cell distribution. Recently adoptive immunotherapy has been delivered by local infusion. In the near future the utilization of LAK/IL-2 may be combined with factors which could improve their distribution, and with some reagents which could enhance LAK cell effectiveness.

The results of this study may lead to improvement in the effectiveness of adoptive immunotherapy, by determining an effective way of delivering effector cells into the tumour-bearing host. The generation of adequate numbers of highly cytotoxic cells, and the efficient delivery of these cells *in vivo*, are key factors that require improvement in

adoptive immunotherapy. This study was aimed at investigating a possible method of increasing LAK cell distribution into particular organs and therefore to tumour sites in order to improve the tumouricidal activity of LAK cells *in vivo*.

1.2 Development of Adoptive Immunotherapy for Cancer Using LAK Cells and IL-2

Adoptive immunotherapy for cancer is a therapy aimed at destroying cancer cells by transfer of activated antitumour lymphocytes into the tumour-bearing host. As early as the 1960's there were some studies which found that the intravenous delivery of lymphocytes from tumour cell-immunised mice into syngeneic tumour-bearing mice could mediate the regression of tumours. For example the treatment of fibrosarcoma in the rat with immune T-lymphocytes (Delorme *et al* 1964). These animal studies suggested that similar therapy could be used in human malignancy, however alternative methods for obtaining tumour-sensitised lymphocytes had to be developed. In 1970's, anti-tumour cytotoxic cells were obtained by sensitising the lymphocytes in *in vitro* culture with tumour cells, MLTC (Treves *et al* 1975; Berenson *et al* 1975; Gillis *et al* 1977; Ruscetti *et al* 1977; Zarling *et al* 1979; reviewed in Wilkinson 1993). In addition, the isolation of the lymphokine that was originally called T Cell Growth Factor (TCGF), now named interleukin-2 (IL-2), revealed that TCGF in the absence of tumour cells could stimulate lymphocytes to become cytotoxic against tumour cells (Aarden *et al* 1979). TCGF made it possible to maintain cytotoxic lymphocytes in long-term culture (Morgan *et al* 1976; Gillis *et al* 1978; Watson *et al* 1979).

In the early 1980's, purified IL-2 was found as a glycoprotein of MW 16,000 (Taniguch *et al* 1983; Louis *et al* 1983), and the IL-2 receptor was isolated (Uchiyama *et al* 1981; Leonard *et al* 1982). IL-2 is synthesised and secreted by mitogen- or antigen- activated T_h lymphocytes (Smith *et al* 1980). The availability of large quantities of highly purified IL-2 (r-

IL-2) was made possible by recombinant-DNA technology. IL-2 was found to promote the proliferation and activation of antigen-stimulated T cells (Yron *et al* 1980; Cheever *et al* 1981) and to stimulate the proliferation of lymphocytes from non-immunised donors, and to provide them with the capacity to recognise and lyse a variety of tumour cells (Lotze *et al* 1980,1981; Rosenstein *et al* 1984). The activated cells which were raised from non-antigen-stimulated lymphocytes were described as Lymphokine-activated Killer (LAK) cells (Grimm *et al* 1982). The possibility now arose of utilizing LAK cells in adoptive immunotherapy. LAK cells could be continuously stimulated *in vivo* by infusion of IL-2 systemically (Ettinghausen *et al* 1985a, 1985b) and could be produced by co-cultivation of peripheral blood lymphocytes with IL-2 *in vitro*. The generation of LAK cells both *in vitro* and *in vivo* has the advantage that it is unnecessary to pre-sensitise the effector cells with tumour cells. LAK cells kill tumour cells in a non-MHC-restricted manner (Hersey *et al* 1987).

In 1984, Rosenberg *et al* at the National Cancer Institute transferred LAK cells into murine tumour models and reported the first demonstration that lymphokine-activated killer cells, activated *in vitro* by IL-2, can inhibit the growth of established melanoma pulmonary metastases (Mazumder *et al* 1984). Systemic administration of xenogeneic IL-2 together with *in vitro*-sensitised and IL-2-expanded lymphocytes in animals resulted in regression of metastases (Donohue *et al* 1984). Both LAK cells and sensitised IL-2-activated lymphocytes have tumouricidal activity *in vivo*. LAK cells and IL-2 infusion has also been used (Mule *et al* 1985).

Administration of high dose IL-2 alone resulted in significant toxicity. In humans, initial systemic administration of recombinant IL-2 at high doses over short periods of time showed the rapid half-life of IL-2 and it's toxicity *in vivo*. There was no detectible LAK cell activity in peripheral blood after short term administration of IL-2. However there was an increase in the number of lymphocytes and their *in vitro* responsiveness to IL-2 (Lotze *et al* 1985; Atkins *et al* 1986). The requirement of high dose of IL-2 (over 500U/ml *in vitro*) to produce LAK cells made it impossible to generate them *in vivo* because of the toxicity. Many efforts have been made to modify the treatment protocol of IL-2 administration to optimise immune effects and decrease toxicity. In murine models it was demonstrated that prolonged administration of IL-2 at tolerable doses could activate sufficient *in vivo* NK/LAK activity compared with a few days administration of very high dose IL-2 (Ettinghausen *et al* 1986; Nishimura *et al* 1987; Talmadge *et al* 1987). It was also shown in humans that prolonged administration of low doses of rIL-2 resulted in selective expansion of NK cells *in vivo* with minimal toxicity (Caligiuri *et al* 1991). However large numbers of LAK cells could be generated *in vitro*. Provision of IL-2 together with administration of large quantities of LAK cells grown *in vitro* was more effective and less toxic (Mule *et al* 1984, 1985, 1986b). The antitumour effect of IL-2 appears to result from the action of LAK cells *in vivo* (Rosenberg *et al* 1985a; Ettinghausen *et al* 1985a, 1985b; Mule *et al* 1985; Hank *et al* 1988; Da Pozzo *et al* 1992).

It was demonstrated in preclinical studies that combined treatment using LAK cells and IL-2 is effective for tumours of distinct histological types such as sarcoma, adenocarcinoma and melanoma, and for both immunogenic and nonimmunogenic tumours (Papa *et al* 1986;

Lafreniere *et al* 1985b; Shu *et al* 1985). Evidence of efficacy of this treatment was also obtained in established metastatic tumors in liver, lung, kidney and brain (Lafreniere *et al* 1985a; Papa *et al* 1986; Salup *et al* 1986; Takai *et al* 1988). A direct relationship existed between the number of LAK cells transferred, the amount of IL-2 given, and the therapeutic effect achieved in murine models (Mule *et al* 1985).

Based on the knowlege from animal experiments, immunotherapy using LAK cells in conjunction with IL-2 was administered for the treatment of selected patients with advanced metastatic tumour. Rosenberg reported the result of their initial clinical trial which resulted in some responses in patients with colorectal cancer, malignant melanoma, renal cell cancer, and lung adenocarcinoma (Rosenberg *et al* 1985a, 1986a, 1986b). In 1987, Rosenberg *et al* reported their progress in treatment of patients with advanced cancer. In many patients the administration of these cells and IL-2 was limited by the toxicity of IL-2. This side effect resulted in a generalised capillary permeability leak syndrome. Further efforts were made to decrease the toxicity and complexity of this therapy.

The role of adoptive immunotherapy in order to achieve significant tumour responses with acceptably low toxicity depends on a successful refinement of methodology and protocols. Such refinement may include more efficient delivery of LAK cells to the target organ. It has been shown that low dose IL-2 plus LAK cells in immunotherapy has greater efficacy (Schoof *et al* 1988). Thompson *et al* 1992 found that a longer maintenance phase of IL-2 at a lower dose is associated with significantly less toxicity without a loss of therapeutic efficacy. It has also been shown that purified populations of A-LAK cells are more

effective in reducing established lung or liver metastases in rat models (Schwarz *et al* 1989). Rosenberg *et al* 1986c showed that LAK cells derived from lymphocytes exposed to tumour cells (such as tumour infiltrating lymphocytes) had greater tumoricidal effect than peripheral blood lymphocyte-derived LAK cells. Local adoptive immunotherapy by direct injection of LAK cells and IL-2 into brain tumours was found effective and had low toxicity (Yoshida *et al* 1988; Ibayashi *et al* 1993). In humans the effect of systemically infused LAK cells is far less in brain tumours (Monod *et al* 1992). In a later murine model with intracerebral metastases it was shown that there was no response to systemic infusion of LAK cells and IL-2 (McCutcheon *et al* 1990). The fact that histological examination of brain tissue did not reveal lymphocyte infiltration indicated that the effector cells were not delivered into tumour sites in brain by systemic infusion. The fact that there was no discernible correlation between *in vitro* target cell lysis by LAK cells and theraputic efficacy *in vivo* (Papa *et al* 1986; Nakano *et al* 1991) suggests that the effector cells may not reach the target organ in sufficient number *in vivo*. Lack of response is not the result of loss of effectiveness *in vivo* because it has been shown that there was corresponding *in vivo* and *in vitro* susceptibility of murine tumours to lysis by LAK cells (Papa *et al* 1986). The tumour cells which appeared to be sensitive to LAK cells *in vitro* do not appear to be resistant to LAK cells *in vivo* (Mule *et al* 1986). It is most likely the result of insufficient LAK cell infiltration into tumour sites. The extravasation of LAK cells into the tumour may be one of the important steps for achieving tumour damage.

The studies of adoptive immunotherapy in humans using LAK cells and IL-2 are continuing. It has been shown that this immunotherapy

results in partial or complete regression (PR and CR) of several types of tumours: prostatic carcinoma and associated pulmonary metastases (Tjota *et al* 1991); advanced renal cell carcinoma (Weiss *et al* 1992; Engelstein *et al* 1992); primary or metastatic liver carcinoma (Miya *et al* 1992; Foon *et al* 1992); non-Hodgkin's lymphoma (Weber *et al* 1992). Local and regional infusion of LAK cells and IL-2 appears to result in more responses because local infusion may be associated with greater accumulation of effector cells at the tumour site. Intrahepatic LAK cells inhibited the growth of metastatic tumours in the liver in some patients with cancers of the gastrointestinal tract (Tsugita *et al* 1990), and resulted in CR and PR in advanced liver metastatic or primary cancer (Han *et al* 1991; Keilholz *et al* 1992; Yamamoto *et al* 1993). Regional arterial administration of LAK cells also resulted in metastatic regression (Hayakawa *et al* 1991, 1992). Local adoptive immunotherapy using A-LAK cells and IL-2 in a nude mouse model of human squamous carcinoma of the head and neck resulted in complete tumour growth inhibition (Sacchi *et al* 1991). In a report on human malignant pleural mesothelioma treated with the local A-LAK immunotherapy, there was a reduction of the malignant pleural effusion (Yanagawa *et al* 1991).

The additional use of different lymphokines or cytokines may result in augmentation of effectiveness of LAK cells and IL-2. The combination with interferon-alpha (IFN-alpha) but not tumour necrosis factor-alpha (TNF-alpha) showed augmentation of the effect of LAK cells *in vitro* (Wanebo *et al* 1991) and *in vivo* (Puri *et al* 1991). Kato *et al* 1991 reported that appropriately timed pretreatment of tumour-bearing mice with TNF-alpha augmented the anti-tumor efficacy of LAK cells. *In vitro* the combined application of IL-2 and IFN-alpha, IFN-gamma or TNF-alpha significantly improved the cytotoxicity of A-LAK cells to

human leukemia cells (Teichmann *et al* 1992). IL-1 and IL-3 may also increase the effect of IL-2 (Marumo *et al* 1992; Okuno *et al* 1992). IL-7 was found to be able to induce significant LAK activity after bone marrow transplantation (Pavletic *et al* 1993).

The combination of chemotherapy with LAK cells and IL-2 adoptive immunotherapy may also result in additive effects (Yamaue *et al* 1991; Wakizaka *et al* 1992; Gazit *et al* 1992). Splenic or hepatic arterial injection of IL-2 with chemotherapeutic agents such as cyclophosphamide (CY), 5 fluorouracil (5FU) and mitomycin C (MMC) resulted in more LAK/NK activity in PBL in comparison to injection of IL-2 alone (Okuno *et al* 1991). Low-dosage CY seems increasing the LAK activity with IL-2 in patients with advanced malignancies of varying types (Abrams *et al* 1993). Lithium can be used as a new immunomodulator for cancer immunotherapy (Wu *et al* 1992). By conjugation of LAK cells with anti-tumour monoclonal antibody, LAK cell killing activity against tumour was specifically enhanced (Shiraiwa *et al* 1991; Takahashi *et al* 1993; Weiner *et al* 1993; Watanabe *et al* 1993).

1.3 *In Vitro* Production and Characterisation of LAK Cells

1.3.1 Introduction

LAK cells are generated by culturing lymphocytes with IL-2 for several days. Since the description of the LAK cell phenomenon there has been extensive investigation of LAK cells including their characterisation and functions. This section summarises work on the morphology, the cell surface characteristics and the tumouricidal activity of LAK cells and LAK cell precursors.

1.3.2 Morphology and cell surface characteristics of LAK cells and LAK precursors

1.3.2a LAK cell morphology

It is generally accepted that among whole lymphocyte populations, LGL/NK cells are the main precursors of LAK cells. NK cells have the same morphology as LGL in the human and rat (Reynolds *et al* 1981a; Timonen *et al* 1981). LGL/NK cells (diameter 10-15μm) are slightly larger than small and medium-sized lymphocytes and they have a relatively high cytoplasmic to nuclear ratio, an eccentric reniform nucleus and weakly basophilic cytoplasm with several azurophilic granules. Human LGL have a very typical lymphocyte staining pattern for tartrate-inhibitable acid phosphatase (Timonen *et al* 1981). Macrophages can be distinguished from LGL on the basis of their large size (15-20μm), a more indented nucleus, and vacuolar cytoplasm. NK cells differ from small lymphocytes in expressing prominent azurophilic granules and their low density (Shau *et al* 1985).

In response to culture in high concentrations of rIL-2 (500-1000U/ml), LGL/NK cells differentiate into highly active cytotoxic cells which have been termed LAK cells. The LAK cells have similar morphology to LGL/NK. Morphological analysis of human A-LAK cells indicate that these cells have the morphology of LGL with abundant cytoplasm and prominent cytoplasmic granules (Melder *et al* 1988), vacuolate cytoplasm and an undulating surface (Vujanovic *et al* 1988c). Sasaki *et al* 1989 have demonstrated that LAK cells have a greater mean diameter than nonactivated LGLs

1.3.2b Cell surface markers

Extensive studies analysing the surface phenotype of LAK progenitors and effector cells have been obtained in three species: human, rat and mouse.

In humans, most of the LAK cell progenitors from peripheral blood lymphocytes have the same characteristics as LGL/NK cells, and the human effector LAK cells also express the same phenotype as activated NK cells. Phillips *et al* 1986 and Ortaldo *et al* 1986 indicated that LAK progenitors in human peripheral blood are LGL/NK cells expressing the surface markers NKH1/Leu19 and CD16. CD16 antigen (Leu11) has been found on almost all activated NK cells (Lanier *et al* 1983). NKH1 antigen (Leu19) has been detected on virtually all NK cells and also on a small subpopulation of $CD3^+$ T cells (Hercend *et al* 1985; Schmidt *et al* 1986). Itoh *et al* 1985a showed that LAK precursor cells are Leu-1.1^+(CD16) lymphocytes with NK activity. The progenitors did not express the pan-T-cell markers CD3 or CD5. The activity of LAK cells developed from cell populations without pan-T marker positive cells

(OKT3 or Leu1) was found to be greatly augmented (Grimm *et al* 1983a). Killer cell precursors were also found in Leu11a$^+$ (CD16) and Leu7$^+$ NK subsets, but not in Leu4$^+$(CD3) or Leu3a$^+$(CD4) T lymphocytes (Itoh *et al* 1986). The majority of LAK activity is mediated by NK cells that express the NKH1/Leu19, but not by T cells that express CD3. Talmadge *et al* 1986 found that the subpopulation of lymphocytes most responsive to high doses of rIL-2 (>100U/ml) comprised LGLs, the morphologic homologue of natural killer cells. Lotzova *et al* 1987 further confirmed that human IL-2-stimulated active NK cells displayed LGL morphology and had CD16 and NKH1/Leu19 (CD56) cell surface phenotype. For human A-LAK cells, phenotypic analysis indicates that a majority of these cells express the CD3$^-$Leu19$^+$ phenotype and a substantial proportion of Leu19$^+$ cells expressed CD16 antigen (Melder *et al* 1988). Thus the progenitors of LAK activity in human peripheral blood are mainly LGL/NK with CD3$^-$, CD16$^+$, NKH1$^+$ (CD56) phenotype.

Grimm *et al* 1983a found that human LAK progenitor cells were present in lymphoid organs containing few active NK cells. They were null cells, distinct from NK cells or classical thymus-derived T-cells. Thymus derived LAK cells are low to medium density lymphocytes, CD3 negative, and they possess the NK-associated marker NKH1/Leu19 (CD56), however they lack most NK-associated markers CD16 (HNK1/Leu11 and B73.1) (Ramsdell *et al* 1987).

In rats, Vujanovic *et al* 1988a showed that the major cell population activated by rIL-2 is the LGL/NK cell. These cells appeared to represent the major population of cells in blood or spleen which generated broad antitumour (LAK) cytotoxicity. Highly purified populations of

peripheral blood LGL express surface markers of rat NK cells such as OX8$^+$, Laminin$^+$, asialo-GM1$^+$, but are OX19$^-$, R1-3B3$^-$, W3/25$^-$, Ia$^-$, SIg$^-$. They are fully capable of generating high levels of LAK activity by 3 to 5 days in culture with rIL-2. Analysis of the LAK effector phenotype by cell sorting demonstrates that the majority of cells with LAK activity are OX8$^+$, Laminin$^+$, asialo-GM1$^+$, OX6$^+$ but not those of mature T or B cells (i.e., OX19, R1-3B3, W3/25, Ia, SIg.). LGLs have been shown to be highly associated with NK activity (Reynolds *et al* 1981a). LGLs express OX8, asialoGM1, L-C, and W3/13 antigens. Very few LGL express the W3/25, Thy1.1, Ia, or SIg antigens (Reynolds *et al* 1981b). Cantrell *et al* 1982 found that LGLs were OX8$^+$ and W3/13$^+$, but W3/25$^-$. Rat LGL are not typical T cells, B cells, monocytes, or PMN. They share some cell surface markers (eg OX18) with T cells (Reynolds *et al* 1981b). Rat cytotoxic T lymphocytes were OX8$^+$, W3/25$^-$, Ia$^-$, Thy1$^-$ (Gilman *et al* 1982). Woda *et al* 1984 demonstrated that rat NK cells do not express the T cell-specific membrane protein OX19. The functional NK population was OX19$^-$ OX8$^+$.

Vujanovic *et al* 1988 described a new method for the purification and rapid expansion of LGL by isolating the plastic adherent IL-2 stimulated LGL and then continuing culture of these cells for several days. These LAK cells are called adherent LAK (A-LAK) cells which are found to have exceedingly high levels of broad antitumour cytotoxicity. They express surface markers of rat NK cells such as OX8(CD8)$^+$, asialoGM1$^+$, laminin$^+$, but are OX19(CD5)$^-$, R1-3B3 (CD5)$^-$, W3/25(CD3)$^-$, OX39(CD25)$^-$, Ia$^-$, and Ig$^-$ (Vujanovic *et al* 1988c). Further investigation of the A-LAK phenotype has also shown that 90-100% of purified A-LAK cells express OX8, asialo-GM1, laminin, and the structure identified by monoclonal antibody 3.2.3, surface markers which are all common to

rat LGL/NK cells. In contrast, neither OX19(CD5), W3/25(CD4), OX41 (macrophage specific), nor Ig was expressed on these cells. All A-LAK cells were LGL's (Schwarz *et al* 1989; Chambers *et al* 1989). It seems that OX8 and 3.2.3 are the most specific for rat LAK cells (for CD8 and CD16) at the present time.

In the mouse, evidence suggests that NK cells are the progenitors of cells with LAK activity. The progenitor cells were asialo-GM1$^+$, Lyt2$^-$, L3T4$^-$, Ia$^-$, Ig$^-$, and Thy-1$^-$, a phenotypic pattern characteristic of mouse NK cells, but not T or B cells (Yang *et al* 1986; Salup *et al* 1987). Hackett *et al* 1986 found in normal adult mice that splenic NK activity was found in the NK-1.1$^+$ fraction and also in asialo GM1$^+$ cells. Purified NK-1.1$^+$ cells showed a homogeneous population, each cell containing one to four cytoplasmic granules. Data on the effector phenotype of mouse LAK cells also indicate that they express surface markers similar to those of activated NK cells (asialo-GM1$^+$, Thy-1$^+$, Ia$^-$, FcR$^+$) (Yang *et al* 1986). Precursors of LAK cells are Thy1$^+$ (Rosenstein *et al* 1984). Both Thy1$^-$ and Thy1$^+$ murine LAK precursors can give rise to LAK cells which are cytotoxic to tumour cells (Ballas *et al* 1987). Rosenstein *et al* 1984 showed that LAK lytic activity was due to Thy1$^+$, Lyt1-2$^+$ cells. Ballas *et al* 1987 and Ting *et al* 1986 delineate at least two different LAK precursors, one Thy1$^-$, Lyt2$^-$, aGM1$^+$ (NK like), and another Thy1$^+$, Lyt2$^+$, aGM1$^+$ (T cell like). However these divergent results probably resulted from the different culture methods for generating LAK cells. Other studies in mice have suggested a distinction between NK cells, cytotoxic T-lymphocytes and cells with LAK activity (Merluzzi *et al* 1985; Andriole *et al* 1985; Ballass *et al* 1986; Merluzzi *et al* 1986).

In humans and in rats, several investigators have suggested that LGL/NK cells are derived from noncytotoxic agranular precursor cells which are called large agranular lymphocytes (LAL). Human thoracic-duct lymphocytes are devoid of NK activity, but when incubated with IL-2, develop into LAK cells (Andriole *et al* 1985). Lotzova *et al* 1987 found that impairment of NK cells tumour-binding and lytic activity in leukemia patients could be corrected by culture of peripheral blood effector cells with IL-2. Thus non-cytotoxic pre-LAK cells exist. That NK activity could be generated from nongranular precursors was first demonstrated by Shau *et al* in 1985. These workers found that after selectively depleting LGL from human PBL with a lysosomotropic amine (LME) and subsequently culturing the LME-resistant cells with IL-2, LAK cells were obtained from the culture. The precursors are probably LAL because these cells have the same low buoyant density as LGL/NK cells, and thus can be selected together with LGLs from Percoll density gradient separation columns. However they are resistant to LeuOMe or LME which depletes LGL/NK cells. These cells are distinct from mature NK cells which express HNK1, OX16 (B73.1, Leu-11b) and OKM1 markers. Gray *et al* 1985 also found that depletion of NK activity from human PBL does not impair the development of LAK activity. That development of LAK activity does not require mature NK cells is shown by the generation of activated LAK cells from fresh thymocytes and lymph node cells which lack natural cytotoxic activity. Thymocyte LAK precursors are of low to medium density, with their phenotypic identification devoid of OX16 and NKH1, T cell surface marker OX1 negative, and predominantly OX3-negative (Gray *et al* 1985; Ramsdell *et al* 1987). These phenomena suggest that the precursors are LAL. LAL are also found to be the immediate precursor cells for LGL/NK cells in rats (Maghazachi *et al* 1988a). These authors found that LAL, LGL/NK,

and LAK cells appear to represent sequential developmental and activation stages. In response to rIL-2, enriched populations of splenic LAL can first differentiate into LGL with NK activity and then these LGL/NK cells can be further differentiated and/or activated by high doses of rIL-2, to become the cells with LAK activity (Maghazachi *et al* 1988a). These LAL express asialo-GM1 but only some of them express OX8 (30%) which are expressed on virtually all rat LGL/NK cells (Vujanovic *et al* 1988c; Woda *et al* 1984; Reynolds *et al* 1981b; Vujanovic *et al* 1988a) and no surface laminin. They lack the T cell surface marker OX19. Kumagai *et al* 1982 also found LAL were asialo-GM1$^+$ but had no NK activity. During the transformation of LAL into LGL/NK, these cells acquire LGL/NK cell surface markers as well as NK activity. Andriole *et al* in 1985 also found that there was no correlation between the presence of NK cells and the capacity to generate LAK cells after *in vitro* incubation of splenocytes with IL-2 in some immunodeficient mouse strains. It has also been shown that active NK cells can be induced from precursors from bone marrow (BM). BM is devoid of, or has a low frequency of cytotoxic NK cells (Hackett *et al* 1985, 1986). Thymocytes and bone marrow cells need a longer time to generate LAK activity in culture with IL-2 (Vujanovic *et al* 1988b). LAL may comprise a major source of LAK progenitors in lymphoid populations having few LGL or mature active NK cells.

1.3.3 Production of LAK cells *in vitro*

1.3.3a Isolation of LAK precursors from whole lymphocyte populations

LAK cells have been generated from a variety of organs and tissue sources in different species. In human LAK activity against tumour has

been generated from lymph node, thymus, thoracic duct cells, bone marrow, spleen and PBL (Grimm *et al* 1983b). Since the LAL and LGL/NK cells are the major precursors of LAK cells, information about their presence in different organs provides a guide for the selection of tissue for LAK cell generation *in vitro*. In rat the LGL precursor frequency in different organs has been shown to be in the order of peripheral blood> spleen> peritoneal exudate> lymph node> thymus or bone marrow (Reynolds *et al* 1981a). The frequency of LGL and NK activity in different strains appears different. The low NK activity in older animals or in strains with low NK cells is not always associated with low number of LGL (Grimm *et al* 1983). Grimm *et al* 1983 found in the W/Fu rat that the frequency of LGL among the spleen cells is 2-6%, and among PBL is 4-14%. Timonen *et al* 1981 also reported the frequency of LGL among human blood leukocytes varied from 2% to 6%.

There are several different techniques available to isolate LGL from mixed leukocyte populations. Nylon wool (NW) was described as a material which could induce B cells and macrophages to adhere to it. LGL/NK cells are of low density because of their relatively high cytoplasmic: nuclear ratio, and can be separated from higher density lymphocytes on Percoll discontinuous density gradients. Different types of gradients have been used. In a 7-step gradient, the highest frequency of LGL (82%) with enriched NK activity was found in fraction 2. In a 4-step gradient, the fraction 2/3 contained 73% LGL (Reynolds *et al* 1981a). Human LGL could be enriched to a purity of >90% by depleting high affinity rosette-forming cells from the cells collected from low density Percoll fractions (Timonen *et al* 1981). Timonen *et al* 1982 found that rat LGL could be further enriched by

depleting high affinity sheep erythrocyte rosette-forming cells from the LGL-enriched Percoll fractions, resulting in >90% purity. Other techniques are available to obtain the LAK precursors. One technique is to deplete the T cells from NWP lymphocytes with monoclonal antibody (Mab). The Mab R1-3B3 can react with T cell membrane antigens but not with that of LGL. Use of this Mab with complement can remove pan T cells. Another technique makes use of LGL-selective antibodies and cell sorting (Ortaldo *et al* 1981). The disadvantages of this technique are the small recovery of LGL and the risk of changing cell membrane structure and function.

In 1988 Vujanovic *et al* first described a new procedure for the purification and rapid expansion of LGL. The antitumour effector cells were generated by isolating IL-2 stimulated plastic adherent LGL then continuously culturing these for several days. They found that one of the first responses of a small subpopulation of cells (LGL) to IL-2 is their adherence to the plastic surfaces of a culture flask. After 24-48h, about 4.5% of input cells could adhere to the plastic surface and 97% of these adherent cells are LGLs. Forty eight hours culture seems to be the optimal time for selecting the adherent cells for expansion. These LGLs express surface markers of rat NK cells. When plastic-adherent LGL/NK cells were cultured over 3-4 days in IL-2 in conditioned medium, these cells expanded 30-100 fold and they comprised highly purified LAK cells with a very high level of broad antitumour cytotoxicity. Schwarz *et al* 1989 showed that purified populations of A-LAK cells have superior effect to LAK cells in reducing established metastases in rat models. It was demonstrated that by culturing human MNC from healthy individuals and cancer patients, A-LAK cells that have more potent antitumour cytotoxic function *in vitro* (compared to

LAK cells prepared in a conventional manner) are obtained. This relatively new technique has been utilised in human A-LAK cell production (Melder *et al* 1988).

1.3.3b Factors influencing LAK cell proliferation

It has been described that LAK cells could be generated by coculture of LAK precursors with IL-2 *in vitro,* and that IL-2 alone directly stimulates LAK precursors to become cytolytic effector cells (Grimm *et al* 1983b). Recombinant human interleukin-2 in the presence or absence of additional stimuli was found to be able to induce and maintain the proliferation of human PBL. However when low concentrations (<10U/ml) of interleukin-2 was used, proliferation was observed only when additional signals (antigen, mitogen) were provided (Talmadge *et al* 1986). Higher concentration (>100U/ml) of IL-2 stimulated the proliferation of LAK cells in the absence of exogenous lectin, antigen or allogeneic serum. Rosenstein *et al* 1984 also reported that IL-2 is the key stimulus for the generation of LAK cells. The lytic capacity of LAK cells is due only to IL-2 and not Con A, fetal calf serum or other lymphokines. However Vujanovic *et al* 1988c suggested that factors in addition to IL-2 present in conditioned medium may be required for optimal expansion of A-LAK cells. LGL synthesise DNA and proliferate very rapidly in response to rIL-2 in culture (Talmadge *et al* 1986). Up to 85% of adherent LGL were synthesising DNA in the culture (Vujanovic *et al* 1988c). The reaction of LGL to IL-2 seems dependent on the expression of the TAC receptor on the cell membrane (Talmadge *et al* 1986). The cells from discontinuous Percoll gradient fraction-2 (>90% LGL) showed expression of the TAC receptor.

There is evidence that the presence of some other cell populations *in vitro* may influence the LGL proliferation. Macrophages have been shown to have suppressive effects on NK activity *in vitro.* Vujanovic *et al* 1988c found that both suppressor T cells and monocytes in bulk culture inhibit LAK cell generation. Triozzi *et al* 1991 found that human monocytes markedly inhibit LAK cell expansion in PBL culture with IL-2. The addition of monocytes to IL-2 stimulated lymphocytes decreased LAK cell activity. This inhibition was enhanced in the presence of rIFN gamma (Weng *et al* 1991).

1.3.4 Tumouricidal activity of LAK cells *in vitro* and *in vivo*

Lymphokine-activated Killer (LAK) cells are immune effector cells which can non-specifically lyse neoplastic cells including NK-resistant tumour cells. *In vitro,* LAK cells have been shown to have broad cytotoxic activity against a wide variety of tumour cells. Rosenstein *et al* 1984 found that murine LAK cells produced by culture of lymphocytes with IL-2 manifested significant lysis of several fresh NK-resistant syngenic cell lines (MCA-102, MCA-106, EL-4 lymphoma) and the NK-sensitive YAC target. Lafreniere *et al* 1985 and Papa *et al* 1986 demonstrated that LAK cells can mediate anti-tumour effectiveness despite the lack of specific antigen recognition by the immune system to the MCA-102 sarcoma which is a non-immunogenic tumour. LAK cells can effectively lyse autologous fresh tumour cells in addition to allogeneic fresh tumours and all cultured tumours tested, including those NK-resistant targets, in a 4h chromium-release assay (Grimm *et al* 1982; Lotze *et al* 1981). Vujanovic *et al* 1988b found that rat LAK cells had very high levels of cytolytic activity to a variety of tumour cells including *in vitro* tumour lines raised from various tissue origins

from rat, mice or human. LAK cells could easily lyse fresh syngenic ascitic or solid tumour cells. LAK cells were unable to lyse fresh normal targets. LAK cells had antitumour activity in standard adoptive transfer (Winn-type) assays to an NK-resistant syngenic adenocarcinoma-MADB106 (Vujanovic *et al* 1988b). Human MNC incubated in the presence of high concentrations of rIL2 develop broad cytotoxic reactivity against autologous and allogeneic fresh tumour cell targets and tumour cell lines (Grimm *et al* 1982; Itoh *et al* 1986). The broad spectrum of tumour lysis distinguishes LAK cells from that of conventional CTL (cytotoxic T lymphocytes) which require the recognition of specific antigens and MHC molecules on tumour cells.

Cytotoxicity of LAK cells has been demonstrated *in vivo*. LAK cells and IL-2 are effective for a variety of histological tumour types (sarcoma, adenocarcinoma, and melanoma) and for both immunogenic and nonimmunogenic tumours (Papa *et al* 1986; Lafreniere *et al* 1985a, 1985b; Shu *et al* 1985). Evidence of tumouricidal activity has also been obtained in different sites aganst established metastatic tumours such as the hepatic metastases (Lafreniere *et al* 1985), pulmonary metastases (Papa *et al* 1986), renal (Salup *et al* 1986), and brain metastases (Takai *et al* 1988). A direct relationship has been shown between the number of LAK cells transferred, the amount of IL-2 given and the therapeutic effect achieved in murine models (Mule *et al* 1985).

The mechanisms responsible for the tumouricidal effect of LAK and A-LAK cells remains obscure, but clearly require cell : cell contact and this is dependent on gaining access to the target cells. *In vivo* this includes the process of LAK cells extravasation through the microcirculation. Basse *et al* 1991b have demonstrated that adoptively transferred A-LAK

cells were able to migrate out of tumour vessels and establish direct contact with tumour cells within 16h of injection. These authors also obtained direct evidence that A-LAK cells migrated to and heavily infiltrated metastases of murine tumours in different organs. Ultrastructural study of LAK cell lysis has shown that LAK cells make close contact with the target cell followed by the delivery of as yet unknown products into the tumour cells (Chen *et al* 1991). Harcel *et al* 1991 found that the frequency of LAK cells in the developing liver metastases was 3-6 times higher than that in the surrounding normal liver tissue, which indicated that LAK cells lyse tumour cells by direct attack. In liver metastasis-bearing animals, very few A-LAK cells were seen in normal and metastatic tissue following systemic injection. Lotzova *et al* 1987 found that following systemic injection, very few LAK cells arrive at the sites of tumour and relatively small fraction of total injected cells localised in tumour. Basse *et al* 1991b indicated that the effectiveness of LAK cells *in vivo* depands on the route of administration. Substantial infiltration of lung metastases was seen after systemic intravenous injection, whilst significant infiltration of liver metastases was seen only after intraportal injection of the A-LAK cells, indicating impaired trafficking of systemically injected A-LAK cells through the lung capillaries. Sasaki *et al* 1989 indicated that increased rigidity of LAK cells coupled with their large cell size may explain the poor localisation of LAK cells into tumour targets *in vivo*, because of trapping of the cells in the lungs. Felgar *et al* 1990 pointed out that A-LAK cells do not actively migrate towards tumour sites. A-LAK cells appeared to enhance suboptimal but ongoing host antitumour effector mechanisms by release of cytokines, functioning as a form of "helper cell". Sasaki *et al* 1991 suggested that A-LAK cells might induce tumour necrosis by impairment of tumour vascular

supply. Jain *et al* 1989 indicated that cytokines produced by A-LAK cells, such as interferon, tumour necrosis factor-alpha (Futami *et al* 1991), IL-2 and granulocyte macrophage colony-stimulating factor, may diffuse into the tumour and produce a toxic effect in tumour cells either directly or by recruitment of other cytotoxic cells to the area of the tumour.

1.4 *In Vivo* Distribution of LAK Cells Infused Systemically or Regionally

Only a few studies of LAK cell distribution following systemic or regional infusion have been reported. Studies of cell distribution in the rat have shown that purified LAK cells are primarily trapped in the lungs 2h after systemic infusion. By 24h, the cells had left the lungs and were largely located in the liver and spleen. Forty seven per cent of ^{51}Cr-labelled purified A-LAK cells injected systemically into syngeneic F344 rats accumulated in the lungs 2h after injection, then redistributed to the liver and the spleen by 24h (Maghazachi *et al* 1988c). Maghazachi *et al* 1988b found that ^{51}Cr-labelled LAK cells also displayed a restricted pattern of distribution, localising mainly in the lungs at 2h after iv. injection but redistributing to the liver and the spleen by 24h. Labelled LAK cells were rarely recovered from the lymphoid tissues, including the peripheral lymph nodes and the mesenteric lymph nodes. Felgar *et al* 1990 analysed tissue distribution patterns of purified A-LAK cells in normal and tumour-bearing rats. These authors also found that A-LAK cells first migrated to the lungs 2h after systemic infusion followed by migration to the liver and spleen by 24h, in both normal and tumour-bearing rats. Liver metastases did not significantly alter the migration pattern of iv. administered A-LAK cells. Maghazachi *et al* 1989 found that preincubation of A-LAK cells with certain carbohydrates resulted in redistribution of A-LAK cells from the lungs to the liver. They suggested that sequestration of A-LAK cells in the lung capillaries might be mediated by cell surface receptors. However there is also evidence that the capillaries of the lungs and the intestinal tract restrict systemically injected LAK cells because of the large size and rigidity of these cells (Sasaki *et al* 1989).

Several investigators have studied the migration pattern and tissue distribution of cytotoxic cells using different labelling radioisotopes. Reynolds *et al* 1984 demonstrated that 2h following intravenous injection, a high percentage of ^{111}In-oxine radiolabelled LGL were found in the liver (25.1%), spleen (17.7%) and lungs (13.5%), and a small proportion remained in blood (9.0%). At 24h following intravenous injection, more radioactivity was detected in the liver (31.9%) and spleen (20.9%), and less in the lungs (6.5%) and blood (4.0%). Very little radioactivity was found in the thymus, CLN, MLN, SI, or kidneys. Rolstad *et al* 1986 showed that 0.5h after iv. injection, ^{111}In-oxine labelled LGL located in liver (31.0%), lung (24.0%), spleen (11.0%) and in the blood (12.8%) in athymic nude rats. By 24h these cells redistributed into liver (38.0%) and spleen (17.0%) with a decreased radioactivity in lung (13.0%) and blood (9.3%). Only a very low percentage of radioactivity was recovered in MLN and kidney (0.3%). The main sites of localisation of [^{3}H]uridine labeled LGL were the alveolar walls of the lungs and the red pulp of spleen. There was no radioactivity detectable in the circulatory areas of T lymphocytes such as lymph nodes and splenic white pulp (Rolstad *et al* 1986). Using microautoradiography, Takai *et al* 1988 administered [^{3}H]thymidine-labelled LAK cells systemically iv. into rats. LAK cells accumulated in the lung shortly after administration and then appeared in the liver and spleen. By labelling LGL with [^{3}H]uridine, it has been shown that 2h after systemic iv. injection, trapping of these cells is noted in the lungs (26.1%), liver (22.2%), and spleen (8.5%). By 24h the percentage of cells decreased to: lung 10.0%, liver 11.5% and spleen 6.0% (Rolstad *et al* 1986). The decreased radioactivity in these organs correlated with the total radioactivity decreasing (from 97.4% in 1/2h to 66.4% in 2h

followed by 33.9%24h after injection), which suggested a rapid release of [^{3}H]uridine from cells (Ettinghausen *et al* 1985; Rolstad *et al* 1986). Rolstad *et al* 1986 demonstrated that both ^{51}Cr and ^{111}In-oxine radiolabells resulted in a similar distribution pattern for LGL. Marincola *et al* 1988 investigated the distribution of human PBL and LAK cell *in vivo* in cancer-bearing nude mice by labelling cells with ^{111}Indium-Oxine. After administration of these cells into human pancreatic cancer-bearing nude mice, they found that LAK cells were taken up predominantly by liver and spleen, but PBL located mainly to lung, kidney, skin and pancreatic tumours.

Maghazachi *et al* 1990 investigated the different distribution of LAK cells labeled with four radioisotopes: ^{51}Cr; ^{111}In-oxine, ^{125}I-dUrd and ^{111}In-Cl. They found that 30min and 2h after systemic iv. injection, cells labelled with ^{111}In-oxine had an equivalent distribution into the lung and liver, those labelled with ^{51}Cr or ^{125}I-dUrd showed a higher accumulation in the lungs, whereas cells labelled with ^{111}In-Cl entered into the liver and blood. Twenty four hours after injection, LAK cells labelled with ^{111}In-Cl, ^{111}In-oxine or ^{51}Cr redistributed to the liver and spleen in variable concentrations. Cells labelled with ^{125}I-dUrd were not detected in any organ tested, probably because of the elution of ^{125}I-dUrd from the labelled cells after iv. administration. But the regional administration of ^{125}I-dUrd labelled cells through portal vein did not show the elution of ^{125}I-dUrd from cells (Basse 1992b, 1992a).

The pattern of *in vivo* distribution of rat LAK cells is similar to that of LGL/NK cells but different from that of activated T cells (Maghazachi *et al* 1988c). Maghazachi *et al* 1988b indicated that a LAK-like migration could be shown by LGL, but both T cells and LAL showed a different

pattern of distribution from LGL/LAK cells. Reynolds *et al* 1984 also showed that the distribution of LGL/NK differed markedly from that of the other leukocytes. By 2h-4h following transfusion, there were significantly more LGL (13.5%) than T cells (6.4%) remaining in the lungs and the difference persisted through 48h (5.4% vs 0.8%). Zoller *et al* 1982 suggested that rat LGL have a lower capacity than T or B cells to traverse the blood-lymph barrier. The migration pattern of cultured pre-immunised CTL is markedly different from immunised cells that had not been cultured with IL-2. With an increased CTL frequency after culture in MLC with IL-2, the cell migration was progressively decreased in spleen, lymph nodes and femurs, while increased levels of radioactivity were detected in the lung and liver (LeFevre *et al* 1987). Lotze *et al* 1980 investigated the migration patterns of human MLC-generated cells and murine CTL clones cultured with IL-2. The results showed that ^{51}Cr labelled murine activated cells were located in the lungs 4h after iv injection and redistributed to the liver and spleen over the next 24h. When compared with labelled uncultured splenocytes, the cells cultured with IL-2 showed an early increased accumulation in the lung. Human activated cells exhibited a similar trafficking pattern to cells of the mouse. It was found that only 5-6% of infused activated cells could be detected in blood 20min after injection, compared to other studies showing 50% of uncultured cells detected in the blood shortly after iv infusion. This difference suggests that the large IL-2 cultured cells are quickly removed from the circulation. It was most likely that activated cells were more easily entrapped by the first pass through the capillary bed of the lung. Injection of ^{51}Cr-labelled cultured CTL resulted in extensive deposition of cells in the lungs and had very little associated radioactivity in lymph nodes after iv. injection (LeFevre *et al* 1984). After systemic infusion LAK cells

were localised to the lungs but not to the tumour site (Hayakawa 1992). These studies generally reveal an accumulation of cells in the lungs and a paucity of CTL in lymphoid sites. There are different views as to what mechanisms are responsible for the differential entrapment of activated cells. Lotze *et al* 1980 suggested that cell membrane alterations may be induced by culturing cells in IL-2 and these changes may explain the altered trafficking pattern. The cultured cells are large and blastic, and may become trapped in the capillary bed of the lung. Comparing the results from studies of *in vivo* LGL or LAK cell distribution, after systemic iv. injection, there is a higher entrappement of LAK cells in rat lungs (Maghazachi *et al* 1988c; Reynolds *et al* 1984). Similarly, more activated-T cells are entrapped in the lungs than fresh-T cells. LAK cells and activated T cells have a relatively large diameter (11.0μm, 9.7μm) compared with LGL (mean diameter 7.2μm) and fresh T cells (6.6μm). LAK cells are significantly less deformable than other cell types such as nonactivated lymphocytes. Cell deformability was independent of cell size. LAK cells contain numerous cytoplasmic granules. Both the cell membrane and the cytoplasmic factors contribute to the rigidity of LAK cells. Structural rigidity is known to influence the behaviour of cells in capillaries, by hindering their passage through small capillaries and altering their haemodynamic behaviour in postcapillary venules (Sasaki *et al* 1989; Worthen *et al* 1989). Maghazachi *et al* 1988b showed that LAK cells may have surface carbohydrate-binding protein which allows them to bind to endothelial cells lining blood vessels. In summary the reason for the early high retention of A-LAK cells in the lung seems to be related to the characteristic irregular morphology of A-LAK cells, the high rigidity of these cells, and altered cell surface characteristics.

The migration of lymphocytes to Peyer's patches and lymph nodes appears to be associated with the presence of receptors on lymphocytes for high endothelial venules (HEV) (LeFever *et al* 1984; Lotze *et al* 1980; Zoller *et al* 1982). The paucity of production of CTL lymph node lymphocytes may result from the loss of HEV receptors and other differences in cell surface carbohydrate composition (Carroll *et al* 1983). McCoy *et al* 1990 found that rat LGL/NK cells cultured with IL-2 results in altered expression of specific cell-surface carbohydrates which alter their lytic function.

It is possible that the limited ability of LAK cells to extravasate could be modified. Migliori *et al* 1987 demonstrated that macrophage activation factor could effectively attract LAK cells from the circulation. By stimulation of macrophages at the sites of tumour growth, more LAK cells were attracted. Marincola *et al* 1988 found that when the nude mice were pretreated with human recombinant tumour necrosis factor (TNF), localisation of LAK cells was enhanced both in implanted tumours in the pancreas and in the surrounding normal tissue.

The administration of IL-2 may be necessary to maintain the cytotoxic activity of injected LAK cells and their proliferation *in vivo* (Ettinghausen *et al* 1985). Administration of IL-2 does not result in the accumulation of LAK cells at the site of IL-2 injection, nor did it result in a modulation of the overall distribution pattern or total recovery of radiolabelled LAK cells in organs (Maghazachi *et al* 1988c). The administration of IL-2 does not alter the anatomic distribution of A-LAK cells when analyzed by tissue sections (Felgar *et al* 1990).

Harcel *et al* 1991 found that the frequency of LAK cells localised in developing liver metastases was 3-6 times higher than that in the surrounding normal liver tissue. Ames *et al* 1989 have shown that the preferential localisation of TIL in tumour sites to normal tissue. Griffith *et al* 1989 demonstrated that transferred human autologous TIL pretreated with IL-2 preferentially localised to metastatic tumour sites. They indicated that this localisation was based on the ability of TIL to distinguish tumour from normal tissue. LAK cells showed increased migration to tumour site compared to splenocytes (Midis *et al* 1992). Morita *et al* 1987 found that the localisation of IL-2-activated PBL to hypernephroma was increased when these cells were given as an intra-operative, intra-arterial infusion just prior to nephrectomy. Hayakawa *et al* 1991, 1992 found that by transarterial regional infusion, LAK cells showed short-term but appreciable accumulation of LAK cells in the tumour site.

There are several reports regarding the regional infusion of LAK cells in tumour immunotherapy and in experimental systems. Keilholz *et al* 1992 showed that the regional administration of LAK cells was essential for successful treatment. Tumour regression was observed only in anatomic areas of the liver into which the LAK cells were locally perfused. Basse *et al* 1991b studied whether a more direct delivery of A-LAK cells to the liver could enhance the uptake of A-LAK cells into the liver and improve infiltration of liver metastases. Using rhodamine-labeled A-LAK cells they found that after these cells were inoculated directly into the portal system, more than five times as many cells were found in the normal liver tissue compared to systemic iv. injection. By 16h, the number of A-LAK cell in the normal liver tissue decreased whereas in tumour tissue the number was more than

5 times as many as after systemic iv. injection. These findings indicate that the route of injection is an important factor in achieving accumulation of LAK cells within liver metastases. Conventional systemic iv. injection could be viewed as regional infusion for the lung. It is found that adoptively transferred cells reach the metastatic lesions in different organs in a time-dependent manner. 0-1h after injection, almost no infiltration of the metastases by A-LAK cells is seen. Many A-LAK cells are located at the nomal lung tissue at the begining, then disappear from nomal lung tissue at 16h whereas they are found in 90%-95% of pulmonary metastases. Kuppen *et al* 1992 found that 2h after local infussion (through hepatic artery for liver and jugular vein for lungs), a high concentration of LAK cells in the first capillary bed can be obtained. They suggested that local administration of LAK cells may be more effective against tumour. Lafreniere *et al* 1985 and Ettinghausen *et al* 1985 have mentioned that the intraportal or intrahepatic administration of LAK cells is significantly more effective than the systemic iv. administration of these cells in treatment of liver metastases. Nelson *et al* 1990 also demonstrated that cultured human PBL distributed to intraperitoneal tumour when administered intraperitoneally, but did not do so when administered systemically intravenously. Basse *et al* 1992b found that a substantial accumulation of A-LAK cells in the liver could be achieved only when the cells were injected directly into the portal vein.

1.5 The Effect of Mannitol on the Blood Brain Barrier (BBB)

In 1990 McCutcheon *et al* reported their investigation of adoptive immunotherapy for intracerebral metastases in mice. They demonstrated that intracerebral tumours are completely resistant to treatment with IL-2 and LAK cells. It has been demonstrated that after intravenous administration, LAK cells can migrate to some extracerebral tumour sites. The demonstration that intracerebral tumours were resistant to treatment with IL-2 and LAK cells suggests that the trafficking pattern of LAK cells may exclude them from the cerebral circulation, or that these cells are unable to breach the BBB. Takai *et al* 1988 reported controversial results suggesting intravenous or intracerebral injections of LAK cells resulted in longer survival of Fischer rats that had received inoculation of T9 gliosarcoma cells into the basal ganglia. Other experiments have failed to demonstrate an increased survival time in rodents with primary brain tumours treated with activated lymphocytes and IL-2.

The BBB can be opened transiently in humans and in experimental animals by infusing a hydrophilic non-electrolyte hyperosmolar solution such as mannitol into the internal carotid circulation. Hiesiger *et al* 1986 investigated the effect of intracarotid infusion of hyperosmolar mannitol solutions on cerebral capillary permeability and blood flow. It was found that capillary sensitivity to osmotic manipulation is both tumour- and concentration-dependent. Before mannitol administration, the blood-to-tissue transfer constant (K) values in brain tumour sites were significantly higher than those in cortex. C6 gliomas were about three times more permeable than W256 tumours. After the administration of 1.6M mannitol, the K values for

both tumours and adjacent tissue were increased. Groothuis *et al* 1990 found that there was no change in the rate of delivery of cells to brain tumours as a result of hyperosmotic BBB disruption, however they found that there was a marked enhancement of delivery to tumour-free brain. It was suggested that the reason is probably due to the type of tumour used for investigation (Rapoport *et al* 1990), i.e. the efficacy of hyperosmolar mannitol depends on the permeability characteristics of the tumor type. In addition there was a direct correlation between tumour size and increased capillary permeability. Mannitol at a concentration of 1.37M did not increase the K values for either tumour or adjacent tissue.

Osmotic shrinkge of cerebrovascular endothelial cells is generally considered to be the prodominant mechanism of BBB breakdown, resulting in passive diffusion across separated interendothelial cell junctions and enhanced endocytosis and vesicular transport across the endothelium (Houthoff *et al* 1982). Franceschini *et al* 1988 demonstrated that treatment with hypertonic solutions of mannitol (0.25M, 0.5M, 1M) caused opening of the blood brain barrier in the newt due to the opening of the tight junctions between the endothelial cells, which was demonstrated by the presence of the tracer in interjunctional regions between adjacent endothlial cells. They found in the specimens treated with hyperosmolar mannitol that increasing the osmolarity of the solution caused more extensive disruption of the BBB. The molecular mechanisms mediating BBB breakdown in rats has been investigated by Koenig *et al* 1989. They reported that rapid stimulation of ornithine decarboxylase (a polyamines rate-regulating synthetic enzyme) activity and polyamine synthesis in cerebral

microvessels is involved in the breaching of the BBB by the intracarotid infusion of 1.6M hyperosmolar mannitol.

Chapter 2 Materials and Methods

2.1 Generation and Characterisation of A-LAK Cells

A-LAK cells were generated according to the procedure described by Vujanovic *et al* in 1988. The characterisation of A-LAK cells was defined in three ways: (i). Cell surface marker analysis by immunofluorescent staining; (ii). morphological analysis by Giemsa-stained cytocentrifuge preparations; (iii). cytotoxicity assay in a standard 4h ^{51}Cr-release microcytotoxicity assay.

2.1.1 Generation of A-LAK cells

Rats were obtained from the animal house of University of Tasmania. They were housed and kept in the animal room in the Clinical School in accordance with NH&MRC guidelines. Young male rats 100g-120g were used for splenectomy to obtain cells from spleen for cell culture. Syngeneic rats about 120g were used for the cell trafficking study.

During the experiments, the rats were anaesthetised with inhalation of ether combined with ip. injection of 1:10 diluted pentobarbitone sodium (Boehringer Ingelheim, Australia). For the inhalation anaesthesia, the rat was placed into a glass box containing with 5ml-10ml ether and kept there until it's whiskers stoped moving. Usually the rat could wake up in 1-5min without continuous inhalation of further ether or ip. injection of pentobarbitone. The Pentobarbitone sodium(60mg/ml) was diluted with PBS-A which was sterilised by filtration (0.22μ filter, millipore Corporation, USA) in the ratio of 1:10. The diluted pentobarbitone was atored at 4-8°C. Before it was injected intraperitoneally into the rat, the dose was determined by the weight of the rat and administered at 30mg/kg (eg. 0.5ml for a 100g rat). This

combined anaesthesia method gave a satisfactory result which usually lasted for more than 30min.

Dose of Pentobarbitone Na used in the experiments (ip.)

weight of rats body	minimum Volume of 1:10 Pentobarbitone Na	Maximum Volume of 1:10 Pentobarbitone Na
100g	0.5ml	0.5ml
110g	0.5ml	0.55ml
120g	0.5ml	0.60ml
130g	0.5ml	0.65ml
140g	0.5ml	0.70ml
150g	0.5ml	0.75ml

Under the anaesthesia, laparotomy was carried out aseptically using standard procedures (Van Dongen *et al* 1990). All the operative instruments were autoclaved at 120°C for 15min and dried for 20min in the autoclave machine. Rat skin was prepared with 70% ethanol and the abdomen was opened through the midline. After the spleen or mesenteric lymph nodes were removed, the abdomen was closed using 4-0 absorbable suture and the skin edges were brought together and secured with clips. Rats which underwent splenectomy were sacrificed by neck spine dislocation immediately after operation and under full anaesthesia.

Each rat spleen was placed into RPMI-1640 (Flow, UK.) in a sterile petri dish in a laminar flow hood to perform the procedure of lymphocyte preparation. Each spleen was gently squashed and single-cell suspensions were obtained, washed in RPMI 1640 three times to remove cell debris (at 250g for 5-10min). Cells were resuspended in 6ml of Tissue Culture Medium (TCM) which consisted of RPMI 1640 with 10% FCS (Flow, UK.). Splenic mononuclear cells were isolated after

centrifugation of splenocytes on Ficoll-Hypaque gradients (Flow, UK, density 1.077g/ml, at 300g for 20min).

To deplete B cells and macrophages, these mononuclear cells were incubated in a nylon wool column (Julius *et al* 1973). The nylon wool (Polysciences, Inc., US.) was previously soaked extensively in 0.9% saline for one week to remove toxic substances. The nylon wool column (20ml syringe containing 0.6g of sterile nylon wool) had been incubated with 15ml warm TCM in 37°C for 1h. $0.5x10^8$ to $1.0x10^8$ cells in 1-2ml were incubated on the columns at 37°C for 1h. Nylon wool non-adherent lymphocytes were collected by gently washing the column through with 20ml-30ml warm TCM.

For A-LAK cell generation, 10-20ml nylon wool nonadherent lymphocytes at a density of $2.0\text{-}3.5x10^6$ cells/ml were cultured in Complete Medium [CM: RPMI 1640 medium (Flow, UK.) supplemented with 10% heat-inactivated FCS (Flow, UK.), 2mM L-glutamine (Flow, UK.), 50µg/ml streptomycin and 100 U/ml penicillin and 1% nonessential amino acids (Flow, UK), Hepes buffer (CSL, Australia), 2-Mercaptoethanol ($7x10^{-4}$ M) and 1000 U/ml human rIL-2 (Boehringer Mannheim, W.Germany)] in $75cm^2$ tissue culture flasks (Corning, US.) using the method described by Vujanovic *et al* 1989. The cells were cultured in a humidified atmosphere of 5% CO_2/95% air at 37°C. After culture with rIL-2, some LGL selectively adhered to the plastic surface of the culture flask. At 48h of culture, the nonadherent cells were decanted from the flask, removed by centrifugation, and the medium passed through a 0.45-µm millipore filter (Flow, UK). In this way the conditioned medium was retained. After washing the flask with warm TCM to clear away the nonadherent cells, the conditioned

medium was added back to the adherent LGL cells in the flask. The adherent cells were then cultured in the pure conditioned medium or in conditioned medium diluted with fresh CM for several more days till the adherent LGL had undergone adequate expansion.

A-LAK cells were harvested after adding 5ml cold 5mM EDTA (BDH, UK.) in PBS to the culture flask. Then cells were washed in RPMI-1640 and resuspended in RPMI 1640 + 10%FCS. Cell viability was assessed by trypan-blue dye exclusion. When cell viability was less than 90%, the cells were centrifuged on Ficoll-Hypaque gradients (density=1.077 g/ml, Pharmacia, USA; 400g for 30min) to remove the dead cells. The viable cells were collected, washed and resuspended in medium for radio-labelling or cell characterisation.

2.1.2 Characterisation of A-LAK cells

2.1.2a Cell surface marker analysis

Cell surface marker analysis was carried out using immunofluorescent staining. A-LAK cell surface phenotype has been discussed in section 1.3.2. The monoclonal antibodies (Mab's) used for A-LAK cell surface marker analysis in this experiment were:

OX8: a murine IgG1 for the surface marker of CD8 on rat NK cells, LAK cells, T suppressor/cytotoxic lymphocytes; **OX19:** murine IgG1 for the surface marker of CD5 on rat pan-T cells; **OX34:** murine IgG2a for the surface marker of CD2; **OX1:** murine IgG1 for rat leucocyte common antigen. These Mab's were obtained from Serotec, Australia. The second-step reagent was sheep-antimouse IgG F(ab')2 fragment conjugated with FITC (Silenus, Australia); Sheep anti-rat immunoglobulin antibody (Silenus, Australia) conjugated with FITC were used for rat B cells; Mouse IgG1 conjugated with FITC (Becton Dickinson, US.) were used as negative controls.

Chambers *et al* 1989 introduced a more specific Mab for rat A-LAK cells and NK cells named 3.2.3 antibody, an IgG1k (CD16). This Mab was not available in Australia when cell surface marker analysis was performed in this study.

The Mab's were stored at -20°C. When used in immunofluorescent staining, 1µl of each of these undiluted antibodies or 10µl of 1:10 freshly diluted antibodies with PBS + 0.5%BSA were mixed with 1.0-2.0x10^5 cells in 100µl staining buffer.

For surface marker analysis, nylon wool nonadherent lymphocytes or A-LAK cells from culture were washed for 3 times in cold PBS-A (phosphate buffered saline; Oxoid limited, UK) and once in staining buffer. The staining buffer comprised PBS-A, 0.1%sodium azide (BDH, UK) and 20% bovine serum albumin (CSL, Australia). Then 1.0-2.0x10^5 lymphocytes or A-LAK cells in 12x75-mm plastic tubes in 100µl cold staining buffer were mixed with 10µl of primary Mab's (1:10) at 4°C for 45-60min. The cells were washed three times in staining buffer and resuspended with 10µl of the second antibody and kept at 4°C for 30min. After three further washes the cells were resuspended in 10µl staining buffer and put on glass slides (Knittel Glaser, Germany). They were fixed in 100% Ethanol after dried on slides at room temperature. 20µl PBS-Azide-Glycerol (Heidelberg, US) was added and cells were covered by a glass cover slip. Cells were counted using alternate incidental or UV light.

2.1.2b Morphological analysis

Giemsa-staining cytocentrifuge preparations were made using of 2.0-4.0x10^5 cells in 200µl to analyse cell morphology. Cells were washed in PBS twice and resuspended in PBS + 10%FCS at 1.0-2.0x10^6 cells/ml. Each slide was made using 200µl (2.0-4.0x10^5 cells). Cytocentrifuge holders were washed in sterilised water and kept dry before use. Special filter cards (Shandon, UK) together with slides and holders were placed into the cytocentrifuge and the cells were added into the holders. After centrifugation at 1000rpm for 3min, the cells were dried on the slides at room temperature. Giemsa stain (BDH, UK) was used at 100% density or diluted 1:1 with PBS to stain the cells on slides for 5min. The slides were rinsed with PBS, dried at room temperature and fixed under

cover slips. The cells were examined using a Lietz Ortheplan microscope and photographic records were made.

2.1.2c Cytotoxicity assay

Cytotoxicity was measured using a standard 4h ^{51}Cr-release microcytotoxicity assay carried out in 96-well, round-bottomed micro-well plates (Corning, USA). Target cells were YAC-1 and P815. YAC-1, a mouse T cell lymphoma, was used as an NK-sensitive target. P815, a murine mastocytoma, was used as an NK-resistant target cells. Target cells were kept in culture medium (RPMI 1640 with 10%FCS, 50µg/ml streptomycin and 50^{u}/ml penicillin, 2mM L-glutamine and HEPES buffer) at 37°C and subcultured 2-3 times per week. Target cells were harvested from the culture when they were needed for cytotoxicity assays. 1.0-2.0x10^6 target cells were labelled with 100µCi of ^{51}Cr at 37°C for 1h, washed, resuspended in RPMI-1640 at a concentration of 5.0x10^4 cells/ml, and seeded into 96-well round-bottomed microplates at 5x10^3 cells/well. Effector cells were harvested from A-LAK cell cultures, washed and resuspended in RPMI 1640 + 10%FCS at concentrations of 2.0x10^6 cells/ml, 5.0x10^5 cells/ml, and 1.25x10^5 cells/ml. A-LAK cells were added to microwells to give effector: target ratios of 40:1, 10:1 and 2.5:1 in a final volume of 200µl per well. Each ratio was set up in triplicate. After 4h incubation at 37°C, 100µl of supernatant was collected from each well and was counted in a gamma counter to determine experimental release. Spontaneous release and maximum release were also measured. Spontaneous release was obtained from wells receiving target cells only. Maximum release was obtained from target cells to which 1%Triton X-100 (BDH, US) was added to cause

complete cell lysis. Percentage cytotoxicity was calculated using the formula:

$$\% \text{ cytotoxicity} = 100 \times \frac{\text{experimental release - spontaneous release}}{\text{maximum release - spontaneous release}}$$

2.2 Short-term *in Vivo* Distribution of Radiolabelled A-LAK Cells in the Rat

2.2.1 Radiolabelling of A-LAK cells

A-LAK cells harvested from culture were labelled with ^{51}Cr. After suspending 1-9x10^6 cells in 1ml RPMI 1640 + 10%FCS, 100μCi ^{51}Cr was added and the cells were incubated at 37°C in a water bath for 1h. The radiolabelled A-LAK cells were washed in RPMI-1640 5 times. 50μl of supernatant of the first, 4th and last washing were taken for gamma-counting to ensure that there was no free ^{51}Cr remaining in the supernatant. After labelling, A-LAK cells were resuspended in serum-free PBS-A, and cell viability was checked by trypan blue exclusion before infusion.

Radiolabelled A-LAK cells were suspended in 1.2ml PBS-A. 1ml labelled A-LAK cells were placed in a 2ml-syringe for infusion into rats for the trafficking study. Three aliquots of 25μl labelled cells were taken at the same concentration as the injected cells and counted on gamma-counter. The radioactivity (cpm) in the total volume of infused cells was obtained by correcting the cpm obtained from 25μl labelled cells to the total injected cell volume. (total injected cpm in 1ml= 40 x cpm in 25μl). The remaining labelled cells were used to check the spontaneous release of ^{51}Cr from the cells. These remaining cells were resuspended in 300μl RPMI 1640 + 10%FCS and put separately into three tubes (100μl for each tube). After centrifugation at 1000rpm for 3min, cells were incubated at 37°C in 5%CO_2/95% air for 2h. The radioactivity in 1/2 supernatant (50μl) and in 1/2supernatant+pellet was counted using

a gamma-counter. Spontaneous release (SR) was calculated using the formula:

$$\%SR = \frac{2 \times \text{cpm in } 1/2 \text{ supernatant} \times 100}{\text{cpm in } 1/2 \text{ supernatant} + \text{cpm in } (1/2\text{supernatant+pellet})}$$

Because the ^{51}Cr may also be released from the injected cells *in vivo*, the total injected cpm measured before infusion could no longer reflect the CPM of injected cells in 2h. Therefore the CPM of A-LAK cells after 2h was obtained using the formula:

CPM of injected cells in 2h = total injected cpm x (1–%SR).

2.2.2 Adoptive transfer of radiolabelled A-LAK cells into recipient syngeneic rats

Trafficking studies were carried out in three groups (eight rats per group). One milliliter of radiolabelled A-LAK cells was infused into each rat. In group (1), A-LAK cells were systemically injected through the tail vein. In group (2), A-LAK cells were regionally injected through the portal vein. In group (3), A-LAK cells were injected through the portal vein following intraportal infusion of 30% mannitol.

For the injection of ^{51}Cr-labelled A-LAK cells, each rat was anaesthetised with inhalation of ether and ip. injection of pentobarbitone sodium as described in section 2.1.1. A 27gar needle (Terumo, Melbourne, Australia) was used for cell injection through the tail vein. The rat was then kept in its cage for 2h. For regional infusion, laparotomy was carried out aseptically, the portal vein was exposed, a PE10 cannula was inserted and the cells were infused under observation. When the cannula was removed, the small hole in the

portal vein was compressed for 1-5min to prevent bleeding. The rat's abdomen was closed and rat was kept in its cage for 2h.

This study was initially planed to inject cells through hepatic artery not the portal vein. Unfourtunitely it appeared impossible due to technical difficulties with PE10 cannula which was the smallest cannula that could be connected to a 27gar needle(smallist available). We realised that the hepatic metastases are via the hepatic arterial but not the portal vein. However with the aim of studing the effect of mannitol on the distribution of LAK cell in tissue level in the no tumour-bearing organs, the potal vein infussion should be able to lead us to obtain the close results about the cell distribution as it would show via hepatic artery.

2.2.3 Determination of tissue distribution of radiolabelled A-LAK cells

Two hours after A-LAK cell infusion, the rats were sacrificed by inhalation of an overdose of ether. A blood sample was taken by heart puncture and 3 MLN, the spleen, both kidneys, liver, lung and brain were removed and each organ was weighed. The radioactivity associated with liver, lung and brain samples were determined by counting weighed pieces of each organ and then correcting counts for the total weight of each organ. The radioactivity associated with the other organs were determined by counting the whole organ. Blood-associated radioactivity was normalised to a total volume of 10ml per rat. Results of the distribution of A-LAK cells were expressed as a percentage of injected CPM in organs. They were also expressed as percentage of radioactivity in every gram of organ which was

specifically used for representing the distribution of A-LAK cells in different tissues without the influence of the organ size.

$$\%\text{distribution of LAK cells in organ} = \frac{\text{CPM in that organ}}{\text{CPM of all cells}} \times 100$$

$$\text{CPM in organ} = \frac{\text{cpm of piece of organ}}{\%\text{counted}}$$

$$\%\text{counted} = \frac{\text{weight of counted piece}}{\text{weight of whole organ}} \times 100$$

$$\%\text{distribution of LAK cells per gram of organ} = \frac{\%\text{distribution in organ}}{\text{weight of organ}}$$

2.3 Administration of Mannitol

Sterile 30% (0.03 g/dl) Mannitol (Multicel, Australia) at the dose of 0.3mg/kg body weight (100µl/100g) was infused into the portal vein 5min before the A-LAK cell injection.

To study the effect of intraportal mannitol on the distribution of A-LAK cells in the rat, the distribution of intraportally infused A-LAK cells after the mannitol was compared with that of intraportal A-LAK cells without mannitol. Thirty% mannitol instead of 20% mannitol was used in this study because it had been demonstrated that the higher osmolal solution had a greater effect in increasing the space between vascular endothelial cells (Franceschini *et al* 1988). At concentration of less than 20%, mannitol had no obvious effect in opening the Blood-Brain Barrier (Hiesiger *et al* 1986). The A-LAK cells were infused through the portal vein cannula 5min after the mannitol injection because it had been shown in these studies that the effect of mannitol commenced after 5-30min.

For the administration of mannitol and A-LAK cells, laparotomy was carried out aseptically. The portal vein was well exposed and a PE10 cannula was inserted. The 30% mannitol was previously warmed in a 37°C water bath to dissolve all the crystals in the mannitol solution. Following the administration of mannitol, 1ml radiolabelled A-LAK cells was infused under observation. The rat was kept in its cage for 2h. The rats were sacrificed, organs were harvested and CPM in organs were determined as per Section 2.2.3.

Chapter 3 Results

3.1 A-LAK Cell Generation and Characterisation

3.1.1 Isolation of A-LAK precursor from spleen mononuclear cells

To study the distribution of A-LAK cells following infusion into the rat it was necessary to obtain the purest possible culture of A-LAK cells as possible. The isolation of LGL was carried out in two stages: firstly by incubation of spleen mononuclear cells in a nylon wool column to eliminate Macrophages and B cells; and secondly by removal of flask non-adherent T cells from the culture flask. The number of mononuclear cells obtained from the spleen following centrifugation on Ficoll-Hypaque (p=1.077) ranged between $6.7x10^7$ and $17x10^7$ cells. These cells were a mixed population including T cells, B cells and Microphages, which were shown in Fig. A. Following nylon wool incubation cell numbers ranged between $2.7x10^7$ and $7x10^7$ cells. The mean percentage of cells removed on NW-column was 57.9%±14.5%. These nylon-wool nonadherent lymphocytes are shown in Fig. B. After 48h of cell culture the culture flask was washed gently. The cell population present in the washing were termed flask non-adherent cells.

Immunofluorescent staining was only possible on a small number of samples. The results are shown in Table 1. The results indicated that the incubation of lymphocytes on nylon wool columns resulted in a modest reduction of B cells in the nylon wool non-adherent cells, and the presence of significant number of OX_{34} and OX_8 cells in the nylon wool non-adherent population. Immunofluorescent staining of the flask-nonadherent cells following 48h of culture showed that majority

of the population were T cells and B cells. These results indicated that the reductions of B and T cells were suitable to allow the culture of flask adherent LGL/LAK cells.

Table 1 Cell surface marker analyses of NW-nonadherent lymphocytes and the flask-nonadherent cells from 2-days culture

Cells	OX1	2ndAb	MouseIg G	SIg	OX19	OX34	OX8
NW-na	100%	8%	0%	36%	NT	NT	NT
NW-na	100%	4%	0%	35%	50%	83%	50%
NW-na	100%	4%	0%	12%	60%	63%	NT

100µl of 1-2 x 10^6/ml NW-nonadherent lymphocytes' surface marker were analysed by immunofluorescent staining. SIg is for B cells. OX19 is a pan T cell marker. OX34 and OX8 are shared by LGL/NK cells and T cells.
NT- non-tested.

3.1.2 Second antibodies

The immunofluorescent staining method, sheep-antimouse IgG $F(ab')_2$ fragment conjugated with FITC was used as the 2nd-Antibody. Unlike sheep anti-rat immunoglobulin antibody (for rat B cells) and Mouse IgG1 (for negative controls), the primer Mab's used in this study are not conjugated with FITC. To make the cells visible under UV microscope it is essential to stain the 2nd-Antibody on the primer Mab's on cells. The 2nd-Antibody should only combine with primer Mab's. Table 1 shows that there was a low rate of nonspecific reaction of the 2nd-Antibody with cells. By preincubating the cells in 20% BSA in 37°C water bath for 30min to decrease the possibility of the cell membrane changing, increasing the cell viability up to nearly 100%, and keeping cells in 4°C temperature during the procedure, diluting the 2nd-antibody before using, it was not possible to further decrease the nonspecific reaction of this 2nd-Antibody with cells. Finally by

incubation of 2nd-Antibody with fresh lymphocytes, the 2nd-Antibody had no nonspecific reaction with A-LAK cells as shown in Table 2.

3.1.3 Culture of A-LAK cells

A-LAK cells were generated by culturing nylon wool nonadherent lymphocytes with IL-2. Initially 5-10ml of $2x10^6$ cells/ml NW-nonadherent cells were cultured in $25cm^2$ small culture flasks with 1000U/ml IL-2. However, after several experiments it was found that cultures grew better in $75cm^2$ culture flasks using 10-20ml of $2x10^6$ to $3.5x10^6$ cells/ml. In final experiments, the A-LAK cell cultures were started with up to $4x10^6$ cells/ml.

During the first 2 days of culture, LGLs adhered to the plastic surface of the culture flask. The number of adherent LGL in the flask after removing flask nonadherent cells was not investigated. We did not collect adherent LGL from flasks for cell counting. However after the flask-nonadherent cells had been decanted, the adherent cells were evaluated by observing the frequency of adherent cells in the flasks under microscopy. The number of adherent cells generated varied from rat to rat. It was shown that the viability of flask-non-adherent cells varied widely from culture to culture. Low cell viability usually indicated that the culture was not successful, and was usually followed by a failure of LGL proliferation.

The adherent LGLs were continuously cultured in the culture flask as shown in Fig. C. In this study, 3-10 days more were needed to obtain adequate expansion of A-LAK cells. The number of A-LAK cells harvested varied between $1x10^6$ and $8.8x10^6$ cells. One interesting

observation was that the cells in the culture flask always proliferated well in one area (mostly in the middle of the flask and slowly in the surrounding area). When the cells in the middle of the flask became fully expanded, those cells in the other area of the flask had not fully expanded. This presented a significant problem of A-LAK cell generation in this study.

The culture medium used for continuing the adherent LGL proliferation was conditioned medium or a mixture of fresh medium with conditioned medium. It was found that using fresh medium only instead of conditioned medium to continue the culture resulted in a slow proliferation of LAK cells. Thus conditioned medium was usually used. However, when there were many dead flask nonadherent cells in supernatant after first 2-days culture, it was necessary to use fresh medium to obtain a better growth of cells. Using conditioned medium could result in failure of cell proliferation in this situation. Adding fresh medium to the conditioned medium could result in a more satisfactory proliferation of these adherent cells. The final methodology for this study was to continue the adherent LGL culture with a mixture of conditioned medium and fresh medium. A-LAK cells harvested from these cultures usually comprised about $5x10^6$ cells.

3.1.4 Identification of A-LAK cells

To confirm that the cells from this culture were A-LAK cells, three cell characterisation methods were used.

i) Immunofluorescent staining of cell surface markers using Mab's OX8 for the LAK cells and OX19 as a pan-T cells marker to exclude the possibility that these OX8 positive cells were T cells. NK cells and A-

LAK cells do not express the T cell surface marker of CD5 (which react with OX19). Sixty seven per cent to 90% of A-LAK cells showed the surface marker phenotype of NK/LGL cells (OX8) (Table 2). The population contained few pan-T positive cells (only 4-6.5% of cells expressing OX19). No B cell surface marker Ig was detectable in the A-LAK cell population.

Table 2 Cell surface marker analyses of A-LAK cells

Cells	OX1	2nd-Ab	MouseIg G	SIg	OX8	OX34	OX19
A-LAK	100%	5%	0%	0%	76%	60%	NT
A-LAK	100%	0%	0%	0%	90%	50%	NT
A-LAK	100%	0%	0%	0%	NT	78.4%	4.4%
A-LAK	100%	0%	0%	0%	67%	28%	6.5%

100µl of 1.5-5x10^6/ml A-LAK cells were used for analysing of their surface phenotype. 10µl Mabs were added into the cells and incubated in 4°C for 60min. After being washed in cold PBS-20%BSA-0.1%Azide, 10µl of 2nd-Ab were added and incubated in 4°C for 30min. After washing extensively with the same staining buffer, cells in 10µl same buffer were put on slides and dry in room temperature. Then the cells were fixed with 100% Ethanol. Cells were counted on UV microscope after covered with slip.

ii) Morphological identification of A-LAK cells was carried out to further confirm the immunofluorescent staining results. Fig. D shows morphological analysis of A-LAK cells using Giemsa-stained cytocentrifuge preparations. Purified A-LAK cells were morphologically large granular lymphocytes (LGL). The A-LAK cells have relatively high cytoplasmic/nuclear ratio (because of abundant cytoplasm), eccentric reniform nucleus, prominent cytoplasmic granules and undulating surface.

iii) Cytotoxicity assays were carried out to confirm the functional integrity of A-LAK cells. A-LAK cells showed high ability to lyse cultured YAC-1 (a mouse T cell lymphoma, was used as an NK-sensitive target) and P-815 (a murine mastocytoma, was used as an NK-resistant target) cells in 4h ^{51}Cr-release cytotoxic assays. In this study, A-

LAK cells lysed 33-40% YAC-1 cells and 44-52% P815 cells at the E/T (effector to target) ratio of 10:1. At an E/T ratio of 40:1 A-LAK cells lysed 70% P815 cells and 100% YAC-1 cells.

A-LAK : YAC-1			A-LAK : P815		
2.5 :1	10:1	40:1	2.5:1	10:1	40:1
15.1%	40.4%	ND	2.9%	52.5%	ND
13%	33%	ND	17%	44%	ND
14.05%	36.7%	101%	9.95%	48.25%	70%

3.2 A-LAK Cell Distribution

3.2.1 ^{51}Cr labelling of A-LAK cells

In this study the A-LAK cell distribution was investigated by counting the radioactivity accumulated in organs after radiolabelled A-LAK cells were infused. A-LAK cells were radiolabelled with ^{51}Cr before infusion.

To ensure that the cells infused into the rat were free of free ^{51}Cr isotope, cells were washed 5 times with PBS-A following labelling. This technique proved to be effective in removing free ^{51}Cr (Table 3). The radioactivity in the supernatant was negligible compared to that of the cells (0.9-7.0% of that in cells).

The efficiency of cell labelling was 0.002-0.018 cpm/cell (Table 4). This level of radioactivity was sufficient for studying the distribution of A-LAK cells in tissues after cell injection.

Spontaneous release of ^{51}Cr from cells after 2h incubation at 37°C was studied. This is required to show the degree of spontaneous leakage of radioactivity from cells after infusion, in order to obtain the true radioactivity within A-LAK cells in each organ. Table 4 shows that the SR% varied from 8.6%-56.0% with mean result of 30.8%±15.5%. In this table "CPM of cells" refers to radioactivity which is estimated to remain within A-LAK cells *in vivo* 2h after infusion (ie after subtraction of spontaneous release).

The viability of infused cells was usually >90%.

Table 3 Radioactivity in supernatant and in A-LAK cells

Group 1	cpm in 50µl 1st washing supernant	cpm in 50µl last washing supernatant	cpm in 50µl cells	free ^{51}Cr / cpm in cells
rat 1	1453	23.5	571	4.1%
rat 2	3037	17.2	560	3.1%
rat 3	891	110#	245	-
rat 4	3114	42.4	928	4.6%
rat 5	470	8	206	3.9%
rat 6	613	16	498	3.2%
rat 7	952	17	680	2.5%
rat 8	1105	54	748	7.2%
Group 2				
rat 1	3975	20	368	5.4%
rat 2	29059	703*	936	-
rat 3	962	19.8	432	4.6%
rat 4	3000	37	1596	2.3%
rat 5	404	20	540	3.7%
rat 6	952	17	680	2.5%
rat 7	1105	54	748	7.2%
rat 8	1105	54	748	7.2%
Group 3				
rat 1	3330	26.1	904	2.9%
rat 2	2939	31	862	3.6%
rat 3	2504	32.3	792	4.1%
rat 4	3114	42.4	928	4.6%
rat 5	470	8	206	3.9%
rat 6	941	4	431	0.9%
rat 7	941	4	431	0.9%
rat 8	951	11	378	2.9%

After labelling with 100µCi ^{51}Cr in a 37°C water bath for 1h, cells were washed in PBS for 5 times to clean out the free ^{51}Cr in the supernatant.
#showed the result from 4th washing. cells were washed once again and resuspended in 1ml PBS for infusion. (The radioactivity in 4th washing supernatant were usually 140-401 for other labelling. Results are not shown in this table)
*It is the radioactivity in the 4th washing supernatant. Cells were then washed twice more. Last time washing was not taken out for counting.

Table 4 A-LAK cell labelling, and spontaneous release of ^{51}Cr in 2h

Group Group 1	LAK cells infused	Total cpm in cells infused	Radioactivity cpm/cell	SR% of cpm in 2h	CPM of cells #
rat 1	0.66 x 10^6 (0.3ml)	3428	0.0052	37%	**2160**
rat 2	2.64 x 10^6 (2ml)	22400	0.0085	56%	**9856**
rat 3	2.1 x 10^6	4896	0.0023	8.6%	**4475**
rat 4	1.5 x 10^6	18552	0.0124	42%	**10760**
rat 5	1.7 x 10^6	4116	0.0024	14.5%	**3519**
rat 6	1.65 x 10^6	9970	0.0060	16%	**8376**
rat 7	1.5 x 10^6	13598	0.0091	10.7%	**12143**
rat 8	2.5 x 10^6	14956	0.0060	45%	**8226**
Group 2					
rat 1	0.85 x 10^6	7364	0.0087	43.6%	**4153**
rat 2	3.5 x 10^6	18720	0.0054	55%	**8424**
rat 3	2.8 x 10^6	8640	0.0031	18.9%	**7007**
rat 4	1.6 x 10^6 (900µl)	28728	0.018	17.2%	**23787**
rat 5	1 x 10^6	10793	0.0108	28.5%	**7718**
rat 6	1.5 x 10^6	13598	0.0091	10.7%	**12134**
rat 7	2.5 x 10^6	14956	0.0060	45%	**8226**
rat 8	2.5 x 10^6	14956	0.0060	45%	**8226**
Group 3					
rat 1	1.02 x 10^6	18080-883*	0.0177	29%	**12210**
rat 2	2.86 x 10^6	17248	0.0060	56%	**7589**
rat 3	1.44 x10^6 (800µl)	12675-1848*	0.0088	35%	**7038**
rat 4	1.5 x 10^6	18552	0.0124	42%	**10760**
rat 5	1.7 x 10^6	4116	0.0024	14.5%	**3519**
rat 6	2.9 x 10^6	8612	0.0030	25.6%	**6407**
rat 7	2.9 x 10^6	8612	0.0030	25.6%	**6407**
rat 8	1.6 x 10^6	7552	0.0047	18.6%	**6147**

#CPM in cells represented the radioactivity in cells after incubated in rat for two hours. It is obtained after subtracted the SR% from the radioactivity of the infused cells.

* this radioactivity was obtained from the cells which were not infused into the portal vein because of leakage. They were soaked in cotton wool and the radioactivity of them was counted.

3.2.2 Distribution of A-LAK cells by systemic and regional infusion and their comparison

To study the distribution of A-LAK cells in organs by systemic and regional infusion, 1.0-3.5x10^6 labelled A-LAK cells in 1ml PBS were infused into syngeneic male rats through the tail vein or portal vein respectively. Both groups comprised 8 rats for the trafficking studies. Two hours after A-LAK cell infusion, rats were sacrificed and the organs (spleen, kidney, lung, liver, MLN, brain) and blood were taken for gamma-counting to study the distribution of the labelled A-LAK cells in these organs.

The distribution of A-LAK cells after 2h is shown in Table 5 and Figure 2, and in Table 6 and Figure 3. Table 5 shows the mean result of labelled cell distribution per organ via systemic and regional infusion and the comparison between the two groups. (The cell distribution in each individual rat is shown in Table D in the Appendix). As shown in Table 5, there was no significant increase in the A-LAK cell distribution to the liver by regional infusion compared to systemic infusion. There is a suggestion of increased early accumulation of A-LAK cells in the liver following portal vein infusion (24.36% vs 16.41%) however this result is not statistically significant ($P>0.05$, Student's t-test). Following regional infusion, fewer cells accumulated in the lungs (6.01% vs 15.65%, $P<0.05$) compared with systemic infusion. The distribution of A-LAK cells to the brain was very small by either systemic or regional infusion (0.12% and 0.08% respectively).

Table 6 shows the mean result of A-LAK cell distribution expressed per gram of organ, comparing systemic to regional infusion. Calculation of the results in this way should give a more accurate representation of

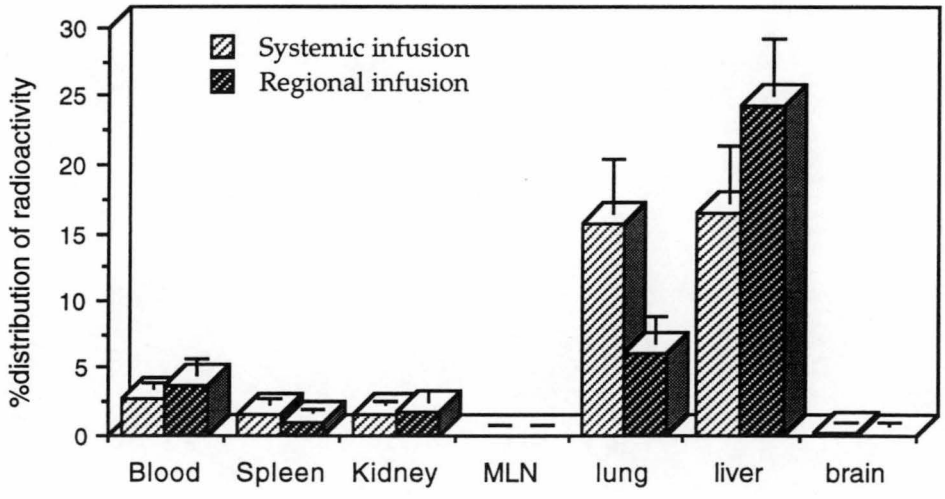
cell distribution because it excludes variation which could result from the different size of organs between rats. These results also show that there was no significant increase in the A-LAK cell distribution to the liver by regional infusion compared to systemic infusion. The results are suggestive of greater distribution of A-LAK cells into liver by regional infusion through portal vein (3.89%/g vs 2.15%/g) however the result is not of statistical significance. In lung the increased early distribution of A-LAK cells which resulted from systemic infusion through tail vein compared to portal vein was again shown (19.02%/g vs 6.55%/g, $P<0.05$, Student's t-test). There was no significant difference in cell distribution per gram in spleen, kidneys and brain by these two routes of infusion. The distribution of A-LAK cells to brain could be neglected on the basis of per gram of tissue. By showing the cell distribution per gram of MLN, it is shown that there was distribution of A-LAK cells to MLN, however there was no significant difference between systemic and regional portal infusion.

Table 5 Comparison of A-LAK cell distribution **per organ** by systemic and regional infusion

ORGAN	SYSTEMIC %D/organ* ± sd	REGIONAL %D/organ* ± sd	P**
blood	2.71 ± 1.23	3.66 ±3.24	0.45
spleen	1.51 ± 1.21	0.97 ±0.80	0.31
kidneys	1.57 ± 0.66	1.78 ±2.24	0.8
MLN	0.04 ± 0.03	0.02 ±0.02	0.18
lung	15.65 ± 11.43	6.01 ± 5.9	0.05
liver	16.41 ± 11.56	24.36 ±11.5	0.19
brain	0.12 ± 0.14	0.08 ±0.11	0.53

*%D/organ= mean percentage of total dose of A-LAK cells infused, taking into accout of spontaneous isotope release, per whole organ (8 rats).
**P- Student's t-test.

Figure 2: Graphical representation of data from Table 5



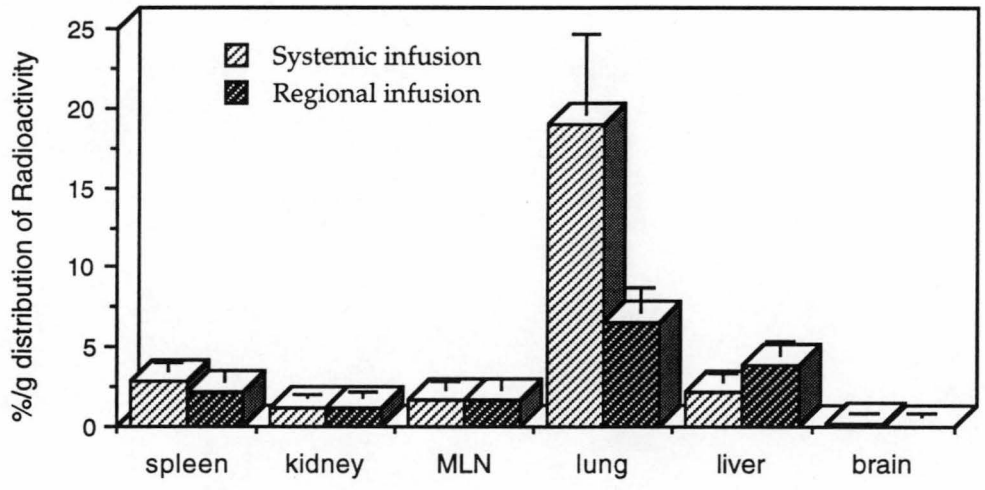
Bars indicate SE.

Table 6 Comparison of A-LAK cell distribution **per gram** by systemic and regional infusion

ORGAN	SYSTEMIC %D/g* ± sd	REGIONAL %D/g *± sd	P**
spleen	2.77 ± 1.76	2.23 ± 1.92	0.56
kidney	1.18 ± 0.65	1.19 ± 1.03	0.99
MLN	1.72 ± 1.16	1.7 ± 1.9	0.98
lung	19.02 ± 14.27	6.55 ± 4.31	0.03
liver	2.15 ± 1.71	3.89 ± 2.52	0.13
brain	0.1 ± 0.11	0.06 ± 0.09	0.51

*%D/g = mean percentage of total dose of A-LAK cells infused, taking into account of spontaneous isotope release, per gram of organ (8 rats).
**P- Student's t-test

Figure 3: Graphical representation of data from Table 6



Bars indicate SE.

Figures 4 and 5 give a graphical representation of the distribution of labelled A-LAK cells per gram of tissues. These calculations are based on the assumption that little radioactivity accumulated elsewhere, ie. in organs not tested, such as muscle, soft-tissue and gut. The whole radioactivity in these tissues is taken as 100%. Because some A-LAK cells must have distributed to these non-tested sites, these graphs can be taken as close approximation of the A-LAK distribution at 2 hours to these tissues. Taking account of this assumption, approximately 70% of radioactivity was recovered in lung, which was 9 times of that in liver tissue following systemic infusion (Figure 4). The tissue distribution by systemic infusion was in the order of: lung> spleen> liver> MLN> kidney> brain. In Figure 5 it is shown that by portal vein regional infusion the radioactivity recovered in lung was approximately 40% of the total recovered activity in detected organs. Following portal vein infusion lung tissue retained about twice the activity of liver tissue. Liver tissue was second in order of tissue uptake of labelled A-LAK cells, compared to other tissues following regional infusion.

These results show that regional infusion increased the distribution of cells to liver and decreased the early accumulation of cells in lung. However irrespective of the route of infusion, the highest cell uptake was in lung tissue.

Figure 4: Graphical representation of the distribution of A-LAK cells per gram in detected organs by **systemic** infusion.

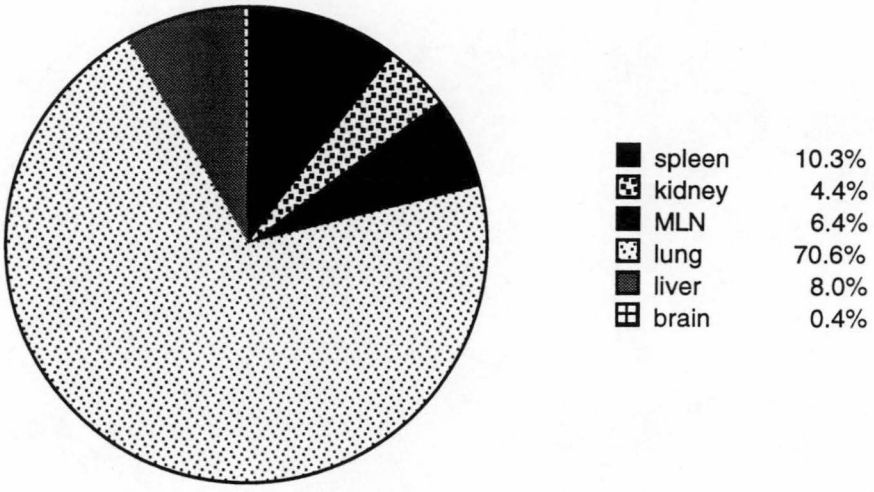
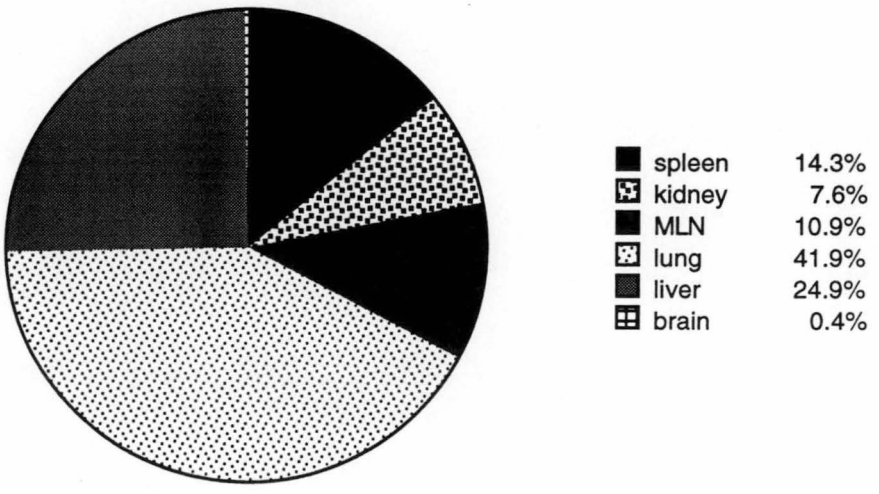


Figure 5: Graphical representation of the distribution of A-LAK cells per gram in detected organs by **regional** infusion.



3.2.3 Effect of mannitol on the distribution of A-LAK cells to the liver infused via the portal vein.

To study the effect of intraportal mannitol on the uptake of A-LAK cells in the liver, a third group of 8 rats was used. In this group, prior to the intraportal infusion of labelled A-LAK cells, 30% mannitol was infused at 0.3mg/kg body weight via the portal vein cannula. Two hours later rats were sacrificed and the organs and blood were taken for radioactivity counting. The results of A-LAK cell distribution with and without mannitol infusion were compared, to study the effect of intraportal mannitol. The results of these experiments are shown in Table 7 and Figure 6. When intraportal mannitol was given just prior to A-LAK infusion, there was a significantly increased distribution of intraportal infused A-LAK cells into liver compared to A-LAK infusion without mannitol (54.05% vs 24.36%, $P< 0.0005$) (Table 7, Figure 6). To exclude the possibility that this effect of intraportal mannitol was a result of increased blood volume in the liver, the results of A-LAK cell distribution per gram of tissue were calculated, and are shown in Table 8 and Figure 7. The results also indicate that the mannitol enhanced the delivery of A-LAK cells into the liver (9.33%/g vs 3.89%/g $P< 0.001$).

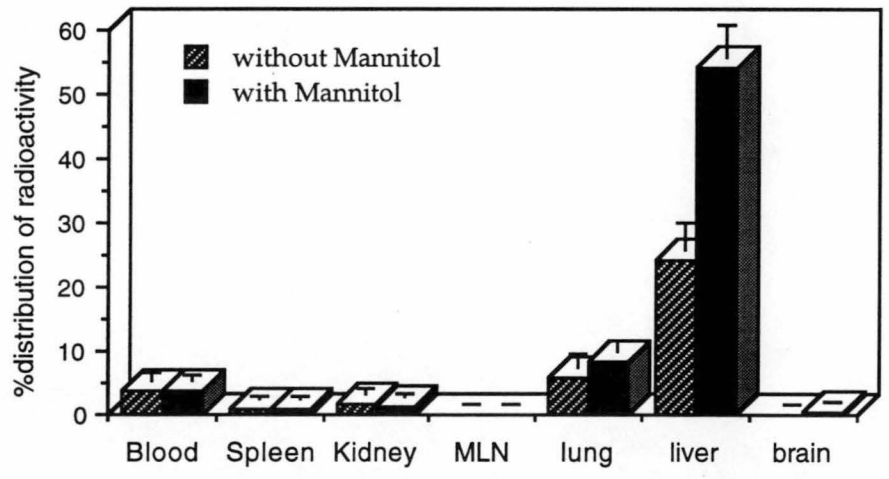
In addition, intraportal mannitol administration resulted in significantly increased A-LAK distribution to MLN (0.05% vs 0.02%, $P<0.05$) and to brain (0.26% vs 0.08%, $P<0.05$). There was no significant difference in A-LAK distribution to lung, spleen, kidneys or blood following intraportal mannitol.

Table 7 Comparison of %distribution of A-LAK cells **per Organ** by regional infusion with and without Mannitol

ORGAN	Without M %D/organ± sd *		With M %D/organ± sd*		P**
blood	3.66	±3.24	3.76	±2.71	0.95
spleen	0.97	±0.80	1.0	±1.0	0.96
kidneys	1.78	±2.24	1.44	±0.81	0.69
MLN	0.02	±0.02	0.05	±0.02	0.04
lung	6.01	± 5.9	8.39	±4.27	0.37
liver	24.36	±11.53	54.05	±14.17	0.0004
brain	0.08	±0.11	0.26	±0.21	0.04

*%D/organ= mean percentage of total dose of infused A-LAK cells, taking into account spontaneous isotope release, per whole organ (8 rats)
**P- Student's t-test

Figure 6: Graphical representation of data from Table 7



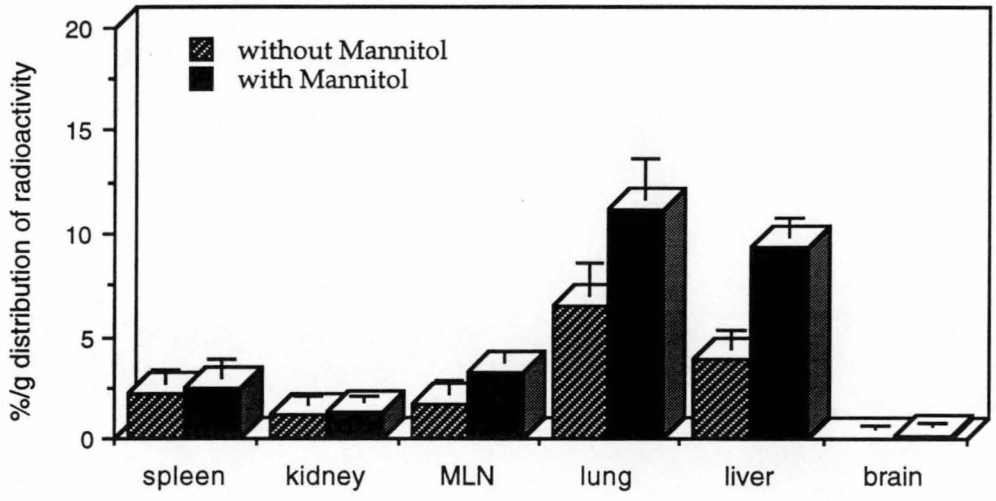
Bars indicate SE.

Table 8 Comparison of %distribution of A-LAK cells **per gram** by regional infusion with and without Mannitol

ORGAN	Without M %D/g ± sd*		with M %D/g ± sd*		P**
spleen	2.23	±1.92	2.41	±2.58	0.87
kidney	1.19	±1.03	1.30	±0.82	0.81
MLN	1.7	±1.9	3.20	±1.70	0.13
lung	6.55	±4.31	11.2	±5.6	0.08
liver	3.89	±2.52	9.33	±2.67	0.0009
brain	0.06	±0.09	0.19	±0.15	0.051

*%D/g= mean percentage of total dose of infused A-LAK cells, taking into account spontaneous isotope release from cells at 2h, per gram of organ (8 rats).
**P- Student's t-test

Figure 7: Graphical representation of data from Table 8



To show that the effect of intraportal mannitol was not due to differences in the radioactivity in blood, Table 9 shows that there was no significant difference in the radioactivity in blood between the two groups (3.76% vs 3.66%). Table 9 also shows that the average weight of liver was 6.83g in group 2 and 5.89g in group 3 indicating that the increased radioactivity within liver in group 3 was not due to increased size of this organ following mannitol.

By comparison of the total radioactivity recovered in these organs between two groups as shown in Table 9, it was found that the percentage of total recovered radioactivity was much higher in the mannitol group than that in the group without mannitol (69.05% vs 35.9%, $P<0.005$). This is mainly because of the increased cell distribution to liver.

Table 9 Comparison of % distribution of A-LAK cell in liver and blood by regional infusion without or with mannitol, organ weight and % radioactivity in whole organs.

	%D* in liver ±sd	weight of liver g	%D* in blood ±sd	%distribution in detected organs
without M	24.36 ± 11.53	6.83 ± 1.73	3.66 ± 3.24	36.18 ± 15.61
with M	54.05 ±14.17	5.89 ± 0.67	3.76 ± 2.71	69.05 ± 17.76
P**	0.0004	0.1716	0.9476	0.0015

*%D= mean percentage of total dose of A-LAK cell infused, taking into account the spontaneous isotope release at 2h (8 rats).
**P- Student's t-test

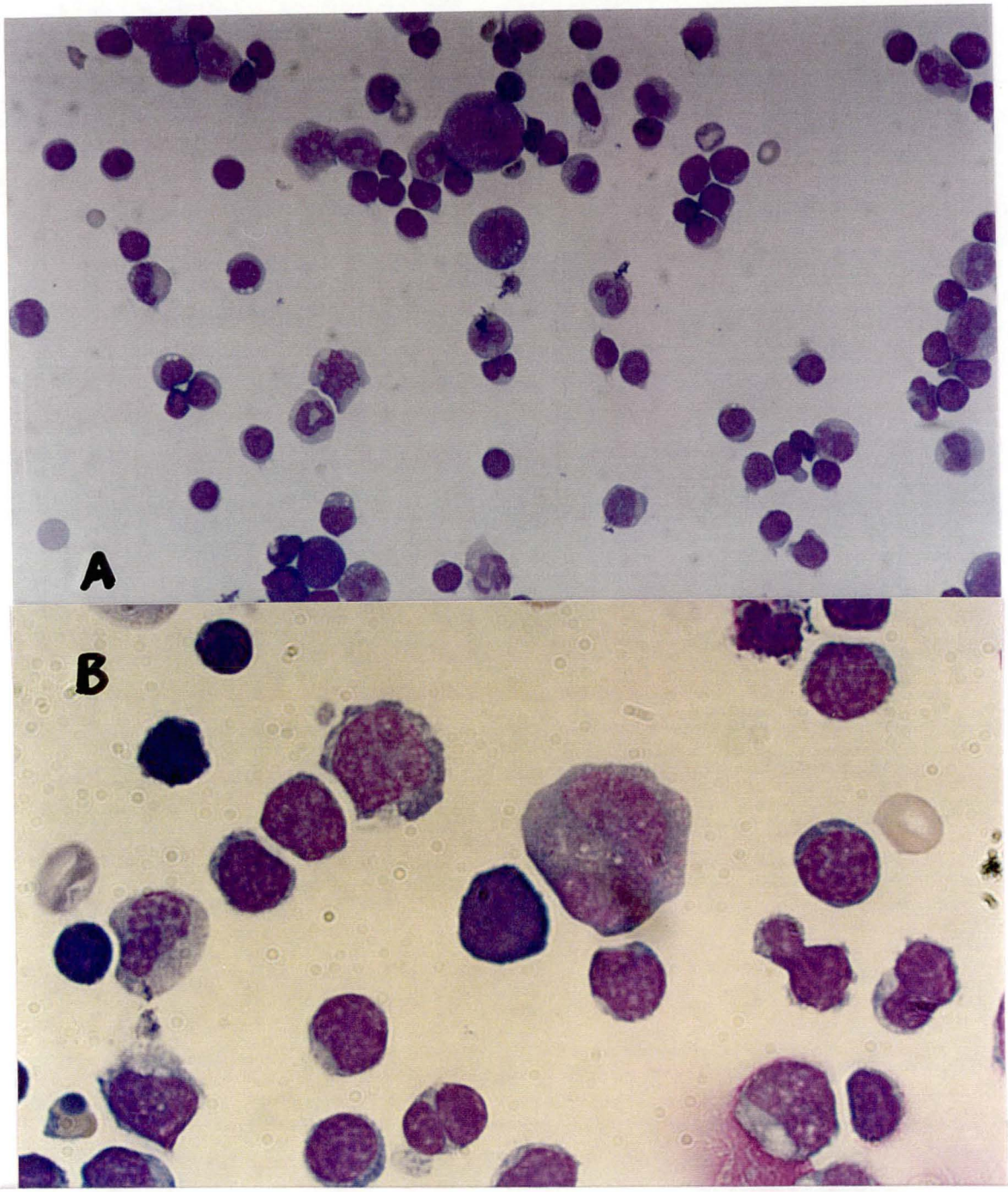


Fig. A Morphology of rat splenocytes. Cells are obtained from the interface of Ficoll-hypaque gradient centrifugation. It shows mixed population of microphage, lymphocytes and some RBC. (A) shows splenocytes under low magnifiction (x500). (B) shows them in a higher magnification (x1250).

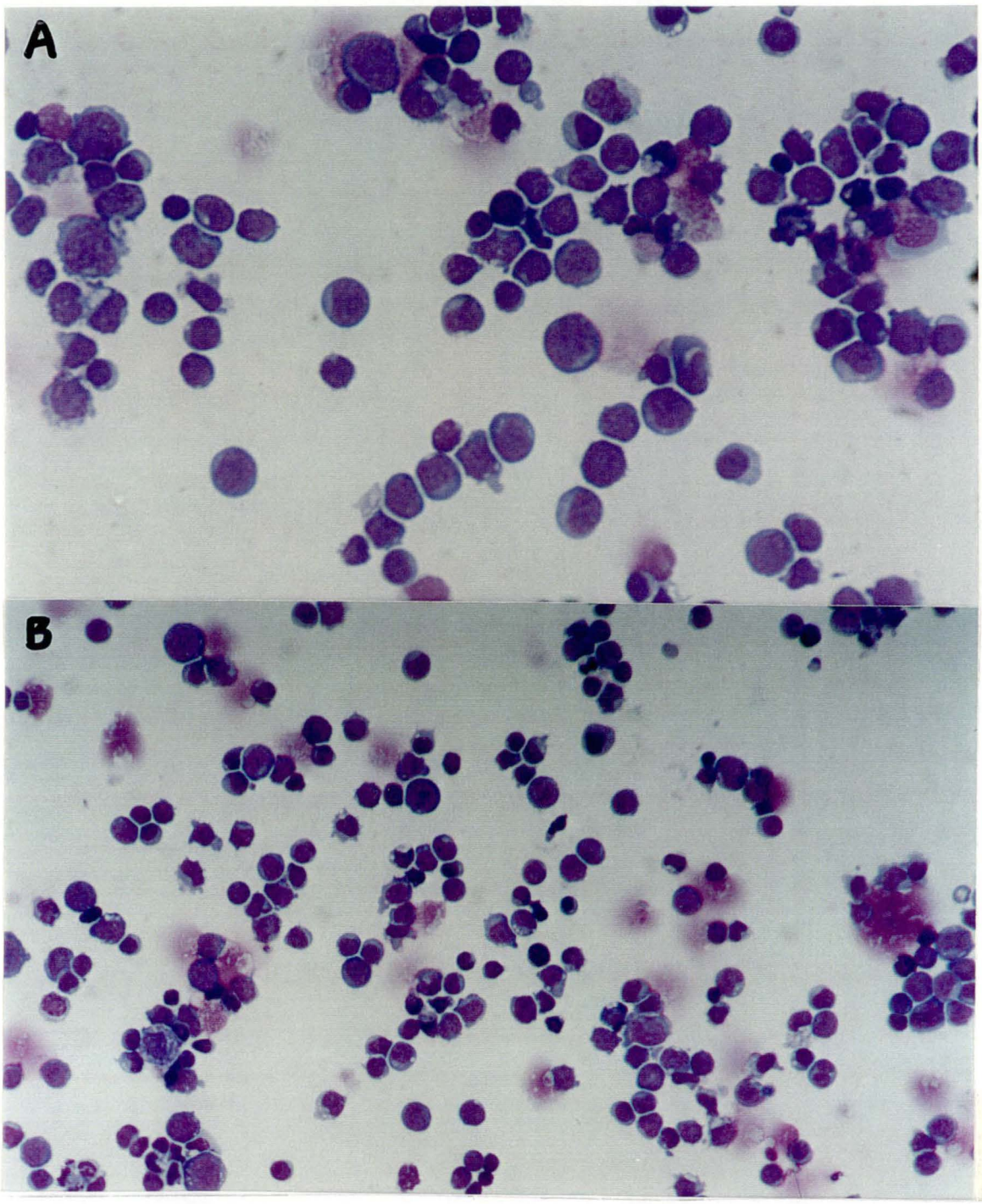


Fig. B Mophology of NWP lymphocytes. These NWP (Nylon-wool unadherent) lymphocytes are collected from NW column after splenocytes have been incubated in it at 37°C for 1hr. These cells are going to be cultured with 1000U/ml IL-2 in T-75 flask. (A) is made under magnification of (x500). (B) is under low panel (x312).

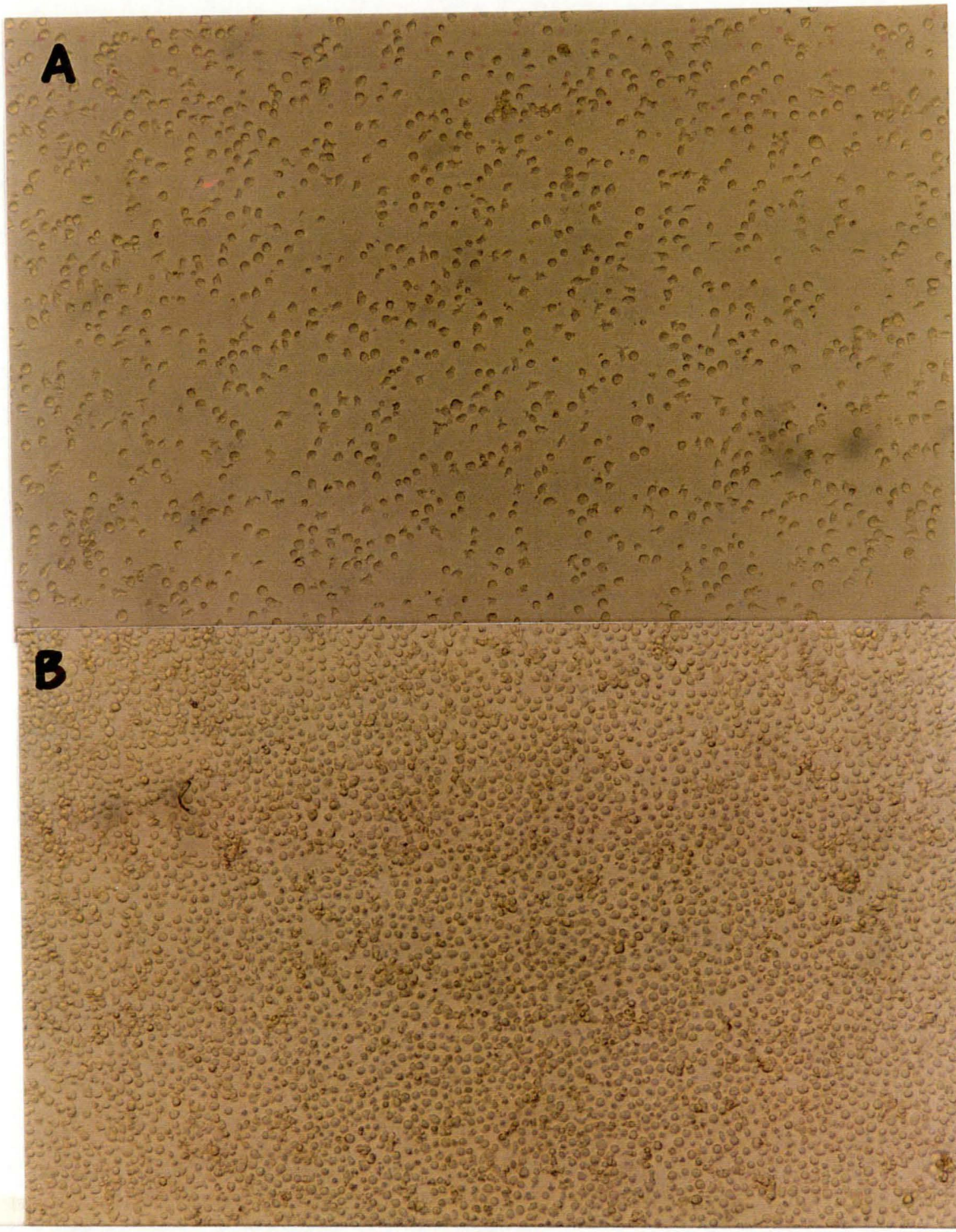


Fig. C The adherent LGL in culture flask. After 2-days culture of NWP lymphocytes with IL-2, plastic unadherent cells were washed away and adherent LGL are continuesly cultured. (A) shows cells are continuing expanding. (B) shows the cells are nearly fully expanded in flask at day-7 of culture just before cell harvest.

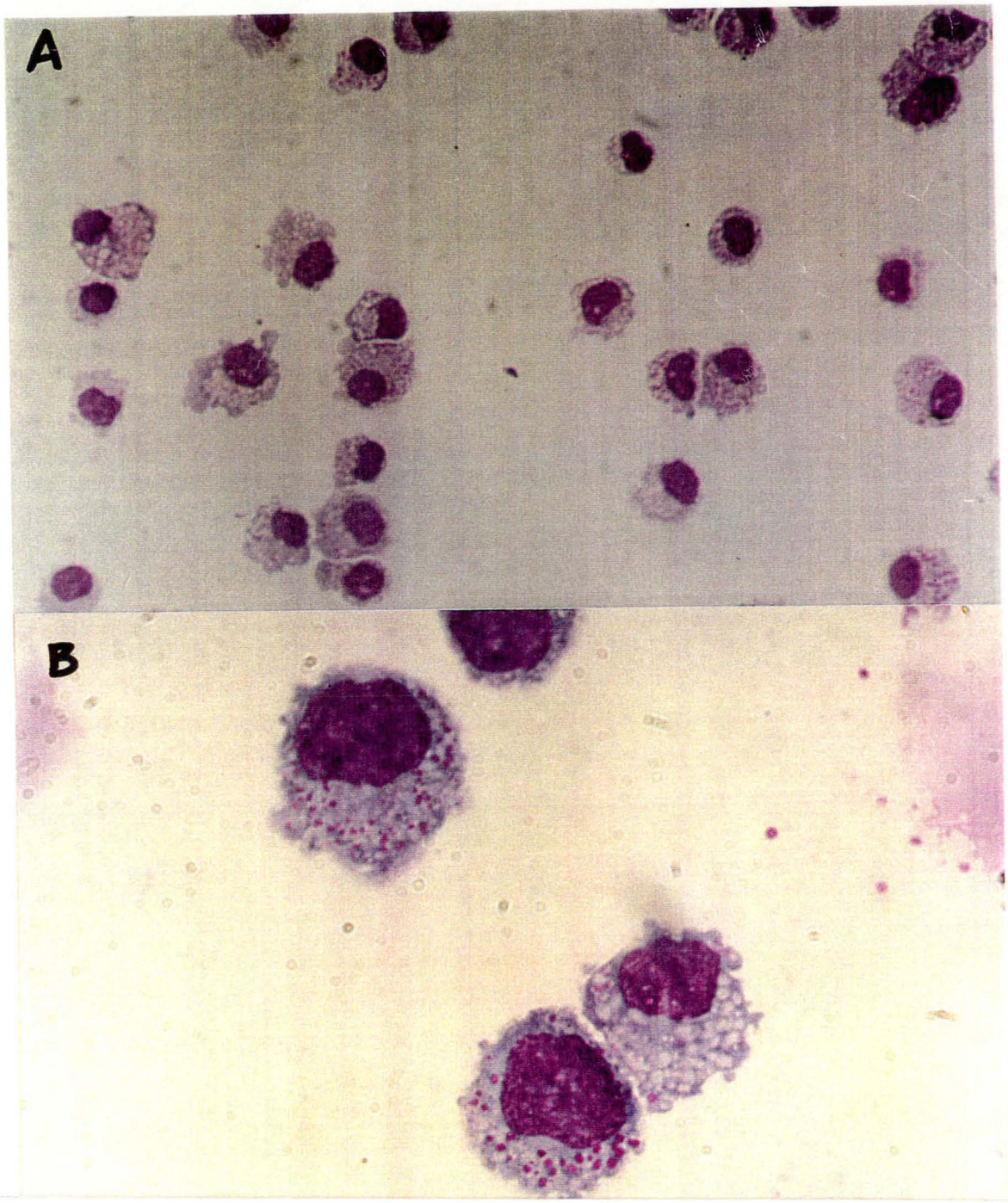


Fig. D Morphology of A-LAK cells. A-LAK cells were harvested from culture. Cytospin slides are fixed in Giemsa stain. These A-LAK cells show a relatively high cytoplasmic: nuclear ratio with abundant cytoplasm, an eccentric reniform nucleus and prominent cytoplasmic granules. (A) is under lower magnification of (x500). (B) shows cells under magnification of (x1250).

Chapter 4 Discussion

4.1 A-LAK Cell Generation and Characterisation

4.1.1 A-LAK cell generation

This study determined the distribution of A-LAK cells following intravenous and intraportal infusion into rats and the effect of intraportal mannitol on the uptake of A-LAK cells in liver. Since T cells have a different migration pattern to LAK cells (Rolstad *et al* 1986; Maghazachi *et al* 1988b, 1988c), it was essential that the LAK cells used in this study be as pure as possible. A-LAK cells were generated using the method of Vujanovic *et al* 1988c: LGL were isolated by stimulating nylon wool non-adherent splenic lymphocytes with IL-2. Following IL-2 stimulation LGL adhere to the plastic surface of a culture flask, and develop into LAK cells. Following the proliferation of these LAK cells, a highly purified A-LAK cell population is obtained.

Incubation of lymphocytes in a nylon wool column is designed to deplete B cells and macrophages from the spleen cell population. This study showed that B cell depletion was not as complete as several published studies have indicated. Schwarz *et al* 1989 and Vujanovic *et al* 1988c reported that use of a nylon wool column could consistently reduce the percentage of B cells to less than 2% and macrophages to less than 0.3%. In our study, the B cells among nylon wool non-adherent lymphocytes were much higher (12%-36%). The reason for the less efficient B cell depletion during nylon wool incubation is not clear. In another work (Wilkinson S. MD thesis) the leaching and preparation of the nylon wool were found to be possible factors. Fortunately there were no B cells detectable in the A-LAK cell population (Table 2), which suggests that the B cells were unable to adhere to the plastic

surface in culture flask. Whether the presence of B cells in the first 2 days culture could influence the later LAK cell proliferation has not been examined.

The immunofluorescent staining study of flask nonadherent cells showed that cytotoxic T lymphocytes appeared in these cultures after 48 hours (Table 1). Cytotoxic T cells were both OX_{19} and OX_8 positive. By removing the flask nonadherent cells, the adherent LGL/NK/LAK cells were separated from the activated T cells. Thus, this culture allowed the collection of purified A-LAK cells (purity 67-90%).

Vujanovic *et al* 1988c reported that by 48h, about 4.5% of NW-non-adherent splenocytes adhere to the culture flask plastic surface. In our study, the percentage of adherent cells at 48h was not determined. The number of culture flask adherent LGL appeared to vary from rat to rat.

The number and concentration of the adherent LGL was an important factor in the later proliferation of A-LAK cells. A low number of adherent LGL resulted in failure of A-LAK cell proliferation. The factors responsible for the number of adherent LGL could be: (1). The percentage of LGL in the starting lymphocyte population, which depends on the organ and strain and possibly the age of rats (Reynolds *et al* 1981); (2). The number of nylon wool non-adherent lymphocytes used at the beginning of the culture. Optimal density of $2x10^6$ cells/ml was suggested by Vujanovic *et al* in 1988. In the present study the production of A-LAK cells varied markedly depending on the starting cell density ($2x10^6$/ml to $3.5x10^6$/ml). A greater concentration of starting cells usually resulted in a better culture of A-LAK cells.

It was observed that the A-LAK cell culture was influenced by the culture flask surface area to which the LGL adhered. A flask of size $75cm^2$ provided a larger area for the flask adherent LGL expansion. In the present study, although the A-LAK cells did not achieve full expansion on all of the available flask surface area, the number of A-LAK cells harvested from the large flask was proportionally more than that from small flasks ($25cm^2$).

Successful A-LAK cell culture was also influenced by the LGL response to IL-2. In this study, there were several instances of failure of cell proliferation to IL-2. Loss of IL-2 receptors could be one reason for this. Another possible reason (Vujanovic *et al* 1988c) is that both suppressor T cells (T_S) and monocytes may inhibit the proliferation of LAK cells in bulk culture. It is possible that the presence of T suppressor cells in the first 2 days of culture could result in a high concentration of T_S products in the culture supernatant which inhibited LGL expansion. However, the use of the conditioned medium in these cultures for subsequent successful expansion of A-LAK cells implied that such factors were not present or were of short half life.

4.1.2 A-LAK cell identification

In this study A-LAK cells were characterised by immunofluorescent staining, morphological identification and cytotoxic analysis.

The phenotypic profile of A-LAK cells in this work is in agreement with previous published studies (Schwarz *et al* 1989; Vujanovic *et al* 1988c; Maghazachi *et al* 1988c). The majority cells from adherent cell culture were OX8 positive A-LAK cells with only about 5% OX19

positive cells. Between 80%-100% of LAK cells should be OX8 positive and these cells should be OX_{19} negative. OX_{19} (CD5) is a pan T cell marker (Vujanovic *et al* 1988a; Chambers *et al* 1989; Ortaldo *et al* 1986). Though some OX8 positive cells could be T cells, T cells should be OX19 positive as well. Therefore the phenotypic profile of cells in this study is consistent with the recognized pattern for A-LAK cells. Though antibody 3.2.3 is the most specific antibody for A-LAK cells at the present time, it was not available nor used in this study. OX_{34} is the Mab which reacts with the CD2 surface marker (Reynolds *et al* 1981b; Ortaldo *et al* 1981; Williams *et al* 1987). The results in this study suggest that OX34 may react with both A-LAK cells and T cells. There were no previous studies found which tested this Mab on A-LAK cells.

Morphological analysis of A-LAK cells in the rat using Giemsa-stained cytocentrifuge preparations indicated that A-LAK cells had a morphology consistent with previously published studies (Melder *et al* 1988; Vujanovic *et al* 1988c; Sasaki *et al* 1989). Morphological study of LGL was not carried out in this study. The comparison of the morphology of LGL with A-LAK, which would show the size of cells before and after stimulation by IL-2, was not carried out. Sasaki *et al* 1989 has demonstrated that LAK cells have a greater mean diameter than nonactivated LGL.

Cytotoxicity assay results in this study confirm that the cells from the A-LAK cell culture were predominantly A-LAK cells, as A-LAK cells showed high ability to lyse cultured YAC-1 and P-815 cells in 4h ^{51}Cr-release cytotoxic assays.

4.2 Distribution of ^{51}Cr-labelled A-LAK Cells in the Rats

4.2.1 ^{51}Cr-labelling of A-LAK cells

To study the distribution of radiolabelled A-LAK cells in rats by systemic and regional infusion, and the effect of intraportal mannitol on the uptake of LAK cells into liver, A-LAK cells were radiolabelled with ^{51}Cr before being infused into rats. ^{51}Cr is a commonly used radioisotope for monitoring the migration pattern of lymphocytes *in vivo*. This isotope has many advantages and few disadvantages compared with other isotopes such as ^{3}H, ^{14}C, ^{75}Se, ^{99}mTc and ^{125}I in lymphocyte trafficking studies (Rannie *et al* 1977).

The presence of radioactivity in the organs following cell infusion may be due to radiolabelled cells, free ^{51}Cr, or spontaneously released ^{51}Cr. In this study it is important to ensure that the radioactivity in the organs really reflects the presence of LAK cells rather than free isotope. To exclude the presence of free ^{51}Cr from the infused cell supernatant the radiolabelled cells were washed thoroughly. The washing eliminated ^{51}Cr from the supernatant of infused cells as shown in Table 3. The other potential source of free isotope is the spontaneous release of ^{51}Cr during the two hour circulation inside the rat. This problem could affect the trafficking study result in two ways. Firstly, once the ^{51}Cr is released from the cells the radioactivity per infused cell will be decreased. The radioactivity of cells detected before infusion would therefore no longer be a reflection of the migrating cell numbers *in vivo*. Since the percentage of cell distribution in organs is calculated based on the total radioactivity of cells injected (formula in 2.2.3), the radioactivity within cells should be known as accurately as possible.

Secondly, if the released ^{51}Cr accumulated in organs the radioactivity in the organs no longer reflects the presence of LAK cells. To obtain the radioactivity in cells 2h following infusion, some cells were incubated for two hours and the percentage of ^{51}Cr released into the supernatant was calculated (see the formula for the SR in 2.2.1). The radioactivity obtained in the SR was then subtracted from the radioactivity of the infused cells (Table 3). In this way the first source of error is excluded. This is based on the assumption that spontaneous release *in vitro* is approximately equivalent to the rate of SR *in vivo*. From this study there was no correlation between the spontaneous release and the % distribution of radioactivity in body organs (Table E of Appendix), which suggests that there was no significant accumulation of released ^{51}Cr in tissues. It has been demonstrated that the free ^{51}Cr is efficiently removed through urinary excretion and not retained in tissues (Rannie *et al* 1977; LeFever *et al* 1987). Basse *et al* 1992b found that 24h following cell infusion, released ^{51}Cr accumulated in the liver. The present study does not show correlation between spontaneous ^{51}Cr release and radioactivity in the liver (Table E of Appendix), which indicates that at 2h the radioactivity in liver is not resultant from accumulation of free ^{51}Cr.

It has been suggested that dead cells may accumulate in the liver after infusion. It is possible that the dead cells still carry some radioactivity after 2h. LeFever *et al* 1987 found a linear correlation between viability of the cell population injected and the amount of radioactivity detected in the kidneys. In the present study the cell viability at infusion was usually greater than 90%. Maghazachi *et al* 1988c found that the radioactivity obtained from the lungs, liver and spleen after injection of intact cells was not due to dead cells or free ^{51}Cr.

4.2.2 A-LAK cell distribution

This study shows that the distribution of A-LAK cells in the liver is slightly higher at 2h following regional portal vein infusion compared to systemic infusion, however results do not reach statistical significance. Bass *et al* 1992b has found increased accumulation of A-LAK cells in the liver 24h after portal infusion. However this study has found that prior portal infusion of mannitol further increases LAK cell distribution to the liver, to a significantly higher level than regional infusion without mannitol.

The early distribution of A-LAK cell in the organs differs with the route of administration, that is, systemic (via the tail vein) and regional (via portal vein). Comparing systemic to regional infusion, it was found that an increased number of A-LAK cells distributed to the liver following regional injection (as shown in Table 5). Other organs, particularly the lung show increased numbers of A-LAK cells via systemic infusion. Results (in Table 5) indicate that portal vein infusion decreases the early accumulation of LAK cells in lungs (6.01% vs 15.65%). The effect of regional infusion is more clearly shown by looking at the percentage distribution of LAK cell per gram of lung tissue (6.55% per gram vs 19.02% per gram, $P<0.05$, Table 6). However, irrespective of the route of infusion, a large proportion of A-LAK cells do accumulate in the lungs (Figure 4 and 5). This result has also been obtained by other investigators (Reynolds *et al* 1984; LeFever *et al* 1984; Maghazachi *et al* 1988c, 1988b; Rannie *et al* 1977; Felgar *et al* 1990). The results shown in Fig. 4 and Fig. 5 indicate that the retention of LAK cells in lungs is not solely related to the route of systemic infusion. Systemic infusion of A-LAK cells results in the early entrapment of

these cells in the lung capillary bed. By regional infusion, the early retention of A-LAK cells in the lung is decreased. However, A-LAK cells are still mainly accumulated in the lungs. LAK cells tend to localise in lungs much more than do non-activated T lymphocytes (Lotze *et al* 1980; Reynolds *et al* 1984; Maghazachi *et al* 1988c; Marincola *et al* 1988). There are several explanations for the preferential accumulation of LAK cells by the lungs. Lotze *et al* 1980 indicated that cell membrane alteration may be induced by the growth of these cultured cells in the presence of IL-2 and may explain their altered migration ability. LAK cells are large and this probably explains their early trapping in the lung. Sasak *et al* 1989 and Worthen *et al* 1989 also showed that A-LAK cells were rigid in comparison with other nonactivated lymphocytes. Structural rigidity is known to influence the behaviour of cells in the capillaries by hindering their passage through small capillaries and altering their haemodynamic behaviour in postcapillary venules. Maghazachi *et al* 1988b indicated that LAK cells may have carbohydrate-binding protein which allows them to bind to endothelial cells lining the lung vessels. Thus, the reasons for the early high retention of A-LAK cells in the lung appear largely related to the morphology of A-LAK cells, the high rigidity of these cells and the nature of the LAK cell surface membrane.

Clinical trials have shown that one side effect of high dose administration of IL-2 and LAK cell therapy is a capillary leak syndrome including pulmonary oedema (Rosenberg *et al* 1987). Damle *et al* 1987 and Aronson*et al* 1988 showed that LAK cells can lyse cultured endothelial cells *in vitro*. Thus LAK cells may damage endothelial cells in the lungs, which may contribute to pulmonary oedema. Infused LAK cells recovered from the lungs are still viable

and cytolytic (LeFever *et al* 1987). Since the regional infusion of LAK cells decreases the early retention of these cells in the lung capillary bed, it is possible that this side effect may be reduced by this route of infusion. Further study is required to determine whether regional infusion merely delays the accumulation of these cells in lungs.

The present study also found that A-LAK cells distributed to MLN following either systemic or regional infusion. The results (Table 6) show that 1.7% of infused radioactivity is recovered per gram of MLN, which is comparable to that in liver (2.15% per gram) and spleen (2.77% per gram). This result is consistent with findings obtained by other investigators which have indicated that LAK cells have the ability to localise in LN (Ettinghausen *et al* 1985; Marincola *et al* 1988; Harcel *et al* 1991). It has been demonstrated that activated T lymphocytes lose the ability to localise to LN (LeFever *et al* 1984). This change has been considered to be attributed to the loss of HEV receptors and other differences in cell surface carbohydrate composition (Dailey *et al* 1982; Carroll *et al* 1983).

The main purpose of present study was to investigate the effect of intraportal mannitol on the distribution of LAK cells to liver. The comparison of the uptake of LAK cells in liver following portal vein administration with and without mannitol showed that intraportal mannitol can significantly increase the uptake of A-LAK cells in liver (Table 7). The mechanism of increased distribution of A-LAK cells to liver following mannitol infusion is most likely due to the effect of mannitol on the endothelial vascular cells. Previous studies have demonstrated that mannitol has the effect of opening the Blood-Brain Barrier. The mechanism by which mannitol achieves this effect is by

increasing the intercellular gap between capillary endothelial cells (Franceschini *et al* 1988). Mannitol as a hyperosmolar solution has been shown to enhance the distribution of chemical agents into brain. The increased space between endothelial cells enables the large and rigid LAK cells to pass through the capillary wall into tissue. The effect of intraportal mannitol is further evidence that the limited pattern of LAK cell distribution *in vivo* is probably due to their large size and rigidity.

Immunohistochemical analysis was not carried out to obtain primary evidence of the histological localisation of adoptively transferred LAK cells. However the isotope trafficking study does suggest that these cells have been delivered into tissue (Table 8 and 9).

Mannitol has the effect of reducing the extravascular fluid in organs, therefore decreasing the interstitial pressure in tissues. The normal difference between vascular pressure and interstitial pressure may be responsible for inhibiting transvascular movement of LAK cells, ie. when interstitial pressure is higher than intravascular pressure, LAK cells have greater difficulty in passing into tissue because they have to move against a pressure gradient. After interstitial pressure is decreased by mannitol, LAK cells may pass through more easily.

The results show that there is only a very limited distribution of LAK cells to the brain even after the administration of mannitol (Table 7 and Table 8). The influence of interstitial pressure on the LAK cell migration is not a satisfactory explanation for poor distribution of LAK cells to brain. Although mannitol can decrease the interstitial pressure, the characteristics of this organ may have a more important role which

limits the distribution of LAK cells to this organ. Rannie *et al* 1977 has found that the central nervous tissue consistently gave the lowest concentration of radioactivity when the distribution of lymphocytes was studied.

Once LAK cells have accumulated in tissue it is possible that the closing of the capillary intercellular spaces may prevent the escape of entrapped A-LAK cells. Mannitol is only able to open the intercellular spaces for a limited time. Thus it is possible that once mannitol ceases to have any effect, the endothelial cells are able to revert to normal and prevent escape of A-LAK cells.

The optimal time between infusion of mannitol and delivery of LAK cells is uncertain. LAK cells infused too late after the infusion of mannitol would be unable to utilise the physiological effects of mannitol efficiently. The continuous infusion of mannitol is not recommended until the effect of prolonged mannitol administration on the uptake of LAK cells has been studied. The escape of LAK cells may occur when the entrapment of cells in the tissue reaches a certain level. Futher study will be required to confirm this.

The effect of mannitol on the delivery of LAK cells to tumour sites and liver metastases is of major interest. The microvasculature in tumour and metastases have different characteristics from normal tissues. In tumours the vessels are dilated, saccular, and tortuous (Jain 1988), and the blood flow is slow and even absent in the centre of the tumour. The interstitial pressure is higher towards the centre of tumour and the intercapillary distance is increased with tumour growth (Jain 1989). It has been demonstrated that LAK cells take significantly longer to

bind to tumour vasculature compared to normal vasculature, probably because of abnormal microvascular surface characteristics of tumours (Sasaki *et al* 1991). These characteristics may limit the direct effects of mannitol on tumour circulation and LAK cells may have difficulty in reaching all tumour sites. Thus in a small tumour lesion the entrapment of cells may be more efficient than in a large one due to the increased high interstitial pressure area in the larger tumour.

The present study shows that the distribution of LAK cells in normal tissue is increased by mannitol. It is possible that mannitol could improve the accumulation of LAK cells within tumour. Little is known about whether tumour micro-circulation characteristics would limit the distribution of LAK cells. Further investigations is necessary to examine the effect of intraportal mannitol on LAK cell uptake over a longer time period, (for example twenty four hours) in normal tissues as well within tumour in liver. In addition it will be important to determine if these protocols result in longer survival for tumour-bearing rats.

In summary, this study provides evidence that intraportal infusion of mannitol may increase the distribution of intraportally infused A-LAK cells to the liver during adoptive immunotherapy. Regional infusion of LAK cells may have the additional effect of limiting the accumulation of A-LAK cells in the lung with a possible reduction in pulmonary oedema.

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Appendix 1 Distribution of A-LAK Cells by Systemic and Regional Infusion and by the Effect of Intraportal Mannitol

After labelled the A-LAK cells with ^{51}Cr, 1ml of cells in PBS were infused into syngeneic male rat through tail vein or portal vein. Two hours later the rats were sacrificed and the organs and blood were taken for gamma-count to study the distribution of the A-LAK cells in different organs. Table A, B and C present the records of the trafficking study in these three groups. CPM is the radioactivity remained in infused cells *in vivo* 2h after infusion and it is obtained by subtracting SR from the total-injected-cpm. CPM of all cells = total injected cpm x (1–%spontaneous release). It is presumed that the released ^{51}Cr had been cleaned from the blood stream through the kidneys. The %distribution per organ = 100 x cpm in organ / CPM. For some organs the radioactivity counts were obtained by counting part of these organs. The weights of the counted pieces and the uncounted remainder are recorded in Table D.

The relevant formulae are summarised below:

$$\%\text{distribution of LAK cells in organ} = \frac{\text{CPM of organ}}{\text{CPM of all cells}}$$

$$\text{CPM of organ} = \frac{\text{cpm of piece of organ}}{\%\text{counted}}$$

$$\%\text{distribution of LAK cells per G} = \frac{\%\text{distribution in organ}}{\text{weight of organ}}$$

$$\%\text{counted} = \frac{\text{weight of counted piece}}{\text{weight of whole organ}} \times 100$$

Table A Distribution of A-LAK cells by tail vein infusion

Rat 1	Blood	Spleen	Kidney	MLN	Lung	Liver	Brain	Total
CPM of all cells								**2160**
cpm of peice of organ	6.9/ml	10.1	9.7	0	28.9	19.0	0.8	
cpm of organ	69	10.1	28.7	0	124.6	358.5	3.36	595
%dis-tion per organ	**3.2%**	**0.5%**	**1.3%**	**0**	**5.8%**	**16.6%**	**0.2%**	**27.5%**
weight of organ (g)		0.4	2.1	0.19	1.12	11.65	1.22	
%dis-tion per gram		1.25%	0.62%	0	5.18%	1.43%	0.16%	

Rat 2	Blood	Spleen	Kidney	MLN	Lung	Liver	Brain	Total
CPM of all cells								**9856**
cpm of peice of organ	35/ml	169.1	234.4	8.5	564.2	268.5	5.3	
cpm of organ	350	169.1	234.4	2.8/LN	1432	1561	11.3	3970
%dis-tion per organ	**3.6%**	**1.7%**	**2.4%**	**0.07%**	**14.5%**	**15.8%**	**0.1%**	**40%**
weight of organ (g)		0.49	1.18	0.04	0.66	6.56	0.96	
%dis-tion per gram		3.47%	2.03%	5.25%	21.9%	2.41%	0.1%	

Rat 3	Blood	Spleen	Kidney	MLN	Lung	Liver	Brain	Total
CPM of all cells								**4475**
cpm of peice of organ	6.9	100.6	42.8	8.5	172.5	134.9	0.3	
cpm of organ	69	100.6	42.8	2.8/LN	638.9	1226.4	2.5	2080
%dis-tion per organ	**1.5%**	**2.2%**	**1%**	**0.06%**	**14.3%**	**27.4%**	**0.06%**	**46.5%**
weight of organ (g)		0.58	1.63	0.05	0.89	8.94	1.26	
%dis-tion per gram		3.79%	0.61%	3.8%	16.1%	3.07%	0.05%	

Rat 4	Blood	Spleen	Kidney	MLN	Lung	Liver	Brain	Total
CPM of all cells								**10760**
cpm of peice of organ	24.8	80.0	163.8	9.4	348.6	132.6	2.0	
cpm of organ	496	80	163.8	3.1/LN	922.2	808.5	5.2	2479
%dis-tion per organ	**4.6%**	**0.7%**	**1.5%**	**0.02%**	**8.6%**	**7.5%**	**0.05%**	**23%**
weight of organ (g)		0.41	1.14	0.04	0.74	5.91	1.05	
%dis-tion per gram		1.71%	1.32%	2.18%	11.6%	1.27%	0.048%	

Rat 5	Blood	Spleen	Kidney	MLN	Lung	Liver	Brain	Total
CPM of all cells								**3519**
cpm of peice of organ	13.5	13.7	50.2	4.2	64.4	17.5	0	
cpm of organ	135	13.7	50.2	1.4	213.9	157.7	0	
%dis-tion per organ	**3.8%**	**0.4%**	**1.4%**	**0.04%**	**6.1%**	**4.5%**	**0%**	**16.2%**
weight of organ (g)	1ml	0.54	1.31	0.1	0.83	7.51	1.26	
%dis-tion per gram		0.74%	1.07%	1.2%	7.3%	0.6%	0%	

Rat 6	Blood	Spleen	Kidney	MLN	Lung	Liver	Brain	Total
CPM of all cells								**8376**
cpm of peice of organ	15.8	114.4	93.4	5.3	303.4	141.4	0.5	
cpm of organ	158	114.4	93.4	1.8	1465.7	1536.9	1.64	3401.8
%dis-tion per organ	**1.9%**	**1.4%**	**1.1%**	**0.02%**	**17.5%**	**18.4%**	**0.02%**	**40.3%**
weight of organ (g)		0.45	1.45	0.09	0.92	8.06	1.31	
%dis-tion per gram		3.1%	0.76%	0.7%	19%	2.3%	0.015%	

Rat 7	Blood	Spleen	Kidney	MLN	Lung	Liver	Brain	Total
CPM of all cells								**12143**
cpm of peice of organ	17.5	129.3	138	8.3	438.2	125	3.8	
cpm of organ	175	129.3	138	2.8	2047.7	477.1	10.8	
%dis-tion per organ	**1.4%**	**1.1%**	**1.1%**	**0.02%**	**16.9%**	**3.9%**	**0.09%**	**24.5%**
weight of organ (g)		0.58	1.45	0.04	0.84	9.5	1.22	
%dis-tion per gram		1.9%	0.76%	1.7%	20%	0.41%	0.07%	

Rat 8	Blood	Spleen	Kidney	MLN	Lung	Liver	Brain	Total
CPM of all cells								**8226**
cpm of peice of organ	6.9	336.7	228.6	14.7	929.6	278.1	8.1	
cpm of organ	138	336.7	228.6	4.9	3417.6	3056	34.6	7225
%dis-tion per organ	**1.7%**	**4.1%**	**2.8%**	**0.06%**	**41.5%**	**37.2%**	**0.42%**	**87.8%**
weight of organ (g)		0.66	1.23	0.06	0.81	6.5	1.28	
%dis-tion per gram		6.2%	2.3%	2.9%	51%	5.72%	0.33%	

Table B Distribution of A-LAK cells by portal vein infusion

Rat 1	Blood	Spleen	Kidney	MLN	Lung	Liver	Brain	Total
CPM of all cells								**4153**
cpm of peice of organ	10.9	14.8	42	7.8	80.5	92.2	0	
cpm of organ	121.1	14.8	42	2.6/LN	281.5	434.9	0	896.9
%dis-tion per organ	**2.9%**	**0.4%**	**1%**	**0.06%**	**6.8%**	**10.5%**	**0**	**21.6%**
weight of organ (g)		0.43	1.05	0.03	0.63	4.72	1.03	
%dis-tion per gram		0.93%	0.95%	6.26%	10.8%	2.25%	0	

Rat 2	Blood	Spleen	Kidney	MLN	Lung	Liver	Brain	Total
CPM of all cells								**8424**
cpm of peice of organ	9.0	138.5	114.5	0	364.9	153.0	0.5	
cpm of organ	900	138.5	602.6	0	1658.6	1700	2.5	5001
%dis-tion per organ	**10.7%**	**1.6%**	**7.2%**	**0**	**19.7%**	**20.2%**	**0.03%**	**59.4%**
weight of organ (g)		0.37	2.16	0.13	1.62	9.75	1.66	
%dis-tion per gram		4.32%	3.33%	0	12.2%	2.07%	0.02%	

Rat 3	Blood	Spleen	Kidney	MLN	Lung	Liver	Brain	Total
CPM of all cells								**7007**
cpm of peice of organ	5.6	48.3	19.7	0	50.0	103.3	0	
cpm of organ	140	48.3	48.9	0	284.1	1122.8	0	1644.1
%dis-tion per organ	**2%**	**0.7%**	**0.7%**	**0**	**0.3%**	**16%**	**0**	**23.5%**
weight of organ (g)		0.48	1.34	0.15	0.91	7.59	1.48	
%dis-tion per gram		1.46%	0.52%	0	0.33%	2.11%	0	

Rat 4	Blood	Spleen	Kidney	MLN	Lung	Liver	Brain	Total
CPM of all cells								**23787**
cpm of peice of organ	11.2	63.0	122.6	16.9	194.4	1236.9	5.5	
cpm of organ	280	63	123	5.6/LN	627	8530	13	9641.6
%dis-tion per organ	**1.2%**	**0.3%**	**0.5%**	**0.02%**	**2.6%**	**36%**	**0.06%**	**40.5%**
weight of organ (g)		0.44	1.25	0.08	0.81	6.13	0.96	
%dis-tion per gram		0.68%	0.4%	0.89%	3.21%	5.87%	0.06%	

Rat 5	Blood	Spleen	Kidney	MLN	Lung	Liver	Brain	Total
CPM of all cells								7718
cpm of peice of organ	16.3	37.9	82.8	4.8	35.6	150.2	6.3	
cpm of organ	163	37.9	82.8	1.6	126.4	1314.1	23.4	
%dis-tion per organ	**2.1%**	**0.5%**	**1.1%**	**0.02%**	**1.6%**	**17%**	**0.3%**	**22.6%**
weight of organ (g)		0.51	1.42	0.04	0.71	8.05	1.3	
%dis-tion per gram		0.98%	0.77%	1.6%	2.29%	2.1%	0.23%	

Rat 6	Blood	Spleen	Kidney	MLN	Lung	Liver	Brain	Total
CPM of all cells								12134
cpm of peice of organ	25.4	104.9	82.4	6.6	122.2	283.1	0	
cpm of organ	254	104.9	82.4	2.2	558	3217	0	
%dis-tion per organ	**2.1%**	**0.9%**	**0.7%**	**0.02%**	**4.6%**	**26.5%**	**0**	**34.8%**
weight of organ (g)		0.4	1.43	0.05	0.73	7.7	1.15	
%dis-tion per gram		2.25%	0.49%	1.1%	6.3%	3.44%	0	

Rat 7	Blood	Spleen	Kidney	MLN	Lung	Liver	Brain	Total
CPM of all cells								8226
cpm of peice of organ	26.2	224.9	170.2	7	143.3	387.4	4.8	
cpm of organ	524	224.9	170.2	2.3	612.4	3761.2	17.45	
%dis-tion per organ	**6.3%**	**2.7%**	**2.1%**	**0.03%**	**7.4%**	**45.7%**	**0.2%**	**57.0%**
weight of organ (g)		0.45	0.96	0.04	0.64	4.95	1.2	
%dis-tion per gram		6%	2.19%	2.1%	11.6%	9.2%	0.17%	

Rat 8	Blood	Spleen	Kidney	MLN	Lung	Liver	Brain	Total
CPM of all cells								8226
cpm of peice of organ	5.0	54.7	78.4	7	19.9	207.9	1.0	
cpm of organ	166.7	54.7	78.4	2.3	108.2	1856.3	2.87	
%dis-tion per organ	**2%**	**0.7%**	**0.95%**	**0.03%**	**1.3%**	**23%**	**0.03%**	**28.0%**
weight of organ (g)		0.59	1.09	0.05	0.76	5.78	1.09	
%dis-tion per gram		1.19%	0.87%	1.7%	1.7%	3.98%	0.03%	

Table C Distribution of A-LAK cells by the effect of intraportal mannitol

Rat 1	Blood	Spleen	Kidney	MLN	Lung	Liver	Brain	Total
CPM of all cells								12210
cpm of peice of organ	19.2	40.7	123.2	5.9	209.3	405.4	2.3	
cpm of organ	192	40.7	123.2	1.97	550.8	3898	8.7	4815.2
%dis-tion per organ	**1.6%**	**0.3%**	**0.5%**	**0.02%**	**4.5%**	**32%**	**0.07%**	**39.4%**
weight of organ (g)		0.46	1.22	0.04	0.74	6.83	1.1	
%dis-tion per gram		0.65%	0.41%	1.21%	6.08%	4.69%	0.064%	

Rat 2	Blood	Spleen	Kidney	MLN	Lung	Liver	Brain	Total
CPM of all cells								7589
cpm of peice of organ	45.4	76.6	132.8	9.8	300.9	513.2	4.0	
cpm of organ	454	76.6	132.8	3.3	999.7	3539.3	14.3	5220
%dis-tion per organ	**5.9%**	**1%**	**1.7%**	**0.04%**	**13.2%**	**46.4%**	**0.2%**	**68.8%**
weight of organ (g)		0.37	1.06	0.03	0.73	5.39	1.5	
%dis-tion per gram		2.7%	1.6%	4.3%	18.1%	8.65%	0.13%	

Rat 3	Blood	Spleen	Kidney	MLN	Lung	Liver	Brain	Total
CPM of all cells								7038
cpm of peice of organ	65.3	50.3	197.8	11.9	302.6	634.7	3.5	
cpm of organ	653	50.3	197.8	3.9	829	3800.6	7.7	5535
%dis-tion per organ	**9.3%**	**0.7%**	**2.8%**	**0.05%**	**11.8%**	**54%**	**0.01%**	**78.6%**
weight of organ (g)		0.44	0.99	0.04	0.74	4.56	0.95	
%dis-tion per gram		1.59%	2.83%	4.23%	16%	11.84%	0.01%	

Rat 4	Blood	Spleen	Kidney	MLN	Lung	Liver	Brain	Total
CPM of all cells								10760
cpm of peice of organ	19.2	363.1	241.4	14.3	369.9	745	4.0	
cpm of organ	192	363.1	241.4	4.7	1185.6	6478.3	9.5	8474.6
%dis-tion per organ	**1.8%**	**3.4%**	**2.3%**	**0.04%**	**11%**	**60%**	**0.09%**	**78.8%**
weight of organ (g)		0.4	1.17	0.04	0.77	6.33	1	
%dis-tion per gram		8.5%	1.97%	3.32%	14.3%	9.48%	0.09%	

Rat 5	Blood	Spleen	Kidney	MLN	Lung	Liver	Brain	Total
CPM of all cells								3519
cpm of peice of organ	10.9	16.3	48.6	6.8	52.6	296.8	3.3	
cpm of organ	109	16.3	48.6	2.27	189.2	2650	11.7	
%dis-tion per organ	**3.1%**	**0.5%**	**1.4%**	**0.06%**	**5.4%**	**75.3%**	**0.3%**	**86.1%**
weight of organ (g)		0.5	1.19	0.08	0.72	5.99	1.25	
%dis-tion per gram		1%	1.18%	2.42%	7.5%	12.6%	0.24%	

Rat 6	Blood	Spleen	Kidney	MLN	Lung	Liver	Brain	Total
CPM of all cells								6407
cpm of peice of organ	19.4	78.9	92.2	24.2	147.6	4474.4	5.9	
cpm of organ	194	78.9	92.2	8.1	833.9	4474.4	24.9	
%dis-tion per organ	**3.0%**	**1.2%**	**1.4%**	**0.1%**	**13%**	**69.8%**	**0.39%**	**88.9%**
weight of organ (g)		0.45	1.12	0.06	0.79	5.99	1.39	
%dis-tion per gram		2.7%	1.25%	6.3%	16.5%	11.7%	0.28%	

Rat 7	Blood	Spleen	Kidney	MLN	Lung	Liver	Brain	Total
CPM of all cells								6407
cpm of peice of organ	26.9	24.1	45.2	4.8	72.3	3316.3	10.9	
cpm of organ	269	24.1	45.2	1.6	361.5	3316.3	41.3	
%dis-tion per organ	**4.2%**	**0.38%**	**0.7%**	**0.03%**	**5.6%**	**51.8%**	**0.65%**	**63.4%**
weight of organ (g)		0.4	1.1	0.06	0.7	5.93	1.4	
%dis-tion per gram		0.95%	0.64%	1.25%	8%	8.7%	0.46%	

Rat 8	Blood	Spleen	Kidney	MLN	Lung	Liver	Brain	Total
CPM of all cells								6147
cpm of peice of organ	7.1	31.8	43	8.1	162.3	2634.5	24.5	
cpm of organ	71	31.8	43	2.7	162.3	2634.5	24.5	
%dis-tion per organ	**1.2%**	**0.5%**	**0.7%**	**0.04%**	**2.64%**	**42.9%**	**0.4%**	**48.4%**
weight of organ (g)		0.41	1.29	0.05	0.81	6.09	1.47	
%dis-tion per gram		1.22%	0.54%	2.7%	3.26%	7.04%	0.27%	

Table D Organ weight records

Group 1 Rat 1	Weight of peice of organ counted(g)	Weight of left organ un-counted(g)	Weight of organ (g)	% Counted
Blood	1ml	9ml		10%
Spleen	0.4		0.4	100%
Kidney	0.71	1.39	2.1	33.8%
MLN	0.19		0.19	100%
Lung	0.26	0.86	1.12	23.2%
Liver	0.62	11.03	11.65	5.3%
Brain	0.29	0.93	1.22	23.8%

Group 1 Rat 2	Weight of peice of organ counted(g)	Weight of left organ un-counted(g)	Weight of organ (g)	% Counted
Blood	1ml	9ml		10%
Spleen	0.49		0.49	100%
Kidney	0.59 0.59		1.18	100%
MLN	0.04		0.04	
Lung	0.26	0.4	0.66	39.4%
Liver	1.13	5.43	6.56	17.2%
Brain	0.45	0.51	0.96	46.9%

Group 1 Rat 3	Weight of peice of organ counted(g)	Weight of left organ un-counted(g)	Weight of organ (g)	% Counted
Blood	1ml		10ml	10%
Spleen	0.58		0.58	100%
Kidney	0.8 0.83		1.63	100%
MLN	0.05		0.05	
Lung	0.24	0.65	0.89	27%
Liver	0.98	7.96	8.94	11%
Brain	0.15	1.11	1.26	12%

Group 1 Rat 4	Weight of peice of organ counted(g)	Weight of left organ un-counted(g)	Weight of organ (g)	% Counted
Blood	0.5ml		10ml	5%
Spleen	0.41		0.41	100%
Kidney	0.57x2		1.14	100%
MLN	0.04		0.04	
Lung	0.28	0.46	0.74	37.8%
Liver	0.97	4.94	5.91	16.4%
Brain	0.4	0.65	1.05	38.1%

Group 1 Rat 5	Weight of peice of organ counted(g)	Weight of left organ un-counted(g)	Weight of organ (g)	% Counted
Blood	1ml		10ml	10%
Spleen	0.54		0.54	100%
Kidney	0.66 0.65		1.31	100%
MLN	0.1		0.1	
Lung	0.25	0.58	0.83	30.1%
Liver	0.83	6.68	7.51	11.1%
Brain	0.34	0.92	1.26	26.9%

Group 1 Rat 6	Weight of peice of organ counted(g)	Weight of left organ un-counted(g)	Weight of organ (g)	% Counted
Blood	1ml		10ml	10%
Spleen	0.45		0.45	100%
Kidney	0.7 0.75		1.45	100%
MLN	0.09			
Lung	0.19	0.73	0.92	20.7%
Liver	0.74	7.32	8.06	9.2%
Brain	0.4	0.91	1.31	30.5%

Group 1 Rat 7	Weight of peice of organ counted(g)	Weight of left organ un-counted(g)	Weight of organ (g)	% Counted
Blood	1ml		10ml	10%
Spleen	0.58		0.58	100%
Kidney	0.69 0.76		1.45	100%
MLN	0.04			
Lung	0.18	0.66	0.84	21.4%
Liver	2.49	7.01	9.5	26.2%
Brain	0.43	0.79	1.22	35.3%

Group 1 Rat 8	Weight of peice of organ counted(g)	Weight of left organ un-counted(g)	Weight of organ (g)	% Counted
Blood	0.5ml		10ml	5%
Spleen	0.66		0.66	100%
Kidney	0.6 0.63		1.23	100%
MLN	0.06			
Lung	0.22	0.59	0.81	27.2%
Liver	0.59	5.91	6.5	9.1%
Brain	0.3	0.98	1.28	23.4%

Group 2 Rat 1	Weight of peice of organ counted(g)	Weight of left organ un-counted(g)	Weight of organ (g)	% Counted
Blood	0.9ml		10ml	9%
Spleen	0.43		0.43	100%
Kidney	0.55 0.5		1.05	100%
MLN	0.03		0.03	
Lung	0.18	0.45	0.63	28.6%
Liver	1	3.72	4.72	21.2%
Brain	0.42	0.61	1.03	40.8%

Group 2 Rat 2	Weight of peice of organ counted(g)	Weight of left organ un-counted(g)	Weight of organ (g)	% Counted
Blood	0.1ml		10ml	1%
Spleen	0.37		0.37	
Kidney	0.41	1.75	2.16	19%
MLN	0.13		0.13	
Lung	0.36	1.26	1.62	22%
Liver	1.41	8.34	9.75	9%
Brain	0.34	1.32	1.66	20%

Group 2 Rat 3	Weight of peice of organ counted(g)	Weight of left organ un-counted(g)	Weight of organ (g)	% Counted
Blood	0.4ml		10ml	4%
Spleen	0.48		0.48	100%
Kidney	0.54	0.8	1.34	40.3%
MLN	0.15		0.15	
Lung	0.16	0.75	0.91	17.6%
Liver	0.7	6.89	7.59	9.2%
Brain	0.45	1.03	1.48	30.4%

Group 2 Rat 4	Weight of peice of organ counted(g)	Weight of left organ un-counted(g)	Weight of organ (g)	% Counted
Blood	0.3ml		10ml	3%
Spleen	0.44		0.44	100%
Kidney	0.59 0.66		1.25	100%
MLN	0.08		0.08	
Lung	0.25	0.56	0.81	31%
Liver	0.89	5.24	6.13	14.5%
Brain	0.4	0.56	0.96	42%

Group 2 Rat 5	Weight of peice of organ counted(g)	Weight of left organ un-counted(g)	Weight of organ (g)	% Counted
Blood	1ml		10ml	10%
Spleen	0.51		0.51	100%
Kidney	0.69 0.73		1.42	100%
MLN	0.04			
Lung	0.2	0.51	0.71	28.2%
Liver	0.92	7.13	8.05	11.43%
Brain	0.35	0.95	1.3	26.9%

Group 2 Rat 6	Weight of peice of organ counted(g)	Weight of left organ un-counted(g)	Weight of organ (g)	% Counted
Blood	1ml		10ml	10%
Spleen	0.4		0.4	100%
Kidney	0.7 0.73		1.43	100%
MLN	0.05			
Lung	0.16	0.57	0.73	21.9%
Liver	0.68	7.02	7.7	8.8%
Brain	0.43	0.72	1.15	37.4%

Group 2 Rat 7	Weight of peice of organ counted(g)	Weight of left organ un-counted(g)	Weight of organ (g)	% Counted
Blood	0.5ml		10ml	5%
Spleen	0.45		0.45	100%
Kidney	0.47 0.49		0.96	100%
MLN	0.04			
Lung	0.15	0.49	0.64	23.4%
Liver	0.51	4.44	4.95	10.3%
Brain	0.33	0.87	1.2	27.5%

Group 2 Rat 8	Weight of peice of organ counted(g)	Weight of left organ un-counted(g)	Weight of organ (g)	% Counted
Blood	0.3ml		10ml	3%
Spleen	0.59		0.59	100%
Kidney	0.53 0.56		1.09	100%
MLN	0.05			
Lung	0.14	0.62	0.76	18.4%
Liver	0.65	5.13	5.78	11.2%
Brain	0.38	0.71	1.09	34.9%

Group 3 Rat 1	Weight of peice of organ counted(g)	Weight of left organ un-counted(g)	Weight of organ (g)	% Counted
Blood	1ml		10ml	10%
Spleen	0.46		0.46	100%
Kidney	0.6 0.62		1.22	100%
MLN	0.04		0.04	
Lung	0.28	0.46	0.74	38%
Liver	0.71	6.12	6.83	10.4%
Brain	0.29	0.81	1.1	26.4%

Group 3 Rat 2	Weight of peice of organ counted(g)	Weight of left organ un-counted(g)	Weight of organ (g)	% Counted
Blood	1ml		10ml	10%
Spleen	0.37		0.37	100%
Kidney	0.54 0.52		1.06	100%
MLN	0.03		0.03	
Lung	0.22	0.51	0.73	30.1%
Liver	0.78	4.61	5.39	14.5%
Brain	0.42	1.08	1.5	28%

Group 3 Rat 3	Weight of peice of organ counted(g)	Weight of left organ un-counted(g)	Weight of organ (g)	% Counted
Blood	1ml		10ml	10%
Spleen	0.44		0.44	100%
Kidney	0.49 0.5		0.99	100%
MLN	0.04		0.04	
Lung	0.27	0.47	0.74	36.5%
Liver	0.76	3.8	4.56	16.7%
Brain	0.43	0.52	0.95	45.3%

Group 3 Rat 4	Weight of peice of organ counted(g)	Weight of left organ un-counted(g)	Weight of organ (g)	% Counted
Blood	1ml		10ml	10%
Spleen	0.4		0.4	100%
Kidney	0.57 0.6		1.17	100%
MLN	0.04		0.04	
Lung	0.24	0.53	0.77	31.2%
Liver	0.73	5.6	6.33	11.5%
Brain	0.42	0.58	1	42%

Group 3 Rat 5	Weight of peice of organ counted(g)	Weight of left organ un-counted(g)	Weight of organ (g)	% Counted
Blood	1ml		10ml	10%
Spleen	0.5		0.5	100%
Kidney	1.19		1.19	100%
MLN	0.08			
Lung	0.2	0.52	0.72	27.8%
Liver	0.67	5.32	5.99	11.2%
Brain	0.36	0.89	1.25	28.8%

Group 3 Rat 6	Weight of peice of organ counted(g)	Weight of left organ un-counted(g)	Weight of organ (g)	% Counted
Blood	1ml		10ml	%
Spleen	0.45		0.45	100%
Kidney	0.55 0.57		1.12	100%
MLN	0.06			
Lung	0.14	0.65	0.79	17.7%
Liver	0.77	5.22	5.99	100%
Brain	0.33	1.06	1.39	23.7%

Group 3 Rat 7	Weight of peice of organ counted(g)	Weight of left organ un-counted(g)	Weight of organ (g)	% Counted
Blood	1ml		10ml	10%
Spleen	0.4		0.4	100%
Kidney	0.54 0.56		1.1	100%
MLN	0.06			
Lung	0.14	0.56	0.7	20%
Liver	0.81	5.12	5.93	100%
Brain	0.37	1.03	1.4	26.4%

Group 3 Rat 8	Weight of peice of organ counted(g)	Weight of left organ un-counted(g)	Weight of organ (g)	% Counted
Blood	1ml		10ml	10%
Spleen	0.41		0.41	100%
Kidney	0.64 0.65		1.29	100%
MLN	0.05		0.05	
Lung	0.22	0.59	0.81	100%
Liver	0.81	5.28	6.09	100%
Brain	0.54	0.93	1.47	100%

Table E summarises the distribution of A-LAK cells in organs in the three groups of rats. The number and viabilities of infused cells are shown in this table. The distribution results are obtained after calculation as shown in Table A-C.

Table E A-LAK cell Distribution in Three Groups of Rats

Cells10^6	Viability	SR%	Blood	Spleen	Kidney	MLN	Lung	Liver	Brain
				Group 1					
0.66	30%	36.9	3.2%	0.5%	1.3%	0	5.8%	16.6%	0.2%
2.64	94%	56	3.6	1.7%	2.4%	0.1%	14.5%	15.8%	0.1%
2.1	97%	8.6	1.5%	2.2%	1.0%	0.1%	14.3%	27.4%	0.1%
1.5	92%	42	4.6%	0.7%	1.5%	0.02%	8.6%	7.5%	0.1%
1.7	92%	14.5	3.8%	0.4%	1.4%	0.04%	6.1%	4.5%	0
1.65	90%	16	1.9%	1.4%	1.1%	0.02%	17.5%	18.4%	0.02%
1.5	85%	10.7	1.4%	1.1%	1.1%	0.02%	16.9%	3.9%	0.1%
2.5	95%	45	1.7%	4.1%	2.8%	0.1%	41.5%	37.2%	0.4%
				Group 2					
0.85	95%	43.6	2.9%	0.4%	1.0%	0.1%	6.8%	10.5%	0
3.5	74%	55	10.7%	1.6%	7.2%	0	19.7%	20.2%	0.03%
2.8	78%	18.9	2.0%	0.7%	0.7%	0	4.1%	16.0%	0
1.6	71%	17.2	1.2%	0.3%	0.5%	0.02%	2.6%	36.0%	0.06%
1	90%	28.5	2.1%	0.5%	1.1%	0.02%	1.6%	17.0%	0.3%
1.5	85%	10.7	2.1%	0.9%	0.7%	0.02%	4.6%	26.5%	0
2.5	95%	45	6.3%	2.7%	2.1%	0.03%	7.4%	45.7%	0.2%
2.5	95%	45	2.0%	0.7%	0.9%	0.03%	1.3%	23.0%	0.03%
				Group 3					
1.02	90%	29	1.6%	0.3%	0.5%	0.02%	4.5%	32%	0.1%
2.86	90%	56	5.9%	1.0%	1.7%	0.04%	13.2%	46.6%	0.2%
1.44	94%	35	9.3%	0.7%	2.8%	0.1%	11.8%	54.0%	0.01%
1.5	92%	42	1.8%	3.4%	2.3%	0.04%	11.0%	60.0%	0.1%
1.7	92%	14.5	3.1%	0.5%	1.4%	0.1%	5.4%	75.3%	0.3%
2.9	92%	25.6	3.0%	1.2%	1.4%	0.1%	13.0%	69.8%	0.4%
2.9	92%	25.6	4.2%	0.4%	0.7%	0.02%	5.6%	51.8%	0.6%
1.6	95%	18.6	1.2%	0.5%	0.7%	0.04%	2.6%	42.9%	0.4%