IMMUNOTHERAPY OF INTRAPERITONEAL CANCER

Effects of Lymphokine Activated Killer Cells and Interleukin-2 in Murine Models

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IMMUNOTHERAPY OF INTRAPERITONEAL CANCER
Effects of Lymphokine Activated Killer Cells
and Interleukin-2 in Murine Models

IMMUNOTHERAPIE VAN INTRAPERITONEALE MALIGNE TUMOR
Effecten van lymfokine geactiveerde 'killer' cellen
en interleukine-2 in muizenmodellen

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR A.H.G. RINNOOY KAN
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Therapeutic efforts are evidence of caring of two sorts, not only the physician's concern for the patient, but also concern for self. I am not criticizing this dual obligation that has so much to do with human motivation when these two concerns are so inseparable, so long as physicians put the patients' needs above their own. Physicians really cannot serve others without some need to serve themselves. Also, when both the doctor and the patient must contend with so much that is bad, it is natural for physicians either to want to make things better or, failing that, to try to make things look better to and for themselves. However, we know that these goals must be tempered for proper balance, if the patient is to get a fair share of caring and concern.

Martin A. Adson

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ABBREVIATIONS

AK	activated killer cell
B cell	bursa or bone marrow derived cell
BRM	biological response modifier
cDNA	complementary deoxyribonucleic acid
c-onc	cellular oncogene
CTL	cytotoxic T lymphocyte
DNA	deoxyribonucleic acid
HBSS	Hank's balanced salt solution
IFN	interferon
IL-1	interleukin-1
IL-2	interleukin-2
i.p.	intraperitoneal
i.v.	intravenous
LAK cell	lymphokine activated killer cell
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NK cell	natural killer cell
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline
PCI	peritoneal carcinomatosis index
PHA	phytohemagglutinin
PMA	phorbol myristate acetate
RNA	ribonucleic acid
TCGF	T cell growth factor
TIL	tumor infiltrating lymphocytes
Tla	thymus leukemia antigen
T lymphocyte, T cell	thymus derived lymphocyte
v-onc	viral oncogene

Chapter 1.

INTRODUCTION

The scope of the cancer problem

While the age-adjusted incidence of most cancers is relatively stable over time, neoplasms in general show an incidence that increases with age. The improving life expectancy of the general population therefore means an increasing absolute number of cancer patients in the coming decades. (Stuurgroep toekomstscenario's gezondheidszorg, 1987)

Surgery, radiation therapy, chemotherapy and combinations thereof, can cure only a proportion of cancer patients. It is estimated that, excluding carcinoma in situ and nonmelanoma skin cancer, about fifty percent of cancer patients will die with disease.
Improved surgical techniques will not markedly impact on this situation. Progress in radiation therapy is more likely to result in a decrease in side effects than in better tumor control. Chemotherapy has shown spectacular progress in its impact on a number of tumors, notably haematologic neoplasms and testicular cancer. Solid tumors, especially those of the digestive tract have however proven rather resistant to chemotherapy.
Primary prevention by reducing known risk factors and early detection by screening and by an increased awareness both in the medical profession and in the laity are approaches that will prove their worth, if any, in the coming years.

Predictions as this are, by their very nature, speculative, but it seems too complacent to trust that improvements in the 'classical' treatment modalities or in their combined use will impact in a meaningful way on the cancer problem as a whole.

Tumor immunotherapy

Tumor immunotherapy has till sofar not come of age. It has enjoyed bursts of interest and a number of human trials have been conducted, none of them strikingly successful. Most of those treatment protocols can be faulted on the basis that they were chosen semi-intuitively. In recent years our understanding of basic immunologic phenomena has

deepened. New techniques, especially the recombinant DNA technique have widened the treatment armamentarium.
Cells that kill tumor cells without the need for recognition of tumor specific antigens have been described and can be manufactured.
This state of affairs provides a rationale for further research in this direction. The ultimate aim of tumor immunotherapy is complete rejection of the tumor.

Animal models

The relevance of animal tumor models for human tumor immunology has been debated (Hewitt 1982, Herberman 1983). Most animal studies have used viral or chemically induced transplantable tumors in young immunocompetent recipients. Clearly this differs from the autochthonous tumor in the human situation.
On the other hand, there is considerable evidence for an important role of chemical carcinogens in the etiology of several common human tumors (e.g. lung, bladder, colon). Also, recent evidence for the frequent expression of oncogenes in human tumors raises the possibility of analogies to virus induced tumors in rodents.

It would be neither feasible nor justifiable to test all possible immunomodulatory approaches in the human situation. A pragmatic attitude is to screen possible tumor immunotherapeutics in animal models. Promising agents should be tried in several animal models, employing a spectrum of tumors, preferably including autochthonous cancers.

With the realization that a limited success in animal models does not preclude effectiveness in the human situation nor marked effectiveness in rodents necessarily translates in success in the human patient, limited human trials can then be conducted. If human trials do not show strikingly positive results, they should be discontinued.

These remarks seem quite self evident, but many human trials have been undertaken on only the flimsiest suggestion of efficaciousness in animal models and have long been continued in the face of substantial side effects and little antitumor activity.

The intraperitoneal model

The experiments described in this thesis were carried out in the Colorectal Cancer Section of the Surgery Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland.

Within the Section, there was a strong clinical interest in the problems of recurrent disease of colorectal cancer, notably peritoneal carcinomatosis.
Not infrequently, implants in the peritoneal cavity are the only manifestation of recurrent disease. At autopsy no metastases outside the peritoneal cavity are found. At present no effective treatment for this condition exists.
It was therefore decided to choose a model of established intraperitoneal tumor.

Immunotherapy of intraperitoneal tumor in murine models.

In Chapter 2, the concept of and approaches to immunotherapy are reviewed. There is an ever increasing list of immunomodulating agents with potential effectiveness. It will be seen that treatment with the lymphokine interleukin-2 (IL-2) and a certain cell type, obtained by incubating lymphoid cells in IL-2 (lymphokine activated killer cells, LAK cells) is theoretically attractive.
In Chapter 3, the literature on IL-2 and LAK cells is reviewed.
Materials and methods are described in Chapter 4.
In four chapters describing experimental results, the LAK cell phenomenon, LAK cell generation, dose-response effects and the applicability of treatment with IL-2 and LAK cells are explored.
The results are discussed in Chapter 9.

Part of these experiments have been published before (Ottow *et al.* 1985, 1986a-b and 1987a-b).
However, some of these results can now be better put into perspective and a number of experiments had not yet been reported. It was therefore decided to rewrite the material in this form.

Chapter 2

CONCEPTS IN IMMUNOTHERAPY

2.1 History

In the last decades of the 19th century the principal mechanisms of immunity against infectious diseases were elucidated. These findings inspired the concept that a neoplasm creates in its host a similar immune response and that such reactivity might be used to therapeutic advantage. In early experiments tumors were excised from one animal and transplanted to other, randombred animals. The new host in most instances rejected the tumor and this was explained by assuming immunity to antigens unique to the cancerous cell.
However, when inbred strains became available, it soon transpired that tumors were rejected only when transplanted to individuals genetically different from the tumor bearing donor. What had been considered a response against 'tumor-specific' antigens, turned out to be a response to the major histocompatibility antigens of the transplanted tumor. This realization led to a loss of interest in tumor immunology.

The first suggestion that immunity to determinants unique to the malignant cell might exist after all, came from the work of Foley (1953). He used a transplantable, methylcholantrene induced tumor in mice. Untreated, these tumors killed the mice. By ligation at the base of a developing tumor, the blood supply to the tumor could be interrupted, inducing tumor necrosis and preventing spread. When these mice were challenged with live cells taken from the same tumor line, the grafts were frequently rejected while similar grafts to naive mice were accepted and killed the host.
These findings were expanded by Prehn and Main (1957). These authors showed that immune animals, while rejecting a challenge with tumor cells, would accept a graft of normal tissue and that transplants of normal tissues did not induce immunity against tumors. These observations ruled out that remaining histoincompatibility in supposedly inbred strains was responsible for the tumor immunity described by Foley.

Final proof of the existence of tumor-associated antigens came from the work of Klein and coworkers (1960). Tumors were induced in a leg of a mouse. The leg was then amputated and tumor cells passed in a syngeneic host. Other cells of this tumor were irradiated to prevent their proliferation and injected into the original donor. After this immunization, life, unirradiated tumor cells were transplanted back to the original donor who now rejected his own tumor.
A next important discovery was made by Old and Boyse (1965), who found that certain oncogenic viruses produced unique cell surface determinants in infected cells.

The first clinical trial of tumor immunotherapy in human patients that drew wide attention was conducted by Mathé and co-workers (1969). A vaccine composed of irradiated allogeneic leukemia cells combined with BCG seemed to prolong the remission rate in acute lymphoblastic leukemia. This trial reawakened interest in the clinical application of tumor immunotherapy. A steady flow of experiments and publications followed. Their impact is briefly discussed in Section 2.6.
The recent discovery of oncogenes has given a new theoretical foundation to the concept that tumor cells might carry unique antigens that can be used for therapeutic purpose.

2.2 Tumor-associated antigens

Tumor immunotherapy presupposes the existence of tumor-associated antigens or the presence of other changes on the tumor cell by which immune cells can recognise it as different from normal cells. Tumor-associated antigens conceptually fall into two categories: true tumor-associated antigens, found on neoplastic cells only and abnormally expressed antigens. These abnormally expressed antigens are also detectable on some normal cells at some stages of differentiation. Included in this category are the oncofetal antigens [e.g. alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA), gross cystic disease protein (GCPD)], the differentiation antigens (e.g. the thymus leukemia antigen) and the lineage associated antigens [e.g. those designated carcinoma-associated antigen 19-9 (CA 19-9) and CA-125].

True tumor-associated antigens are often referred to as tumor-specific antigens. As complete restriction of antigens to tumor cells is difficult to prove in the human situation, this designation will be avoided.
Tumor-associated antigens can be demonstrated by a variety of techniques. Tumor transplantation experiments showing the rejection of tumor cells by the immunized host, have been mentioned above. Antigens so detected are sometimes referred to as tumor-associated transplantation antigens. In in vitro experiments, lysing of tumor cells by immune lymphocytes can be demonstrated. Alternatively, tumor-associated antigens can be detected by a variety of serological techniques e.g. immunofluorescence and complement mediated cytotoxicity.
Tumor-associated antigens can be generated by synthesis of new molecules, exposure of antigens that are normally covered or modification of existing molecules. IFN-gamma for example can increase the expression of antigens.

As reviewed by De Klein (1986), oncogenes can be defined as normal cellular genes that have the potential to bring about morphologic transformation or tumor formation. They form a group of genes which has been highly conserved throughout evolution. Quantitative (enhanced or constitutive expression) and qualitative (mutations, truncations) alteration of those genes will interfere with the normal cellular functions and vould ultimately lead to transformation. The majority of these cellular oncogenes was identified because they represent the cellular homologues (c-onc) of the transferring genes of acute RNA tumor viruses. These viruses contain on extra host cell derived sequence, v-onc, which is responsible for the transformation. Activation of oncogenes could lead to generation of new membrane antigens that are not expressed on normal cells.

2.3 Immune surveillance theory

Ehrlich (1909) was maybe the first to suggest the idea that one of the functions of the immune system might be to destroy cells that had undergone malignant transformation. The concept was popularised by

F.Macfarlane Burnet (1957, 1970), who coined the term immunologic surveillance. This attractive theory has not conclusively been proven. Arguments cited to support it (Klein 1982), include the following. Occasionally, spontaneous regression of tumors is seen (Everson and Cole, 1966). Experimental carcinogenesis is in some models enhanced in immune suppressed animals (e.g. Stutman, 1983a). Patients with immune disorders or receiving immunosuppressive treatment show an increased incidence of neoplasms, mainly lymphomas (Stutman, 1983b). Arguments against the immune surveillance theory comprise the following.

The hypothesis predicts that in immunodepressive conditions an increased incidence in spontaneous or induced tumor development would be seen. But the enhanced experimental carcinogenesis in immunosuppressed animals, mentioned above, is seen only in selected models, and does not appear to be a general phenomenon (e.g. Pirofsky et al., 1980).

Furthermore, in the category of patients with immune disorders or on immunosuppressive treatment, mentioned above, the hypothesis would predict an increase in all kinds of tumors, rather than just lymphoid tumors. The increase in lymphomas can also be explained by disbalances in the immune regulation (Stutman, 1983b).

Finally, the fact that in tumor transplantation, small tumor inocula sometimes take, while larger inocula are rejected (Old et al., 1962) is difficult to explain within the framework of the immune surveillance theory.

2.4 Effectors of immunologic reactivity against neoplasm

T lymphocytes were traditionally thought to be the most important effectors of tumor cell killing. Transfer of immunity to naive hosts by injection of T cells has been shown in a variety of murine models. Different subsets of T lymphocytes seem to be operative in different tumor-mouse strain combinations (Rosenstein et al., 1984a). Tumor cytotoxicity of T lymphocytes can be studied in vitro in cell mediated lymphocytotoxicity experiments. Both in in vivo and in in vitro studies, T cell mediated immunity is antigen specific.

Direct contact between target cells and effector cells is needed. The mechanisms proposed include release of a lymphotoxin, direct membrane-membrane interaction and the activity of T cell-associated enzymes.

Natural killer (NK) cells were discovered as a result of the finding that cells from patients without cancer could inhibit growth of tumor cell colonies (Herberman and Ortaldo, 1981). NK cells are probably identical with the so-called large granular lymphocytes. NK cells can lyse various tumors without previous exposure. Not all tumors however are NK sensitive. The category of cells designated **killer (K) cells, null cells**, or **natural cytotoxic cells** form an ill-defined system that kills a spectrum of tumors that appear different from those destroyed by NK cells (Stutman, 1983b). In contrast to the cytotoxic T lymphocyte, this reactivity is not based on a specific immune reaction. **Macrophages** can display both specific and aspecific killing. Specific killing is carried out by the so called armed macrophage. Normal macrophages can be armed by contact with specifically sensitized lymphocytes or by serum and are cytotoxic only to the specific target to which they have been sensitized (Schulz et al., 1983). Hibbs and coworkers (1972) described nonspecifically activated macrophages showing reactivity to a variety of tumor cells. Interferon (IFN) gamma appears to be the most important activator of macrophages (Nathan et al., 1983).
Antibodies binding to cell surface antigens can make a tumor cell susceptible to complement dependant cytotoxicity. Transfer of immunity to naive hosts by administration of antibodies is difficult to achieve and enhanced tumor growth rather than rejection is sometimes observed (Kaliss, 1958; Hellström and Hellström, 1974).

The **lymphokine activated killer cell** and the question of its nature will be discussed in Section 3.6.

2.5 Mechanisms by which malignant cells escape tumor immunity

Several mechanisms by which malignant cells escape tumor immunity can be hypothesized.
Tumor-associated antigens might not be expressed on certain tumor cells. Alternatively, certain tumors might be heterogenous for tumor-associated antigen expression and subclones not displaying antigens might be selected.
The phenomenon of temporary loss of antigens after exposure to specific antibodies has been termed antigenic modulation. This effect was first

observed in murine systems during studies of the thymus leukemia antigen (Tla). When mice immunized against this antigen were inoculated with Tla positive leukemia's, the tumor cells became Tla negative. On passage of leukemic cells to naive hosts, the Tla antigen reappeared (Old et al., 1963).

In some situations, inoculation with small numbers of tumor cells leads to progressive tumor growth while inoculation with large amounts of tumor cells leads to tumor rejection (Old and Boyse, 1965).

Immunologic enhancement refers to the observation that tumors show enhanced growth in the presence of specific antibodies. It can be hypothesized that antibody binding to tumor-associated antigen prevents development of specific cellular immunity (afferent enhancement). Alternatively it could prevent sensitized lymphocytes to bind to tumor cells (efferent enhancement).

Tumor bearing individuals show decreased responses in a variety of immunologic tests. Other reasons of decreased immunological competence are cytotoxic drugs, primary immune disorders and malnutrition. All these situations potentially interfere with efficient immune surveillance.

Apart from the aspecific immune suppression in tumor bearing animals mentioned above, tumor cells may also induce specific suppressor cells (Berendt and North, 1980). These suppressor cells inhibit antitumor effects of cytotoxic lymphocytes.

2.6 Approaches to tumor immunotherapy

If malignant cells have escaped putative mechanisms of immune surveillance and a progressively growing tumor is established, attempts at mediating tumor rejection can be considered. Several approaches can be distinguished. The therapeutic agent can exert a direct influence on the tumor cell (= passive immunotherapy) or act indirectly, via the host's immune system. A further distinction is between agents showing a reactivity to tumor cells that is immunologically specific and agents lacking such specificity. In Table 2.1 examples of these approaches are presented.

TABLE 2.1

APPROACHES TO IMMUNOTHERAPY

Approach	Example	Reference
passive-specific	injection of cytotoxic T cells to mice carrying a lymphoma resulting in cure	Rosenstein and Rosenberg 1984
passive-aspecific	administration of activated macrophages intraperitoneally for peritoneal carcinomasosis	Stevenson et al. 1984
active-specific	administration of irradiated tumor cells resulting in rejection of original tumor	Klein et al., 1960
active-aspecific	administration of the interferon inducer-ABPP to rats, carrying a transplantable adenocarcinoma resulting in growth retardation	Eggermont et al., 1986

The term 'adoptive immunotherapy' has been in use for forms of passive immunotherapy. In recent publications, contradictory definitions have been given. For example, it has been defined as passive immunotherapy with sensitized cells (Rosenberg, 1984). In contrast, some authors do not restrict the definition to cells but include antibodies, 'immune' RNA and transfer factor (Morton and Wells, 1977). In further contrast, sometimes both direct (= passive) and indirect mechanisms are included (Ettinghausen and Rosenberg, 1986).

One can theorise potential advantages of tumor immunotherapy, including

1. activity especially towards tumor cells, leaving normal tissues uninjured.
2. low morbidity
3. full immunocompetence of the host not required
4. no immunosuppression in the host
5. easily combined with other modalities (surgery, radiation therapy, chemotherapy)

The recent advances in basic immunology, notably the recombinant technology and the hybridoma technique have led to an explosive growth in the number of agents that can be considered or have been used for tumor immunotherapy experiments.
The term biological response modifier (BRM) is used to describe agents, capable of affecting the host's immune response towards tumors and includes biological substances that are produced by the cell genome (Smalley and Oldham, 1984). In Table 2.2 a selection of BRMs is presented.

Evidence for the efficacy of tumor immunotherapy in numerous animal models has been reviewed elsewhere (Eberlein et al., 1982; Fefer et al., 1982; Rosenberg, 1984). In most of these animal models, immunogenic, chemically or virally induced tumors to which specifically sensitized cells can be generated with relative ease, have been used. It has been difficult to translate the successes in these experiments to the human situation. Oldham and Smalley (1983) reviewed randomised studies of immunotherapy in man. Twentyfour studies suggested some benefit. Repeat studies often did not confirm these results. Twentythree studies were considered negative and nine equivocal[1]. The lack of highly purified biologic reagents and the heterogeneity of tumors and patients were limitations in the design of these trials. A major problem is the poor expression of tumor-associated antigens on spontaneous human neoplasms. This has thwarted efforts at raising cells reacting specifically to these antigens.
Almost a century after the awakening of interest in tumor immunology and after a veritable avalanche of publications on experimental and

---

1 Interferon treatment of juvenile papillomatosis and hairy cell leukemia might be the only examples where tumor immunotherapy has left the experimental phase.

TABLE 2.2

EXAMPLES OF BIOLOGICAL RESPONSE MODIFIERS

	Reference	
Immunomodulating agents		
BCG	Morton *et al.*	1974
Corynebacterium parvum	Israel and Edelstein	1974
"immune" RNA	Pilch and Ramming	1970
levamisole	Amery	1975
DNCB	Klein *et al.*	1976
picibanil (OK432)	Micksche *et al.*	1982
Interferons and interferon inducers		
ABPP	Eggermont *et al.*	1986
Poly A:U	Lacour *et al.*	1980
IFN (alpha, beta and gamma)	Goepfert *et al.*	1982
Thymosins		
thymosin alpha-1	Schulof *et al.*	1983
Lymphokines and cytokines		
interleukin-2	chapters 5-8	
transfer factor	Lawrence	1969
tumor necrosis factor	Carswell *et al.*	1975
Antigens		
(modified) tumor cells	Klein *et al.*	1960
(modified) tumor-associated antigens	Holmes *et al.*	1970
Antibodies		
(monoclonal) antibodies reactive to tumor-associated antigens	Foon *et al.*	1982
. via antibody dependant cellular cytotoxicity	Morton and Wells	1977
. via complement	Ohanian and Schlager	1981
Effector cells		
Macrophages	Hibbs *et al.*	1972
NK cells	Uchida and Micksche	1983
cytotoxic T cells	Old *et al.*	1962
LAK cells	chapters 5-8	

clinical studies in the last decade, tumor immunotherapy only has a marginal role to play. Past efforts have centered around attempts to either stimulate aspecifically the whole immune apparatus or to develop cells reacting specifically to tumor-associated antigens. These modalities appear, both on theoretical and empirical grounds, to hold little promise.
New approaches seem necessary.

As will be further discussed in Section 3.6 it has become clear recently that cells can be nonspecifically activated to become reactive to a variety of tumor cells, including 'NK cell resistant' lines. This reactivity appears not to be directed at tumor-associated antigens. Administration of these cells is a novel approach, different from the modalities mentioned in the previous paragraph. Such cells have been designated activated killer cells (AK cells). One way of activating is exposure to the lymphokine, IL-2. For cells so activated the term lymphokine activated killer (LAK) cells has been coined. Experiments, exploring this approach in murine models, are described in this thesis.

Chapter 3

INTERLEUKIN-2, A SURVEY OF THE LITERATURE

3.1 Discovery

For a long time, lymphocytes were thought to be terminally differentiated cells incapable of self renewal. Nowell (1960) however, established that plant lectins, such as phytohemagglutinin caused lymphocytes to undergo mitosis. Later, Kasakura and Lowenstein (1965) and Gordon and MacLean (1965) reported the presence of mitogenic factors in the supernatants of human leukocyte cultures. Subsequently, many mitogenic factors thought to be of T cell origin were described. For the most part, unfractionated supernatants were assayed for biological activity and the mitogenic factors were named according to the assay used to detect the activities. Thus, there are reports of mitogenic factors designated thymocytic mitogenic factor, thymocyte activating factor, killer helper factor, thymocyte stimulating factor, lymphocyte mitogenic factor, etc.
Because different lymphoid sites from different species were utilized as cellular targets for assays, it was difficult to ascertain whether a factor described by one group of investigators was similar or divergent from a factor described by others. Additionally, because mixed populations of target cells were employed in the assays, it was never clear whether or not the activity detected was due to the presence of macrophage derived factors (Smith and Ruscetti, 1981).
With the discovery of a method that could select and maintain the continuous proliferation of normal T cells in culture (Morgan et al., 1976) it became possible to construct a specific, quantitative assay for the mitogenic factor responsible for T cell growth. For this factor the designation T cell growth factor (TCGF) was suggested (Gillis et al., 1978a).

At the Second International Lymphokine Workshop (Ermatingen, Switzerland, 1979) a group of investigators made an attempt to end the prevailing semantic confusion (van Aarden et al., 1979). The neologistic term interleukin-2 (IL-2) was proposed and informally

accepted for the murine factor that promotes and maintains long term in vitro cultures of T cells. This designation replaced the variety of terms then in use, among them TCGF.
IL-2 is now also used for nonmurine compounds. The macrophage derived lymphocyte-activating factor was designated Interleukin-1. More recently lymphokines have been described for which the designations IL-3 and IL-4 were proposed (Hapel et al., 1981, Noma et al., 1986).

Apart from its mitogenic properties, IL-2 mediates, as will be reviewed in Section 3.4, a variety of effects, among them antitumor activity. This prompted the evaluation of tumor immunotherapy with IL-2 in experimental models.

3.2 Structure and biochemistry

Human and murine IL-2 have been isolated and purified to apparent homogeneity by several groups both from normal lymphocytes and from neoplastic cell lines (Welte et al., 1982; Robb et al., 1983a; Chang et al., 1985).
Murine and human IL-2 have distinct isoelectric points and molecular weights (Mier and Gallo, 1980). Heterogeneity in size and charge of IL-2 is mainly attributed to variable glycosylation (Gillis et al., 1982). The activity of human IL-2 on lymphoid cells crosses interspecies boundaries, while murine IL-2 has no proliferative effect on human T cells.

Human IL-2 consists of a 133 amino acid polypeptide (molecular weight approximately 15,000) containing a single intramolecular disulfide bridge (Figure 3.1). The sequence of amino acids was originally derived by examination of complementary DNA (cDNA) prepared from transformed and non-transformed T cells (Taniguchi et al., 1983; Devos et al., 1983). In both cases, the cDNA coded for a molecule of 153 amino acids, the first 20 of which were predicted to form a signal sequence Sequence analysis of the mature protein confirmed that these 20 residues were removed prior to secretion (Robb et al., 1983a).
When comparing various forms of IL-2, mainly the amino acid at position 3 displayed heterogeneity (Robb et al., 1983b).

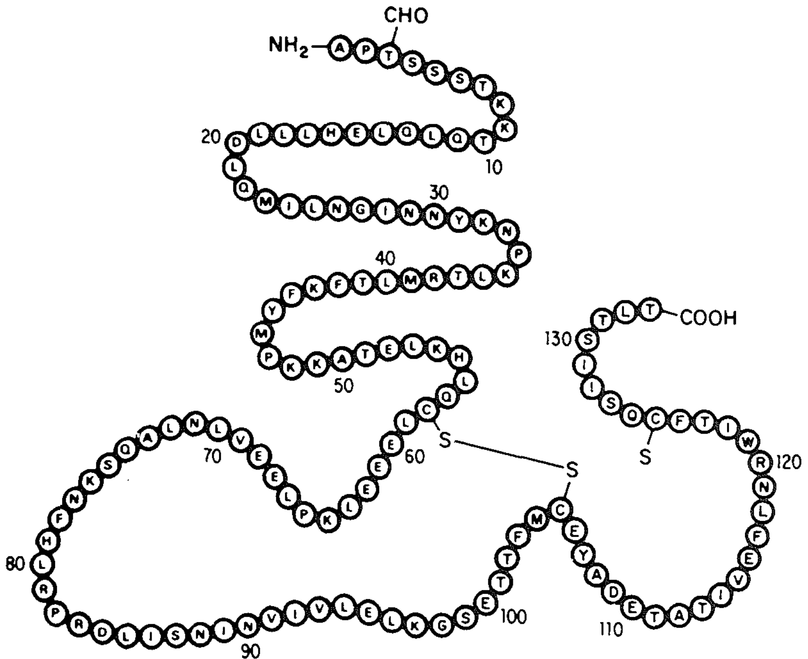


Figure 3.1 The amino acid sequence of human IL-2 arranged in an arbitrary folding pattern. (The studies of Ju et al. (1987) suggest that the NH_2 terminus, the COOH terminus and the internal 30-to 60-region are juxtaposed to form the binding site) (reproduced from Robb, 1984).

Highly purified natural IL-2 preparations are functionally distinct from other cytokines, including interferon, granulocyte-macrophage colony stimulating factor, B cell growth factor, B cell differentiating factor, IL-1 and macrophage activating factor at concentrations that supported T cell growth in vitro (Welte et al., 1982).

Studies of IL-2 were initially hampered by its limited availability. Lectin-stimulated normal mononuclear cell populations produced very low amounts of IL-2. Use of phorbol-myristate-acetate improved the yield some fivefold. The introduction of murine (Farrar et al., 1980) and human (Gillis and Watson, 1980) neoplastic T cell lines increased the production about fiftyfold.

Even using these lines, the technique was relatively cumbersome and availability of IL-2 a limiting factor in the conduction of experiments. Using recombinant DNA techniques, the gene for IL-2 was inserted into Escherichia coli and expressed at high levels, thereby ensuring virtually unlimited quantities of the lymphokine (Rosenberg et al., 1984). This Jurkat derived IL-2 has methionine replacing the amino-terminal alanine of native IL-2. No loss of biological activity has been detected.

The proliferative response of IL-2 dependant lymphocytes in the presence of exogenous IL-2 is the basis for the most commonly utilized and reproducible biological assay (Gillis et al., 1978a). Log_2 dilutions of samples containing unknown concentrations of IL-2 are added to the target population and the proliferative response is measured by determining the ^{3}H-thymidine uptake. Different IL-2 preparations, when supplied at optimal concentration, provide an equal maximal thymidine incorporation. Actual incorporation is dependant upon the concentration of IL-2. The dose-response curve is symmetrically sigmoid when the response is plotted against the $\log_2$ of the IL-2 dose. The more a certain sample can be diluted before losing activity, the more IL-2 activity per volume was present. (Section 4.4 and Figure 4.1 further clarify this point).

Different investigators have used different systems to assign IL-2 units. In concord with several previous publications (e.g. Rosenberg et al., 1984), we defined the titer, in units per milliliter as the reciprocal of the dilution required to sustain one-half of the maximum thymidine uptake. In earlier work, 10% had been used as the determining point (Rosenberg et al., 1983). Other systems are used by Gillis et al. (1978a) and Farrar et al. (1980).
A reference reagent arbitrarily said to contain one unit/ml has been developed by the Biological Response Modifiers Program (BRMP), National Cancer Institute. Unitage of study samples can be determined by comparing the activity of a sample with this reference unit.

An advantage of this IL-2 bioassay is that a number of biological molecules affecting T cell activation do not influence the assay (Ruscetti and Gallo, 1981).
While a radioimmunoassay for IL-2 has been described (Smith et al.,

1983) its lack of specificity and reproducibility have led to continued reliance on the bioassay.

After intravenous injection, IL-2 is rapidly cleared. Serum half life in mice has been reported between 1.6 and 3.7 minutes after intravenous injection (Donohue and Rosenberg, 1983; Chang et al., 1984).
Intraperitoneal or subcutaneous administration results in lower peak values but prolonged serum activity. The serum half life after i.p. injections in mice has been estimated 2-3 hours (Mulé et al., 1985). In humans the serum half life of recombinant IL-2 after i.v. injections has been reported as 6-9 minutes with a second component of clearence of 30 to 120 minutes (Lotze et al., 1985a). A partially purified IL-2 preparation demonstrated a 22.5 minute half life (Bindon et al. 1983).

3.3 The IL-2 receptor

The presence of a specific IL-2 receptor had long been surmised (Farrar et al. 1982). The availability of a monoclonal antibody, designated anti-Tac, has helped clarifying this point. Anti-Tac competitively blocks IL-2 driven T cell proliferation and inhibits binding of ^{3}H-IL-2 to the cell. It immunoprecipitates a membrane glycoprotein with an approximate molecular weight of 50 kiloDalton (Leonard et al., 1982).
Binding experiments in which radiolabelled membrane preparations from detergent-solubilized human T cell blasts were passed sequentially through affinity support columns coupled with IL-2 and anti-Tac demonstrated that the same glycoprotein was bound by both columns (Robb and Greene, 1983). The fact that the number of anti-Tac binding sites in lymphoid populations greatly exceeds the number of binding sites for radiolabelled IL-2 led to the demonstration of distinct high and low affinity IL-2 receptors (Robb et al., 1984).
The high affinity receptor appears to mediate the immune response (Leonard et al., 1985); the functional significance of the low affinity IL-2 receptor remains to be clarified.

Following binding to the receptor, IL-2 could be recovered by elution with a pH 4 buffer as long as internalization was blocked by a low temperature. At 37°C however, labelled factor rapidly entered a pH 4 resistant state, presumably through internalization of the complex of

IL-2 and its receptor. Thereafter, the factor underwent lysosome-dependant degradation.

The effect of IL-2 binding is to cause the cell to progress from the late G1 phase of the cell cycle into the S phase. (Klaus and Hawrylowicz, 1984).

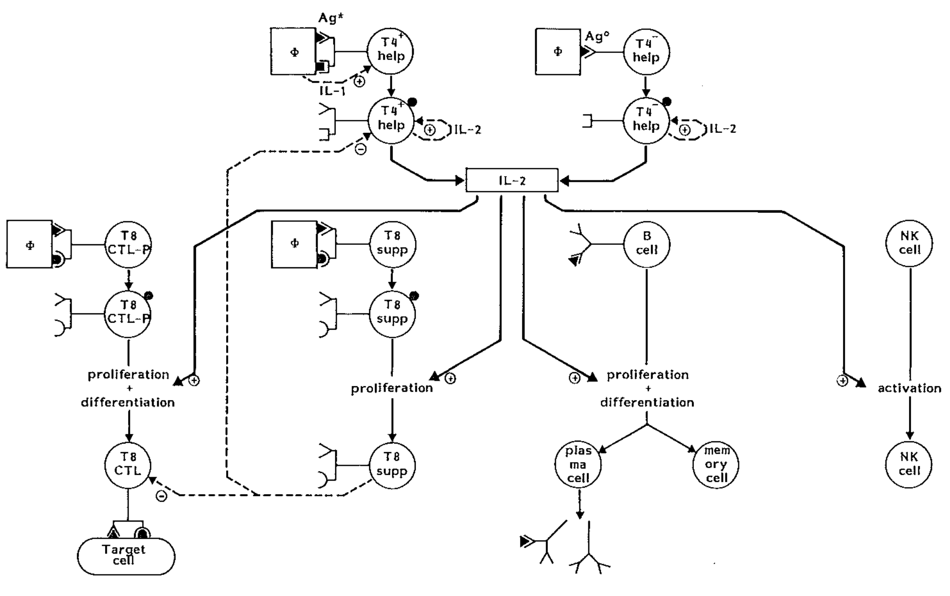
Only a small percentage, approximately 5% of resting T cells display the IL-2 receptor (Taylor et al., 1986). If such cells are stimulated with IL-2, the subpopulation already expressing the receptor expands. There seems to be no induction of IL-2 receptors on cells previously lacking this structure. In contrast, a much higher fraction of T cells stimulated with antigen or mitogen does express IL-2 receptors [approximate 50% (Taylor et al., 1986)]. The effect of IL-2 on the expression of its receptor is controversial. Smith and Cantrell (1985) in a model using a preactivated, synchronized population of T cells found that IL-2 diminished the expression of the high affinity receptor while at the same time enhancing the expression of the Tac antigen. At variance, Welte et al. (1984) using fresh PBL found that induction of Tac antigen paralleled an increase in T cell proliferation in the presence of exogenous IL-2.

Not all effects of IL-2 are mediated via the IL-2 receptor. B cells may differentiate in response to IL-2 in a manner not related to expression of the IL-2 receptor (Zubler et al., 1984; Le thi and Fauci, 1985).

NK cells show several IL-2 driven phenomena of which only one is inhibited by anti-Tac antibody (Mukaida et al., 1986).

3.4 Physiological functions and the effects of pharmacological doses

The physiological role of IL-2 is only partly unraveled. Figure 3.2 depicts a schematic view that accomodates some of the relevant findings (Morgan et al., 1976; Gillis and Smith, 1977; Farrar et al., 1981; Robb, 1984; Brooks et al., 1985; Emmrich et al., 1985; Itoh et al., 1985a; Le thi and Fauci, 1985; Mingari et al., 1985; Schmidt et al., 1985; Le thi et al., 1986; Mizuochi et al., 1986; Shirakawa et al., 1986; Heeg et al., 1987).



▶ Ag
MCH I
MCH II
● IL-2 R

CTL-P: cytotoxic T lymphocyte precursor
CTL: cytotoxic T lymphocyte
Φ: macrophage

NK: natural killer cell
help: helper cell
supp: suppressor cell

* includes virus, MCH I alloantigen, MCH II alloantigen
° only MCH I alloantigen

Figure 3.2 Possible role of IL-2 in the immune response.

Antigen is presented to T4 helper cells by macrophages in the context of MHC II determinants.[1] In addition the macrophages release IL-1. The dual stimulus of antigen and IL-1 causes the clone of helper cells reactive to this antigen to express the IL-2 receptor (upper part of figure). Furthermore, the helper cells produce IL-2, stimulating their proliferation in an autocrine fashion. (T4 negative helper cells have also been described. They are triggered only by macrophages bearing MHC I alloantigens). The IL-2 so produced influences several arms of the immune response (lower part of figure).

1. Antigen is presented to the precursors of cytotoxic T lymphocytes in the context of MHC I determinants (lower part of figure, left column). The clone of cytotoxic T lymphocyte precursors reactive to this antigen is stimulated and expresses the IL-2 receptor. IL-2, produced by helper cells causes proliferation and differentiation. The resulting cell is cytotoxic for the target cell.
2. Antigen is presented to suppressor cells by macrophages in the context of MHC I determinants (lower part of figure, 2nd column). Upon stimulation with antigen, suppressor cells display the IL-2 receptor. Helper cell-produced IL-2 mediates suppressor cell proliferation. These cells display an inhibitory action on cytotoxic T lymphocytes and helper cells, thus providing a mechanism to dampen the immune response after a suitable interval.
3. B cells, activated by antigen, proliferate and differentiate (3rd column). Several immune modulators play a role, among them IL-2. The B cells differentiate to memory cells and plasma cells. These last cells produce antibodies capable of binding with the relevant antigen.
4. Finally (right hand column) NK cells are activated by IL-2.

The effects of pharmacological doses of IL-2 are rather different from the physiologic role. Tables 3.1-3.3 enumerate some of the more salient effects found in <u>in vitro</u> and <u>in vivo</u> experiments in the mouse and in <u>in vitro</u> studies in humans.
The immune stimulating properties of IL-2 prompted the search for possible antitumor effects.

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[1] The recognition of an antigen in the context of MHC molecules is known as the Zinkernagel-Doherty phenomenon (Zinkernagel and Doherty, 1974).

TABLE 3.1

EFFECTS OF PHARMACOLOGICAL DOSES OF IL-2 IN *IN VITRO* MOUSE EXPERIMENTS

	Reference
- Expands CTL cultures	Farrar *et al.* 1982
- Augments cytotoxic activity of cultured CTLs	Ting and Yang 1982
- Provides helper activity in the generation of CTLs	Rosenberg *et al.* 1984
- Augments natural killer cell activity	Henney *et al.* 1981
- Induces LAK cells	Yron *et al.* 1980

TABLE 3.2

EFFECTS OF PHARMACOLOGICAL DOSES OF IL-2 IN *IN VIVO* MOUSE EXPERIMENTS

	Reference
- Increases primary alloimmune response	Rosenberg *et al.* 1983
- Increases secondary alloimmune response	Chang *et al.* 1984
- Induces growth of activated T cells	Cheever *et al.* 1985
- Augments antitumor effect of cultured T lymphocytes	Cheever *et al.* 1982
- Stimulates endogenous lymphoid cell proliferation	Ettinghausen *et al.* 1985a
- Restores responsiveness to allogeneic challenge of immunosuppressed recipients	Conlon *et al.* 1985
- Enhances NK activity	Talmadge 1985
- Helps viral clearence independant of NK cells	Rouse *et al.* 1985
- Induces LAK cells	Ettinghausen *et al.* 1985a

TABLE 3.3

EFFECTS OF PHARMACOLOGICAL DOSES OF IL-2 IN IN VITRO EXPERIMENTS WITH HUMAN CELLS

	Reference
- Expands CTL cultures	Gillis et al. 1978b
- Provides helper activity in the generation of CTLs	Rosenberg et al. 1984
- Corrects some acquired immunologic abnormalities	Rook et al. 1983
- Provides B cell differentiation	Le thi and Fauci 1985
- Mediates proliferation of NK cells	Schmidt et al. 1985
- Induces LAK cells	Grimm et al. 1982

3.5 IL-2 inhibitor and counterregulatory mechanisms

There are conflicting reports on the subject of IL-2 inhibitors. Hardt et al., (1981) described an IL-2 inhibitor in serum of normal mice and rats. Itoh et al. (1985b) demonstrated a suppressive factor in the serum of melanoma patients. This factor hampered the induction of LAK cells. Ravikumar and coworkers (1985), in a model of experimental colon cancer in rats, found a decreased interleukin production in tumor bearing animals. After tumor resection and adjuvant active specific immunotherapy, tumor recurrence was associated with persistent low levels of IL-2 production. Conversely, freedom of tumor recurrence was associated with normal IL-2 levels.

Hersey et al. (1983) showed inhibition of IL-2 production by supernatants of cultured melanoma cells. These inhibitors did not affect mitogenic activity of IL-2.

Burger et al. (1984) suggested the presence of a tumor induced

suppressor T cell which decreased IL-2 activity. After stimulation of mouse spleen cells with Con A, the supernatant contains an IL-2 inhibitor effectively neutralizing the biological activity of IL-2 (Honda et al., 1985).

However, Donahue et al., (1984) could not confirm the presence of inhibitory factors in normal mouse serum. Neither did Hersey and coworkers (1981) find inhibitory activity in autologous serum of melanoma patients.

This area certainly deserves further study.

3.6 The LAK cell phenomenon

3.6.1 Discovery of the LAK cell

Yron and coworkers (1980), were the first to explore the properties of the cell type that later became known as the LAK cell. Suspensions of solid murine tumors were placed in IL-2 resulting in proliferation of lymphoid but not of tumor cells. The cells grown in IL-2 were found to lyse tumor cells but not normal syngeneic cells. Similar cytotoxic cells could be produced by incubating normal splenocytes in IL-2. It was later shown that these cytotoxic cells could be produced from normal human lymphocytes as well (Lotze et al., 1981). The designation LAK cell was proposed by Grimm et al (1982).

Cells, akin to the LAK cell had been described before. By a variety of stimuli, but in the absence of antigen, lymphocytes could be made cytotoxic for a broad range of targets. These stimuli included bacterial extracts, fetal calf serum, allosensitization, mixed lymphocyte cultures and lectin stimulation. The resulting cells were variously called anomalous, activated, alloactivated, nonspecific, promiscuous, or PHA activated killer cells. For a review see Grimm et al. (1982) and Sondel et al. (1986). The culture conditions used are known to involve IL-2 production. It seems plausible that IL-2 is the final common pathway in all these modalities, either supplied exogenously or generated in the culture. Addition of exogenous IL-2 is the most effective way of generating these killer cells. The

designation LAK cell usually is restricted to cells so activated.

3.6.2 LAK cell cytotoxicity

In Table 3.4 an overview is presented of targets to which LAK cells show cytotoxicity in *in vitro* experiments (Kedar *et al.*, 1982; Ballas and Ahmann, 1983; Grimm *et al.*, 1985).

TABLE 3.4

TARGETS SUSCEPTIBLE TO LAK CELLS

- fresh tumor cells
  cultured tumor cells

- autologous tumor cells
  syngeneic tumor cells
  allogeneic tumor cells
  xenogeneic tumor cells

- primary tumor
  metastatic tumor

- solid tumor
  lymphoid tumor

- NK sensitive targets
  NK resistent targets

- hapten-modified autologous cells

It appears that LAK cells react to all forms of 'altered self'. There is no consensus on the effect on unmodified autologous cells. Some workers report that normal PBL, lung, liver and kidney cells are not lysed by LAK cells (Grimm *et al.*, 1985). This would of course be the most attractive state of affairs from a therapeutic point of view. Others however, report *in vitro* experiments in which LAK cells appear cytotoxic for normal, fresh autologous

cells albeit at a low level (Kedar et al., 1982; Sondel et al., 1986). It can not be excluded that either during the ^{51}Cr incubation step or during the cytotoxicity assay, a modification ensues that is recognized by LAK cells and that the susceptibility of normal cells is an in vitro artefact.

It is not clear, how the LAK cell recognizes its target. It has been shown that one clone or subclone, isolated by limiting dilution techniques, lysed multiple targets (Rayner et al., 1985a). Conversely, tumor targets were each lysed by multiple clones. Cold target inhibition studies have shown that any LAK sensitive tumor population can interfere with the LAK mediated destruction of other LAK sensitive targets (Grimm and Wilson, 1985).
In summary, each LAK cell shows cytotoxicity to a variety of 'altered-self' cells, without prior exposure to such cells. The mechanism of recognition is unclear. This activity has been described as 'polyspecific'.

Cytotoxicity of LAK cells has also been examined in in vivo experiments.
Gorelik et al. (1981) administered mixtures of murine tumor cells and LAK cells into the foot pads of normal mice and similar mixtures of human cells into the foot pads of nude mice. They found limited and transient cytotoxic effects and suggested that the short survival period of LAK cells limited their effectiveness.
Kedar et al. (1982) also found that in Winn-type neutralization assays, LAK cells delayed or inhibited growth of lymphomas and carcinomas.
In a somewhat more rigorous model Mazumder and Rosenberg (1984) injected melanoma cells in a tailvein to induce pulmonary tumor foci in mice. Three days later LAK cells were administered i.v. Mice treated with LAK cells were reported to show a reduced number of pulmonary tumor nodules. We have however not been able to reproduce these results.
Similarly, Mulé et al. (1984) using the pulmonary inoculation model, found neglegible effects of LAK cells alone but striking effects when LAK cells were combined with IL-2 administration.
Further support that in general LAK cells alone are not effective but LAK cells plus IL-2 are effective is given in Sections 5.3 and 9.5.

3.6.3 The nature of LAK cells

LAK cells originally were described as a unique class of cells, distinct from both the classical CTL and the NK cell. Differences were thought to exist in such characteristics as target recognition, stimulus, serologic type

TABLE 3.5

INITIAL CHARACTERIZATION OF LAK CELLS, LEADING TO THE FORMER CONCEPT OF AN UNIQUE LAK CELL TYPE

	LAK	NK	CTL
Target recognition	recognizes 'altered-self', mechanism not elucidated	recognizes NK sensitive targets, mechanism unknown	immunologically specific antigen-antibody reaction
Stimulus	IL-2	IFN augments IL-2 augments	specific antigen
Precursor location	thoracic duct +	thoracic duct -	thoracic duct +
Serologic type effector			
Human	OKT 3 + OKT 8 + OKM 1 - OKT 11 -	OKT 3 - OKM 1 + OKT 11 + Leu 7 +	OKT 3 + OKT 8 + OKM 1 -
Mouse	Thy 1 + Ly 1 - Ly 2 - and + asialoGM - MCH II - gamma FcR +	Thy 1 - Ly 1 + Ly 2 - asialoGM + gamma FcR +	Thy 1 + Ly 1 - Ly 2 - asialoGM -
Serologic type precursor			
Human	OKM 1 - OKT 3 - OKT 11 - Leu 1 - Leu 7 -	unknown	OKM 1 - OKT 3 + OKT 11 +
Mouse	Thy 1 - (? and +) MCH II - asialoGM +	asialoGM +	

of the effector cell, serologic type of the precursor cell and precursor location as presented in Table 3.5 (Grimm et al., 1982, 1983a, 1983b; Grimm and Rosenberg 1984; Rosenstein et al., 1984b; Merluzzi et al., 1984, 1985; Yang et al., 1986).
However, this concept has come under considerable challenge as reviewed by Herberman (1987) and Lotzová (1987). It now appears that LAK activity should be thought of as a phenomenon: destruction of tumor cells by (various) effector cell populations after activation by IL-2. Most of this activity now seems attributable to augmentation and/or activation of NK cells. The term LAK cells is used here only operationally, to refer to cell populations with LAK activity.

3.6.4 IL-2 and LAK cell generation

As discussed previously, incubation of lymphocytes in IL-2 results in LAK cell generation. Other cytokines tested, including IL-1, migration inhibition factor (MIF) and several IFN preparations did by themselves not result in LAK activity (Grimm et al., 1983b).

3.6.5 Kinetics of LAK cell development

After incubation in IL-2, LAK activity can be detected after 2 days. Peak activity is seen after 3-7 days (Grimm and Rosenberg, 1984; Itoh et al., 1985a; Mule et al., 1984). It is not necessary that IL-2 is present during the whole culture period. Incubation in IL-2 during 1 hour, followed by culture in a standard medium also generates LAK cells, although less efficiently (Grimm and Rosenberg, 1984). The

frequency of LAK precursors in C57BL/6 spleen cells and lymph node cells is estimated at 1 in 12.000 and 1 in 7.000 respectively.

3.6.6 Alternative IL-2-activated cells

As has been reviewed, LAK cells can be generated by exposure to IL-2, without exposure to the target cell. It could be surmised that dual stimulation of IL-2 in conjunction with the relevant tumor cell, results in a more efficient effector cell. With the use of the MCA-105 tumor, Shu et al. (1987) cultured lymphocytes from tumor bearing mice in vitro with viable tumor cells in the presence of IL-2. They obtained therapeutically effective cells, capable of tumor regression and apparently more potent than the LAK cell. As discussed in Section 2.6, it might very well be that such an approach would fail in human tumors. Supportive to this surmise is the fact that the above approach does not work in all murine tumors tested.

As discussed in Section 3.6.1 LAK cells initially were produced from tumor infiltrating lymphocytes and later from normal lymphocyte populations. Rosenberg et al. (1986) reported on the effects of tumor infiltrating lymphocytes (TIL) expanded in IL-2. The data in this paper comparing LAK cells and TIL are difficult to interpret as it is not known how many cells with LAK activity actually were present in the cell mixtures given. It could be that TIL are more potent than LAK cells against the tumor from which they derive. But again, as reviewed above, it seems possible that this can not be exploited in the clinical situation.

3.7 Toxicity

The toxicity of recombinant IL-2 in mice has received little systematic attention.
Doses of up to approximate 100.000 U/day ($5x10^6$ U/kg/day) are usually well tolerated. At higher doses, mice appear wasted after several days of treatment and mortality is seen.

Matory et al. (1985) systematically studied IL-2 toxicity in rats. Low dose ($\leq$ 3,000 U/kg/day) continuous intravenous infusion was associated with normal serum chemistry and organ histology. Intermittent intravenous bolus injections up to 10^6 U/kg/every other day were similarly tolerated. However, animals that received higher dose (> 10,000 U/kg/day) continuous i.v. infusion died with signs of hepatocellular necrosis.

Lotze et al., (1985 a, b) studied natural and recombinant IL-2 toxicity in human patients with cancer or AIDS. The maximal dose of natural IL-2 was limited only by the supply of this agent. In 23 patients treated with recombinant IL-2, dose limiting toxicity was reached with doses of 10^6 units/kg as a single bolus or 72,000 U/kg/day when given as continous infusion. Minimal renal or hepatic toxicity was noted. Mild anaemia, thrombocytopenia and eosinophilia were seen. Pronounced weight gain due to fluid retention was seen in a majority of patients. Administration by continuous infusion is less well tolerated than by intermittent bolus injection. However, the first mode of administration appears to have a better antitumor activity (Section 7.4).

In humans treated with LAK cells plus IL-2 the major side effect

appears to be a capillary leak syndrome, resulting in major fluid retention (Rosenberg *et al.* 1985b, 1986).

Chapter 4

MATERIALS AND METHODS

4.1 Mice

9-14 Week old female C57BL/6 (BL/6) mice, 18 week old female DBA/2J mice and 14 week old male C57BL/10 nude mice (BL/10 nude) were obtained from Jackson Laboratory, Bar Harbor, ME, and 16 week old female C3H/Hen mice from the Animal Genetics and Production Branch, Frederick, MD. They were maintained on laboratory chow and acidified water ad libitum in a pathogen free environment.

4.2 Tumors

In BL/6 mice, a series of tumors was induced in the Surgery Branch laboratory by i.m. injection of 0.1 ml of 0.1% 3-methylcholanthrene in sesame seed oil. Following their appearance, these tumors were harvested and inoculated i.m. into other syngeneic mice. Tumor samples were then cryopreserved from the first transplant generation. After 6 transplant generations, samples from the first generation were thawed and serially transplanted. From the tumors so generated, the lines designated MCA-101, MCA-102 and MCA-105 were used for studies described in this thesis.

Attempts to immunize syngeneic animals by tumor growth and excision were ineffective. However, mice could be immunised to MCA-105 by intradermal injection of tumor cells admixed with Corynebacterium parvum. This immunisation procedure resulted in a period of tumor growth, followed by regression in about 75% of mice. A proportion of mice free of tumor, rejected tumor challenge (Shu and Rosenberg, 1985). We have not been able, by a variety of methods, to immunize mice against MCA-101 and MCA-102.

Single cell suspensions of these tumors were obtained by excising the tumor, mincing the tissue in Hank's balanced salt solution (HBSS) (Biofluids, Rockville, MD) followed by repeated treatment at 37° with 0.25% trypsin without calcium or magnesium (Biofluids, Rockville, MD).

After three minutes of stirring, the supernatant was discarded and an equal volume of fresh trypsin was added to the flask. For the next 3 eight minute periodes the supernatants, containing released tumor cells, were collected and fresh trypsin again added back to the minced tumor. The supernatants were pooled in ice-cold HBSS. The cells were passed through 100 gauge nylon mesh (Tobler, Ernst and Traber Co., Elmsford, NY) and washed three times in HBSS.

M-3, a carcinogen induced amelanotic melanoma in C3H mice was serially passaged in syngeneic hosts in our laboratory. Its use was not restricted to an early transplant generation. Single cell suspensions were prepared as described above.

MCA-38, a dimethylhydrazine induced murine colon adenocarcinoma in BL/6 mice, (gift from Dr. S.D. Deodhar, Cleveland Clinic, OH) was passaged subcutaneously in syngeneic hosts in our laboratory. Single cell suspensions were prepared as described above.

P815, an ascitic DBA/2 mastocytoma was serially passaged in syngeneic hosts in our laboratory. Single cell suspensions were obtained by washing the abdominal cavity with phosphate buffered saline (PBS). The cells obtained were washed 3 times in HBSS.

EL-4, a lymphoma syngeneic to BL/6 mice, was passaged serially as an ascites tumor in our laboratory. (This line, studied for possible susceptibility to tumor immunotherapy is different from the subline, used to generate IL-2 [see Section 4.3.2]). Cells were obtained as described for the P815 mastocytoma.

B16, a carcinogen induced melanoma syngeneic to BL/6 was serially passaged in culture media consisting of RPMI 1640 (Biofluids, Rockville, MD), $5x10^{-5}$ M 2-mercapto-ethanol (Aldridge Chemical Co., Milwaukee, WI), 100 U/ml penicillin (Media Unit, NIH, Bethesda, MD), 100 microgram/ml streptomycin (Media Unit NIH, Bethesda, MD), 0.03% glutamine (Media Unit NIH, Bethesda, MD) and 7.5% fetal calf serum (Gibco Laboratories, Grand Island, NY). Single cell suspensions were prepared by brief digestion in 0.05% trypsin, 0.02% EDTA without calcium or magnesium.

Live cells of all tumor cell suspensions were counted in 0.08% trypan blue. Live cells exclude the dye. Counting and adjustment of concentration were done immediately prior to inoculation.

All tumors used were free of viral pathogens and mycoplasma.

4.3 IL-2 preparations

4.3.1 Recombinant IL-2

Recombinant IL-2 (Cetus Corporation, Emeryville, CA) was generously made available by Dr. S.A. Rosenberg. This product and its engineering have been described in detail (Rosenberg et al., 1984). Briefly, human peripheral blood lymphocytes (PBL) and a subclone of the human Jurkat leukemia line were stimulated for production of IL-2, and cytoplasmic RNA extracted. Polyadenylate containing RNA (messenger RNA) was chromatographically purified and fractionated. Fractions founds to contain the peak IL-2 mRNA activity were pooled and used for construction of cDNA banks in E. coli. The cDNA inserts from 2 clones of the Jurkat bank and 2 clones of the PBL bank that were found to have restriction enzyme maps consistent with IL-2, were completely sequenced and found to be almost identical. A plasmid for the expression of one of the Jurkat clones was engineered and E. coli, harboring this plasmid, indeed did produce IL-2.
The recombinant IL-2 was provided as a lyophilized powder in vials and was reconstituted with sterile water to $5x10^5$ U/ml. (Determination of activity is described in Section 4.4). Each vial contained approximately 0.3 mg IL-2 (specific activity $3-5x10^6$ U/mg). The endotoxin present in the preparations was less than 1.0 ng per 10^5 U, as measured in a standard Limulus assay. Vehicle preparations for the IL-2 contained mannitol and sodium dodecylsulphate to facilitate solubilization of the IL-2.

4.3.2 Natural IL-2

For production of natural IL-2, an EL-4 subline (gift from J.J. Farrar, NIH) was used (Farrar et al., 1982). This line is different from the one described above onder 4.2.
The EL-4 lymphoma subline has been found to produce IL-2 in response to phorbolesters, notably 4-phorbol-12-myristate-13-acetate (PMA). Addition of PMA to a culture of the EL-4 subline leads to decreased proliferation, DNA synthesis, RNA synthesis and protein synthesis in conjunction with an increased concentration of IL-2 in the supernatant. Two mechanisms-of-action can be hypothesized. The EL-4 cells might produce and utilize IL-2 constitutively. PMA might act by inhibiting proliferation and reducing utilization of IL-2, thereby allowing accumulation of IL-2 in the supernatant. Alternatively PMA might selectively enhance synthesis of IL-2. These two possibilities are not mutually exclusive.
Alternative methods to produce natural IL-2 include lectin stimulation of human or murine lymphocytes. Inherent to such protocols is the potential lectin contamination of the final product. In evaluating immune activity, lectin rather than IL-2 mediated effects have to be considered. For this reason such protocols were not used.

EL-4 thymoma subline cells were grown to approximately 10^6 cells/ml in culture medium consisting of RPMI 1640 (Biofluids, Rockville, MD), $5x10^{-5}$ M 2-mercapto-ethanol (Aldridge Chemical Co., Milwaukee, WI), 100 U/ml penicillin (Media Unit, Bethesda, MD), 100 microgram/ml streptomycin (Media Unit NIH, Bethesda, MD), 0.03% glutamin (Media Unit NIH, Bethesda, MD), 25 mM Hepes (Biofluids, Rockville, MD) and 7.5% fetal calf serum (Gibco Laboratories, Grand Island, NY). Cells were then harvested, washed 3 times in HBSS and resuspended in serum free medium (RPMI 1640, penicillin, streptomycin, glutamine and Hepes in concentrations as above) at 10^6 cells/ml and cultured for 48 hours in the presence of 40 ng/ml PMA (Sigma Chemical Company, St. Louis, MO). Supernatants were concentrated approximately 100 fold using a Pellicon Millipore concentrator with a 10,000 dalton exclusion limit (Millipore Corporation, Bedford, MA) and were filtered (0.45 microm. filter, Nalgene Labware, Rochester, NY).
A volume of 100% saturated ammonium sulphate solution was added to an equal volume of the concentrate at room temperature and stirred for 2

hours. The solution was spun at 2700xg for 20 minutes and the precipitate was discarded. Additional ammonium sulfate was added to form a 75% saturated solution and this was stirred for 2 more hours. A second precipitate was obtained by centrifugation as above and the precipitate dissolved in PBS.

The resuspended precipitate was dialyzed against distilled water using dialysis membranes with a 10,000 molecular weight exclusion limit followed by dialysis against PBS at pH 7.4. Each dialysis was carried out for 24 hours at 4°C with constant stirring and two bath changes. The dialyzed sample was passed through a 0.45 microm. filter and assayed for IL-2.

4.3.3 Administration

The IL-2 preparations were diluted as needed in PBS for exogenous administration.

4.4 Biological assay for IL-2

IL-2 levels were estimated using the capacity to maintain growth of a long term IL-2 dependant cell line as the discriminating assay. Serial two-fold dilutions of 0.1 ml of the test sample were made in a 96 well flat bottom microtiter plate (NR 3596, Costar, Cambridge, MA). Dilutions were performed horizontally across the plate to the 23rd row; control medium was added to row 24.

The IL-2 dependant lymphocyte cell line used, designated M53, was maintained in our laboratory. M53 cells were washed free of IL-2 by triple centrifugation and resuspension in HBSS. Life cells were counted by 0.08% trypan blue exclusion and cells resuspended at the desired concentration in culture medium (RPMI 1640, Biofluids, Rockville, MD), $5x10^{-5}$ M 2-mercapto-ethanol (Aldridge Chemical Co., Milwaukee, WI), 100 U/ml penicillin (Media Unit, NIH, Bethesda, MD), 100 microgram/ml streptomycin (Media Unit NIH, Bethesda, MD), 0.03% glutamine (Media Unit NIH, Bethesda, MD), 25 mM Hepes (Biofluids, Rockville, MD) and 7.5% fetal calf serum (Gibco Laboratories, Grand Island, NY). To each well of the microtiter plate 0.1 ml containing $5x10^{3}$ cells was added. Plates were incubated for 20 hours and then pulsed with 2 microCurie

^{3}H-thymidine (50-80 Ci/mM) (New England Nuclear, Boston, MA) and cultured for another four hours. The well contents were harvested (MASH II, MA Bioproducts) passed onto filter papers, and the supernatants rinsed away. Radioactivity retained on the filter paper (i.e. ^{3}H-thymidine incorporated into the cellular DNA) was determined with an automatic counter. Mean counts for duplicate samples were determined and plotted against the dilution. An IL-2 preparation of known activity was used as control.

For the studies reported here, the titer in units per milliliter was defined as the reciprocal of the dilution required to sustain one half of the maximum thymidine uptake.

In Figure 4.1 a simultaneous assay of a batch of recombinant IL-2 and a batch of EL-4 derived IL-2 is depicted. Titers were estimated at 500 U/ml for the control sample, 4000 U/ml for the recombinant IL-2 and 75,000 U/ml for the EL-4 derived IL-2.

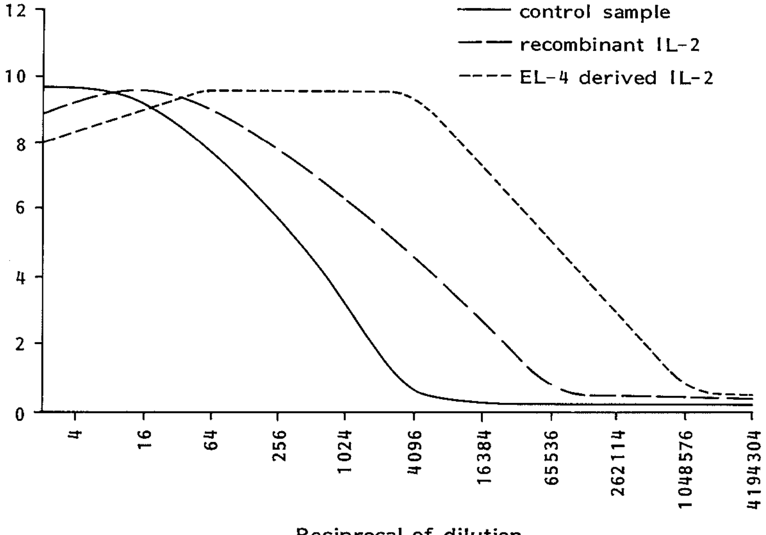


Figure 4.1 ^{3}**H-thymidine uptake (vertical axis) of an IL-2 dependant cell line under the influence of 3 samples of IL-2. Based on the dilutions at which half maximum incorporation is found, titers are estimated as 500, 4000 and 75,000 U/ml for the control, recombinant IL-2 and EL-4 derived batch respectively.**

4.5.1 LAK cells

Donors were killed in a CO_2 chamber. Spleens were harvested aseptically, passed through a 60 gauge wire mesh, the erythrocytes lysed osmotically with ACK buffer (Media Unit, NIH, Bethesda, MH) and the remaining lymphocytes washed three times in HBSS. LAK cells were generated by placing $5x10^8$ splenocytes in 175 cm^2 (750 ml) flasks (Falcon, Oxnard, CA) in 175 ml of complete medium [RPMI 1640 (Biofluids, Rockville, MD) with 0.1 mM nonessential amino acids (Gibco Laboratories, Grand Island, NY) 1 mM sodium pyruvate (Gibco Laboratories, Grand Island, NY), $5x10^{-5}$ M 2-mercapto-ethanol (Aldrich Chemical Company, Milwaukee, WI), 100 U/ml penicillin (Media Unit NIH, Bethesda, MD), 100 microgram/ml streptomycin (Media Unit, NIH, Bethesda, MD), 0.03% glutamine (Media Unit, NIH, Bethesda, MD), 25 mM Hepes buffer (Biofluids, Rockville, MD), and 10% fetal calf serum (Gibco Laboratories, Grand Island, NY)] with 250,000 U IL-2. The flasks were incubated supine for 72 hours unless otherwise noted (in three experiments, cells incubated for 96 hours were studied as well). The cells were then harvested into sterile 250 ml centrifuge tubes (Falcon), passed over Ficoll (Lymphocyte-M; Cederlane Laboratories Ltd, Hornby, Ontario, Canada) to remove dead cells and washed 3 times in HBSS. Life cells were counted by 0.08% trypan blue exclusion. The resulting cell suspension is referred to as LAK cells, although not all cells will actually have LAK activity.

In the experiment in which tumor bearing donors and donors in whom the tumor had been resected, were studied, mice were injected with 10^5 MCA-105 cells i.m. in the right hind leg. One week later, the tumor bearing extremity was amputated in part of these animals. Two weeks after tumor inoculation spleens of both groups were harvested and prepared as above.

4.5.2 Cultured lymphocytes

Cultured lymphocytes were splenocytes incubated in complete medium without IL-2.

4.6 Culture conditions

All in vitro tumor passages and cultures were done at 37° in a moist atmosphere with 5% CO_2.

4.7 Tumor therapy experiments

In all experiments, mice received tumor prior to random allocation into a treatment group. Tumor cells were injected in 2 ml HBSS unless otherwise noted. Animals not receiving LAK cells and/or IL-2 received injections of an identical volume of HBSS. At the conclusion of an experiment, animals were sacrificed.

4.7.1 Solid intraperitoneal tumor

For these neoplasms, intraperitoneal tumor mass was scored in a blinded fashion as described below, on a scale from 0 through 3. Representative examples of tumor mass and corresponding score are shown in Figure 4.2. It should be noted that the difference between score 1 and 3 is exceedingly large. The score is termed the Peritoneal Carcinomatosis Index (PCI). Alternatively, the animals were followed for survival.
Scoring was performed in a blinded fashion using the following methodology. On the day of sacrifice, all mice were ear-tagged and their numbers recorded. All groups of mice were mixed together. Mice were taken from the pool without reference to their ear tags and scored after thorough inspection of the entire abdominal cavity. Mice of similar scores were placed in groups of peritoneal carcinomatosis index of 0,1,2 or 3. After all mice were scored, placed in their groups, and checked by a second observer, the ear tag was read and the data were analyzed.

PERITONEAL CARCINOMATOSIS INDEX			
0	1	2	3
NO TUMOR DETECTABLE			
NO TUMOR	MINIMAL TUMOR (3 or less foci ⩽ 1 mm)	MODERATE TUMOR	EXTENSIVE TUMOR (most intraperi- toneal space replaced)

Figure 4.2 Peritoneal carcinomatosis index (PCI). To score the intraperitoneal tumor mass, mice were sacrificed and the abdominal contents carefully inspected. A PCI of 0 was given to mice without detectable tumor, 1 indicated minimal tumor with 3 or fewer foci of cancer ⩽ 1 mm in diameter; moderate tumor was scored as 2; if most of the peritoneal cavity was replaced by tumor this was scored as 3.

4.7.2 Intrahepatic tumor

To generate hepatic tumor we developed a model of intrasplenic injection. Mice were anaesthesized with penthotal i.p. and the skin prepped with alcohol 70%. Via a short incision in the left flank, the spleen was mobilised and isolated from the abdominal cavity with a

sterile gauze. Tumor cells were injected in 1 ml via a 27 G needle. The needle was left in situ for 1 minute to minimise leaking of tumor cells and then withdrawn. Bleeding usually was minimal. The spleen was returned to the abdominal cavity and the abdomen closed with 1 or 2 metal clips. One day later the spleen was removed under similar anaesthesia through the same incision. Three days after tumor inoculation survivors were randomly allocated to a treatment group. Fourteen days after tumor administration, the mice were sacrificed, the livers harvested and the tumor load scored in a blinded fashion as described above for intraperitoneal tumor. Tumor load was estimated as follows.

0 = no tumor detected
1 = 0 - 25% }
2 = 25 - 50% } of liver replaced by tumor
3 = 50 - 75% }
4 = > 75% }

4.7.3 Subcutaneous tumors

Midway between xyphoid and umbilicus 0.1 ml of tumor cells was injected intradermally with a 27 G needle, raising a small wheal. Tumor size was estimated by measuring the tumor with a caliper. The largest diameter and the one perpendicular to it were measured. The product of these two figures is reported as estimate of the tumor size.

4.7.4 Ascites tumors

Tumor load was assayed by estimating the number of tumor cells. Animals were sacrificed and 3 ml PBS was injected into the peritoneal cavity and thoroughly mixed with the contents of the abdomen by external massage. After aspiration of 1.5 - 3 ml of fluid, an identical volume of PBS was injected. After repeated external massage, a second sample was drawn. Life cell concentrations in both samples were determined by trypan blue exclusion.

The total number of cells was estimated with the following formula:

$$\text{Total cells} = \frac{(\text{concentration } 1^{st} \text{ sample})^2 \times \text{volume } 1^{st} \text{ sample}}{\text{concentration } 1^{st} \text{ sample} - \text{concentration } 2^{nd} \text{ sample}}$$

4.8 In vitro assay of LAK cell cytotoxicity

Serial two fold dilutions of 0.1 ml of LAK cells were made in a 96 well microtiter plate (Linbro Scientific Corp., Hamden, Conn.). Target cells were washed and suspended in culture medium. Approximately 2×10^7 cells were incubated with 200 microCi of ^{51}Cr (Radiochemical Center, Amersham, United Kingdom) for 45 minutes at 37° with occasional stirring. Cells were washed to remove unbound ^{51}Cr and 0.1 ml of the target cell suspension containing 10^4 cells was added to each well. Effector to target ratio varied from 100:1 to 1.6:1. All test were run in triplicate. To estimate the maximum ^{51}Cr release, target cells were incubated with 0.1 ml of 0.1 N hydrochloric acid. Spontaneous release was estimated by adding 0.1 ml medium to target cells. After 4 hours (MCA-105, MCA-38, EL-4) or 16 hours (B16) incubation at 37°, plates were spun at 2000g for 20 minutes and supernatants harvested with a Titertek supernatant collection system (Flow Labs, Rockville, MD). Radioactivity in the supernatants was counted with an automatic counter and is expressed as counts per minute (cpm). Specific release was calculated with the formula

$$\frac{cpm_{experimental} - cpm_{spontaneous}}{cpm_{maximal} - cpm_{spontaneous}} \times 100$$

4.9 Statistics

There was a minimum of 6 animals per treatment arm. Overall significance of differences in an experiment was examined with the Jonckheere test for trend (Hollander and Wolfe, 1973). If this test showed a one-sided p value ≤ 0.05, pairwise comparisons of differences in tumor load were examined with the Wilcoxon rank sum test with a

correction for ties (Hollander and Wolfe, 1973). A two sided p value $\leq$ 0.05 was considered significant. Differences in survival were evaluated with the generalized Wilcoxon (Breslow) test (Breslow, 1970), a two sided p value $\leq$ 0.05 was considered significant. All p values presented are two-tailed.

Chapter 5

THE LAK CELL PHENOMENON

5.1 Introduction

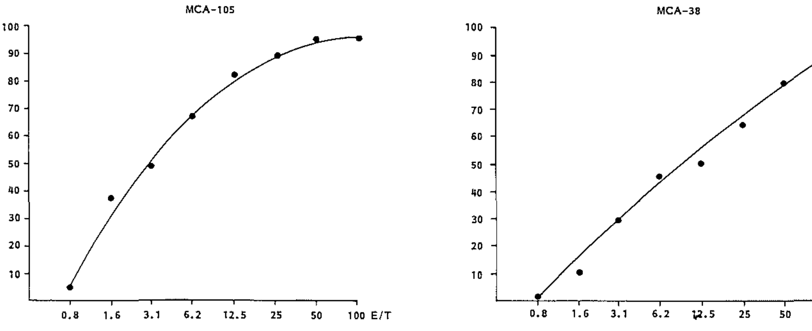
In Section 3.6, the literature on activated killer cells has been reviewed. It was seen that a cell type has been described, that can be activated by a variety of stimuli to become cytotoxic for 'non-self' cells. One way of activation is in vitro incubation of lymphocytes with the lymphokine, IL-2. This modality is dependable and reproducible. Cells so activated have been termed LAK cells. In this section, the antitumor effects of LAK cells and IL-2 in treating established intraperitoneal tumor are explored in a murine model.
The choice for an intraperitoneal model has been discussed in Chapter 1.

The experiments conducted, were designed to clarify the following points.

a. Is an antitumor effect seen of LAK cells alone, IL-2 alone or LAK cells given in conjunction with IL-2 in this i.p. model ?
b. Does in vitro incubation suffice for cell activation or is the addition of exogenous IL-2 to the culture medium mandatory ?
c. Does growth retardation translate in prolonged survival ?
d. Is site-specific treatment superior to systemic treatment ?
e. Are in vivo antitumor effects of recombinant IL-2 similar to those of natural IL-2 ?

5.2 LAK cells mediate **in vitro** cytotoxicity in short term ^{51}Cr release assays

Initially, aliquots of LAK cells used for in vivo experiments were assayed for in vitro cytotoxicity against the relevant target in ^{51}Cr release assays.



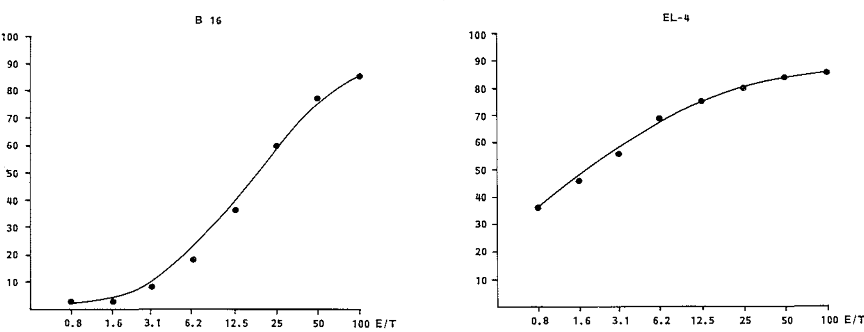


Fig. 5.1 Cytotoxicity in ^{51}Cr release assay of LAK cells against MCA-105, MCA-38, B16 and EL-4. Percentage specific release and effector to target ratio are shown.

Representative examples of cytotoxicity against 4 different tumor cells, MCA-105, MCA-38, B16 and EL-4 are shown in Figure 5.1 . Marked cytotoxicity is seen against all targets. In view of the constant and predictable *in vitro* effects, later *in vivo* experiments were performed without parallel *in vitro* testing.

5.3 Optimal reduction of established intraperitoneal tumor requires LAK cells plus IL-2

BL/6 mice were injected with MCA-105 tumor cells i.p. on day 0. Treated mice received injections of LAK cells i.p. on day 3 and 6 and/or they were given IL-2 i.p. every 12 hours on days 3 through 8. Experimental groups were treated with LAK cells alone, IL-2 alone or LAK cells plus IL-2. The results are shown in Table 5.1. LAK cells alone were not effective. Administration of IL-2 alone provided significant tumor reduction in two of five experiments. Transfer of LAK cells in conjunction with IL-2 gave a significant tumor reduction over control animals in 5 of 5 experiments. LAK cells plus IL-2 are more effective than IL-2 alone in 5 of 5 comparisons; in 3 experiments this difference is significant.
To address the point if *in vitro* lymphokine activation of lymphocytes is essential for mediation of antitumor effects, LAK cells were compared with fresh splenocytes (normal lymphocytes) and cells cultured under identical situations as the LAK cells but without IL-2 (cultured lymphocytes).
Mice received i.p. tumor injections and IL-2 as before. On day 3 and 6 experimental groups were injected i.p. with normal lymphocytes, cultured lymphocytes or LAK cells. The results are shown in Table 5.2. LAK cells plus IL-2 significantly reduced tumor growth when compared with IL-2 alone. Normal and cultured lymphocytes, given in conjunction with IL-2 were not more effective than IL-2 alone.

TABLE 5.1

OPTIMAL REDUCTION OF ESTABLISHED INTRAPERITONEAL TUMOR REQUIRES LAK CELLS PLUS IL-2[a]

PCI[b]

	A Control	B LAK	C IL-2	D LAK + IL-2
Exp 1	3	2.3 ± 0.4 (NS)	1.6 ± 0.6 (0.04)	1.4 ± 0.4 (0.003)
Exp 2	1.8 ± 0.2	1.2 ± 0.4 (NS)	0.8 ± 0.2 (0.003)	0 (0.0003)
Exp 3	2.8 ± 0.2		2.8 ± 0.2 (NS)	1.6 ± 0.5 (0.02)
Exp 4	2.8 ± 0.2	2.8 ± 0.2 (NS)	2.2 ± 0.6 (NS)	0.7 ± 0.4 (0.007)
Exp 5	2.7 ± 0.3	2.5 ± 0.3 (NS)	1.8 ± 0.7 (NS)	1.0 ± 0.3 (0.02)

[a] BL/6 mice received 5×10^4 (Exp 4&5) or 10^5 (Exp 1-3) live MCA-105 tumor cells i.p. on day 0; 10^7 (Exp 1&2) or 3×10^7 (Exp 3-5) live LAK cells i.p. on day 3 and 6 and 10,000 (Exp 1&2) or 50,000 U (Exp 3-5) IL-2 every 12 hours on day 3 through 8. Mice were sacrificed on day 13 (Exp 1) or day 14 (Exp 2-5).

[b] Mean Peritoneal Carcinomatosis Index ± SEM. In parentheses the significance of difference with the control group.

Statistically significant further pairwise comparisons:

Experiment 2 - B vs D 0.02 ; C vs D 0.03
Experiment 3 - C vs D 0.04
Experiment 4 - B vs D 0.007 ; C vs D 0.05
Experiment 5 - B vs D 0.02

TABLE 5.2

LAK CELLS ESSENTIAL FOR THERAPEUTIC EFFECTS

	A IL-2	B NL[c] + IL-2	C CL[d] + IL-2	D LAK + IL-2
Exp 1	2.3 ± 0.3	2.6 ± 0.2 (NS)	2.4 ± 0.2 (NS)	1.0 ± 0.4 (0.05)
Exp 2	1.5 ± 0.5	2.2 ± 0.2 (NS)	2.5 ± 0.3 (NS)	0.3 ± 0.2 (0.05)

[a] BL/6 mice received 10^5 MCA-105 tumor cells i.p. on day 0; $3x10^7$ life cells on day 3 and 6 and 25,000 U IL-2 i.p. every 12 hours from day 3 through 8. Mice were sacrificed on day 14.

[b] Mean Peritoneal Carcinomatosis Index ± SEM. In parentheses the significance of differences with the IL-2 group.

Statistically significant further pairwise comparisons:

Experiment 1 - B vs D 0.02 ; C vs D 0.03
Experiment 2 - B vs D 0.004; C vs D 0.004

[c] Normal lymphocytes

[d] Cultured (without IL-2) lymphocytes

5.4 Immunotherapy with IL-2 prolongs survival

BL/6 mice were injected with MCA-105 tumor as before and followed for survival. All animals were autopsied to determine whether death was tumor related. For statistical analysis nontumor related deaths were excluded. In the experiment shown in Figure 5.2, treatment started on day 3 after tumor inoculation. There was a significant prolongation of survival in mice given IL-2 alone (median 20 days vs control 14 days).

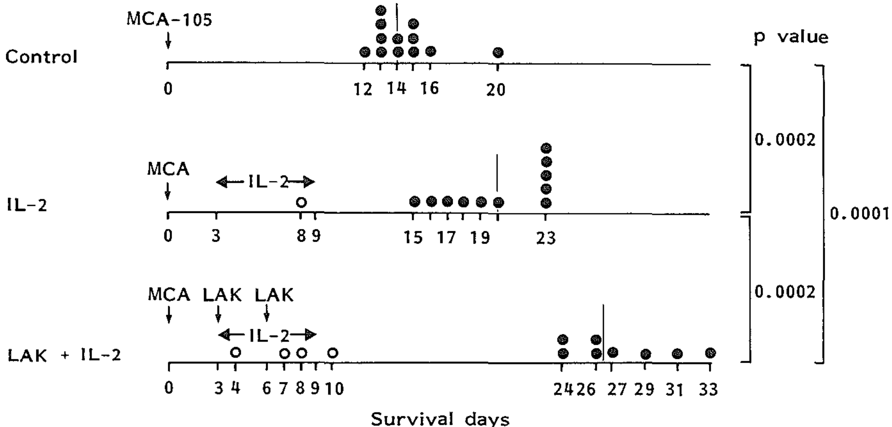


Fig. 5.2 **Treatment with IL-2 and LAK cells increased survival in tumor bearing mice. Mice were given 10^5 MCA-105 tumor cells intraperitoneally on day 0. They received 10^8 LAK cells i.p. on day 3 and 6 and 50,000 U IL-2 i.p. every 12 hours on day 3 through 8. Mice were followed for survival and autopsied at death. Death with tumor is shown with a closed circle, death without tumor as an open circle. Treatment with IL-2 plus LAK cells resulted in a significant survival benefit over both the control group and the IL-2 alone group.**

Even more pronounced effects were seen when IL-2 plus LAK cells were used (median 26 1/2 days). In the survival experiments shown in Figure 5.3, treatment started on the same day as the tumor inoculation. Median survival of the control group (11 1/2 days) is significantly prolonged by IL-2 alone (17 days) and by LAK cells plus IL-2 [(25 days and 24 1/2 days for 3-day and 5-day LAK cells (see Section 6.4) respectively].

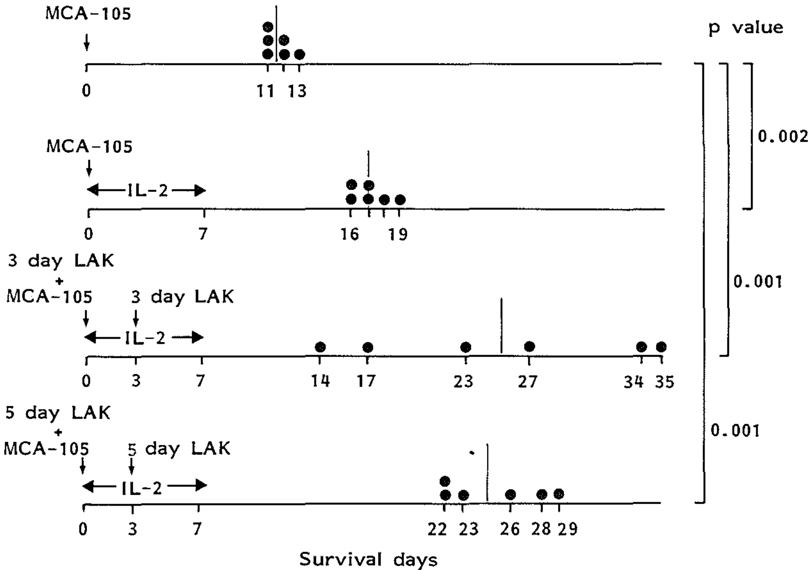


Fig. 5.3 In this experiment treatment started on the day of tumor inoculation (10^5 live MCA-105 tumor cells); $3x10^7$ LAK cells i.p. were given on day 0 and 3, 10,000 U IL-2 i.p. was given every 12 hours from day 0 through 6.

In a similar experiment mice were randomized to start with treatment on day 0, day 3 and day 9. LAK cells were given twice and IL-2 for 6 days as before. All treatment schedules prolonged survival, even when started at day 9 and in view of the fact that control animals started dying from day 12 onwards.

5.5 Site-specific treatment of intraperitoneal tumor provides better tumor reduction than systemic treatment

All treatment to this point had been administered site-specific i.e. LAK cells i.p. and IL-2 i.p. for intraperitoneal tumor. Efficacy of intravenous (i.v.) administration of LAK cells and subcutaneous (s.c.)

TABLE 5.3

EFFECT OF SITE-SPECIFIC AND SYSTEMIC IMMUNOTHERAPY OF ESTABLISHED INTRAPERITONEAL CANCER[a]

	PCI[b]						
	A	B	C	D	E	F	G
Exp.	Control	IL-2 s.c.	IL-2 i.p.	LAK i.v.+ IL-2 s.c.	LAK i.v.+ IL-2 i.p.	LAK i.p.+ IL-2 s.c.	LAK i.p.+ IL-2 i.p.
1	3.0 ± 0.0			3.0 ± 0.0 (NS)			0.8 ± 0.3 (0.004)
2	1.9 ± 0.3			0.6 ± 0.3 (0.01)	1.0 ± 0.4 (NS)	0.8 ± 0.4 (NS)	0.0 ± 0.0 (0.002)
3	1.0 ± 0.2			0.4 ± 0.2 (NS)	0.3 ± 0.3 (NS)	0.3 ± 0.2 (0.05)	0.0 ± 0.0 (0.005)
4	2.2 ± 0.3	1.7 ± 0.6 (NS)	2.0 ± 0.5 (NS)		1.3 ± 0.9 (NS)	1.3 ± 0.3 (NS)	0.8 ± 0.2 (0.02)
5	3.0 ± 0.0	3.0 ± 0.0 (NS)	1.4 ± 0.6 (0.003)	2.7 ± 0.2 (NS)	1.8 ± 0.5 (0.003)	2.2 ± 0.3 (0.008)	1.3 ± 0.3 (0.0004)

a BL/6 mice received 10^4 (Exp 1-3) or 10^5 (Exp 4&5) live MCA-105 tumor cells i.p. on day 0; $3x10^7$ LAK cells on day 3 and 6 and 50,000 U IL-2 i.p. every 12 hours from day 3 through 8. Peritoneal Carcinomatosis Index was scored on day 14.

b Mean Peritoneal Carcinomatosis Index ± SEM. In parentheses the significance of differences as compared to the control group.

Further statistically significant pairwise comparisons:

Experiment 1 - D vs G 0.004
Experiment 5 - B vs C 0.01; B vs E 0.01; B vs F 0.02; B vs G 0.002; D vs G 0.01

injection of IL-2 (systemic treatment) on intraperitoneal tumor was compared with the site-specific approach. Intravenous injections were given in a tail vein.
Experimental groups received IL-2 alone s.c., IL-2 alone i.p., LAK cells i.v. and IL-2 s.c., LAK cells i.v. and IL-2 i.p., LAK cells i.p. and IL-2 s.c. and both LAK cells and IL-2 i.p.
The results of 5 separate experiments are detailed in Table 5.3.
In all experiments, the most marked tumor reduction was seen when both LAK cells and IL-2 were given in a site-specific fashion. This reduction was always significantly different from the control group. The other modalities gave a less impressive and less consistent tumor reduction.

5.6 Recombinant IL-2 and EL-4 subline derived IL-2 are equally effective in treating intraperitoneal tumor

The early experiments, pointing to a possible antitumor effect of interleukin-2 had been performed with natural IL-2 usually only partially purified (Donohue et al., 1984; Mazumder and Rosenberg 1984). The *in vivo* efficacy of recombinant IL-2, used in all experiments described above and natural IL-2, derived from a murine tumor line and partially purified, was compared.
The potency of a batch of recombinant IL-2 and a batch of EL-4 subline derived IL-2 (E-IL-2) was assayed in one ^{3}H-thymidine-uptake assay (Figure 4.1). MCA-105 bearing mice received LAK cells generated by incubation with either recombinant IL-2 or E-IL-2 and exogenous IL-2 from the same source. In all instances, groups treated with LAK cells plus IL-2 had a lower PCI than did control animals (Fig. 5.4). There were no significant differences between the effects of IL-2 and E-IL-2.

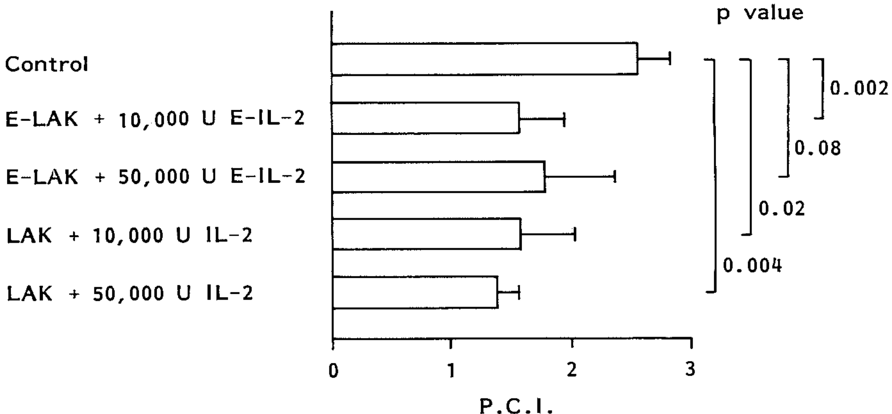


Fig. 5.4 **Recombinant IL-2 and EL-4 subline derived IL-2 (E-IL-2) are equally effective. Mice received 10^5 MCA-105 tumor cells i.p. on day 0, $3x10^7$ LAK cells on day 3 and 6 and IL-2 i.p. every 12 hours from day 3 through 8. Mice were sacrificed on day 14. Horizontal bars show the mean PCI per group ± SEM. Significance of pairwise comparisons is indicated. E-LAK cells are splenocytes activated with E-IL-2.**

5.7 Conclusion

In the experiments described above, mice were injected i.p. with tumor. Untreated animals started to die around day 14. At that time a considerable tumor load could be detected in the abdomen of sacrificed animals.

Treatment with LAK cells in conjunction with IL-2 gave a marked and significant reduction in tumor load in every single experiment. LAK cells alone did not provide tumor growth retardation. IL-2 alone provided smaller and less consistent effects than IL-2 plus LAK cells. Parallel results can be seen in survival experiments. LAK cells and IL-2 gave a marked and significant prolongation of survival compared to control animals. The results of IL-2 alone were intermediate.

Fresh lymphocytes or lymphocytes cultured without exogenous IL-2 did not provide any antitumor effect. It appears then, that the LAK cell phenomenon is a particular one.

On the basis of these results and similar results in other models, it can be hypothesized that IL-2, given alone, leads to some in vivo LAK cell formation and that IL-2, given in conjunction with LAK cells sustains their activity. Experiments, testing this hypothesis, will be discussed in Section 9.5.

There are a number of clinical situations in which tumor recurrence or metastases are expected mainly in one body compartment. In these situations, the utility of local, site-specific treatment can be considered.

In the experimental model, site-specific treatment appeared superior to systemic treatment, possibly due to differences in trafficking of the LAK cells, and in the concentration of IL-2 to which they are exposed. In vitro immune effects of recombinant IL-2 have been found similar to those of natural IL-2 (Rosenberg et al., 1984). These findings could be expanded to in vivo antitumor effects.

Chapter 6

LAK CELL GENERATION

6.1 Introduction

In the experiments described in Chapter 5, LAK cells were generated by incubating splenocytes from normal, syngeneic donors. In the clinical situation, such donors will not generally be available.
Either (allogeneic) donor lymphocytes could be considered, or the patient's own lymphocytes. In the latter situation, the patient will either have demonstrable tumor or the visible tumor will have been resected and the immunotherapy given in an adjuvant setting.

A further question is the optimal period of in vitro incubation when generating LAK cells. In this section, experiments adressing these points are described.

6.2 Allogeneic and syngeneic LAK cells are similarly effective

To examine possible MHC restriction in in vivo antitumor efficacy of LAK cells, allogeneic and syngeneic cells were compared. BL/6 mice (H-2^b) received (syngeneic) MCA-105 tumor cells i.p. as before. On day 3 and 6 allogeneic (H-2^d) or syngeneic LAK cells were administered i.p., IL-2 was given as before. The results are shown in Table 6.1. Both allogeneic and syngeneic cells mediate significant tumor reduction. There were no significant differences between these two types of LAK cells.

6.3 LAK cells can be generated from tumor bearing donors and from donors in whom the tumor has been resected

Relevant for extrapolation to the clinical situation is the feasibility of LAK generation from donors in whom the tumor is either still present or resected. To address this point, donors were injected with 10^5 MCA-

TABLE 6.1

ALLOGENEIC LAK AND SYNGENEIC LAK CELLS SIMILARLY EFFECTIVE[a]

PCI[b]

	A	B	C	D
	Control	IL-2	Allo LAK[c] + IL-2	Syn LAK[d] + IL-2
Exp 1	2.9 ± 0.1	2.3 ± 0.3 (NS)	2.0 ± 0 (0.0008)	1.0 ± 0.4 (0.0003)
Exp 2	2.8 ± 0.2	2.4 ± 0.4 (NS)	0.8 ± 0.3 (0.0003)	1.2 ± 0.4 (0.0005)

a BL/6 mice (H-2[b]) received 10^5 MCA-105 tumor cells i.p. on day 0; on day 3 and 6 3×10^7 life LAK cells were injected i.p.; allogeneic LAK cells were DBA/2J (H-2[d]) derived, syngeneic LAK cells were BL/6 derived; 25,000 U IL-2 was given every 12 hours from day 3 through 8. Tumor load was determined on day 14.

b Mean Peritoneal Carcinomatosis Index ± SEM. In parentheses the significance of differences with the control group.

Statistically significant further pairwise comparisons:

Experiment 1 B vs D 0.05
Experiment 2 B vs C 0.01; B vs D 0.04

c Allogeneic LAK cells

d Syngeneic LAK cells

105 cells i.m. in the right hindleg. One week later the tumor bearing extremity was amputated in part of these animals. Two weeks after tumor inoculation spleens of both groups were harvested. Lymphocytes from normal, from tumor bearing and from tumor resected donors were incubated with IL-2 and administered i.p. to mice with established intraperitoneal tumor. IL-2 was given as before. All three types of LAK cells conferred significant tumor reduction when compared to no treatment or administration of IL-2 only (Table 6.2). No significant differences were seen among the various LAK cells.

6.4 Lymphocytes incubated for 3 or 5 days in IL-2 are equally effective

After *in vivo* incubation of lymphocytes with IL-2, LAK activity appears around day 2 and possibly peaks around day 3-7 (Section 3.6.5). In the experiments presented above, cells incubated for 72 hours had been studied. To examine potential enhanced tumor reactivity of cells incubated for a longer period the following experiment was conducted. Mice received tumor and IL-2 i.p. as before. Splenocytes incubated with IL-2 for 72 hours (3 day LAK) or 120 hours (5 day LAK) were administered i.p.

Results are detailed in Table 6.3. No significant differences are found between 3 day LAK and 5 day LAK either when given alone (column B vs C) or in conjunction with IL-2 (column D vs E).

In the survival experiment shown in Figure 5.3 similarly no differences between 3 and 5 day LAK cells were found.

6.5 Conclusion

Effective LAK cells can be generated from several sources. In the experimental model, no differences in antitumor activity were found between allogeneic and syngeneic LAK cells. Allogeneic LAK cells could give rise to graft versus host reactions. Such effects would need further study, but in the leucopenic patient, who cannot effectively be used as his own donor, administration of allogeneic LAK cells could be contemplated.

In the tumor bearing state, a number of immunological functions are

TABLE 6.2

TUMORBEARING DONORS CAN GENERATE LAK CELLS[a]

PCI[b]

A	B	C	D	E
Control	IL-2	LAK + IL-2 (normal donors)	LAK + IL-2 (tumor bearing donors)	LAK + IL-2 (tumor resected donors)
2.8 ± 0.1	3 (NS)	2.3 ± 0.2 (0.06)	2.2 ± 0.2 (0.03)	1.8 ± 0.2 (0.004)

[a] Mice received 10^5 MCA-105 tumor cells i.p. on day 0. On day 3 and 6 they received 10^8 LAK cells i.p. LAK cells were derived from normal donors or from donors injected on day -14 with 10^5 MCA-105 cells i.m. in the right hind leg either with tumors in place (column D) or after amputation of the tumor on day -7 (column E). IL-2 25,000 U was given i.p. every 12 hours from day 3 through 8.

[b] Mean Peritoneal Carcinomatosis Index ± SEM. In parentheses significance of differences with the control group.

Further significant pairwise comparisons:

B vs C 0.02; B vs D 0.006; B vs E 0.002

depressed. It could be hypothesized that less efficient LAK cell generation occurs in the tumor bearing donor or in the donor in whom the tumor is resected. Alternatively, the population of LAK cell precursors might be expanded.
However, in the present experimental model, LAK cells generated from tumor bearing or tumor resected donors appeared as effective as LAK cells generated from healthy donors.

As no consistent differences in in vivo antitumor effects were found between 3 and 5 day LAK cells, in further studies, for logistical reasons, 3 day LAK cells were used.

TABLE 6.3

3 DAY AND 5 DAY LAK CELLS EQUALLY EFFECTIVE[a]

PCI[b]

	A	B	C	D	E
	Control	3 day LAK[c]	5 day LAK[d]	3 day LAK[c] + IL-2	5 day LAK[d] + IL-2
Exp 1	3	2.3 ± 0.4 (NS)	2.3 ± 0.5 (NS)	1.4 ± 0.4 (0.003)	1.3 ± 0.6 (0.02)
Exp 2	1.8 ± 0.2	1.2 ± 0.4 (NS)	0.7 ± 0.3 (0.004)	0 (0.0003)	0.5 ± 0.3 (0.003)

[a] BL/6 mice received 10^5 live MCA-105 tumor cells i.p. on day 0; 10^7 live LAK cells i.p. on day 3 and 6 and 10,000 U IL-2 every 12 hours on day 3 through 8.

[b] Mean Peritoneal Carcinomatosis Index ± SEM. In parentheses significance of differences with the control group.

[c] Splenocytes incubated for 3 days with IL-2

[d] Splenocytes incubated for 5 days with IL-2

Statistically significant further pairwise comparison:

Experiment 2 - B vs d 0.02

Chapter 7

DOSE-RESPONSE EFFECTS

7.1 Introduction

As was shown in Chapter 5, treatment with LAK cells in conjunction with exogenous IL-2 reduces tumor load and prolongs survival. However, treated animals do not survive indefinitely and their PCI is not usually zero.
In efforts to optimize treatment results, the dose of LAK cells and the dose of IL-2 was varied.
As reviewed in Section 3.2, IL-2 has a short serum half life. It was conceivable that efforts to maintain some IL-2 activity during longer periods by increased dose fractioning would improve the results.
Another approach is administration of repeated courses of LAK cells and IL-2. Experiments, exploring these approaches are described in this section.

7.2 Increasing the number of LAK cells given in combination with IL-2 results in increased tumor control

In this experiment mice received on day 3 and 6 varying doses of LAK cells, from 0 and $3x10^6$ cells stepwise increased to 10^8 cells. Higher doses were not considered as the amount of spleen donors required, becomes prohibitive.
Tumor inoculation and IL-2 administration were as before. The results are depicted in Figure 7.1. Doses of $3x10^6$ through $3x10^7$ plus IL-2 in this experiment did not provide tumor reduction over that provided by IL-2 alone. Administration of 10^8 cells appeared significantly better, and no tumor could be detected on gross inspection. This experiment was repeated and similar results obtained.

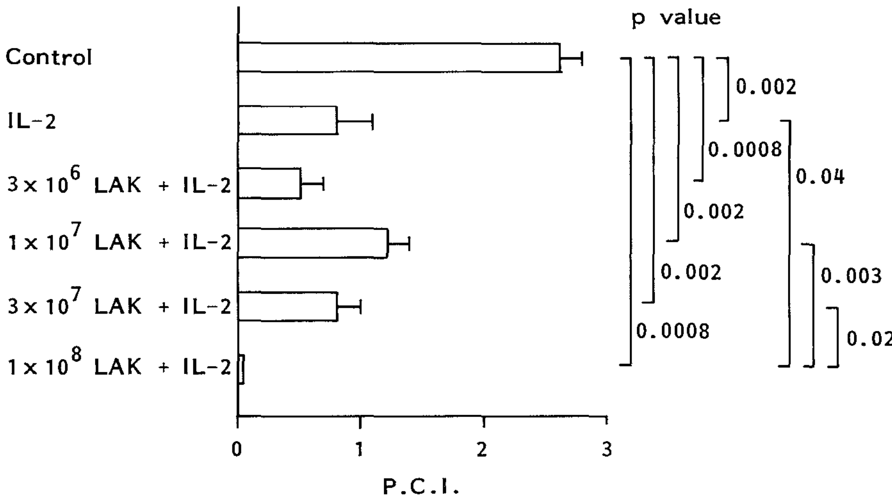


Fig. 7.1 Dose titration of LAK cells given in combination with IL-2. Increasing numbers of LAK cells were given in combination with IL-2 to different groups of mice. BL/6 mice received 10^5 MCA-105 tumor cells i.p. on day 0, LAK cells in designated doses i.p. on day 3 and 6 and 50,000 U IL-2 i.p. every 12 hours on day 3 through 8. Tumor load was scored on day 14. Significant pairwise comparisons are shown. If 10^8 LAK cells were used in addition to exogenous IL-2 , none of the animals were found to have intraperitoneal tumor.

7.3 Effect of IL-2, given in combination with LAK cells is dose-dependant

The optimal amount of IL-2 was similarly sought in a dose titration study. In the experiments described above, doses of IL-2 ranging from 10,000 U to 50,000 U every 12 hours had been used. Varying doses of IL-2 from 0 and 3000 stepwise increased to 100,000 U b.d. were used. Mice received i.p. tumor and LAK cells as before.

In three experiments, 50,000 U twice a day provided optimal tumor reduction (Table 7.1). Increasing the dose to 100,000 U every 12 hours did not provide further tumor reduction. In these experiments, no tumor reduction was seen at doses lower than 50,000 U twice daily. (The apparent discrepancy with experiments 1 and 2 in Table 5.1 is considered in Section 9.5).

TABLE 7.1

DOSE TITRATION OF IL-2, GIVEN IN COMBINATION WITH LAK CELLS[a]

PCI[b]

	A	B	C	D	E	F
	Control	LAK	LAK + IL-2 3000 U b.d.	LAK + IL-2 10.000 U b.d.	LAK + IL-2 50,000 U b.d.	LAK + IL-2 100,000 U b.d.
Exp 1	2.8 ± 0.2	2.8 ± 0.2 (NS)	2.2 ± 0.6 (NS)	2.0 ± 0.5 (NS)	0.7 ± 0.4 (0.007)	
Exp 2	3.0 ± 0.0				1.0 ± 0.4 (0.004)	2.0 ± 0.6 (NS)
Exp 3	2.8 ± 0.2		2.3 ± 0.4 (NS)	2.0 ± 0.5 (NS)	1.6 ± 0.5 (0.02)	2.0 ± 0.5 (NS)

a BL/6 mice received $5x10^4$ (Exp 1&2) or 10^5 (Exp 3) live MCA-105 tumor cells i.p. on day 0; $3x10^7$ live LAK cells i.p. on day 3 and 6 and IL-2 i.p. every 12 hours on day 3 through 8. Tumor load was scored on day 14.

b Mean Peritoneal Carcinomatosis Index ± SEM. In parentheses the significance of differences with the control group.

Statistically significant further pairwise comparisons:

Experiment 1 - B vs E 0.007

7.4 Increased tumor control is seen with increased dose-fractioning of IL-2

In the experiments described till sofar, IL-2 had been administered every 12 hours. In view of the short half life of IL-2, the antitumor effects of increasing dose fractioning were examined. A total daily amount of 100,000 U was given in 1, 2 or 4 doses (100,00 U every 24 hours, 50,000 U every 12 hours or 25,000 U every 6 hours) from day 3-8. Tumor and LAK cells were administered as before. As shown in Figure 7.2 better tumor reduction was seen with frequent low-dose injections.

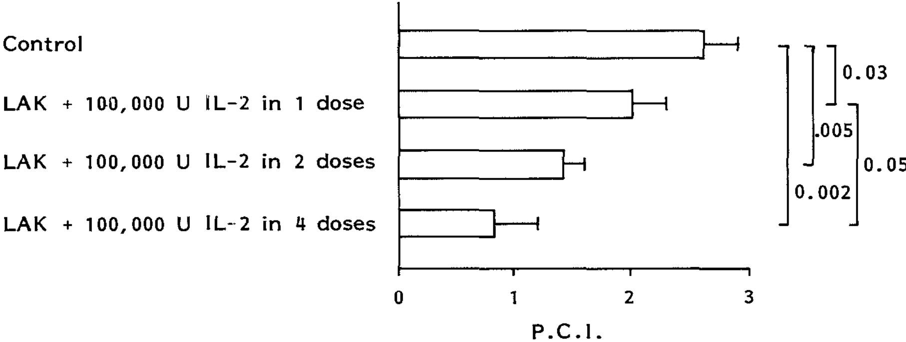


Fig. 7.2 Divided doses of IL-2 were more effective than a single daily injection. 10^5 MCA-105 tumor cells were injected i.p. on day 0. On day 3 and 6, $3x10^7$ LAK cells were injected i.p. On day 3 through 8 100,000 U IL-2 was given i.p. in a single dose, two doses, or four doses. Significant pairwise comparisons are shown. Injecting the daily dose in fractions was more effective than a single injection.

An alternative approach to provide prolonged IL-2 activity is injection of the pharmacon in gelatine from which it is slowly released (Donohue and Rosenberg, 1983). This approach was explored as well. However, although i.p. gelatine injections (vehicle only or with IL-2) were reasonably tolerated in non tumor bearing mice or in mice with tumor outside the peritoneal cavity, a prohibitive mortality was seen in mice bearing intra-abdominal tumor given i.p. gelatine injections. In

surviving mice treated with IL-2 in gelatine no improvement was seen compared to mice treated with an equal dose of IL-2 reconstituted in PBS (data not shown).

7.5 Multiple courses not superior to single course

To examine if multiple treatment courses provide better tumor reduction than single courses, the following experiment was conducted.
Mice, bearing established intraperitoneal tumor received 1, 2 or 4 courses of either IL-2 alone or of LAK cells plus IL-2. Treatment courses started at day 3, 10, 17 and 24 after tumor inoculation. The results are depicted in Figure 7.3.

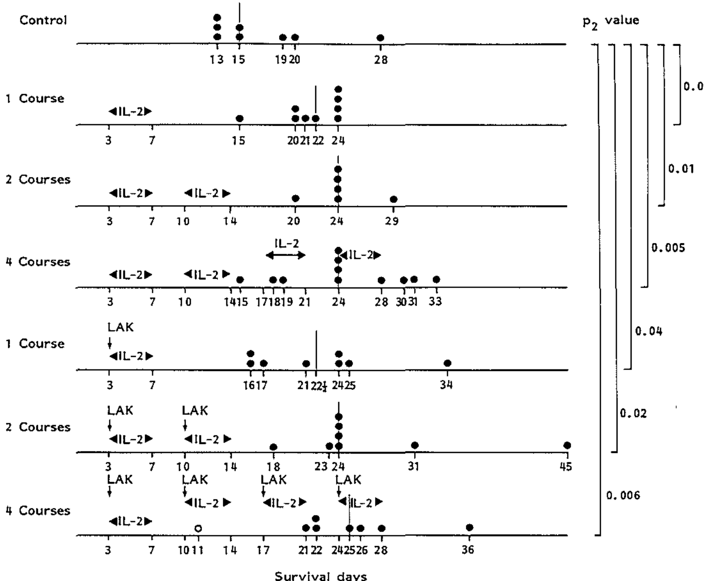


Fig. 7.3 **Multiple courses not superior to single course.**
10^5 MCA-105 tumor cells were injected i.p. on day 0. Depending on the treatment group 10^8 LAK cells were injected i.p. on day 3, day 3 and 10 or day 3, 10, 17 and 24. IL-2 was similarly given from day 3-7, day 10-14, day 17-21 and day 24-28 in a dose of 25,000 U b.d. i.p. Mice were followed for survival and autopsied at death. Death with tumor is shown with a closed circle, death without tumor as an open circle. Significant pairwise comparisons are shown.

All treatments significantly prolonged survival compared to control groups. No significant differences were found between 1, 2 or 4 courses of IL-2 (median survival 22, 24 and 24 days respectively) or between 1, 2 or 4 courses of LAK cells plus IL-2 (median 22 1/2, 24 and 25 days).

7.6 Conclusion

Considering modifications to improve the results of treatment with LAK cells and IL-2, the following seems to apply. Optimal tumor reduction was seen at high doses (10^8) of LAK cells. Lower doses gave less growth retardation. It is cumbersome to administer higher doses because of the number of spleen donors required.

Dose-response effects of IL-2 were somewhat erratic. Low doses do not provide an antitumor effect. Consistent tumor reduction is seen at doses of 50,000 U b.d. Higher doses do cause a notable toxicity. In this model, doses over 50,000 U b.d. did not provide a better tumor reduction. Weese et al. (1987) reported that at very high doses of IL-2, rats had a greater chance of developing tumor following injection of tumor cells than did control animals, while reduced incidence of tumor was seen at lower doses IL-2.
Increased dose fractioning of IL-2 improved the activity of LAK cells. IL-2 has a short in vivo half life. Multiple small peaks apparently are more efficacious than one large peak.

The effect of a single course of LAK cells and IL-2 could not be improved by administration of multiple courses. One could hypothesize that the remaining tumor has lost its susceptibility for LAK cells. Alternatively, antibody formation against LAK cells or IL-2 could explain the results. This is further discussed in Section 9.7.

Chapter 8

BROADER APPLICABILITY OF TREATMENT WITH IL-2 AND LAK CELLS

8.1 Introduction

In the experiments, described in the previous sections, one single tumor, MCA-105, was studied with the tumor inoculated in one location. The question arose if treatment with LAK cells and IL-2 had a broader applicability. To examine this point, a number of other tumors were studied.

In separate experiments, tumor was inoculated subcutaneously or intrahepatically and the efficacy of treatment with LAK cells plus IL-2 tested.

Apart from their tumor bearing state, mice till sofar had a normal immunocompetence. This in contrast to the clinical situation in which immunotherapy could be considered. The patient's immune apparatus might be exhausted by previous radiotherapy or cytostatic drugs. Therefore, IL-2 immunotherapy was tested in the immunocompromised host.

8.2 Immunotherapy with LAK cells and IL-2 is effective in a variety of murine tumors in several mouse strains

To examine if the effects seen with MCA-105 were part of a broader phenomenon, six other tumors were studied in the syngeneic host. The tumors were of varied histology: sarcoma (MCA-101, MCA-102, MCA-105), adenocarcinoma (MCA-38), melanoma (B16), amelanotic melanoma (M3) and mastocytoma (P815). Six tumors were solid, P815 grew as an ascitic tumor. MCA-101 and MCA 102 are nonimmunogenic. The M3 tumor was tested in C3H mice, P815 in DBA mice.

Syngeneic hosts received i.p. administration of a lethal tumor inoculum, and received LAK cells and IL-2 as detailed in Table 8.1.

At 50,000 U every 12 hours [the dose previously (Section 7.3) found to be optimal] given in conjunction with LAK cells a marked and

TABLE 8.1

TREATMENT OF ESTABLISHED INTRAPERITONEAL TUMOR WITH LAK CELLS AND IL-2 IN DIFFERENT MOUSE STRAINS AND TUMORS[a]

			Intraperitoneal Tumor Mass[b]					
			A	B	C	D	E	F
	Mouse Strain	Tumor	Control	LAK	IL-2 10,000 U bd	LAK + IL-2 10,000 U bd	IL-2 50,000 U bd	LAK + IL-2 50,000 U bd
Exp 1	BL/6	MCA-105	2.8 ± 0.2	2.8 ± 0.2 (NS)	2.2 ± 0.5 (NS)	2.0 ± 0.5 (NS)	2.2 ± 0.6 (NS)	0.7 ± 0.4 (0.007)
Exp 2	BL/6	B16	2.7 ± 0.2	2.7 ± 0.2 (NS)			1.7 ± 0.4 (NS)	1.0 ± 0.3 (0.005)
Exp 3	BL/6	MCA-38	1.4 ± 0.3	1.2 ± 0.4 (NS)	1.2 ± 0.5 (NS)	1.0 ± 0.6 (NS)	0.6 ± 0.4 (NS)	0.2 ± 0.2 (0.02)
Exp 4	C3H/Hen	M-3	1.3 ± 0.3	1.0 ± 0.5 (NS)	0.8 ± 0.6 (NS)	1.0 ± 0.5 (NS)	0.8 ± 0.8 (NS)	0.2 ± 0.2 (0.02)
Exp 5	BL/6	MCA-101	3				2 ± 0.5 (0.04)	1 ± 0.4 (0.0006)
Exp 6	BL/6	MCA-102	3				1.5 ± 0.5 (0.003)	1.7 ± 0.4 (0.003)
Exp 7	DBA/2	P815	276 ± 53	225 ± 72 (NS)			37 ± 18 (0.006)	62 ± 9 (0.01)

[a] Dose of tumor used: 5×10^4 (Exp 1&2), 10^5 (Exp 3-7). 3×10^7 LAK cells (Exp 1-4) or 10^8 (Exp 5&6) were given i.p. on day 3 and 6; from day 3 through 8, IL-2 was administered i.p. every 12 hours. In Experiment 7, 10^8 LAK cells were given on day 6 and 9, IL-2 was administered from day 6 through 11. Tumor load was determined on day 14 (Exp 1-6) or day 15 (Exp 7).

[b] Mean Peritoneal Carcinomatosis Index ± SEM (Exp 1-6). Number of cells x 10^5 is given for experiment 7. In parentheses the significance of differences as compared to the control group.

significant tumor reduction was seen in all tumors. IL-2 alone in this dose did not provide consistent tumor reduction. No antitumor effects were seen of LAK cells given alone or with lower doses of IL-2. The nonimmunogenic tumors were equally susceptible to immunotherapy with LAK cells and IL-2 as the immunogenic tumors.

8.3 IL-2 immunotherapy is detrimental in the EL-4 bearing host

The EL-4 lymphoma is known to bear IL-2 receptors (Farrar et al., 1982) and IL-2 generally stimulates growth of cells bearing its receptor (Chapter 3).

In vivo effects of adoptive administration of LAK cells and IL-2 on this tumor were examined. Syngeneic hosts were injected with $5x10^5$ EL-4 cells on day 0 and received LAK cells i.p. on day 3 and 6. The total number of tumor cells was estimated on day 14. Results are depicted in Figure 8.1. Treatment with LAK cells and/or IL-2 appears to increase tumor load roughly one ^{10}log, rather than retard tumor growth as seen with other tumors.

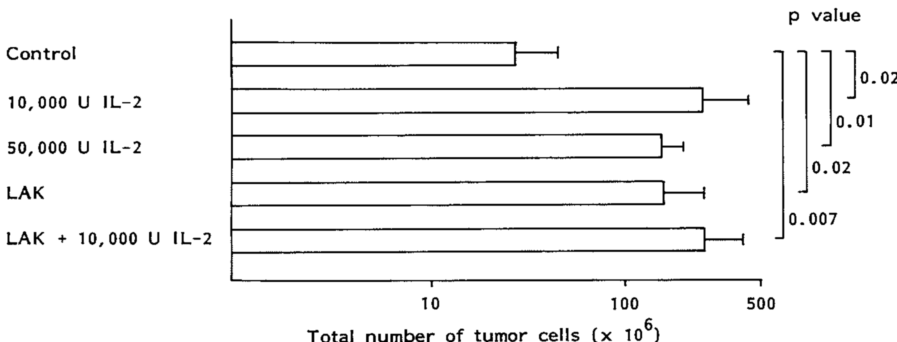


Fig. 8.1 IL-2 immunotherapy is detrimental in the EL-4 bearing host. Mice received $5x10^5$ EL-4 i.p. on day 0, 10^8 LAK cells i.p. on day 3 and 6 and IL-2 every 12 hours on day 3 through 8. The number of intraperitoneal tumor cells was estimated on day 14. Horizontal bars show the mean number of cells ± SEM on a logarithmic scale.

TABLE 8.2

IL-2 IMMUNOTHERAPY IS DETRIMENTAL IN THE HOST, BEARING EL-4 TUMOR

	Control	LAK	IL-2 10,000 U bd	IL-2 50,000 U bd	LAK + IL-2 10,000 U bd	LAK + IL-2 50,000 U bd
Died during treatment	0/8	2/6	6/6	6/6	6/6	6/6

[a] BL/6 mice received $5x10^5$ EL-4 cells i.p. on day 0 and received $3x10^7$ LAK cells i.p. on day 3 and 6. IL-2 was given every 12 hours from day 3 through 8.

In a similar experiment all animals receiving IL-2 at 10,000 U or higher and 2 of 6 animals receiving LAK cells alone, died during treatment (i.e. before day 8) (Table 8.2). The number of tumor cells in animals succumbed during treatment could not reliably be estimated. The number of tumor cells in the surviving animals estimated at day 14 did not differ significantly among the two groups (control and LAK only) (data not shown).

8.4 Treatment with LAK cells and IL-2 reduces intrahepatic tumor

In the above experiments, malignancy established in the peritoneal cavity was studied. To expand these findings, intrahepatic and subcutaneous (Section 8.5) tumor were studied. Intrahepatic tumor nodules were generated in BL/6 hosts by intrasplenic injection of MCA-105 on day 0 and subsequent splenectomy. On day 3 and 6 LAK cells were given i.p. and from day 3 through 8 IL-2 was given i.p. At day 14 the total intrahepatic tumor load was scored on a scale from 0-4. Results are depicted in Table 8.3. LAK cells, given in conjunction with IL-2 significantly reduced tumor growth. LAK cells alone or IL-2 alone were not effective.

8.5 Growth retardation of subcutaneous tumor effected by treatment with LAK cells and IL-2

On day 0, MCA-105, $5x10^4$ cells in 0.1 ml, was injected intradermally halfway between xiphoid and umbilicus, raising a small wheal. LAK cells and IL-2 were given as detailed in Table 8.4. Subcutaneous injections of IL-2 were given in the neck area, away from the growing tumor. Treated mice showed a significant growth retardation of subcutaneous tumor. In this model, no differences were seen between LAK cells administered i.v. or i.p. or between IL-2 given s.c. on i.p.

TABLE 8.3

REDUCTION OF INTRAHEPATIC TUMOR BY LAK CELLS AND IL-2[a]

	Control	IL-2	LAK	LAK + IL-2
Tumorload[b]	2.8 $\pm$ 0.4	2.5 $\pm$ 0.6	2.0 $\pm$ 0.5	1.4 $\pm$ 0.2

a Intrahepatic tumor nodules were generated in BL/6 mice by intrasplenic injection of MCA-105 tumor on day 0; on day 3 and 6 10^7 LAK cells were injected i.p., from day 3 through 8 10,000 U IL-2 was given i.p. every 12 hours.

b Tumorload was scored on a scale from 0-4. The mean score $\pm$ SEM is indicated. In parentheses significance of differences with the control group.

TABLE 8.4

REDUCTION OF SUBCUTANEOUS TUMOR BY LAK CELLS AND IL-2[a]

Tumorsize[b]

	A	B	C	D	E
	Control	LAK i.v.+ IL-2 s.c.	LAK i.v.+ IL-2 i.p.	LAK i.p.+ IL-2 s.c.	LAK i.p.+ IL-2 i.p.
Exp 1	29 ± 3	12 ± 4 (0.04)	11 ± 4 (0.012)	14 ± 5 (0.06)	19 ± 2 (0.02)
Exp 2	30 ± 3	14 ± 2 (0.004)	11 ± 3 (0.004)	12 ± 2 (0.002)	9 ± 1 (0.002)

a BL/6 mice were injected intradermally (belly area) with $5x10^4$ MCA-105 cells in 0.1 ml at day 0. On day 3 and 6 $3x10^7$ LAK cells were given i.v. or i.p. and from day 3 through 8 50,000 U IL-2 were injected s.c. (neck) or i.p.

b Tumor size at day 10 ± SEM. In parentheses significance of difference with control group.

8.6 Treatment with IL-2 and LAK cells is effective in nude mice

In this experiment the efficacy of treatment with LAK cells and IL-2 in an immunocompromised host (nude mice) was studied.
BL/10 nude mice were injected with $5x10^4$ MCA-105 tumor cells. The standard regimen of LAK cells on day 3 and 6 plus IL-2 on day 3 through 8 every 12 hours was used. The results shown in Figure 8.2 reveal that IL-2 plus LAK cells was effective in reducing the intraperitoneal tumor mass. Repeat experiments showed similar results.

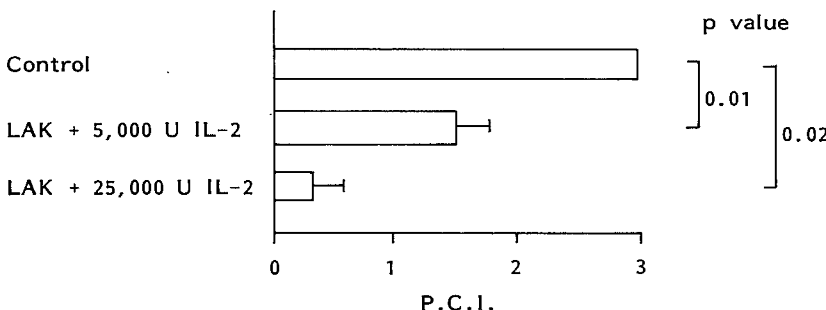


Fig. 8.2 **Adoptive immunotherapy is effective in an immunocompromised host. BL/10 nude mice received $5x10^4$ live MCA-105 tumor cells in 1 ml i.p. on day 0, $3x10^7$ BL/6 LAK cells i.p. on day 3 and 6 and IL-2 i.p. 5,000 U every 12 hours from day 3 through 8. Mice were sacrificed on day 14. Horizontal bars show the mean PCI per group ± SEM. Significance of pairwise comparisons is indicated.**

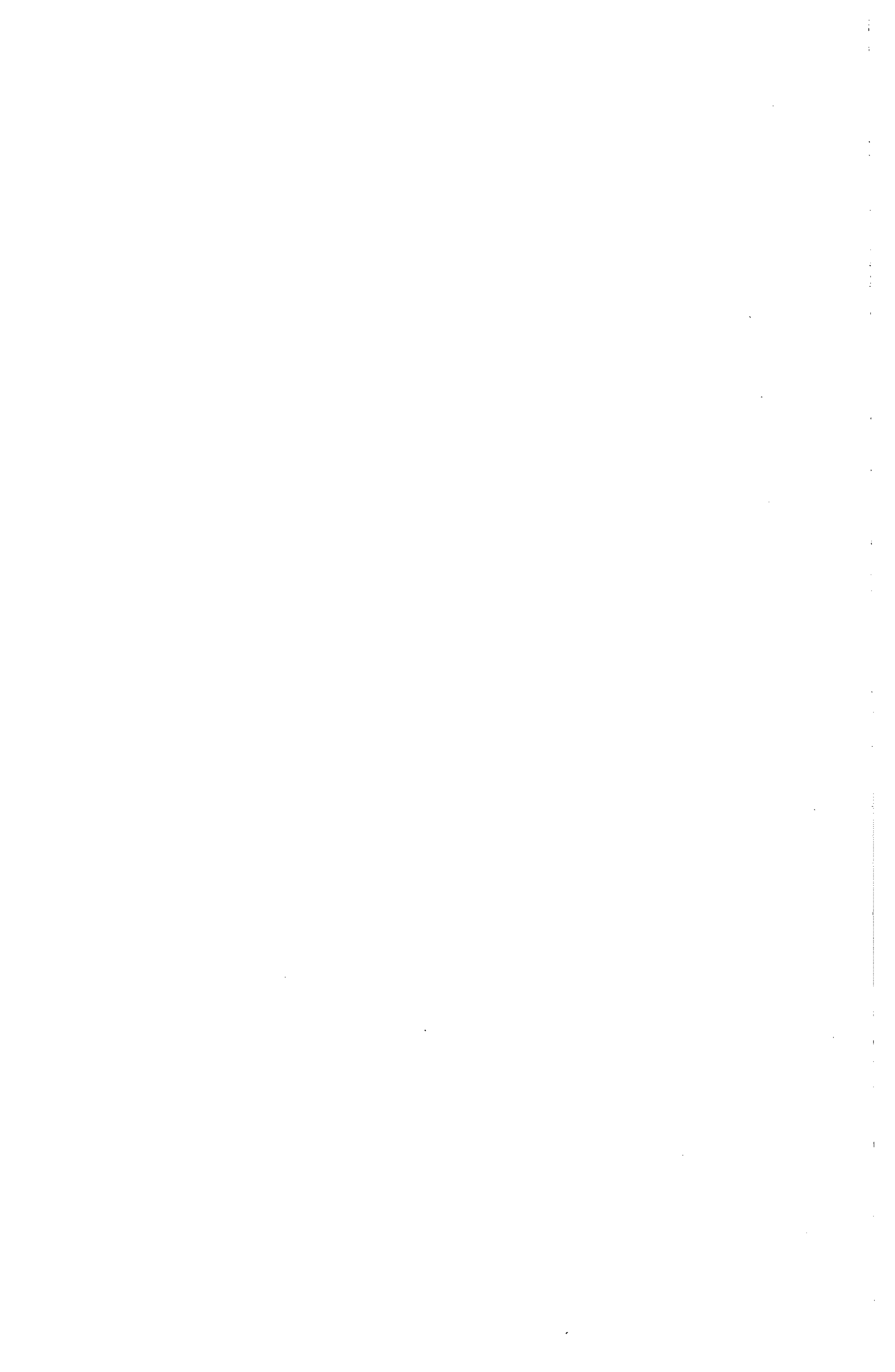
8.7 Conclusion

The experiments described in this section, taken together, suggest that the <u>in vivo</u> effects of LAK cells and IL-2 are part of a broad phenomenon.
Tumors of varied histology in different mouse strains were found to be

susceptible. Solid as well as ascitic tumors, both immunogenic and nonimmunogenic tumors could succesfully be treated.
In treating tumors bearing the IL-2 receptor either growth retardation, as seen in the case of other tumors, or enhanced tumor growth due to direct stimulation of tumor cells by IL-2, could be surmised. When IL-2 was given in the experimental model we used, tumor stimulation appeared to outweigh the first effect, both when counting tumor cells and when studying survival. It is not understood why administration of lymphoid cells with LAK activity by itself seems to promote tumor growth. In _in vitro_ experiments LAK cells were cytotoxic for EL-4 cells (Section 5.2). It could be that some of the transferred cells released IL-2.
There are incidental reports of tumors resistant to LAK cell activity (Bubenik and Indrova, 1987) apparently by other mechanisms, but in general treatment with LAK cells and IL-2 is effective against a broad range of tumors.

Apart from the intra-abdominal tumor, two other locations were studied. Intrahepatic tumor nodules could be treated succesfully by intraperitoneal administration of LAK cells plus IL-2. Subcutaneous tumor as well showed significant growth retardation. No differences were apparent between the four treatment groups: LAK cells i.v. and IL-2 s.c., LAK i.v. and IL-2 i.p., LAK i.p. and IL-2 s.c. and both LAK cell and IL-2 i.p. This suggests that LAK cells can traffic to the subcutaneous compartment and that in this model similar stimulation of LAK cells is seen when IL-2 is given in the same or a different compartment.

Relevant for extrapolation to the clinical situation is the feasibility of this form of immunotherapy in the immunocompromised host. In congenitally athymic mice, LAK cells plus IL-2 gave a tumor reduction similar to that in immunocompetent animals. Experiments in animals with other forms of impaired immunocompetence are reviewed in Chapter 9.2.



Chapter 9

GENERAL DISCUSSION

9.1 The rationale for the experimental model used

IL-2 and LAK cells are theoretically attractive compounds for consideration in tumor immunotherapy.

Past efforts of immunotherapy have concentrated on either an aspecific stimulation of the immune apparatus or on raising cells or antibodies specifically reacting to tumor cells. The inconsistent expression of tumor-associated antigens on human neoplasms is a problem with the latter approach and neither of these approaches has shown impressive clinical results (Section 2.6).

Under certain stimuli, lymphocytes can give rise to so-called activated killer cells. In vitro incubation with the lymphokine IL-2 is the most feasible method of procurement. Cells, so activated, are called LAK (lymphokine activated killer) cells.

LAK cells are cytotoxic for autologous, syngeneic, allogeneic and xenogeneic tumor. To normal autologous or syngeneic cells, LAK cells display a limited or no reactivity. Prior exposure to tumor cells is not necessary for activation (Section 3.6).

Tumor immunotherapy can be considered as sole therapy or in combination with other forms of therapy (surgery, radiation therapy, chemotherapy). One can distinguish situations in which these other forms of therapy are employed with curative intent and immunotherapy is given in an adjuvant setting or alternatively the immunotherapy can be given because it is known that the standard approach will not lead to cure.

In a number of clinical situations, metastatic or recurrent disease has a predilection for a certain body compartment. In the abscence of systemic dissemination, local, or site-specific immunotherapy can then be contemplated. In a considerable proportion of patients with gastro-

intestinal and ovarian malignancy the peritoneal cavity is a prominent site of surgical treatment failure (Ottow et al., 1987a). This led to the choice of a model of intraperitoneal tumor for the experiments described in this thesis. Attention was focused on intraperitoneal (i.e. site-specific) administration of agents examined for tumor immunotherapeutic effects. In general, immunotherapy started three days after tumor inoculation. Experiments in which tumor cells are given together with cytotoxic cells as e.g. the Winn-assay seem even more artificial.

9.2 Requirements for successful immunotherapy

In the experiments described in Section 5.3, it was seen that the combined administration or LAK cells and IL-2 consistently led to a much lower tumor burden as scored by the peritoneal carcinomatosis index (PCI) than in control animals. LAK cells alone were not effective. IL-2 alone showed intermediate results.
The biological significance of the PCI is supported by the parallel results in survival experiments. Treatments that reduce the PCI similarly prolong survival (Section 5.4). As has been reviewed (Section 3.6) incubation in fetal calf serum per se can produce activated killer cells. It was examined if addition of IL-2 to the culture medium was necessary to obtain cells providing tumor reduction. Furthermore, it would be conceivable that in a model in which cells and IL-2 are injected in the same compartments, lymphocytes would undergo in situ activation into LAK cells, as it has been shown that syngeneic lymphoid cells, injected i.p. remain sequestered in the peritoneal cavity for at least 24 hours (Mathisen and Rosenberg, 1980). In addition the initial concentration of IL-2 in the abdominal cavity after exogenous administration is much higher than the concentration used for in vitro LAK production. Generation of LAK cells after short exposure to a high dose of IL-2 has been observed (Section 3.6.5).
For these reasons we compared fresh splenocytes (normal lymphocytes), cells cultured in complete medium without exogenous IL-2 (cultured lymphocytes) and cells cultured in complete medium to which IL-2 was added (LAK cells). Normal and cultured lymphocytes, administered in conjunction with IL-2 were not more effective than IL-2 alone and significantly less than LAK cells plus IL-2 (Table 5.2). It can be

concluded that generation of activated killer cells ex vivo by incubation with IL-2 is more effective than by incubation in xenogeneic serum only or by intraperitoneal IL-2 stimulation.
As seen in Section 8.6, the combined treatment of LAK cells plus IL-2 was effective in congenitally athymic mice. The same applies for mice, immunocompromised by sublethal total body irradiation (Mulé et al., 1985) and in adult, thymectomized, irradiated (at a lethal dose), bone marrow-reconstituted mice (Mulé et al., 1986a).
Taken together these results argue that in a patient with a depressed immune apparatus, therapy with LAK cells plus IL-2 is not automatically ruled out.

In early experiments that pointed to a potential role in tumor immunotherapy, cell-derived IL-2, often only partially purified, was used. Recombinant IL-2 is attractive for experimentation and possibly therapy due to its purity and its availability on a much larger scale. Use of recombinant IL-2 might have some specific limitations. Recombinant IL-2 is not posttranslationally modified as are natural preparations. In addition, it could not be ruled out that the effects seen with cell-derived products were in part based on remaining impurities. In vitro and some in vivo effects of recombinant IL-2 have been examined, and were thought to be similar to those of native IL-2 (Rosenberg et al., 1984; Roifman et al., 1985; and Doyle et al., 1985). However, we were not aware of experiments, directly comparing in vivo antitumor efficacy. If native IL-2 would have been found to be more effective, this should have prompted a search for potentiating cytokines present in the partially purified cellular derived IL-2 preparation.
However, no differences were found (Section 5.6) and it can be concluded that IL-2 of either source fulfills the requirements.

9.3 Site-specific versus systemic treatment

To adress the question of site-specific versus systemic treatment a set of experiments was conducted in which the mode of administration was varied.
Animals with i.p. tumor were treated with LAK cells i.p. and IL-2 i.p. (site-specific treatment) or with LAK cells i.v. and IL-2 s.c.

(systemic treatment). Further control groups consisted of the crisscrosses LAK i.p. + IL-2 s.c. and LAK i.v. + IL-2 i.p.. In addition the effects of IL-2 only given either i.p. or s.c. were compared. The results, as detailed in Table 5.3 show that with the doses of LAK cells and IL-2 used, only the site-specific approach was consistently beneficial. If one of the biologicals was given outside the peritoneal cavity, less tumor reduction was seen. One could entertain the idea that in this model IL-2 alone given i.p. would be more effective than IL-2 alone s.c. hypothesizing a population of LAK cell precursors resident in the peritoneal cavity that would be more efficiently driven to LAK cell formation by the i.p. administration. As seen in Table 5.3, no such differences were found.
The superiority of site-specific treatment is most readily explained by less effective trafficking of LAK cells administered intravenously and less effective sustaining of LAK activity when IL-2 is given in another compartment.

Site-specific treatment in the human situation is technically feasible. LAK cells can be given intraperitoneally via a Tenckhoff catheter, an approach that has been used for administration of activated macrophages (Stevenson et al., 1984). In treatment of hepatic metastases, cells could be administered via a catheter in the hepatic artery. Local treatment has been given intraoperatively after debulking procedures in patients with malignant glioma (Jacobs et al., 1986).
Steis et al. reported objective responses in four of five evaluable patients with peritoneal carcinomatosis following the local use of LAK cells and interleukin-2.

9.4 Amelioration of LAK cell treatment

In attempts to optimize treatment results, the dose of LAK cells, the dose of IL-2 and the dose fractioning of IL-2 were varied.
Increasing tumor control was seen with increased doses of LAK cells (Section 7.2). The maximal dose used was a suspension of 10^8 cells, containing LAK cells. It would be difficult to increase this dose markedly by extrapolation of the methods employed, as the number of spleen donors becomes prohibitive. When considering systemic administration of LAK cells, it should be noted that mice do not

tolerate much higher cell numbers at one time, when given i.v.
A possible approach would be a long term in vitro expansion of LAK cells possibly after cloning. Along this line, Ochoa et al. (1987) described methods for in vitro expension of LAK cells and for their activation by addition of other BRMS. An alternative approach is improving the 'homing' of LAK cells (Migliori et al., 1987).

In the intraperitoneal model, dose-response effects of IL-2, given in combination with LAK cells were less straight forward (Section 7.3). A dose of 50,000 U b.d. provided significant and consistent tumor reduction. Lower doses were less effective, higher doses did not provide better tumor control. In separate experiments (exp. 1 + 2, Table 5.1) doses of 10,000 U b.d. were quite effective. These apparent discrepancies in the dosimetry of IL-2 are explained by the inherent variability present in animal models and by the difficulties in standarizing the units of activity of IL-2, using a titration methodology based on twofold dilutions. (Different batches of IL-2 were used in the experiments in Table 5.1 and those in Table 7.1). When testing one batch, a dose-response effect seems to be present (Table 7.1).

In a model of 3 day-established intrapulmonary tumor, treated with i.v. LAK cells in conjunction with i.p. IL-2, Mulé et al., (1985) found the best tumor control with 30,000 U given three times daily. They did, in that experiment, not test higher doses.
Higher doses do result in a considerable morbidity and mortality in treated mice and dose reduction or dose withholding is sometimes necessary.

In Section 3.2 the fate of IL-2 after in vivo administration and the relatively short half life, approximate 2-3 hours after i.p. injection, were reviewed. The question arose, if increased dose fractioning would lead to a better tumor control. As seen in Section 7.4, dividing a total dialy dose of 100,000 U in four fractions given 6-hourly was more beneficial than two fractions given 12-hourly or one fraction given once daily.
Concurrent results were found by Cheever et al. (1985) who showed that frequent low dose injections of IL-2 were more effective than less frequent, high dose injections in inducing T cell growth in vivo.

Similarly, Donahue et al. (1984) in preliminary results, found an increased response to alloantigen with increased dose fractioning.
In experiments comparing viability and cytotoxicity of LAK cells, superior results were seen when a mini osmotic pump was compared with a single daily injection (Nishimura et al., 1986).

9.5 A possible mechanism by which IL-2 exposed LAK cells operate

In this section we will recapitulate some of the pertinent data on immunotherapy with LAK cells and IL-2, as discussed above, and formulate a hypothesis putting these data into perspective.

The combination of LAK cells and exogenous IL-2 reduces tumor load and prolongs survival, while LAK alone does not work and IL-2 alone gives a limited tumor reduction. Normal lymphocytes and cultured lymphocytes cannot replace LAK cells. Higher doses of LAK cells lead to increased tumor control. Site-specific treatment appears to be better than systemic treatment, and sustained low levels of IL-2 work better than short peak levels. Treatment with LAK and IL-2 is also effective in the immunocompromised host.

It is tempting to speculate along the following line. LAK cells traffic to the tumor cells and kill (part of) them. Exogenous IL-2, preferably at a continuous level, is needed to sustain the activity of the LAK cells and possibly to mediate their proliferation. IL-2 given by itself can lead to some in vivo LAK formation but at the maximal doses tolerated, it is difficult to achieve sufficient LAK activation.
There is now considerable evidence to support such a hypothesis.

In an elegant study, Ettinghausen et al. (1985b) transferred LAK cells to syngeneic and congenic mice and measured cell proliferation by in vivo labeling of DNA with a radioactive thymidine analog. IL-2 was found to promote the in vivo proliferation of transferred LAK cells. Substitution of irradiated LAK cells abolished this proliferative effect.
Similarly, Mulé et al. (1985) found that the in vivo antitumor capacity of LAK cells was significantly reduced or eliminated when gamma-

irradiated (3000 rad) before transfer. In contrast, this radiation dose did not alter the in vitro lytic capacity in ^{51}Cr release assays.
Taken together, these studies suggest that IL-2-mediated in vivo proliferation of transferred LAK cells occurs and is important for the antitumor effects.
Systemic administration of IL-2 alone leads to some in vivo LAK formation from the endogenous lymphoid cell pool (Ettinghausen et al., 1985a). This effect is not seen in pre-irradiated mice. Similarly in some models an antitumor effect has been seen from high dose IL-2 administration without transfer of donor LAK cells (Rosenberg et al., 1985a).

In a host, immunocompromised by sublethal total-body irradiation or by T cell depletion, treatment with IL-2 only is not successful, but treatment with LAK cells plus IL-2 is (Section 8.6; Rosenberg et al., 1985a; Mulé et al., 1985, 1986a). This is what one would expect as treatment with IL-2 only, requires participation of host components in contrast to treatment with LAK cells plus IL-2.

IL-2 toxicity has been reviewed in Section 3.7. It was seen that continuous infusion is less well tolerated than intermittent bolus injections. In Section 7.4, it was seen that increased dose-fractioning had better antitumor effects than intermittent bolus injections.
The possibility that LAK cells have some cytotoxicity to normal cells has been discussed in Section 3.6.2.
Taken together, this argues that the toxicity seen with IL-2 might be due to a (low level) reactivity of LAK cells to normal body cells.

9.6 Versatility of treatment with LAK cells and IL-2

As stated in Section 2.6, one of the problems with previous attempts of immunotherapy has been the raising of cells specifically reacting with tumor cells. As seen in Section 3.6.2, a single LAK cell displays in in vitro tests a 'polyspecific' cytotoxic reaction to a variety of tumor cells while leaving normal syngeneic cells (relatively) uninjured. The mechanism of recognition is not known.

In vitro reactivity to target cells does not necessarily translate in in vivo effectiveness (Section 8.7; Rosenstein and Rosenberg, 1984). It was thought of interest therefore, to examine a panel of tumor cells for their susceptibility to immunotherapy with LAK cells.
Seven tumors in three different mouse strains were successfully treated with LAK cells plus IL-2. Tumors of varied histology, solid tumors and an ascitic line, immunogenic and nonimmunogenic tumors were all similarly reduced. In view of the poor immunogeneity of most spontaneous human tumors, the susceptibility of the nonimmunogenic tumors MCA-101 and MCA-102 is of particular note.

We hypothesized that tumors bearing the IL-2 receptor might be stimulated by IL-2 administration. The EL-4 tumor is known to bear the IL-2 receptor (Farrar et al., 1982), although it is not known if all the several sublines do so. EL-4 bearing hosts, treated with IL-2 showed an increase in number of tumor cells and a decreased survival as compared to control animals (Section 8.3). The results argue for a stimulation of proliferation mediated by the exogenous IL-2. These results should lead to caution when considering IL-2 protocols in patients. Transfected B cells (Korsmeyer et al., 1983) and T cell leukaemias (Lando et al., 1983) may produce the IL-2 receptor.

Our results are at variance with those of Nishimura et al. (1986) who successfully treated EL-4 bearing mice by a combination of LAK cells and slowly released IL-2. It was not stated whether the line they used, displayed the IL-2 receptor.

The tumor immunotherapy approach under study is effective, not only in the intraperitoneal compartment but also in models of pulmonary and hepatic inoculation and in subcutaneous tumor (Sections 8.4 and 8.5; Mulé et al., 1984).

It was found that LAK cells derived from spleens from normal donors, donors about to succumb to tumor growth in their hind leg and donors in whom such a tumor was previously resected are equally effective in this i.p. model. Concurrent results have been published by Hersey et al. (1981). They studied cytotoxicity induced by natural IL-2 against a panel of melanoma cells. They found no difference between lymphocytes from melanoma patients and from normal subjects. In contrast, Balch et

al. (1985) found decreasing LAK activity correlated with increasing clinical stage of melanoma patients. These different conclusions are not readily explained.
Mazumder et al. (1983) could easily generate PHA-activated killer cells from peripheral blood lymphocytes from patients with a variety of malignancies.

9.7 Mechanism by which tumor cells escape immunotherapy with LAK cells and IL-2

Treatment with LAK cells plus IL-2 has a theoretically attractive basis. As discussed above, it prolongs survival and reduces tumor growth in a series of tumors in a variety of locations. However, the PCI is not usually 0, and neither are animals cured in survival experiments.

One could hypothesize that tumor cells escaping this form of tumor-immunotherapy have become LAK resistent. Tumor heterogeneity with diversities including susceptibility to non-LAK-immunotherapy has been well documented (Bosslet and Schirrmmacher, 1982; Uyttenhove et al., 1983).
Mulé et al. (1986b) tested this hypothesis. Tumor cells from pulmonary foci that had escaped treatment with LAK and IL-2 were lysed by LAK cells in ^{51}Cr release assays. When transferred i.v. to new hosts, reduction of pulmonary tumor by LAK cells and IL-2 could again be achieved. Similarly, melanoma cells that had survived two successive treatments with LAK in vitro remained as susceptible to LAK cells lysis in ^{51}Cr release assays as untreated melanoma cells.
Taken together these results argue against selection of inherently LAK cell resistent clones.

In the experiments presented in Section 7.2 it was shown that increasing the number of LAK cells given in a short period of time, increases tumor control. It was also seen that treatment with LAK cells and IL-2 starting on either day 3 or day 9 similarly prolonged survival (Section 5.4). However, multiple courses were not superior to a single course (Section 7.5) although more LAK cells were given and it is possible to treat older tumors. Taken together, these results argue

that counteracting mechanisms ensue after the first course of treatment.

Several possibilities may explain the present inability to eradicate all tumor foci.

1. A fraction of tumor cells (possibly those in a certain phase of their individual cell cycle) may not display the unknown determinant(s) by which LAK cells recognize it as 'non-self', whereas the progeny of these cells is again recognized. Such a mechanism would be reminiscent of 'antigen modulation' reviewed in Section 2.5.
2. LAK cells may not traffic efficiently enough to reach all tumor foci.
3. Serum inhibitors of IL-2 as reviewed in Section 3.5 may play a role (Eggermont and Sugarbaker, 1987).
4. Counteracting mechanisms, possibly antibodies to (absorbed antigens on) LAK cells (Eggermont and Sugarbaker, 1987) and/or IL-2 may negate the effects of repeated treatments. Alternatively, the presumed epitope recognized by LAK cells might be masked by host factors. Such a mechanism would be reminiscent of 'efferent enhancement' (Section 2.5).
5. Present protocols of LAK cells and IL-2 treatment may be suboptimal.

These possibilities are not mutually exclusive. Unless this problem is solved, it is unrealistic to expect real benefit in human trials.

9.8 IL-2 and LAK cell therapy in the human situation

In the above, a number of extrapolations of animal data to the human situation have been alluded to.
Lymphoid cells of tumor bearing hosts can be used to generate LAK cells. Alternatively in a host with a severe lymphopenia, allogeneic LAK cells could be considered.

LAK precursors can be obtained from the patient by lymphocytapheresis and after in vitro incubation with IL-2, LAK cells can be given back intravenously or in a site-specific manner. It seems that the more LAK cells are given in a first course, the more effective the treatment.

The number of LAK cells will be restricted by the techniques of harvesting lymphocytes and in vitro expansion. The dosis of IL-2 will be restricted by the toxicity of the drug.
The daily IL-2 requirement found to consistently reduce tumor in the animal studies presented here was 100,000 U or approximately $5x10^6$ U/kg/day, given in divided doses. In these and other experiments (Mulé et al., 1985) sometimes an effect was seen at lower doses. A treshold seems to lie around 750.000 U/kg/day, given in divided doses. This contrasts with a maximum dose reached in humans of 300.000 U/kg/day before limitation by toxicity (Rosenberg et al., 1985b).

Early human trials of administration of activated killer cells alone or IL-2 alone have shown no tumor regression (Mazumder et al., 1984; Lotze et al., 1985 a+b). [However, in more recent reports, regression of malignant melanoma in response to IL-2 alone was seen (Rosenberg et al. 1987; West et al., 1987)].
Based on data in the murine model, the combined administration of LAK cells and IL-2 is more likely to be efficacious. A report on 157 patients with metastatic cancer in whom standard therapy had failed shows a small number of objective responses (Rosenberg et al., 1987). In a subsequent letter to the editor it was stated that complete responses in 13 of 196 patients with metastatic cancer were seen and that 10 of 13 patients were still in complete regression at intervals ranging from 27 to 3 months (median 7.5) (Rosenberg, 1987).
This experimental treatment has in humans a considerable toxicity and morbidity. The expenses are so high, and the techniques of lymphocyte recovery and LAK formation so demanding that in its present form, it can not be considered for general use.
Notwithstanding the fact that in experimental models permanent survival has proven an elusive goal and notwithstanding the sobering results in human patients, the basic properties of LAK cells and IL-2 are so attractive that further research seems justified.
This research should focus on two points. Firstly on the mechanism by which tumor cells escape LAK cell immunotherapy (Section 9.7) and on methods to overcome this evasion. Secondly on ways of in vivo LAK cell formation obviating the need for cytapheresis and in vitro incubation. As reviewed, in vivo LAK cell formation is possible at high doses of IL-2 but this approach is limited by IL-2 toxicity. A possible approach would be a study of combinations of BRMs. Nishimura et al. (1987)

reported favorably on combining tumor necrosis factor and IL-2 in a murine model.

If these problems could be solved, immunotherapy might dramatically alter the prospects of the cancer patient.

Chapter 10

SUMMARY

Present treatment possibilities for cancer are insufficient for a large number of patients and new approaches therefore seem necessary. Human immunotherapy trials till sofar have shown only modest results. However, the vast field of tumor immunology is only very partly explored. Recent advances in basic immunology open up this field and enable us to unravel the underlying mechanisms (Chapter 1).

Immunotherapy is potentially attractive as it might be reactive especially to tumor cells, leaving normal tissues uninjured, might not require full immunocompetence of the host and can be combined with other treatment modalities.

In past human trials it was mainly attempted either to aspecifically stimulate the whole immune apparatus, or to raise cells reacting to tumor cells with immunologic specificity. The first approach usually had a very simplistic theoretical basis and the choice of agents tested was rather arbitrary. The second approach was frustrated by the poor expression of tumor-associated antigens (Chapter 2).

A cell type, designated activated killer cell, has been described that is cytotoxic to a variety of 'non-self' cells, including tumor cells. This reactivity does not require previous exposure to tumor cells and is not directed at tumor-associated antigens. Unmodified cells are not lysed in any notable degree. On this basis those cells are attractive candidates for consideration in immunotherapy (Chapter 3).

A dependable way of producing those cells is *ex vivo* incubation in interleukin-2 (IL-2). Cells so activated have been designated lymphokine activated killer (LAK) cells.
IL-2 is the name given to a factor discovered because it could maintain long term cultures of T cells. Later on it was found to mediate a variety of other effects among them the production of LAK cells. IL-2 may be produced by recombinant techniques (Chapter 3).

Because LAK cells were judged to be promising candidates for immunotherapy and as they could be easily obtained using recombinant IL-2, it was decided to systematically evaluate these compounds in animal experiments.
A model of peritoneal carcinomatosis was chosen as this entity is presently largely untreatable. Mice were injected i.p. with a dose of lethal syngeneic tumor cells and either followed for survival or evaluated for intraperitoneal tumorload at a predetermined interval. Treatment usually started three days after tumor inoculation when the tumor was established in the peritoneal cavity.

Experimental animals treated with LAK cells and IL-2 showed a marked retardation of tumor growth and a longer survival compared with controls. LAK cells alone were not effective. IL-2 only gave intermediate results. Non-LAK-lymphocytes did not confer benefit.
Site-specific treatment was superior to systemic treatment. IL-2 of different sources (native, recombinant) was similarly effective (Chapter 5).

Allogeneic and syngeneic LAK cells were equally effective. Efficient LAK cells could be raised from donors about to succumb to their tumor and from donors in whom a tumor had previously been resected (Chapter 6).

Optimal tumor reduction was seen at high doses of LAK cells. High doses of IL-2 are quite toxic. The best effects were seen from doses just under the toxic level. A continuous low level of IL-2 was more effective (and more toxic) than intermittent high levels. Repeat courses of LAK cells and IL-2 were not more effective that a single course (Chapter 7).
Immunotherapy with LAK cells and IL-2 was effective in a spectrum of tumors of different histology and immunogenicity and in different mouse strains. Intrahepatic and subcutaneous tumor could also effectively be treated. Similar effects were seen in immunocompetent and in immunodeficient mice (Chapter 8). After administration of LAK cells plus IL-2, tumor cells are killed by the LAK cells. IL-2 operates by sustaining the LAK cell activity. Administration of IL-2 alone leads to some in vivo LAK formation and therefore to some antitumor effect. Administration of LAK cells alone is not effective as the LAK cells are

not stimulated.

As reviewed in Chapter 9, human patients have been treated with IL-2 and LAK cells. Lymphocytes were removed by cytapheresis, incubated *in vitro* with IL-2 and given back in conjunction with IL-2. Experimental data suggests that LAK cells can be generated from tumor bearers and that immunotherapy with LAK cells can be effective in immunocompromised patients. Some antitumor effects were seen, but this treatment is still highly experimental.

Further research should concentrate on the problem of tumor escape and on methods to avoid the *in vitro* step by generating LAK cells *in vivo*.

Samenvatting

De thans ter beschikking staande behandelingsmogelijkheden van kanker zijn ontoereikend voor een groot aantal patienten. Innoverende benaderingswijzen lijken derhalve noodzakelijk. Pogingen tot immunotherapie zoals die in het verleden zijn ondernomen, hebben op zijn best heel bescheiden resultaten laten zien. Het uitgebreide gebied van tumorimmunologie is echter slechts zeer ten dele in kaart gebracht. De laatste tijd zijn er basale vorderingen gemaakt in de immunologie die het mogelijk maken het gebied van de tumor immunologie verder te verkennen en mechanismen die eraan ten grondslag liggen te ontwarren (Hoofdstuk 1).

Immunotherapie is potentieel aantrekkelijk omdat zij in het bijzonder werkzaam zou kunnen zijn tegen tumor cellen, terwijl normale cellen niet beschadigd worden. Voorts is wellicht geen volledige immunocompetentie van de gastheer nodig en kan deze therapie gecombineerd worden met andere behandelingswijzen.

In klinisch-experimenteel onderzoek werd voorheen voornamelijk geprobeerd ofwel op aspecifieke wijze het hele immuunapparaat te stimuleren, of cellen te produceren die met immunologische specificiteit reageren met tumorcellen.
De eerste benadering had gewoonlijk een heel simplistische theoretische basis en de keuze van de onderzochte stoffen was nogal willekeurig. De tweede benadering werd gefrustreerd door de geringe expressie van tumor-geassocieerde antigenen (Hoofdstuk 2).

Er is een cel beschreven, de zogenaamde geactiveerde 'killer' cel, die cytotoxisch is voor cellen die op enige manier van de normale lichaamscel afwijken ('non-self' cellen) waaronder tumorcellen. Het is hiervoor niet nodig dat de 'killer' cel eerder in contact is geweest met de tumorcellen en het mechanisme verloopt niet via de tumor-geassocieerde antigenen. Gewone lichaamscellen worden niet of nauwelijks beschadigd. Op grond van deze kenmerken komen killer cellen in aanmerking voor experimenten met immunotherapie (Hoofdstuk 3).

Een betrouwbare wijze om deze cellen te verkrijgen is ex vivo incubatie in interleukine-2 (IL-2). Op deze wijze verkregen cellen worden

lymfokine geactiveerde 'killer' (lymphokine activated killer) (LAK) cellen genoemd.
IL-2 werd ontdekt als een pharmacon dat celkweek van T cellen over langere perioden mogelijk maakte. Later werd gevonden dat IL-2 een verscheidenheid van andere effecten teweeg brengt, waaronder de produktie van LAK cellen. IL-2 kan gemaakt worden met behulp van recombinant technieken (Hoofdstuk 3).

Aangezien LAK cellen aantrekkelijk leken voor experimenten met immunotherapie en omdat ze makkelijk kunnen worden verkregen met behulp van recombinant IL-2 werd een systematisch onderzoek gedaan naar de effecten in het dierexperiment. Er werd gekozen voor een model van peritonitis carcinomatosa omdat dit ziektebeeld momenteel nagenoeg onbehandelbaar is.

Cellen van een syngene, lethale tumorlijn werden bij muizen intraperitoneaal ingespoten. Behandeling startte gewoonlijk drie dagen later, wanneer de tumor was aangeslagen. Er werd of gekeken naar overleving, of de intraperitoneale tumormassa werd op een tevoren vastgesteld moment bepaald.

Proefdieren die behandeld werden met LAK cellen en IL-2 toonden een minder snelle tumorgroei en een langere overleving in vergelijking met onbehandelde dieren. IL-2 alleen had wel effect doch minder uitgesproken. LAK cellen alleen waren niet effectief. Gewone lymfocyten konden de LAK cel niet vervangen. Toediening van de immunotherapie in het compartiment van de tumor gaf betere effecten dan systemische toediening. Verschillende IL-2 preparaten (natuurlijk, recombinant) hadden een vergelijkbaar effect (Hoofdstuk 5).

Allogene en syngene LAK cellen bleken even doeltreffend. Wanneer tumordragende dieren, die op het punt stonden dood te gaan ten gevolge van hun neoplasma of dieren bij wie de tumor in een eerder stadium was gereseceerd als donor werden gebruikt, werden werkzame LAK cellen verkregen (Hoofdstuk 6).

Optimale tumorremming werd gezien bij hoge doseringen LAK cellen. Hoge doseringen IL-2 zijn vrij toxisch. De beste resultaten werden verkregen met doseringen net onder het toxische niveau. Een voortdurende lage

spiegel IL-2 leek effectiever (en toxischer) dan intermitterende hoge spiegels. Herhaalde kuren LAK cellen en IL-2 waren niet effectiever dan een enkele kuur (Hoofdstuk 7).
Immunotherapie met LAK cellen en IL-2 was werkzaam tegen een verscheidenheid van tumoren van uiteenlopende histologie en immunogeniciteit en in verschillende muizenstammen. Intrahepatische en subcutane tumor kon eveneens op effectieve wijze behandeld worden. Deze vorm van immunotherapie was werkzaam zowel in immunocompetente als in immunodeficiente muizen (Hoofdstuk 8). Na toediening van LAK cellen plus IL-2 worden tumorcellen gedood door de LAK cellen. IL-2 onderhoudt de LAK cel activiteit. Toediening van IL-2 alleen leidt tot enige LAK cel vorming in vivo en derhalve tot enig antitumor effect. Toediening van LAK cellen alleen is niet doelmatig omdat de LAK cellen dan niet tot activiteit worden aangezet.

LAK cellen en IL-2 zijn gebruikt bij de behandeling van patienten, zoals besproken in hoofdstuk 9. Lymfocyten werden verkregen middels cytapheresis, in vitro geincubeerd met IL-2 en teruggegeven aan de patient in combinatie met IL-2. Experimenteel werk suggereert dat LAK cellen verkregen kunnen worden van tumordragende donoren en dat immunotherapie met LAK cellen effectief kan zijn in patienten met stoornissen van het immuunsysteem. Bij de patienten werd welliswaar enige antitumor activiteit gevonden, maar deze behandeling is nog zeer experimenteel.
Verder onderzoek dient zich vooral te richten op het mechanisme waarmee tumorcellen aan deze vorm van immunotherapie kunnen ontsnappen en voorts op methoden om de in vitro stap te vermijden en LAK cellen in vivo te genereren.

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