ENHANCEMENT AND ABROGATION

Modifications of Host Immune Status Influence IL-2 and LAK Cell Immunotherapy

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ENHANCEMENT AND ABROGATION

Modifications of Host Immune Status Influence IL-2 and LAK Cell Immunotherapy

THESIS

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ERNST PHILIP STELLER

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Manipulatie van de Immuun Status van de Gastheer Beinvloedt IL-2 en LAK Cel Immunotherapie

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TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
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PROF. DR. A. H. G. RINNOOY KAN
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For Santje

To Willemine, Ernst, Tjeerd, and Eerke

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LIST OF ABBREVIATIONS

ADCC	antigen-dependent-cellular-cytotoxicity
B-16	B-16 melanoma (H-2^b)
BCG	bacillus Calmette-Guerin
BL/6	C57black/6 mice (H-2^b)
C'	complement
C. parvum	*Corynebacterium parvum*
cpm	counts per minute
^{51}Cr	^{51}Chromium
Cs	Cesium
CSF	colony stimulating factor
CTL	cytotoxic T lymphocytes
Cy	cyclophosphamide
DBA/2	DBA/2J mice (H-2^d)
E. coli	*Escherichia coli*
EL-4	EL-4 Lymphoma (sulbine producing IL-2)(H-2^b)
E : T	effector to target ratio
FACS	fluorescence-activated cell sorter
FUdR	5-fluoro-2'-deoxyuridine
Gy	gray (100 rad)
H-2	locus on the major histocompatibility complex in mice
HBSS	Hanks' balanced salt solution
[^{3}H]	[^{3}H]-thymidine
i.c.	intracutaneous
i.p.	intraperitoneal
i.v.	intravenous
IFN	interferon
IL-1, -2, -3, -4, -5, -6	interleukin 1, 2, 3, 4, 5 , 6
^{125}IUdR	5-[^{125}I]-iodo-2'-deoxyuridine
tIUdR	'total' (tumor + intestine + mesenteric fat) ^{125}IUdR uptake
K	kilo (1,000)
LAK cell	lymphokine-activated killer cell
LU	lytic units
M-53	IL-2 dependent cell line
MCA-102 / -105	methylcholanthrene induced fibrosarcoma 102 / 105 (H-2^b)
MHC	major histocompatibility complex

MLC	mixed lymphocyte culture
(μ)Ci	(micro)curie
(m)M	(milli)molar
MPS	mononuclear phagocyte system
MVE-2	maleic anhydride-divinyl ether-2
NC cell	natural cytotoxic cell
NCI	National Cancer Institute
NIH	National Institutes of Health
NK cell	natural killer cell
P815	P815 mastocytoma ($H\text{-}2^d$)
PBL	peripheral blood lymphocyte
PBS	phophate buffered saline
PCI	peritoneal cancer index
PEC	peritoneal exudate cell
s.c.	subcutaneous
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
TCGF	thymocyte cell growth factor
TIL	tumor infiltrating lymphocyte
TMF	thymocyte mitogenic factor
TNF	tumor necrosis factor
TSF	thymocyte stimulating factor
U	unit

PREFACE

Surgery plays a central role in the treatment of solid tumors. It is in treating the sequelae of systemic spread, metastatic disease, inoperable locations and residual tumor that the team approach of medical oncologist, radiotherapist and surgeon made some progress in prolongation of survival and disease free interval. Colo-rectal cancer is one of the leading causes of cancer related deaths in the Western World. It is with this form of cancer as an example that we will discuss the present treatment options and new developments.

Colo-rectal carcinoma is a cancer that is treated primarily by surgical excision. For recurrence, be it distant or loco-regional, no other adequate answer is available that will either cure patients, prolong disease free interval or increase survival in a majority of cases.

Chemotherapy with 5-Fluorouracil (5-FU) alone or in combination with Methyl-CCNU or Mitomycin-C has a dismal response rate of ± 20 % and a marginal benefit on survival.[1] Although initially reduced recurrence was reported when 5-FU was given into the portal vein as an adjuvant directly after the primary resection in patients with stage Dukes B and C colon cancer, no significant long term effect could be detected.[2]

Radiotherapy might be of value before and / or after resection of rectum cancers and may have some beneficial palliative effect on inoperable pelvic recurrence and bone metastases.[3] However, in view of the short and long term radiation damage to the susceptible intestinal mucosa, it is not an option for primary or adjuvant treatment of intra-abdominal colon cancers.

Although colo-rectal cancer therapy has not progressed much, fine-tuning as well as combination of different treatment options might result in an increased disease free interval and prolonged survival in the future. There might still be hope for 'the one magic cancer cure'; however, reality has shown us to aim at the 'combined effort approach'. Once systemic disease has been detected, the treatment approach should take into account the natural history of the disease[4] as well as the failure patterns.[5] In the case of colo-rectal cancer, therapy of systemic disease should be directed either towards distant metastases or tailored towards the loco-regional recurrence and / or metastases. The latter can be divided in 1. liver metastases, 2. resection-site recurrence and 3. peritoneal seedings. Although our tools have not improved yet, a multi-modality therapy based on careful staging of the patient and knowledge of the natural course of the disease will make better use of

the advantages of surgical, chemotherapeutic and radiotherapeutic treatment modalities, while taking into account the disadvantages.[6]

Liver resection for 1-4 uni- or bi-lobar metastases with post-operative intraperitoneal (i.p.) or intraportal adjuvant chemotherapy[7 8 9] may prolong survival. Adjuvant (post-operative) 5-FU instillations in the abdominal cavity after primary resection will be less toxic for the mononuclear phagocyte system (MPS) and the susceptible mucosa of the gut, while much higher loco-regional levels can be reached. This loco-regional treatment strategy did change the recurrence pattern in a prospective randomized trial.[10]

Peri-operative radiotherapy to the rectum and pelvic area may increase operability and post-operative disease free interval and survival,[11] while damage to the gut epithelium and MPS is minimized.

The toxicity of chemo- and radiotherapy for the MPS and bone marrow will always result in suppression of the immune system of the host.[12] Surgery, and peri-operative factors like blood transfusion, too, act by themselves as a suppressor of immune functions.[13 14 15 16] This in addition to the more or less suppressive effects the tumor has on the immune system. All of this results in a poor functioning immune system at a time when circulating tumor cells and microscopic tumor deposits should be recognized as 'altered self' and should be destroyed by an optimally functioning immune system.[17]

Modern immunology has not only learned us more about the pathways through which the immune system works and is stimulated or suppressed, but also brought us the technology to produce the factors through which the immune system can be regulated: the so called 'biological response modifiers'. Recombinant DNA techniques made interferon, interleukins 1,[18] 2,[19] 3,[20] 4,[21 22] 5,[23 24] 6,[25] tumor necrosis factor,[26] colony stimulating factors[27] and others, available in apparent homogeneity and in large enough quantities as to enable *in vitro*, and *in vivo* experiments as well as phase I and phase II clinical trials on a large scale.[28]

Recently, immunotherapy of cancer has received much attention. Commercially available human recombinant interleukin 2 (IL-2) activates leukocytes into 'omnipotent' lymphokine-activated killer (LAK) cells *in vitro* and *in vivo*. The immunotherapeutic regimen consists of daily injections of interleukin 2 with or without the adoptive transfer of *in vitro* activated LAK cells.[29] In laboratory models[30 31 32] significant cancer reduction and prolongation of survival was demonstrated. In phase I and II clinical trials[29 33] the maximal tolerable dose was determined and clinical effects were demonstrated.

For a cancer to be detected, usually years have passed since the first mutant cell was able to escape the normal control mechanisms of the host. Thus it can

hardly be expected that mere stimulation of the killer cell population of the host can overcome the profoundly suppressed immune system and can do away with a large volume of cancer cells. As the past has proven, single treatment options will not bring definitive cure in most instances. The effects biological response modifiers and activated cells have shown on cancer may ultimately give immunotherapy a definite place in cancer therapy as the 'fourth modality'. In this way multimodality therapy might increase cancer control and overall survival.

IL-2 will induce activated killer cells and cancer cells may be lysed by these activated killer cells. In homeostatic systems negative and positive feedback will keep the system balanced. With exogenous IL-2 the aim is not only to restore the balance of the suppressed immune system, but also to wipe out the uncontrolled cancer cells. This overshoot will have toxic side effects in the host, and will also have side effects on the host immune system. Since the toxicity of IL-2 is prominent, we looked for ways to reduce the IL-2 doses and to increase its immunotherapeutic effects. In this thesis we investigated how the immunotherapeutic effect of IL-2 and LAK cells can be enhanced or abrogated by manipulating the host immune system. This was done in a study using mice as experimental animals. Specific activation of the immune system can boost endogenous IL-2 production and produce other cytokines and helper cells loco-regionally. However, this dynamic process of specific activation may later on produce cells that compete for IL-2, as well as suppressor cells. These suppressive effects can be 'selectively' wiped out by low dose cyclophosphamide thus restoring the immunotherapeutic effects of IL-2 and LAK cells.

By optimizing immunotherapy of experimental animals with IL-2 and LAK cells, especially loco-regionally, this fourth modality will find its place in multimodality cancer therapy of patients. In colo-rectal cancer, immunotherapy might be the ideal modality to restore the immune system after surgical resection of the primary or liver metastases.[34] It could prove the ideal therapy for local control of residual tumor cells in the abdomen and in the liver after chemotherapeutic 'priming' of the host. Furthermore, immunotherapy could be used as an adjuvant to chemotherapy for distant metastases.

In this thesis we will show the delicate influence the host immune status has on the outcome of immunotherapy with interleukin 2 and lymphokine-activated killer cells.

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Chapter 1

GENERAL INTRODUCTION

1.1 The immune system

Man and animal in general are rather stable functioning entities. The stability is preserved by different (homeostatic) systems that maintain an equilibrium between the outer and the inner world. The basis for these systems is laid down in the DNA sequence of the different chromosomes. Through expression of certain sequences during embryologic and fetal life, cells will be committed to special functions, organs and systems. One of these systems is the immune system,[1] developed to control invading organisms and substances. Before control is possible the invading organism or protein has to be recognized and distinguished from man's own cells and proteins as to prevent 'self' destruction. The immune system takes charge of these tasks through recognition of antigens and reacting to these antigens with - humoral and / or cellular - immune responses. These responses are made up by the different cells within the immune system of the host and a complicated interactive regulating system.

We will briefly discuss the parts that comprise the immune system:

1. The humoral immune response is effected by immunoglobulins IgA, IgG, IgM, IgD and IgE. These five different Ig classes are secreted by plasma cells in response to antigens. B cells, produced in the bone marrow, are the precursors of plasma cells and recognize the antigens by surface membrane immunoglobulins.
2. The cellular immune responses are effected by:

a. T cells - T for thymus which is their site of origin. These T cells have helper (induction), specific-cytolytic, suppressor and killer cell functions.

b. Macrophages that play an important role in the afferent limb of the immune response. In an active state these cells are responsible for antigen presentation, delayed hypersensitivity, non-specific tumor cell lysis and destruction of intracellular pathogens.

3. Cytokines like interleukin 1 - 6, interferons, tumor necrosis factor, colony stimulating factors, growth factors and many others constitute regulatory elements affecting the different cells of the immune system as well as 'self' and 'non-self' cells.

1.2 Tumors and the immune system

1.2.1 *Immune surveillance*

In order to safeguard against 'self' destruction from within, the system should control for erroneous cells arising from - either spontaneous or induced - mutants within the basic DNA structure and escaping normal growth and specialisation patterns.[2] The pathways through which this immunologic surveillance system is activated are not yet clearly understood.

1.2.2 *Tumor associated antigens*

Tumor cells are mutant cells that escape the normal regulatory mechanisms of cell growth, but still have the same genetic basic structure as other host cells. Although they may express a variety of oncofetal-, lineage-associated-, differentiation- and histo-compatibility antigens, these antigens are also associated with various non-malignant tissues.[3] As mentioned above the system is safeguarded against self destruction and normally no immune actions are undertaken against these common antigens on tumor cells. Consistent with this is the fact that only very few spontaneous tumor cells will elicit a specific immune response by the host' own immune system. In devising experimental models thus care should be taken to select non-immunogenic, 'spontaneous' tumors in order to mimic the clinical situation as much as possible.
When tumor cells do express non-common antigens the elicited specific immune response may be suppressed by these tumor cells through ways not yet clearly understood. Oncogenes are probably present in the cellular DNA sequence and in that state referred to as proto-oncogenes. They need quantitative and / or qualitative changes - for instance by viruses or carcinogens - that affect expression and thus lead to malignancy.

1.2.3 *Specific and non-specific immunotherapy*

As mentioned above, tumor cells may express antigens common to normal cells at some stages of differentiation. Expression on tumor cells may be abnormal at that stage of development or abnormal in number. Since these antigens are not recognized as 'non-self', they elicit no specific immune response. Only few tumor cells express true tumor associated antigens and are thus susceptible to lysis by specific immune responses. Specific-cytotoxic T lymphocytes (CTL) lyse antigen

presenting cells and only cells with this antigen. Besides these CTLs, T-helper cells and antibodies also mediate the antigen-specific immune responses.[4] 'Dormant' (memory) CTLs are committed T cells in a resting state after repeated antigenic challenges (vaccinations) and are capable of quick and effective immune responses after re-exposure to the antigen.

Besides the specific T cell cytotoxicity the immune system provides for a subset of non-specific cytotoxic cells. This group consists of (natural) killer cells[5] that can lyse tumor targets without previous exposure to tumor cells or their antigens. These killer cells exhibit a broad specificity: a variety of syngeneic, allogeneic and even xenogeneic targets are lysed, although not all tumors are equally susceptible. *In vivo* and *in vitro* the killer cells can be activated by interferon, interferon inducers, immunostimulants such as BCG and *Corynebacterium parvum* (*C. parvum*) and by interleukin 2 (IL-2).

Macrophages, too, are part of the group of non-specific immune competent cells. Gamma interferon will activate macrophages.[6] Activated macrophages can produce interleukin 1 that will mediate tumor cytostasis either directly or through activation of T and B cells.[7] Macrophages may slow down T lymphocyte proliferation and suppress natural killer (NK) cell activity.[8] Possible mediators for these suppressive effects of macrophages may be prostaglandins. Figure 1.1 gives a simplified schedule for the cellular and cytokine interactions aimed at killing the cancerous cell.

1.2.4 *Active and passive immunotherapy*

Active nor passive immunotherapy so far has yielded great results. Passive immunotherapy - transfer of antibodies or mononuclear cells - may be feasible in the experimental setting; in man syngeneic, passive immunotherapy on a large scale is not possible because of the diversity of tumor associated antigens and for ethical reasons. Antigen-specific immunotherapy through vaccination with tumor extracts has not proven effective either. Tumor cells usually are low in antigenic capacity, the host' immunocompetence is low or suppressed and the tumor burden is high.

Other means of immunotherapy are non-specific stimulation of killer cells and macrophages and arrest of the general suppressive effects of suppressor T cells. Non-specific immune modulating agents are: BCG, *C. parvum*, endotoxin, levamisole, allogeneic immunization and prostaglandin inhibitors (aspirin, indomethacin), to name a few. Although systemic, loco-regional or locally increased immune activity of one sort or another can be measured, depending on the route of administration; no major breakthroughs in cancer control were reported yet. The same is true

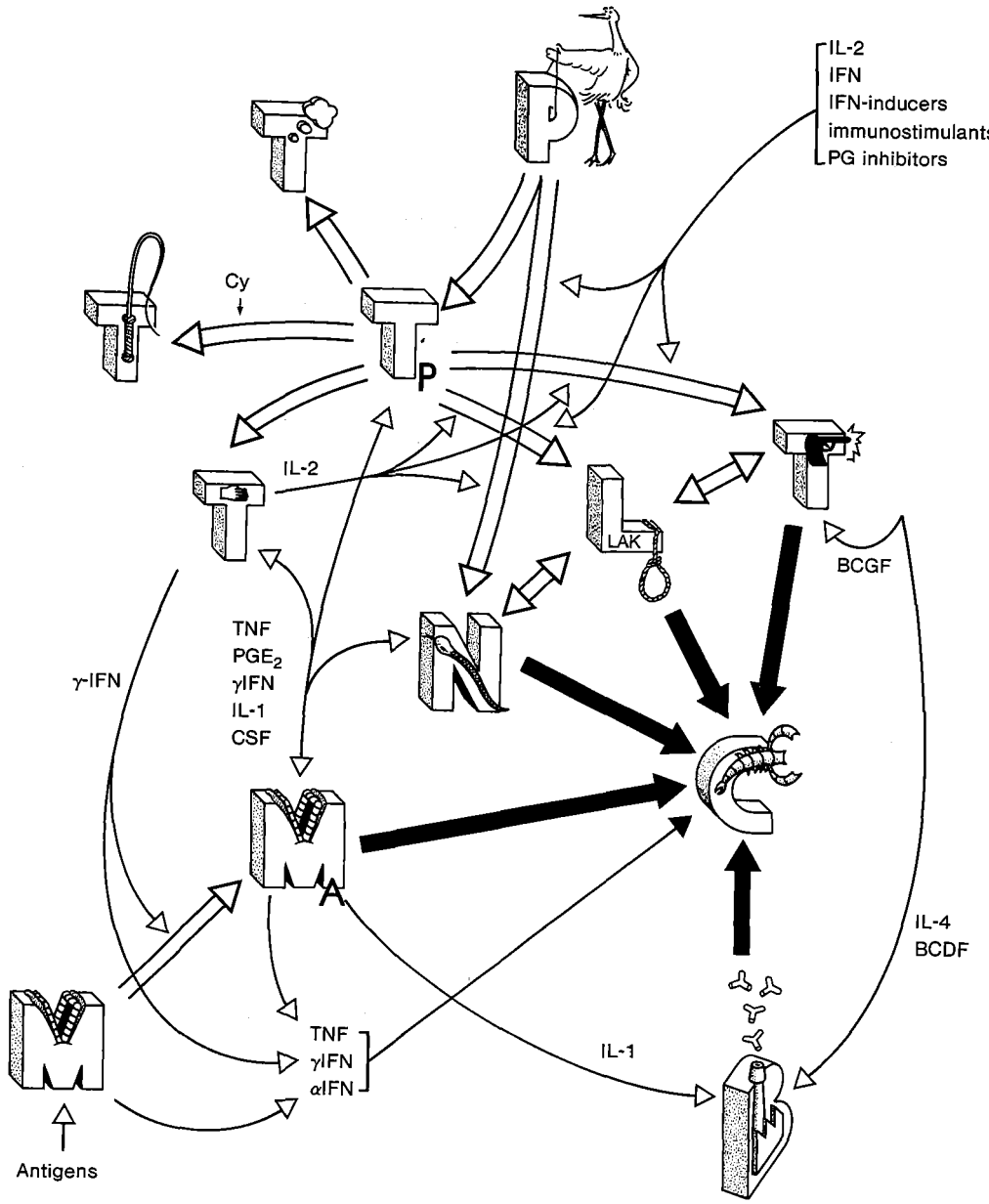
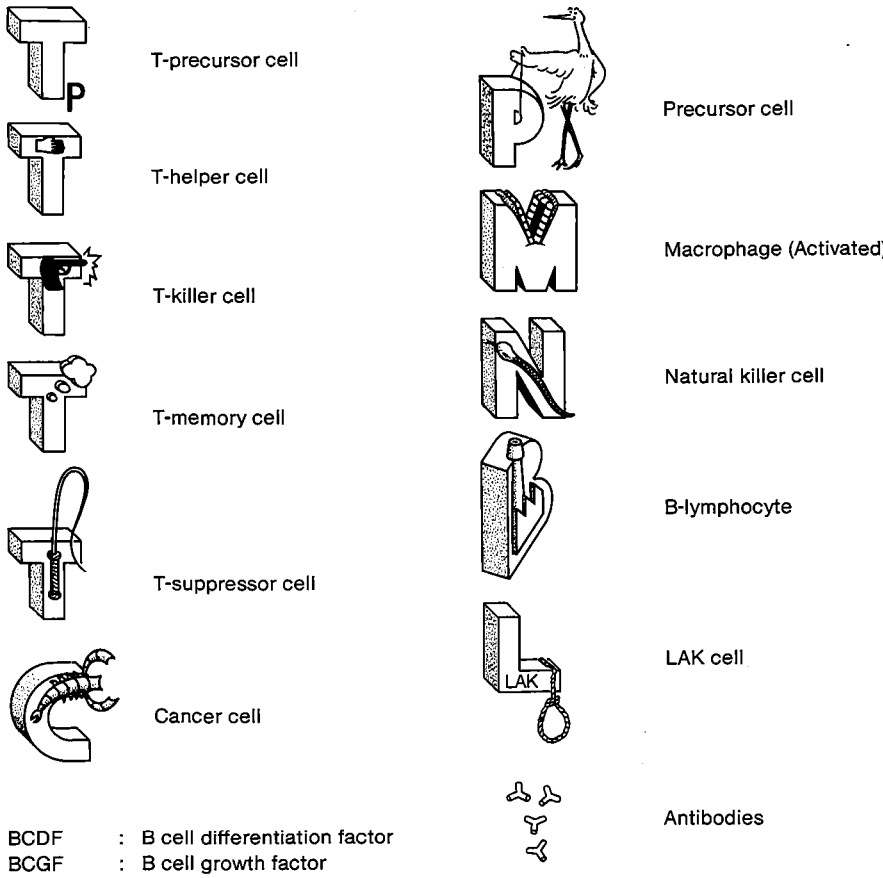


Figure 1.1 A simplified schedule for the cellular and cytokine interactions aimed at death of the cancer cell.

Legend to Figure 1.1



BCDF	:	B cell differentiation factor
BCGF	:	B cell growth factor
CSF	:	Colony stimulating factor
Cy	:	Cyclophosphamide
α, γIFN	:	α, γ Interferon
IL-1, -2, -4	:	Interleukin 1, 2, 4
PG E_2	:	Prostaglandin E_2

for the interferon inducers like poly A:U, poly I:C and MVE-2.

Although cyclophosphamide (Cy) is known to reduce 'selectively' suppressor T cells; a lasting arrest has not been achieved. If reduced immune competence by suppressor T cells is responsible for uncontrolled tumor cell growth, prolonged functional inhibition of suppressor T cells is required. Even then it is hardly conceivable that arrest of suppressor T cells is sufficient for cancer cure without the help of other treatment modalities.

Recently recombinant DNA technology has provided us with the tool for

biosynthesis and subsequent mass production of an increasing number of biological response modifiers. These lymphokines / cytokines are molecules secreted by a variety of cells and provide one means through which the cells involved in the immune process 'communicate' with one another and direct the overall process.[9]

1.3 Interleukin 2 (IL-2)

Chen and Di Sabato[10] were the first to describe a murine spleen cell product that stimulated thymocytes: TSF, thymocyte stimulating factor. Others used different names for this factor: thymocyte mitogenic factor (TMF),[11] T cell growth factor (TCGF),[12] co-stimulator,[13] killer cell helper factor (KHF)[11] and secondary cytotoxic T cell-inducing factor (SCIF).[14] In 1979 most of these researchers met at the Second International Lymphokine Workshop in Ermatingen, Switzerland and decided on a revised nomenclature and proposed the name interleukin 2.[15] This IL-2 has a molecular weight of 30-35,000; an iso-electric point between 3.5 and 5.5; is mercapto-ethanol insensitive and does not contain Ia receptors. Interleukin 2 production requires T cells and macrophages; its activity is MHC unrestricted; it promotes and maintains *in vitro* long-term cultures of T cells[12] and induces primary cytotoxic T cell responses.[13 16 17]

In 1983 Taniguchi *et al*[18] isolated a human IL-2 complementary DNA clone from the Jurkat cell line and determined its nucleotide sequence. Rosenberg and others described the complementary DNA clone of the gene for IL-2 from the Jurkat cell line as well as from normal human peripheral blood lymphocytes.[19] Both cell lines were induced for IL-2 production by the mitogens phorbolmyristate acetate and phytohemagglutinin. After insertion in *Escherichia coli* (*E.coli*) IL-2 was expressed at high concentrations and purified to apparent homogeneity. Analyses on sodium dodecyl sulfate (SDS) gels showed the production of a new protein by the *E. coli* bacteria with an approximate molecular weight of 15,000 daltons, as opposed to the 30-35,000 daltons described by Aarden *et al* for IL-2.[15] This difference probably is accounted for by the reversible denaturing of SDS.[16] The endotoxin level was routinely less than 0.1 ng per 10^5 units of IL-2, as measured in a standard limulus assay. This recombinant human IL-2 is active *in vitro* and *in vivo*; in murine as well as in human cell lines; and in murine models as well as in man.[20 21 22]

In vitro IL-2 acts as helper factor in B and T cell responses, augments the generation of cell-mediated cytotoxic T lymphocytes;[17] maintains longterm proliferative and cytotoxic cell cultures;[23] stimulates lymphokine-activated killer cell activity;[24] and mediates the recovery of the immune function of lymphocytes in

selected immunodeficient states.[12]

In vivo IL-2 will enhance natural killer cell function; augment alloantigen responsiveness;[25] improve the recovery of immune function in acquired immunodeficient states;[22] induce immune functions in nude mice;[26] and mediate anti-tumor effects when administered in conjunction with immune lymphocytes.[27]

The production of large amounts of purified, recombinant IL-2 has intensified research into its role as a regulator in the immune response. These recombinant DNA techniques also enabled animal experiments testing anti-tumor activities of IL-2 on a large scale. Finally, phase I and II trials are underway in patients unresponsive to standard therapy.[20 28]

Toxicity studies in rats[29] revealed no abnormal serum chemistries or organ pathology after intermittent intravenous (i.v.) bolus injections of recombinant IL-2 of doses up to 10^6 units/kg every other day for 2 weeks; nor with continuous i.v. infusions of IL-2 at doses of $\leq$ 3,000 units/kg/day. Continuous infusions of IL-2 at doses > 10,000 units/kg/day were lethal to the rats. At autopsy hepatocellular necrosis with mononuclear cell infiltration, lymphocytic depletion of the thymus and lymphoid infiltrations in liver, lung and spleen were seen.

In man dose limiting toxicity was reached with doses of 10^6 units/kg as a single bolus or 3,000 units/kg/hr as a continuous infusion via the i.v. as well as via the intraperitoneal (i.p.) route.[30] Toxicity consisted of pronounced weight gain (> 20% weight gain over pretreatment weights) due to fluid retention, mild anemia, thrombocytopenia, marked reversible eosinophilia, and minimal renal and hepatic toxicity. Patients experienced dose related fevers, chills, malaise, arthralgias, and myalgias.

1.4 Lymphokine-activated killer (LAK) cells

Natural killer (NK) and natural cytotoxic (NC) cells will lyse tumor targets without previous exposure to tumor cells and their antigens. Although cultured human tumor cell lines have been shown to be susceptible to lysis by NK cells and spontaneous cell-mediated cytotoxicity,[31] the lysis of fresh, uncultured human tumor cells been published only incidentally. Rosenberg and co-workers[32] reported the *in vitro* activation by IL-2 of peripheral blood lymphocytes (PBL) from cancer patients into cytotoxic cells and the lysis of fresh autologous tumor cells in short term ^{51}Chromium (^{51}Cr) release assays in > 90% of cancer patients tested. This NCI research group was convinced it had discovered a unique, non-specific cytolytic cell system distinct from the NK and CTL cytolytic cell systems: These IL-2-activated cytotoxic cells would kill NK-resistant, fresh and cultured tumor targets;

and this activation was interferon independent. And also because there was the suggestion that the precursor of this cytotoxic cell was neither a monocyte nor a NK cell and did not express the OKM-1 monocyte / NK marker. They called this IL-2-activated peripheral blood lymphocyte: lymphokine-activated killer (LAK) cell,[24] which term has found wide acceptance by now. LAK cells can be generated from normal individuals as well as from tumor-bearing patients.

Initially the LAK cell was considered a unique cell: bearing T cell markers and differing functionally from NK cells based on the broader target cell spectrum and the different kinetics of response to IL-2.[33] Further research phenotyped the LAK cell precursor better and elucidated the LAK cell phenomenon (1.4.1-2). So far the reported experimental and clinical results of immunotherapy with IL-2 and LAK cells were achieved with the adoptive transfer of *in vitro* activated, non-selected splenocyte or PBL populations. Discernment of the most pertinent population of cancer-directed killer cells might lead to significant improvement of clinical responses and might result in elimination of side effects associated with administration of high cell inocula.

1.4.1 *The LAK cell precursor*

Much controversy exists about the LAK cell precursor: is it 'NK- like' or of the T cell lineage? Andriole *et al*[34] underscore the differences between the NK cell and the LAK cell precursor: LAK cells can be generated from NK-deficient strains of inbred mice and certain LAK-deficient strains show fresh NK activity. Others find no or minimal LAK activity generated by IL-2 from peripheral blood mononuclear populations depleted of NK cells.[35] In mice the Lyt-2$^-$, L3T4$^-$, ASGM-1$^+$ lymphocyte subset, 98% purified by monoclonal antibody treatment, gave rise to both augmented NK and LAK cell activity when stimulated with IL-2.[36] Ballas *et al*[37] fractionated normal spleen cells on a Percoll density gradient and looked at two fractions giving rise to LAK activity when stimulated with IL-2. In fraction 3, which is enriched for NK cell activity but depleted of the ability to generate cytotoxic T lymphocytes, the precursors were Thy-1$^-$, Lyt-2$^-$, ASGM-1$^+$ ('NK-like'). Fraction 5 with precursor phenotype Thy-1$^+$, Lyt-2$^+$, ASGM-1$^+$ ('T-like') had no NK activity, but was enriched for the ability to generate CTL. The precursors generating LAK cells cytolytic for 2,4,6-trinitrobenzene-sulfonic-acid-modified autologous lymphoblasts had the Thy-1$^+$, Lyt-2$^+$, ASGM-1$^+$ phenotype in both fractions . Both authors find a faster LAK cell induction from the NK enriched population (± 24 hours) than from the 'T-like' population. This in contrast with Lotzová[38] who needed 2 weeks at least for induction of IL-2-activated NK-cell-

mediated anti-leukemia effects. Binding cells with a specific antigen with a monoclonal antibody and complement leaves only the cells without these specific binding sites for analyses. Separation of both antigen-negative and antigen-positive cells by fluorescence-activated cell sorter (FACS) and testing for activity circumvents this problem. Yang *et al*[39] could induce LAK activity from Thy-1,2$^-$ spleen cells and hardly any from Thy-1,2$^+$ splenocytes. Treatment of splenocytes with anti-ASGM-1 heterosera and complement markedly decreased their ability to generate LAK activity. They thus concluded that the precursors of murine LAK cells are 'null' lymphocytes bearing neither T nor B cell surface markers. Contrary to this is the finding that LAK activity can only be induced from FACS sorted CD5$^-$ human PBL (predominantly CD16$^+$ NK cells), and not from CD5$^+$ T lymphocytes.[40]

These data together lead us to conclude that IL-2 can activate 'T-' and 'NK-like' precursors into non-specific killer cells, be it of a classical NK- or LAK-nature. The role of co-factors and -cells as well as the phenotypic characterization of the tumor target cell remains to be elucidated.

1.4.2 *The LAK cell effector*

The LAK cell will kill autologous and allogeneic human tumor cells - irrespective of site or kind of cancer - without cytolysis of normal PBL *in vitro*.[41] The LAK cell effector, too, was considered a unique, newly discovered cell type by the fact that it would lyse fresh, NK-resistant tumor cells in a short (4 hour) ^{51}Cr release assay. Characterization of the effector cell and cytotoxicity of the IL-2-activated NK cell for a wide range of tumor cells complementary to LAK cell susceptibility has cast doubt on the unique features of the LAK effector cell. FACS analysis *in vitro* showed the LAK effector cell to be Thy-1.2$^+$, Ia$^-$, gamma Fc receptor$^+$ and predominantly Lyt-2$^+$.[39] Kalland *et al*[42] showed by negative (antibody and complement) and positive (FACS) selection that 40% of the spleen-derived IL-2-activated effector cells were NK-1$^+$ and ASGM-1$^+$ ('NK-LAK') and 60% expressed the T cell marker Lyt-2, lacking the NK-1 marker, and expressing only small amounts of ASGM-1 ('T-LAK'). Cells with potent LAK activity against fresh tumor targets *in vitro* were identified in the lungs of IL-2-treated mice and showed the Thy-1$^+$, L3T4$^-$, Lyt-2$^-$, ASGM-1$^+$ phenotype by flow cytometry analysis.[43] These authors also found a difference in effector phenotype: ASGM-1 depletion *in vivo* before the onset of IL-2 administration eliminated the successful therapy of 3-day pulmonary metastases from non-immunogenic sarcomas; while Lyt-2$^+$ cells were the predominant effectors in the elimination of both pulmonary micro- and macro-metastases from weakly immunogenic sarcomas.

In the human situation some T lymphocytes may develop LAK activity, but purified blood T lymphocytes do not develop LAK function when cultured with IL-2 alone.[40] Especially cells with low density expression of CD8 and no expression of CD4 were enriched for LAK effector cells.

To conclude we must say evidence is accumulating that there is no unique LAK cell phenotype; LAK seems to be a phenomenon - a function of the cells - and may be mediated by various effector cell populations. In this thesis the term LAK cell is used operationally to refer to the IL-2-activated lymphocyte population; be it 'T-' or 'NK-like' in precursor or effector phenotype.

1.4.3 *The LAK cell: induction*

Interleukin 2 will induce LAK cells *in vivo*,[44 45] as well as maintain the activity of adoptively transferred LAK cells and induce their proliferation *in vivo*.[46] The *in vitro* induction in complete medium needs 3-7 days for peak LAK activity; although an IL-2 pulse for 1 hour and continued culture in complete medium thereafter also induces - less potent - LAK activity.[47]

1.4.4 *The LAK cell: effect*

Adoptive transfer of *in vitro* activated LAK cells will reduce established pulmonary melanoma metastases and prolong survival.[48] Our group[49] did not achieve the same results in an i.p. model; nor did Mulé[50] in a pulmonary inoculation model: LAK cells by itself did not reduce tumor load in a significant way, but combination with IL-2 did. The importance of proliferation of LAK cells for their cytolytic effects is not clearly understood.

So far the combination therapy of i.v. or i.p. adoptive transfer of *in vitro* induced LAK cells together with daily IL-2 injections i.p. has proven most successful in reducing pulmonary,[50] intraperitoneal[49] and liver[51] inoculations with different experimental murine cancer cells as well as prolonging survival. There is a clear dose-response effect of tumor cytolysis for the number of LAK cells as well as for the dose of IL-2. Both in man and in murine models IL-2 toxicity has been dose limiting. Although this immunotherapy has brought definite reduction of tumor and prolongation of survival, total cures have not exceeded 50%.

1.4.5 *Aim of the present study*

This thesis will discuss the role immune cells and the host immune system

can play in enhancement and abrogation of this novel immunotherapy with interleukin 2 and lymphokine-activated killer cells. Chapter 3 and 4 will discuss the scoring methods in this intraperitoneal cancer and immunotherapeutic model. Chapter 5 will show the effect of immune cells from a mixed lymphocyte culture on LAK cell generation and LAK cell effect. In Chapter 6 the immune cells were generated *in vivo* and harvested from the spleen. Their role on LAK cell generation and LAK cell effect are discussed. The effect the host immune system has on IL-2 and LAK cell therapy was tested in a loco-regional - i.p.- tumor model (Chapter 7). Finally Chapter 8 shows the effects of recruitment of immune cells to another - intracutaneous - loco-regional tumor model.

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Chapter 2

MATERIALS AND METHODS

2.1 Mice

C57BL/6 (BL/6) female mice (H-2^b) were obtained from Jackson Laboratory (Bar Harbor, ME) and used 9-10 weeks old.

DBA/2J (DBA/2) female mice (H-2^d) were obtained from Jackson Laboratory (Bar Harbor, ME) and used at a minimal age of 8 weeks.

These mice were sacrificed for spleen donation and used as experimental animals and syngeneic tumor carriers. The mice were maintained on laboratory chow and acidified water *ad libitum*. There were 6 mice to a cage. The cage tops were covered with special filter paper. Together with air-conditioning, protective clothing for animal handlers, airway masks and non-sterile gloves this resulted in a pathogen-free environment. Regular checks ruled out the common murine virus infections (Sendai, Reo 3, MVM, and GDVII).

2.2 Tumors

MCA-105, a weakly immunogenic fibrosarcoma was passaged subcutaneously in the BL/6 host. This sarcoma was originally induced in the C57BL/6 mouse (H-2^b) with 3-methylcholanthrene. Tumor chunks were stored frozen after some initial expansion passages. After thawing the tumor was passaged once before experimental inoculation of the mice. The fibrosarcoma was only used in the 6 following transplant generations.

Single cell suspensions were prepared as follows. After cervical dislocation of the tumor bearing mouse the subcutaneous tumor was excised aseptically. Only viable tumor parts were transferred to ice cold HBSS and minced with a razor blade into 1 mm^3 cubes. The supernatant was discarded and after transfer to an Erlenmeyer flask 10 ml of 0.25% trypsin without calcium or magnesium (Biofluids, Rockville, MD) was added and magnetically stirred. After 3 minutes the supernatant was again discarded and an equal volume of fresh trypsin was added to the flask. For the next 3 eight minute periods the tumor chunks were stirred with fresh

trypsin added each time. Everytime the supernatant, with released tumor cells, was passed through a 100 gauge nylon mesh (Tobler, Ernst & Traber Co., Elmsford, NY) and pooled in ice-cold HBSS. The tumor cell suspension was washed three times in HBSS and live cells were counted (2.4.8).

MCA-102, a non-immunogenic, 3-methylcholanthrene induced fibrosarcoma in BL/6 mice (H-2^b) was passaged subcutaneously (s.c.) in the syngeneic host and used in the first six transplant generations. Single cell suspensions were prepared as described above for the MCA-105 tumor. Fresh tumor cell suspensions were prepared for each ^{51}Chromium (^{51}Cr) release assay from s.c. tumor nodules.

B-16 is a melanoma syngeneic to the BL/6 host (H-2^b) and passaged s.c. The tumor cells were stored away frozen and used in the first six transplant generations. Single cell suspensions were prepared as described above for the MCA-105 tumor. For easy access the B-16 melanoma cells were maintained in culture in complete medium (2.4.1). Single cell suspensions were harvested after 4-6 days in culture by flooding the 250 ml culture flasks with 0.25% trypsin in HBSS. Tumor cells were washed three times in complete medium and counted (2.4.8).

P815 is a mastocytoma of the DBA/2 host (H-2^d); passaged intraperitoneally (i.p.) in syngeneic mice as an ascites tumor. Single cell suspensions of this mastocytoma were obtained by washing the abdominal cavity repeatedly with phosphate buffered saline (PBS). The cells obtained were washed three times in HBSS and after the final wash live cells were counted (2.4.8).

2.3 Interleukin 2

Recombinant interleukin 2 (IL-2) was kindly supplied by the Cetus Corporation (Emeryville, CA). The gene coding for IL-2 was isolated from a high producer Jurkat cell line, and after insertion in *Escherichia coli* was expressed at high levels.[1] The IL-2 was purified to apparent homogeneity and has the *in vitro* and *in vivo* biological activities of native IL-2 produced directly by human lymphocytes. The endotoxin level in the purified preparation was routinely less than 0.1 ng per 10^5 units of IL-2, as measured in a standard Limulus assay. Vehicle preparations for the recombinant IL-2 contained 5% mannitol and 131 µg sodium dodecyl sulphate (SDS) per mg IL-2.

IL-2 was used for the *in vitro* studies, for LAK cell preparation and for exogenous intraperitoneal IL-2 injections. 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 [^{3}H]-thymidine incorporation into 5 x 10^3 cells from a long-term IL-2 dependent murine cell line.[1] The serum half-life of IL-2 is 1.6 ±

0.3 minutes in mice after intravenous injection and a serum IL-2 titer of ≥10 μm/ml could be sustained for 1.3 hours. Intraperitoneal and subcutaneous injection of IL-2 could sustain titers ≥10 μm/ml for 3.0 and 4.8 hours which could be prolonged to 7.5 and 11.0 hours, respectively, when the IL-2 was admixed with gelatin (15% final concentration).[2] The specific activity of IL-2 was 3 - 5 x 10^6 U/mg. Interleukin 2 was used immediately following reconstitution of the lyophilized powder with sterile water and injected i.p. after dilution in HBSS.

2.4 *In vitro* procedures

2.4.1 *Complete culture medium*

The complete medium used was made up with RPMI 1640 media (Biofluids, Rockville, MD), 0.03% glutamine (Media Unit, NIH, Bethesda, MD), 5 x 10^{-5} M 2-mercapto-ethanol (Aldridge Chemical Co, Milwaukee, WI), 100 units/ ml penicillin (Media Unit, NIH, Bethesda, MD), 100 μg/ml streptomycin (Media Unit, NIH, Bethesda, MD), 50 μg/ml gentamicin (Schering, Kenilworth, NJ), 0.5 μg/ml Fungizone (Flow Labs, McLean, VA), 25 mM HEPES buffer (Biofluids, Rockville, MD), and 10% fetal calf serum (Gibco Laboratories, Grand Island, NY).

2.4.2 *Mixed lymphocyte culture (MLC)*

BL/6 and DBA/2 spleens were harvested aseptically and mashed with the hub of a syringe. After lysing the erythrocytes osmotically with ACK lysing buffer (Media Unit, NIH, Bethesda, MD), the remaining lymphocytes were washed with HBSS. BL/6 responder cells (4 x 10^6 cells/ml) in 50 ml complete medium (2.4.1) were incubated with 12.5 ml irradiated (20 Gray (Gy)) DBA/2 stimulator cells (4 x 10^6 cells/ml) in 250 ml flasks (Corning, Corning, NY) (responder to stimulator ratio of 4:1). Alternatively, 0.25 ml of irradiated (100 Gy) P815 tumor cells (4 x 10^6 cells/ml) were added as stimulator cells as indicated in individual experiments (responder to stimulator ratio of 200:1). Cells were cultured for different time periods at 37° C and 5% CO_2. After culture cells were centrifuged at 200 G for 10 minutes and the number of live cells counted in 0.08% trypan blue (2.4.8). Proliferation was checked in a ^{51}Cr release assay (2.4.3) with P815 tumor as target cell.

2.4.3 *^{51}Chromium release assay*

One milliliter of complete medium (2.4.1) with target cells at a concen-

tration of 5 x 10^7 cells/ml was placed in culture with 200 microCurie (μCi) ^{51}Cr (specific activity 250 - 2,500 mCi/mM) (New England Nuclear, Boston, MA) for 30 minutes at 37° C. Labeled cells were washed three times in complete medium and resuspended at 1 x 10^5 cells/ml. A volume of 0.1 ml labeled tumor cells was added per well to doubling dilutions of effector lymphocytes in round-bottomed microtiter plates (Costar, Cambridge, MA). The plates were centrifuged at 200 g for 2 minutes and incubated for 4 hours at 37° C, and 5% CO_2. After culture the plates were centrifuged at 900 g for 10 minutes. The supernatant of the microtiter wells was harvested with SCS harvesting frames and Macrowell strips (Skatron Inc, Sterling, VA). Spontaneous release was measured after incubation with complete medium only; maximal release was determined by addition of 0.1 N HCl. Spontaneous release was ≤30% of maximal release in all data presented. All tests were run in triplicate. The percentage of specific lysis is calculated for each lymphocyte to target cell ratio tested using the fomula:

$$\frac{[\text{experimental } ^{51}Cr \text{ release} - \text{spontaneous } ^{51}Cr \text{ release}]}{[\text{maximal } ^{51}Cr \text{ release} - \text{spontaneous } ^{51}Cr \text{ release}]} \times 100$$

Cytotoxicity is expressed in lytic units (LU) per 10^7 effector cells. A lytic unit being defined as the number of effector cells that cause 33% lysis of ^{51}Cr-labeled target cells (normalized for 10^4 target cells, assuming a constant percentage of lysis at a constant effector to target (E:T) ratio).

2.4.4 *T cell depletion*

Lymphocytes were harvested from MLC (2.4.2) after 6 days of incubation. Dead cells were removed using separation medium (Lympholyte M, Cedarlane Inc, Westbury, NY). Immune lymphocytes at 1 x 10^7 cells/ml were placed in RPMI 1640 plus 25 ml HEPES buffer and 0.84 % bovine albumen (Pathocyte 4, Miles Laboratories, Elkhart, IN). Rabbit anti-mouse T cell serum (Cedarlane Inc, Westbury, NY) at a concentration of 1:20 was added to an equal volume of cell suspension and was incubated for 60 minutes at 4° C. Cells were centrifuged and resuspended to their original volume with rabbit complement 1:12 in media. Incubation was for 60 minutes at 37° C. Cells were washed 3 times, passed through a tuft of nylon wool in a Pasteur pipette and viable cells adjusted to 4 x 10^6 cells/ml.

2.4.5 *Interleukin 2 bioassay*

Triplicate samples of IL-2 containing solutions were added to a 96-well, flat bottom microtiter plate (Costar, Cambridge, MA). M-53 cells, a name given to an IL-2 dependent cell line maintained in our laboratory, were washed free of IL-2 and suspended in complete medium at 5 x 10^4 cells/ml. To each well of the microtiter plate 0.1 ml cell suspension was added. Plates were incubated for 20 hours and then pulsed with 2 μCi [^{3}H]-thymidine (New England Nuclear, Boston, MA). Activity of the [^{3}H]-thymidine was 50 to 80 Ci/mM. Pulsing lasted for 4 hours prior to harvesting on a MASH II unit (M. A. Bioproducts, Walkersville, MD). After washing the cells the [^{3}H]-thymidine uptake was determined as a measure of proliferation of the IL-2 dependent cell line.

2.4.6 *Spleen cell preparation*

Spleen cells from BL/6 mice were harvested aseptically and mashed in ice cold complete media with the hub of a syringe to produce a single cell suspension. Erythrocytes were lysed osmotically with ACK buffer (Media Unit, NIH, Bethesda, MD). The remaining lymphocytes were washed three times in HBSS.

2.4.7 *LAK cell preparation*

Lymphokine activated killer (LAK) cells were generated by placing 5 x 10^8 spleen cells in 175 cm^2 (750 ml) flasks (Falcon, Oxnard, CA) with 175 ml of complete media (2.4.1) to which was added 0.1 mM nonessential aminoacids (Gibco Laboratories, Grand Island, NY), and 1 mM sodium pyruvate (Gibco Laboratories, Grand Island, NY). IL-2 was added at a concentration of 1,000 U/ml. The flasks were incubated supine at 37° C in 5% CO_2 for 72 hours. Cell cultures were collected, washed three times in ice-cold HBSS and resuspended. Viable cells were counted (2.4.8) and a final dilution before injection or *in vitro* assay was prepared in HBSS.

2.4.8 *Cell viability*

In all cell preparations and experiments viable cell numbers were determined by 0.08% trypan blue exclusion. For high cell suspensions an aliquot of the cell suspension was diluted 1:20 with the trypan blue solution; for low cell suspensions an aliquot of the cell suspension was diluted 1:5 with trypan blue solution.

After thorough mixing, a drop was placed on the microscopic counting chamber and the cell concentration of viable (white) cells was determined.

2.5 *In vivo* procedures

2.5.1 *Peritoneal carcinomatosis model*

All BL/6 mice were injected i.p. with 10^5 live MCA-105 tumor cells suspended in 2 ml HBSS on day 0. Around day 14 some control animals died of i.p. tumorload and accompanying cachexia. At this point the experiment was terminated and the animals sacrificed by cervical dislocation. After eartagging, the i.p. tumorload of the mice was scored blindly as described under peritoneal cancer index (2.5.4).

2.5.2 *Allogeneic tumor challenge*

At different time intervals before syngeneic MCA-105 (H-2^b) tumor injection BL/6 mice were pretreated with 10^7 allogeneic P815 cells (H-2^d) i.p. These cells were either live or irradiated as a single cell suspension with 100 Gy (^{137}Cesium (Cs) source, 15 Gy/min exposure rate). Control groups were injected with the same volume of HBSS.

2.5.3 *Intracutaneous tumor model*

MCA-105 and P815 tumor cell suspensions were prepared as described above. After admixture of syngeneic tumor cells with or without the appropriate number of allogeneic tumor cells in HBSS, BL/6 mice were injected a total volume of 0.2 ml intracutaneously (i.c.) in the right flank. Mice were treated from day 0 till day 7 with 50,000 U IL-2, dissolved in 1 ml HBSS, i.p. every 12 hours. At the completion of treatment the mice were eartagged by an independent observer who stored the code. Measurements on the i.c. tumor nodules were done without knowledge of the treatment modality or assigned code. Beginning on the tenth day after tumor inoculation, tumor growth was assessed every 3 or 4 days. The right flank was wetted with a gauze soaked in alcohol. Then the largest diameter of the i.c. tumor and the one perpendicular to it were measured with calipers. The average diameter was taken as the measure of tumor size. For survival mice were followed until death occurred. All animals were autopsied to ascertain the cause of death was tumor growth.

2.5.4 *Treatment regimen*

After syngeneic injection with 10^5 MCA-105 tumor cells i.p. BL/6 mice were randomly allocated to treatment groups. For the intraperitoneal tumor experiments mice were injected 10^8 LAK cells in 2 ml HBSS i.p. with a 25 x 0.6 mm needle on day 3. From day 3 till day 7 mice received their IL-2 dose in 1 ml HBSS every 12 hours i.p. with a 16 x 0.5 mm needle.

Controls had always 12 mice to a group, treatment groups consisted of 6 mice. Animals not receiving LAK cells or IL-2 were injected with an identical volume of HBSS i.p.

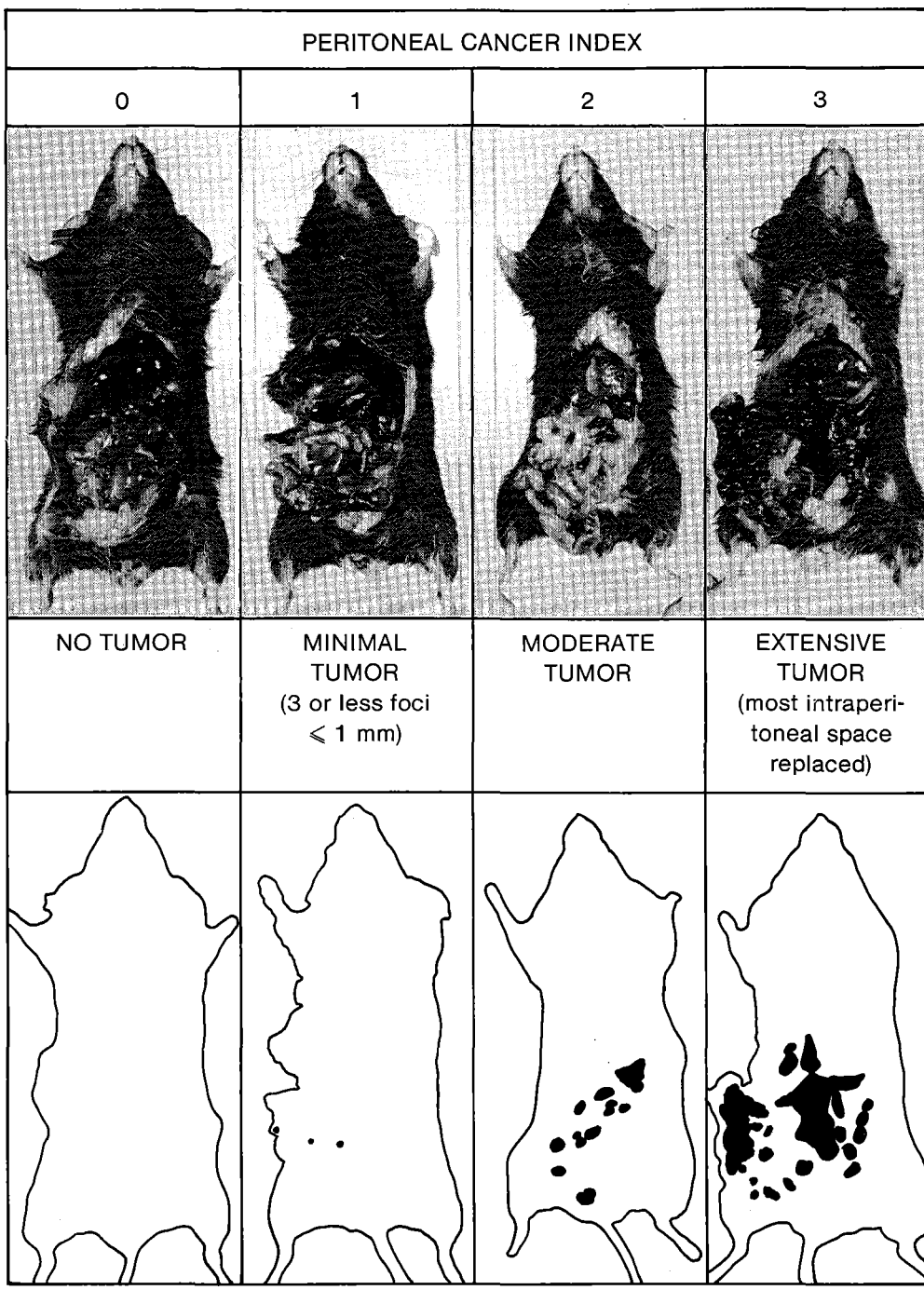
For the intracutaneous tumor experiments too, mice were randomly allocated to treatment groups before injections started with 50,000 U IL-2 i.p. every 12 hours from day 0 through day 6. Again non-treated controls were injected with an identical volume of HBSS i.p.

2.5.5 *Peritoneal cancer index (PCI)*

At the conclusion of an experiment, animals were sacrificed by cervical dislocation and eartagged. An independent researcher recorded which ear tagged mouse belonged to which treatment group. The mice were mixed together. They were taken at random and the abdominal cavity was opened. After thorough inspection of the abdomen the intra-abdominal tumor present was scored and the mouse was assigned to groups 0 through 3 (Figure 2.1). Mice without detectable intraperitoneal tumor were scored 0. When 3 or less tumor foci 1 mm or less in diameter were present the score was 1. With moderate intraperitoneal tumor a score of 2 was given. The score was 3 when most of the intraperitoneal space was replaced by tumor nodules. We called these four levels of intraperitoneal tumor load the peritoneal cancer index (PCI). After assigning all mice to the four different groups they were checked again by the same observer and if necessary changed to a different group. Then a second observer checked all groups again. Rarely this second observer changed the score assigned to a mouse. Only then the code was broken and the mean PCI score ± standard error of the mean (SEM) for every treatment group as well as the control group was determined.

Figure 2.1 Peritoneal cancer index: After termination of the experiment the intraperitoneal tumor load was visually scored without knowledge of the treatment. A score of 0 was given to mice without detectable tumor; 1 indicated minimal tumor with 3 or fewer tumor foci 1 mm in diameter or less. Mice with moderate tumor were scored 2; when most of the peritoneal cavity was replaced

by tumor this was scored as 3.



2.5.6 *Tumor mass*

After visual scoring all tumor nodules were excised from peritoneal surfaces. Gut and mesenteric fat was excised seperately. The wet weight was determined in grams without breaking the assigned code. These same tissues were used to obtain ^{125}IUdR counts in tumor tissue and ^{125}IUdR counts of the intestine and mesenteric fat.

2.5.7 *^{125}IUdR uptake*

24 Hours before sacrifice, mice were given 25 μg of 5-fluoro-2'-deoxyuridine (FUdR) (Sigma, St. Louis, MO) in 1 ml of HBBS i.p. to inhibit thymidylate synthetase activity thereby decreasing endogenous thymidine monophosphate.[3,4] Thirty minutes later, 1 μCi of 5-[^{125}I]-iodo-2'-deoxyuridine (^{125}IUdR) (2,200 Ci/mM) (New England Nuclear, Boston, MA) was administered in 1 ml of HBBS i.p. This gamma-emiting thymidine analog is integrated into DNA after phosphorylation by thymidine kinase.[3] Following sacrifice of mice, tumor nodules were carefully dissected away from the mesenteric fat and the intestine. Stomach, small bowel and large bowel were seperated from the liver and spleen. Radioactivity in counts per minute (cpm) in tumor tissue and in the viscera was then quantitated with a Searle gamma scintillation counter.

2.6 **Statistics**

After assigning ranks to the measured data, the overall significance of differences in an experiment was examined with the Jonckheere test for trend.[5] Two sided p values smaller than or equal to 0.05 were considered significant. With a significant Jonckheere test only, the pairwise comparisons between treatment groups were analysed using the Wilcoxon rank sum test. In the i.p. tumor model the ranked differences of i.p. tumor load were compared and in the cutaneous tumor model the ranked i.c. tumor size was compared, both with a correction for ties.[5] Two sided p values smaller than or equal to 0.05 were considered significant.

2.7 **References**

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Chapter 3

THE PERITONEAL CANCER INDEX

3.1 Introduction

In order to optimize conditions for immunotherapy of neoplastic growth we developed an intraperitoneal tumor model.[1] We expected to increase the immunotherapeutic effects of IL-2 and LAK cells and to reduce the toxicity through maximal cell-to-cell contact between activated lymphocytes and tumor cells as well as easy access of immune competent cells and immune factors of the host to this body compartment.

This was not the only reason for pursuing loco-regional immunotherapeutic strategies. Study of the recurrence patterns of gastro-intestinal and ovarian cancer affirm that tumor implants on peritoneal surfaces are a common site for recurrent tumor.[2 3] Furthermore, in those cancers where systemic treatments have not induced prolonged survival and / or increased the 'no-evidence-of-disease' interval; interest in loco-regional techniques has been aroused.[2]

Traditionally, in peritoneal tumor models the tumor load was not scored, but one looked at survival to grade the result of the experimental therapy.[4 5 6] Sugiura[7] describes three ways to determine the effectiveness of a compound against an ascites tumor: One is to examine the ascites tumor cells cytologically after injection of the compound; another is to measure the increase in survival time resulting from therapy; a third is to measure the amount of ascites formed after treatment. Armstrong[8] is the only author found in the literature who checked for the abdominal tumor load at a certain time point after tumor cell inoculation: The i.p. solid tumor nodules were scored blindly by a second observer on a scale of 0 to 4+ and the malignancy was confirmed histologically.

To score the effect of loco-regional immunotherapy in our intraperitoneal solid tumor model we developed the peritoneal cancer index (PCI). In this chapter this simple visual method for scoring immunotherapeutic effects of solid tumor that has been inoculated as a single cell suspension into the peritoneal cavity, is validated for the first time by objective criteria.

3.2 Methods

In the adoptive immunotherapy strategy employed in these studies BL/6 mice were injected with a cell suspension of 10^5 viable MCA-105 sarcoma or B-16 melanoma cells. Immunotherapy with IL-2 and LAK cells was administered i.p. between day 3 and day 9.[19] Usually on day 14 after tumor inoculation some mice in the control group died and the others appeared sick. These experiments were terminated by day 16.

At this time point intraperitoneal tumor load was scored visually in four groups called the peritoneal cancer index (PCI) (2.5.5 and 3.3.1). Then the tumor nodules were excised and the wet tumor mass was determined (2.5.6 and 3.3.2). The ^{125}IUdR uptake (2.5.7) was measured, accounting for the fast dividing tumor cells (3.3.3). Finally, the 'total' ^{125}IUdR uptake of tumor, intestine and mesenteric fat was determined as a faster and easier way of measuring tumor growth in the abdomen (3.3.4).

The relationship between the different methods of tumor load assessment were determined by least squares linear regression.[10] The scatterplot and the regression equation will give us an idea about the form of the relation between the two variables. When the correlation coefficient approaches 1, then the relation is most likely linear.

3.3 Assessment of the amount of intraperitoneal cancer by four different methods

3.3.1 *Visually scored peritoneal cancer index*

After eartagging the mice were sacrificed by cervical dislocation. A second observer recorded the eartags of the different treatment groups. The intraperitoneal tumor load was assessed blindly by visual inspection as discussed in Chapter 2.5.5. Briefly, the abdomen was opened and the intraperitoneal tumor load scored. A PCI of 0 was given to mice without detectable tumor. The score was 1 when 3 or less tumor foci $\leq$ 1 mm in diameter were seen. Mice with moderate tumor were scored 2; when most of the peritoneal cavity was replaced by tumor, the PCI was 3 (Figure 3.1).

3.3.2 *Wet tumor mass*

After visual scoring, all tumor nodules were excised from the peritoneal

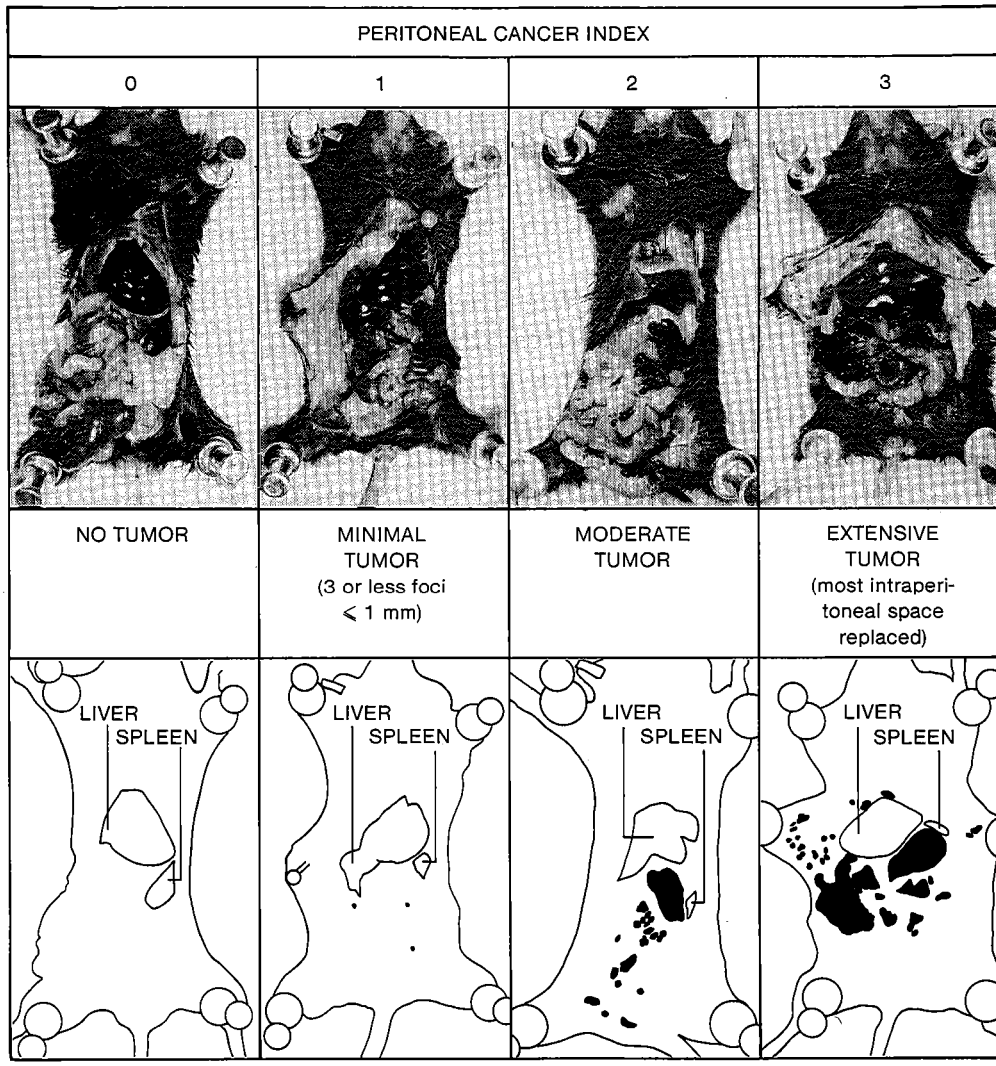

Figure 3.1 Peritoneal cancer index: After termination of the experiment the intraperitoneal tumor load was visually scored without knowledge of the treatment. A score of 0 was given to mice without detectable tumor; 1 indicated minimal tumor with 3 or fewer tumor foci 1 mm in diameter or less. Mice with moderate tumor were scored 2; when most of the peritoneal cavity was replaced by tumor this was scored as 3.

surfaces. Gut and mesenteric fat was excised separately. Without breaking the assigned code, the wet tumor mass was determined in grams. The weights of the excised tumors for the different visually inspected groups are given in Table 3.1. It

should be noted that the mean tumor mass of group 1 and 2 differs by a factor 10 and group 2 and 3 by a factor 5.6. Similar differences were seen in a repeat experiment. The integrity of the groups scored by visual inspection was maintained by weighing the tumors.

Table 3.1 Peritoneal cancer index (PCI), tumor mass, ^{125}IUdR uptake of tumor and 'total' ^{125}IUdR uptake of tumor, intestine and mesenteric fat.

	N	TUMOR MASS (gram) (mean ± SEM)	^{125}IUdR UPTAKE (cpm, mean ± SEM) x 10^3	
			TUMOR	TUMOR + INTESTINE + MESENTERIC FAT (tIUdR)
NORMAL	5	-	-	205 ± 34
PCI 0	14	0	0	116 ± 7
PCI 1	11	0.06 ± 0.02	5.6 ± 1.5	121 ± 13
PCI 2	5	0.6 ± 0.2	104.4 ± 37	238 ± 38
PCI 3	7	3.4 ± 0.5	514 ± 73	630 ± 69

3.3.3 *^{125}IUdR incorporation in tumor*

After determining the wet tumor mass, these same tissues were used to obtain ^{125}IUdR counts in tumor tissue. The mean incorporation of ^{125}IUdR in the tumor was a factor 20 different in PCI-group 2 as compared to PCI-group 1 and a factor 5 different in PCI-group 3 compared to PCI-group 2 (Table 3.1).

3.3.4 *'Total' ^{125}IUdR incorporation in tumor, intestine and mesenteric fat (tIUdR)*

In order to include sub-clinical metastases and in an attempt to simplify this ^{125}IUdR incorporation method of assessing tumor load, we excised the intraperitoneal organs (excluding liver and spleen) and measured gamma emission. The data

in Table 3.1 show the combined ^{125}IUdR counts of tumor plus the ^{125}IUdR uptake of the viscera for each mouse (tIUdR). Using this method for determining the amount of intraperitoneal cancer, no significant differences between visually scored groups 0 and 1 occured. Also, groups of mice with little or no i.p. tumor as a result of IL-2 plus LAK cell therapy showed markedly reduced ^{125}IUdR uptake by the viscera as compared to normal mice. Only mice with a PCI of 3 (for the most part not IL-2 treated mice) showed an increased ^{125}IUdR uptake compared to normal mice. Question remains, if it is the treatment that is causing this visceral atrophy (3.6 and 4.6)? The integrity of the visually inspected groups was not preserved when counting the complete abdominal contents.

To summarize the data contained in Table 3.1: We found that tumor mass and ^{125}IUdR counts indicated a progressive and significant increase in the amount of tumor in the groups created as a result of visual inspection of the abdominal cavity. When 'total' ^{125}IUdR counts of tumor and intestine were measured the progressive increase in the amount of tumor was not observed as clearly. Tumor mass and ^{125}IUdR counts validated the visual scoring system, but 'total' ^{125}IUdR uptake by tumor plus viscera did not.

3.4 Correlation of tumor mass, ^{125}IUdR uptake of tumor and 'total' ^{125}IUdR counts of the abdominal contents

Before taking wet tumor mass as the so-called golden standard for total intra-abdominal tumor load, we have to exclude the possible influence of necrotic tumor mass (3.4.1). Also, for tumor load and tumor progression microscopic tumor deposits are essential. Tumor mass and the PCI will not account for these microscopic tumor deposits; 'total' ^{125}IUdR uptake by the abdominal contents might (3.4.2).

3.4.1 *Tumor mass and ^{125}IUdR incorporation*

If necrotic tumor adds to the mass of the larger i.p. nodules, tumor mass will over-estimate cancer load. Then the ^{125}IUdR uptake test might be a better alternative, since it is a measure of fast dividing (tumor) tissue, and not of dead tissue. When necrotic tumor tissue accounts for a considerable share of the wet tumor mass, we expect a non-linear relation between tumor mass and ^{125}IUdR uptake (*e.g.* an exponential instead of a linear relation). In order to test this hypothesis we have to detemine the relation between ^{125}IUdR and tumor mass by regression analysis. The scatterplot (Figure 3.2) does not give any indication for this alternative hypo-

thesis. Furthermore the correlation coefficient of 0.983 is very high. Finally, the regression equation:

tumor mass = (0.007 ± 0.0002) x ^{125}IUdR uptake + (0.01 ± 0.05)

gives us no reason to exclude a linear relation between tumor mass and ^{125}IUdR uptake: both are 'equivalent' in assessing intra-abdominal tumor load.

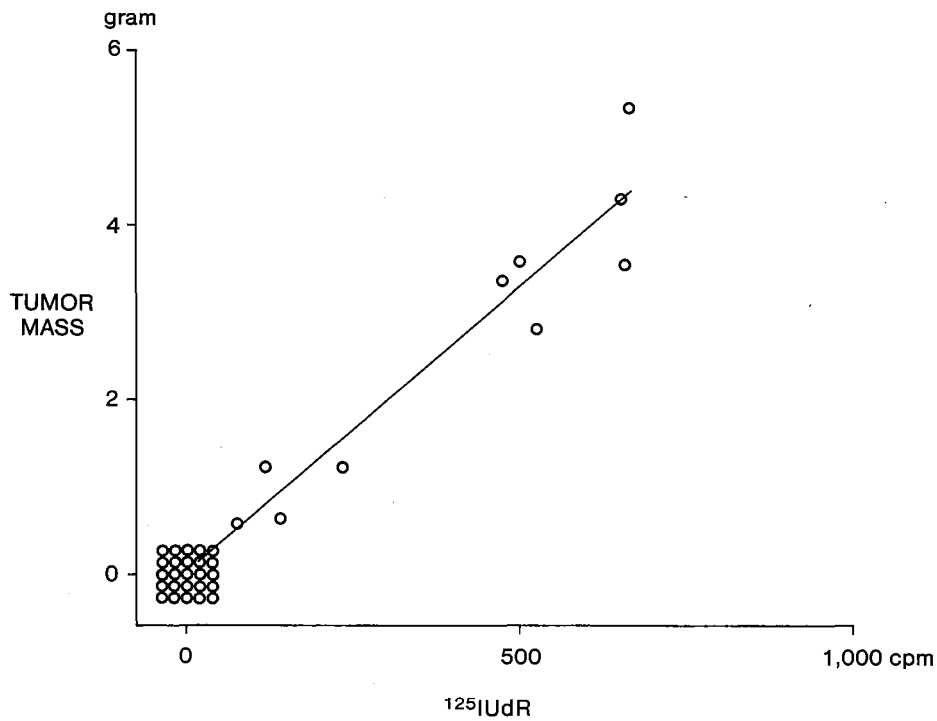

Figure 3.2 Scatterplot showing the linear relation between the wet weights of dissected tumor nodules and their ^{125}IUdR uptake. Correlation coefficient: 0.983.
Regression equation: tumor mass = 0.007 x ^{125}IUdR + 0.01.

3.4.2 *Tumor mass and 'total' ^{125}IUdR uptake of tumor, intestine and mesenteric fat*

Excision of macroscopic visible tumor deposits will leave out microscopic tumor nodules. These microscopic tumor deposits are important for the total tumor burden and tumor progression. In order to include the microscopic tumor nodules, we measured 'total' ^{125}IUdR uptake of i.p. tumor, gut and mesenteric fat (tIUdR). Cutting out the abdominal contents without liver and spleen; and counting 'total'

^{125}IUdR uptake might also be an easier method of assessing i.p. tumor growth. We presume tIUdR is as good an assessment of i.p. tumorload as tumor mass is.
The scatterplot (Figure 3.3) shows a fairly linear relation. The correlation coefficient is 0.976. The regression equation:

tumor mass = (0.007 ± 0.0002) x tIUdR - (0.8 ± 0.08)

We conclude that the hypothesis is correct. tIUdR is equal to tumor mass in assessing i.p. tumor load.

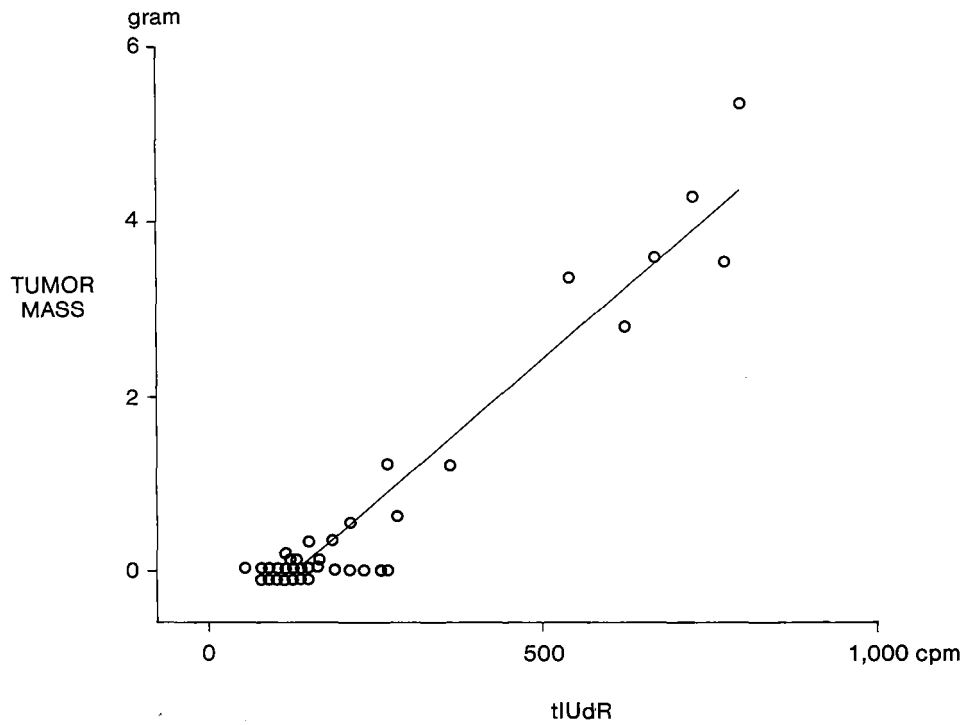

Figure 3.3 Scatterplot showing the linear relation between the wet weights of dissected tumor nodules and the 'total' ^{125}IUdR (tIUdR) uptake of tumor nodules, intestine and mesenteric fat. Correlation coefficient: 0.976. Regression equation: tumor mass = 0.007 x tIUdR - 0.8.

3.5 Comparison of objective tumor mass and ^{125}IUdR tumor incorporation with subjective visual scoring.

3.5.1 *Peritoneal cancer index and tumor mass*

To answer the question if the PCI is much worse in assessing the abdominal

tumor load than the objective method of determining the tumor mass; we have to look at Table 3.2. There is no overlap in tumor mass in the different PCI groups. The PCI-group classifies tumor mass in a significant way as indicated in Table 3.2 ($p_2 \leq 0.03$, Student t test for unpaired samples).

Table 3.2 Peritoneal cancer index (PCI) and tumor mass.

PCI	TUMOR MASS (gram)					P_2 value
	Mean	SD	SEM	Min	Max	
0	0	0		0	0	0.003
1	0.05	0.05	0.01	0	0.15	0.03
2	0.6	0.4	0.2	0.32	1.22	0.0005
3	3.4	1.3	0.5	1.23	5.33	

The scatterplot (Figure 3.4) suggests a non-linear relation between the PCI and tumor mass, accordingly we find a low correlation coefficient (0.81). The regression equation is:

$$\text{tumor mass} = (0.99 \pm 0.01) \times \text{PCI} - (0.3 \pm 0.2)$$

also suggestive for a non-linear relation.

Since the PCI is dependent of the visual perception of tumor diameters in the abdominal cavity and tumor mass is directly related to the volume of the tumor ($\approx$ diameter^3), we may as well compare PCI^3 and tumor mass. The scatterplot (Figure 3.5) shows a more linear relation, as indicated by a higher correlation coefficient (0.92). The regression equation:

$$\text{tumor mass} = (0.127 \pm 0.008) \times \text{PCI}^3 - (0.07 \pm 0.1)$$

underscores the linear relation.

3.5.2 *Peritoneal cancer index and ^{125}IUdR incorporation of the tumor*

The high correlation coefficient of 0.983 between the wet tumor mass and ^{125}IUdR uptake (3.4.1); as well as the more linear relation between PCI^3 and tumor mass (correlation coefficient 0.92) (3.5.1), suggest a parallel relation between PCI^3 and ^{125}IUdR. In view of the correlation coefficient of 0.92 and the regression equation:

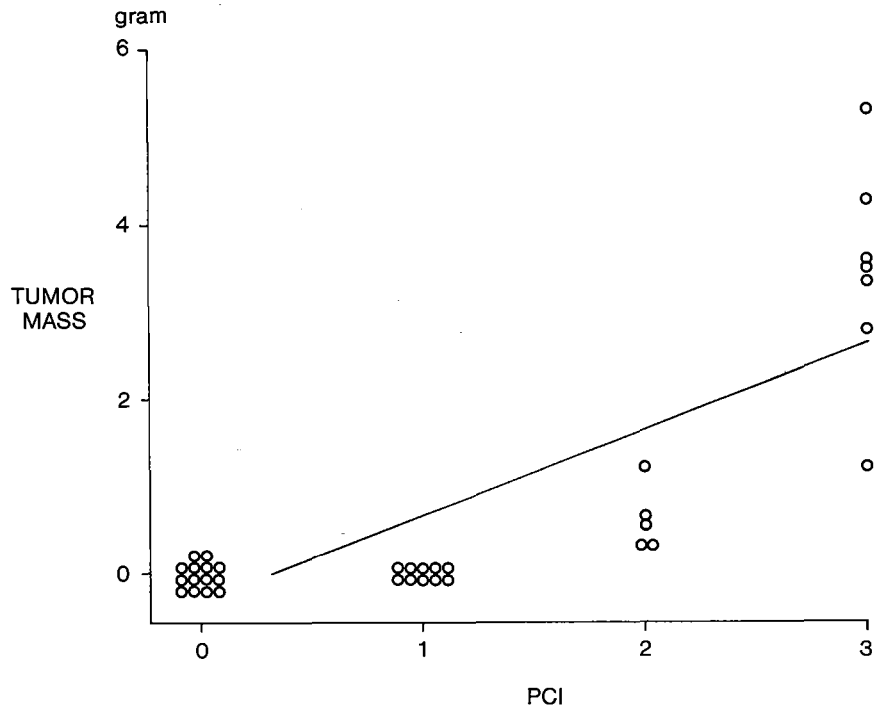

Figure 3.4 Scatterplot showing the non-linear relation between the wet weights of dissected tumor nodules and the visual scored intraperitoneal tumor load. Correlation coefficient: 0.81. Regression equation: tumor mass = 0.99 x PCI - 0.3.

$$^{125}\text{IUdR uptake} = (19 \pm 1) \times \text{PCI}^3 - (11 \pm 17)$$

we must conclude that there is also a linear relation between the ^{125}IUdR uptake of the tumor nodules and the 'cubed' PCI.

3.6 Discussion

The progression or regression of tumor in experimental animals is usually assessed by measurement of subcutaneous tumor in two dimensions. Alternatively the amount of tumor may be assessed by dissecting the tumor nodule from its host and determining the wet weight. Also, volume may be measured by displacement of water. In view of a rising interest in loco-regional therapeutic options, the intra-peritoneal tumor model we developed seems ideal. The subjective peritoneal cancer index we developed to score intraperitoneal tumor load has been validated for the

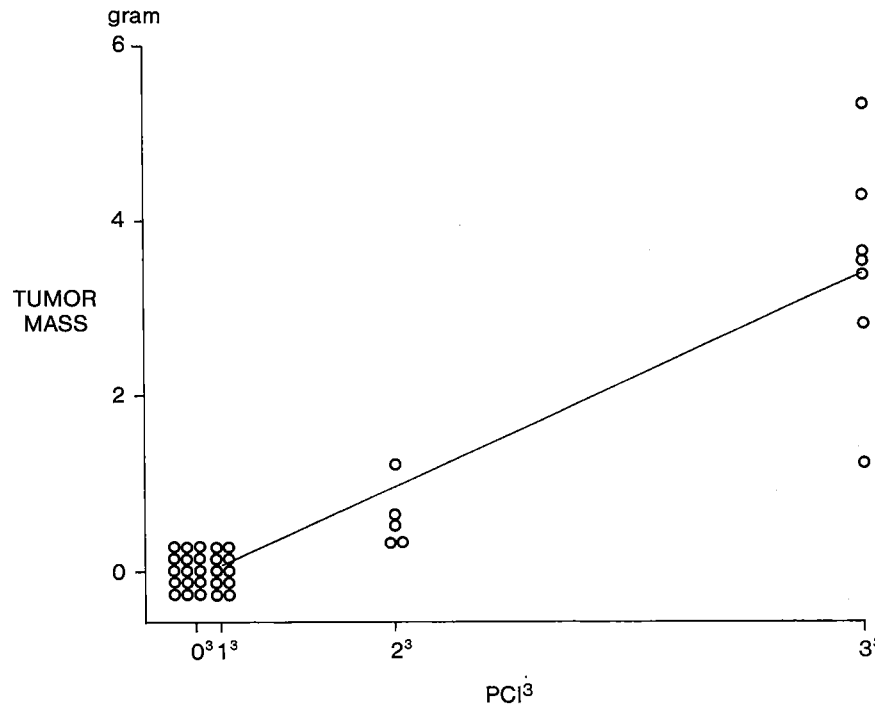

Figure 3.5 Scatterplot showing the linear relation between the wet weights of dissected tumor nodules and the ('cubed') PCI to the third power. Correlation coefficient: 0.92.
Regression equation: tumor mass = 0.127 x PCI^3 - 0.07.

first time by objective scoring methods.

The integrity of the groups defined by visual inspection was established in Table 3.1. Tumor mass differed ten fold between visually scored groups with PCI of 1 and 2; ^{125}IUdR tumor uptake differed twenty fold. The visually scored PCI group 2 and 3 differed five fold by tumor mass and by ^{125}IUdR tumor counts. The integrity of the experimental groups was not maintained when 'total' ^{125}IUdR counts in tumor plus intestine were analyzed. The 'background' counts introduced by the ^{125}IUdR counts in intestine and mesenteric fat abrogated differences previously defined between groups 0 and 1. Also, the difference between groups 1 and 2 was only two fold and groups 2 and 3 differed only three fold. This establishes that 'total' ^{125}IUdR counts in tumor plus intestine is a less sensitive means of quantitating intraperitoneal tumor.

As discussed in Chapter 4, it should be noted that 'normal' - not-injected - mice showed marked increase in the ^{125}IUdR counts in the intestine and mesenteric

fat as compared to IL-2 treated mice (found mainly in PCI groups 0 and 1). We suggest that intraperitoneal interleukin 2 causes some atrophy of the intestine or reduces bowel function in some other way to suppress the normal uptake of ^{125}IUdR by intestinal mucosa. It is quite possible that as part of the activation and proliferation cascade of cytolytic cells set in motion by interleukin 2, cachectin is activated and catabolin / IL-1 is released[11].

Judged by the wet weights of dissected i.p. tumor nodules, ^{125}IUdR uptake of these same tumor nodules is as good an assessment of i.p. tumor load (Figure 3.2). The same holds true for the 'total' ^{125}IUdR uptake of tumor nodules, intestine and mesenteric fat (Figure 3.3). No linear relation could be established between wet tumor mass and the PCI (Figure 3.4). However, this linear relation did exist between PCI^3 and wet tumor mass (Figure 3.5) respectively ^{125}IUdR. It may seem somewhat surprising that the unaided eye can visually score the extent of intraperitoneal cancer as accurately as wet tumor mass or ^{125}IUdR tumor counts. Visual scoring is based mainly on perception of tumor diameters, while tumor mass is on volume ($\approx$ diameter^3). Thus it is only natural to compare PCI^3 with tumor mass and ^{125}IUdR. The three dimensional volume expansion of tumor growth is appreciated by the eye and the eye can discriminate between groups of mice.

3.7 Summary

Comparable experimental results were obtained when visual scores (peritoneal cancer index), tumor mass and ^{125}IUdR tumor uptake were quantitated. Either of these three assessments can be recommended for quantitating intraperitoneal tumor load after immunotherapy. However, practical considerations must also be mentioned. The PCI is easily performed and is inexpensive. It does not require the dissection of tumor nodules from peritoneal surfaces as does determination of wet tumor mass and determination of ^{125}IUdR tumor incorporation. It was hoped that simple counting of gamma emission of the intestinal block which contained tumor would be an accurate and more objective alternative to visual inspection. However, our studies showed lack of sensitivity of this methodology when small amounts of tumor were present in the abdominal cavity. Also, interleukin 2 may have induced a rather surprising suppression of normal ^{125}IUdR uptake by the intestine. These confounding effects could make interpretation of experimental results extremely confusing in some immunotherapeutic situations. The mechanism whereby IL-2 induces intestinal atrophy over such a short time period has yet to be determined.

We conclude that when simplicity of the methodologies, accuracy, and

practicality of the four methods are considered simple visual inspection is the optimal scoring system for abdominal tumor load.

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Chapter 4

COMPARISON OF FOUR SCORING METHODS FOR AN INTRAPERITONEAL IMMUNOTHERAPY MODEL

4.1 Introduction

Systemic immunotherapy with interleukin 2 (IL-2) and lymphokine-activated killer (LAK) cells has proven effective in a lung[1] and a liver inoculation model.[2] Rosenberg[3] showed some effect in man, but toxicity was dose limiting. In developing a loco-regional immunotherapy model we aimed at reduced systemic toxicity through reduced systemic levels of IL-2 and LAK cells. This loco-regional model would optimize conditions for successful immunotherapy by maximizing cell-to-cell contact between tumor cells and cytotoxic lymphocytes. This can be done by inoculating activated lymphocytes into the abdominal cavity together with sufficient quantities of biological response modifiers to maintain tumor cytotoxicity of the in vitro activated lymphocytes.[4] Mathisen and co-workers[5] showed that these activated cells will stay in the peritoneal cavity for an extended time period. Although IL-2 gained nearly immediate systemic access, i.p. IL-2 does maintain LAK cell activity and will generate tumor cytotoxic peritoneal exudate cells (PEC).[6 7 8]

Chapter 3 demonstrated that the subjective PCI score developed for this loco-regional tumor model was supported by the objective methods of scoring by tumor mass or by ^{125}IUdR uptake. In this chapter we will score the result of the instituted immunotherapeutic regimen visually (PCI), by tumor mass, by ^{125}IUdR uptake of tumor nodules and by 'total' ^{125} IUdR uptake of tumor nodules, intestine and mesenteric fat. By analyses of variance we will determine if all four methods perform equally in scoring the effect of i.p. immunotherapy.

4.2 Methods

4.2.1 *Immunotherapeutic regimen*

BL/6 mice were injected with 10^5 viable MCA-105 cells i.p. in 1 ml HBSS on day 0. Treated mice received 10^8 LAK cells i.p. in 1 ml HBSS on day 3 and /or 25,000 units of IL-2 i.p. every 12 hours in 0.5 ml HBSS from day 3 through day 7. On day 15 all mice were injected i.p. with 25 μg of 5-fluoro-2-deoxyuridine (FUdR) to decrease the endogenous thymidine monophosphate production followed 30 minutes later by injection of 1 μCi of ^{125}IUdR (2.5.7). The experiment was terminated on day 16 by sacrificing the experimental animals. After eartagging and recording their treatment all mice were mixed together and their abdominal tumor load scored blindly by one subjective and three objective methods as described under 4.2.2 - 5.

IL-2 and IL-2 plus LAK cell treatment groups consisted of 6 mice. The 12 'control' mice were injected the same volume of HBSS. In order to control for the effect of handling and injecting of the experimental mice, a group of 5 littermates was included at day 15 of the experiment to receive the FUdR and ^{125}IUdR injections. These 'normal' mice did not receive i.p. tumor suspension nor any other i.p. injection. The ^{125}IUdR uptake of the viscera was determined on day 16.

4.2.2 *Peritoneal cancer index*

After opening the abdominal cavity the abdominal tumor load was judged and the mice were assigned to PCI groups 0 through 3 as described earlier (2.5.5). Briefly, mice with no intraperitoneal tumor were scored 0. The score was 1 when 3 or less tumor foci 1 mm or less in diameter were present. With moderate intraperitoneal tumor, a score of 2 was given. Extensive tumor was scored as 3 (Figure 3.1).

4.2.3 *Tumor mass*

Still identifying the mice by their eartag number, the visible tumors were excised and the wet mass was determined in milligrams (2.5.6).

4.2.4 *^{125}IUdR incorporation in tumor*

The excised tumor nodules from every mouse were put into scintillation vials and the ^{125}IUdR counts in tumor tissue were obtained without breaking the

assigned code (2.5.7).

4.2.5 *'Total' ^{125}IUdR uptake in tumor, intestine and mesenteric fat (tIUdR)*

After scoring the PCI and subsequent excision of all abdominal tumor nodules from every eartagged mouse, the viscera excluding liver and spleen were also excised and ^{125}IUdR uptake scored blindly. For the 'total' ^{125}IUdR uptake the counts of tumor nodules were added to the counts per minute (cpm) of the viscera of each mouse and recorded (2.5.7).

4.2.6 *Statistics*

The effect of IL-2 treatment and additional LAK treatment was assessed by the four scoring methods, and analyzed by analysis of variance. Independent variables in these analyses were: injection with tumor (yes / no); treatment with IL-2 (yes / no); and IL-2 plus LAK cell treatment (yes / no).

4.3 **Results**

In table 4.1 the mean scores ± SEM of the normal and control mice as well as of the treated mice are given for the subjective peritoneal cancer index (PCI), and for the objective wet tumor mass, ^{125}IUdR uptake and 'total' ^{125}IUdR uptake. It should be noted that the clear cut difference for the treated mice compared to control mice is shown well by PCI, wet tumor mass and ^{125}IUdR tumor uptake, but not by 'total' ^{125}IUdR uptake. The effect of IL-2 and IL-2 plus LAK cells is a near total absence of i.p. tumor growth compared to no treatment. This is demonstrated by the three scoring methods.

Tumor load of IL-2-treated mice was reduced significantly compared to control mice according to all four scoring methods (PCI: $p_2 < 0.0001$; tumor mass: $p_2 < 0.02$; ^{125}IUdR: $p_2 < 0.02$; tIUdR: $p_2 < 0.04$). Treatment with IL-2 plus LAK cells had no significant effect on tumor load as compared to IL-2 treated mice (all methods $p > 0.1$).

Scoring the experiment by ^{125}IUdR uptake of the tumor yielded similar results as scoring by PCI and tumor mass (Table 4.1). 'Total' ^{125}IUdR uptake also showed significant reduction of uptake of IL-2 and IL-2-plus-LAK treated groups, but the score was affected to a greater degree than could be expected from tumor reduction alone. The 'total' ^{125}IUdR counts of tumor, intestine and mesenteric fat showed a strong discrepancy between treated (IL-2 and IL-2 plus LAK) and non-

Table 4.1 Immunotherapy and four scoring methods of intraperitoneal tumor load: peritoneal cancer index (PCI), wet tumor mass, ^{125}IUdR uptake of tumor and 'total' ^{125}IUdR uptake of tumor, intestine and mesenteric fat.

TREATMENT GROUPS	N	PCI (mean ± SEM)	TUMOR MASS (gram) (mean ± SEM)	^{125}IUdR UPTAKE (cpm) (mean ± SEM) x 10^3 TUMOR	TUMOR + INTESTINE + MESENTERIC FAT (tIUdR)
NORMALS	5	-	-	-	205 ± 34
CONTROLS	12	2.0 ± 0.4	1.2 ± 0.8	156 ± 83	284 ± 88
IL-2	6	0	0	0	99 ± 10
IL-2 + LAK	6	0.3 ± 0.2	0.01 ± 0.01	0.9 ± 0.9	156 ± 16

treated ('normal' and control) mice. Control mice and non-injected-'normal' mice also demonstrated a remarkable difference in tIUdR score, not accounted for by tumor deposits only. Table 4.2 demonstrates the profound depressive effect i.p. injections *persé* (HBSS and IL-2) had on the mean mesenteric fat and lymph node uptake (7 and 9 *versus* 25 for 'normal' mice). Strangely, IL-2 plus LAK cells restored the uptake nearly to normal (23 *versus* 25). Handling and i.p. injections reduced the intestinal ^{125}IUdR uptake somewhat in control mice. IL-2 gave a remarkable depression of intestinal proliferation compared to the control and IL-2 plus LAK cell group.

In view of the discrepancy between the ^{125}IUdR uptake test and the tIUdR test (Table 4.1), we analysed the influence of other variables on tIUdR and ^{125}IUdR by analysis of variance. The 'total' ^{125}IUdR uptake appeared to be determined by the fact the mice belonged to the injected group ($p_2 < 0.001$) as well as by tumor mass ($p_2 < 0.001$). Likewise ^{125}IUdR uptake was completely determined by tumor mass ($p_2 < 0.001$).

Table 4.2 Immunotherapy and ^{125}IUdR uptake of tumor, intestine and mesenteric fat. * - *, And # -# depict the only significant pairwise comparison ($p_2 < 0.05$).

TREATMENT GROUPS	N	^{125}IUdR UPTAKE (cpm, mean ± SEM) x 10^3			
		TUMOR	INTESTIN	MESENTERIC FAT	TUMOR + INTESTINE + MESENTERIC FAT (tIUdR)
NORMALS	5	-	179 ± 32 *	25 ± 19	205 ± 34
CONTROLS	12	156 ± 83	113 ± 15 *	7 ± 1	284 ± 88 #
IL-2	6	0	90 ± 10	9 ± 1	99 ± 10 #
IL-2 + LAK	6	0.9 ± 0.9	135 ± 7	23 ± 13	156 ± 16

4.4 Discussion

A useful immunotherapy model was described by Wexler and colleagues[9] and has been extensively employed by Rosenberg and co-workers.[10] In this model the number of lung implants growing after injection of tumor into the tail vein of the mouse is scored visually. Also, injection into the spleen with resultant hepatic metastases was described by Kopper and colleagues[11] and has been utilized by Rosenberg and co-workers as a hepatic implantation model.[12] These various models have been used with reliable data being produced in numerous immunotherapy settings. Perhaps the most sensitive method by which to assess the antitumor effects of primed lymphocytes may be the mixing of activated cells with tumor cells followed by subcutaneous inoculation of the mixture. This has been commonly referred to as the Winn-assay or tumor neutralization test.[13 14] This model allows intimate access of cytotoxic lymphocytes to tumor cells by establishing direct effector cell to tumor cell contact.

There may be some definite advantages to the peritoneal cancer model for studying immunotherapy in the experimental animal. In the models previously

mentioned repetitive therapy over the course of several days is difficult or impossible. Repetitive immunotherapy manipulations can only be performed in the Winn-assay, for example, if one attempts to repeatedly inject the tumor site. These injections are difficult and placement of effector cells or drugs is extremely inaccurate. In other models repeatedly injecting activated cells in the tail vein of a mouse for more than one day has its limits. For example, in the hepatic metastases model repetitive injection of the portal vein to increase the number of treatments directed at the liver is technically demanding. The peritoneal cancer model described in this manuscript allows one to repeatedly deliver activated cells or activated cells plus other treatments into the area in which the tumor exists and to deliver these treatments on several different occasions. Mixing of effector cells, tumor cells, and drugs within the peritoneal cavity over many days enables the delivery of combination treatments without difficulty. If a fine gauge needle is used, the trauma to mice is minimal.

Another advantage of the peritoneal cancer model is that direct contact of adoptively transferred lymphocytes and tumor cells is assured as in the Winn-assay. The loco-regional nature of tumor tissue and effector cells promotes cell-to-cell contact and should maximize the anti-tumor effects. One of the major problems with assessment of the effects of tumor cytotoxic lymphocytes concerns the requirement for adequate access of lymphocytes to tumor tissue. Such access may be extremely limited because of the reduced blood supply that tumors receive as they undergo necrosis from outgrowing their vasculature. With intraperitoneal cancer models the loco-regional effects of biologic response modifiers, activated cells, or chemotherapeutic agents can be directly assessed and thereby maximized. It is important to note that the number of effector cells or concentrations of drugs may be markedly increased within the peritoneal cavity as compared to the systemic circulation.

This chapter demonstrates that the reduction of i.p. tumor by immunotherapeutic regimens can be scored objectively as well as by a subjective visual scoring method. The objective scoring methods that validated the integrity of the PCI groups also classify the result of immunotherapy well: objective wet tumor mass and ^{125}IUdR uptake perform about equal as the PCI. 'Total' ^{125}IUdR uptake was more practical than tumor mass and ^{125}IUdR; but it was not a good scoring method since it was largely determined by the mice being injected. Since control mice were injected with HBSS, 'total' ^{125}IUdR incorporation demonstrated no clear difference between control and treated mice.

IL-2 seemed to reduce intestinal proliferation even more than mere i.p. injections did. This reduced proliferation was not seen when mice were treated

with IL-2 and LAK cells. It could be postulated that by simultaneous injection of LAK cells and IL-2, the LAK cells will bind IL-2 preferentially to systemic access and thus reduce the anti-proliferating effect on the intestines.
Definitely, 'total' ^{125}IUdR uptake can be ruled out as a better scoring method. Apparently micro-metastases do not contribute substantially to total tumor load. Although the method seemed better and quicker, it is not a good scoring method since too many other disturbing variables are introduced.

4.5 Summary

The compartmental approach to treating intraperitoneal cancer with IL-2 and LAK cell immunotherapy loco-regionally has proven worthwhile by reducing tumor load. This effect could be monitored objectively (tumor mass and ^{125}IUdR uptake) as well as subjectively (PCI) with all methods performing equally. The more practical objective method of determining 'total' ^{125}IUdR uptake of tumor, intestine and mesenteric fat did prove to be biased by the mice being injected and treated; only in second instance to be determined by the i.p. tumor mass.

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Chapter 5

ALLOGENEIC CHALLENGE AND LAK CELL GENERATION AND MAINTENANCE BY INTERLEUKIN 2 *IN VITRO*

5.1 Introduction

Interleukin 2 (IL-2) will induce so-called lymphokine-activated killer (LAK) cells *in vitro* and *in vivo*. The LAK cell is *in vitro* highly cytotoxic for a wide variety of tumor- and blast cells; but not for normal peripheral blood lymphocytes (PBL).[1] Because of the toxic side effects of IL-2 *in vivo* we were interested in the influence of an on-going specific immune response on LAK cell generation and possible synergistic tumoricidal effect with IL-2 and LAK cell immunotherapy. *In vitro* systems are ideal for testing the influence of seperate components of the immune system on the generation of LAK cells by IL-2 and on LAK cell cytolysis. In a mixed lymphocyte culture (MLC) specific cytolytic T lymphocytes (CTLs) are elicited by incubating irradiated stimulator cells with fresh lymphocytes as responder cells. Since this induction of CTLs is a dynamic phenomenon between CTL precursors and T helper cells, cells from the MLC were harvested sequentially.

This chapter will look at the potential of MLC cells: 1. Are MLC cells capable of lysis of an allogeneic tumor target? 2. Can IL-2 enhance the lysis of this allogeneic tumor target? 3. Can these MLC cells still be induced into LAK cells and lyse a syngeneic tumor target (5.3.1)? 4. What is the influence of MLC cells on the induction of LAK cells from normal BL/6 splenocytes by IL-2 (5.3.2)? 5. Do MLC cells enhance or abrogate LAK cell maintenance by IL-2 (5.3.3)? The rest of the chapter deals with the co-factors and the subsets of cells that account for the effects of MLC cells on LAK cells.

5.2 Methods

BL/6 splenocytes (H-2^b) were incubated for 0, 2, 4, 6, and 10 days with

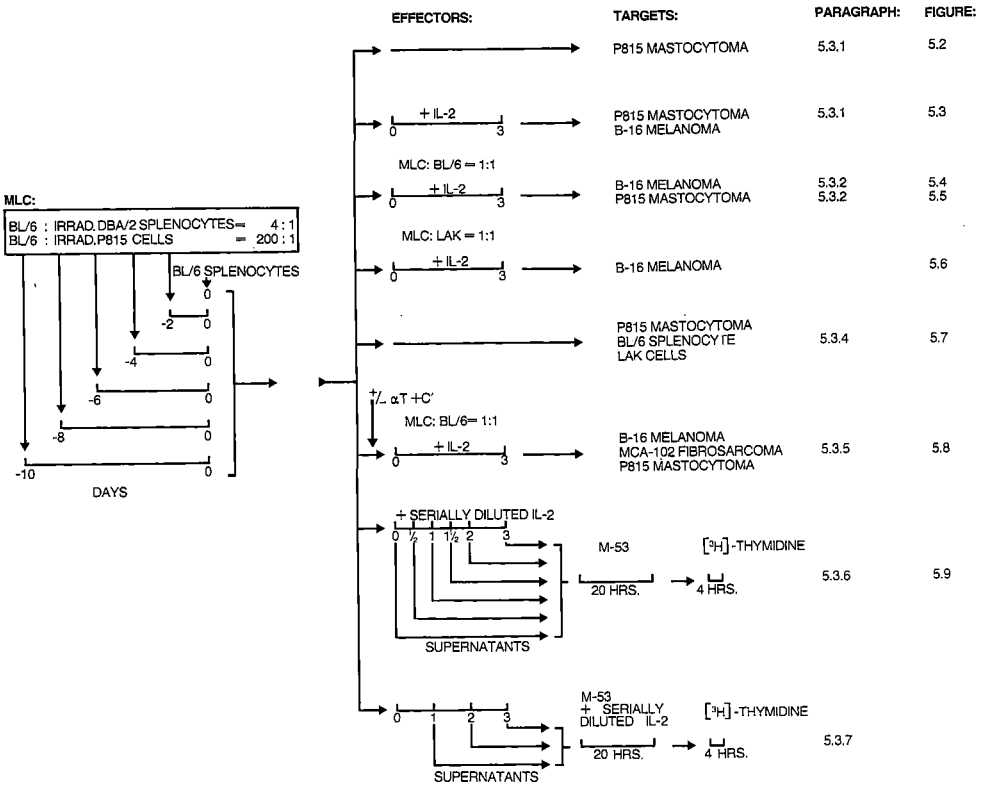

Figure 5.1 Flow chart mixed lymphocyte culture (MLC) cells [BL/6 responder cells and DBA/2 stimulator cells (ratio 4:1), and P815 stimulator cells (ratio 200:1) respectively] taken out of culture after their respective culture time and cultured with IL-2 (1,000 U/ml); BL/6 lymphocytes (1:1) and IL-2; or LAK cells (1:1) and IL-2. Cytotoxicity was tested in a ^{51}Chromium release assay against P815 (H-2^{d}) mastocytoma cells and B-16 (H-2^{b}) melanoma cells.

irradiated DBA/2J splenocytes (H-2^{d}) in a mixed lymphocyte culture with a responder to stimulator ratio of 4:1 as described in Chapter 2.4.2. Alternatively BL/6 splenocytes were stimulated with irradiated P815 mastocytoma cells (H-2^{d}) at a ratio of 200:1. After their respective culture time cells were taken out of culture, washed and counted (Figure 5.1). These MLC cells were cultured for another 3 days with IL-2 (1,000 U/ml) in complete medium and tested for CTL enhancement against the P815 mastocytoma target and for LAK cell induction against the B-16 melanoma target in a 14 hour ^{51}Cr release assay. Alternatively these MLC cells were mixed with normal, fresh BL/6 splenocytes in a ratio of 1:1 and cultured for

three days with IL-2 (1,000 U/ml). Again their cytolytic activity was tested in a ^{51}Cr release assay with P815 and B-16 cells as targets. Finally these MLC cells were mixed with *in vitro* activated LAK cells in a ratio of 1:1 and cultured for another 3 days with IL-2 (1,000 U/ml). These cells were tested against the B-16 target in a 14 hour ^{51}Cr release assay. Before cultured cells were used in culture again dead cells were removed using separation medium.

5.3 Results

5.3.1 *Allo- and syngeneic cytotoxicity of MLC cells cultured in IL-2*

To determine maximal cytolytic responses of BL/6 responder cells to H-2^d alloantigens, mixed lymphocyte cultures were initiated and harvested at day 2, 4, 6, and 10. Responses to both irradiated P815 tumor cells (responder to stimulator ratio 200:1) and DBA/2 lymphocytes (responder to stimulator ratio 4:1) were tested. The cytotoxicity of these MLC cells was tested against the allogeneic P815 tumorcells as target in a ^{51}Cr release assay. The results are shown in Figure 5.2. High levels of cytotoxicity were generated by day 4, peaked at day 6 and 8, and were decreasing by day 10. No essential difference was seen when DBA/2 splenocytes or P815 tumorcells were used as stimulator cells in the MLC.

BL/6 responder cells were incubated with DBA/2 stimulator cells. 2, 4, 6, And 10 days after initiation of these cultures they were harvested on the same day. The cells were counted and placed in culture with IL-2 (1,000 U/ml) for three days. Normal BL/6 splenocytes served as control. Following three days of incubation with IL-2, the cultures were harvested and the cytotoxicity tested against the allogeneic P815 tumorcells and the syngeneic B-16 melanoma cells. In this ^{51}Cr release assay the primed cells from the MLC cultured in IL-2 showed an increased cytolysis of the allogeneic P815 target (Figure 5.3). However, the cytolysis of the syngeneic target was reduced, compared to the cytotoxicity of normal BL/6 splenocytes cultured in IL-2. Especially on day 2 the induction of LAK cells from the MLC was profoundly depressed. This was still true for day 4 and 6 of MLC. The cytotoxicity of the cells from MLC in Figure 5.3 was markedly increased from the cytotoxicity seen in Figure 5.2. This can be attributed to the IL-2 incubation. Day 2 responder cells from MLC were profoundly depressed in LAK cell induction and killing of the syngeneic target. However, these same day 2 responder cells could be induced into markedly increased cytotoxicity towards the allogeneic target by IL-2.

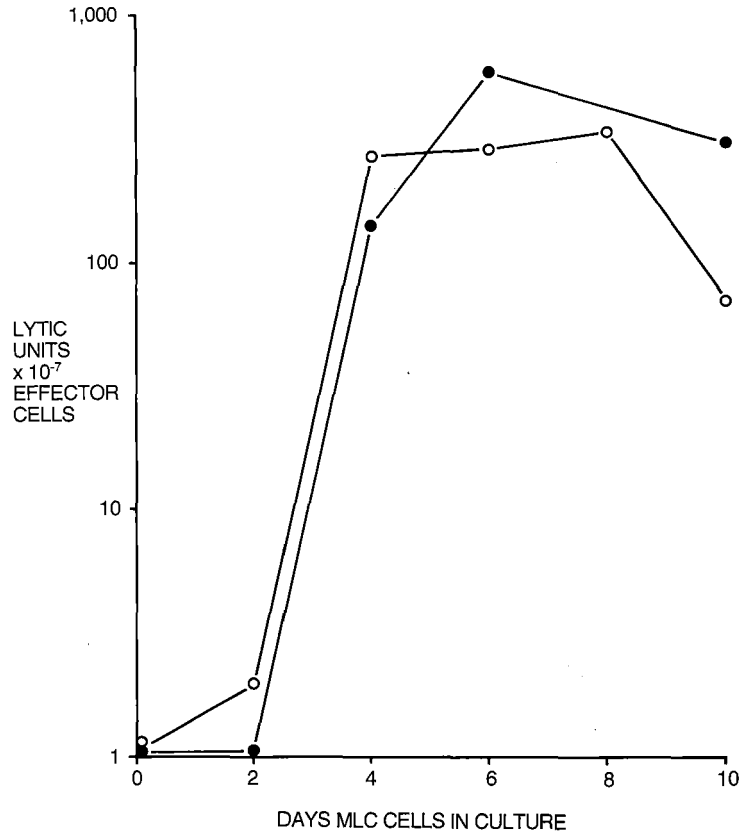

Figure 5.2 BL/6 anti H-2^d alloimmune responses *in vitro*. Mixed lymphocyte cultures using BL/6 responder cells and irradiated DBA/2 lymphocyte stimulators (open circles, ratio 4:1) or P815 stimulator cells (closed circles, ratio 200:1) were initiated. Cultures were harvested on days 2, 4, 6, 8, and 10. The levels of cytotoxicity generated *in vitro* were determined against H-2^d alloantigens (P815 mastocytoma cells).

5.3.2 *Influence of MLC cells on the in vitro induction of LAK cells tested in an allo- and syngeneic cytotoxicity assay*

Three day culture with IL-2 induces normal BL/6 splenocytes to proliferate into LAK cells. To answer the question whether normal BL/6 splenocytes, induced into proliferation by alloantigens, could influence the induction of LAK cells by IL-2, we designed the following experiment. Normal BL/6 splenocytes were maintained in culture for 2, 4, 6 and 10 days with irradiated DBA/2, respectively P815 stimulator cells. On day 0, BL/6 splenocytes were cultured with the different res-

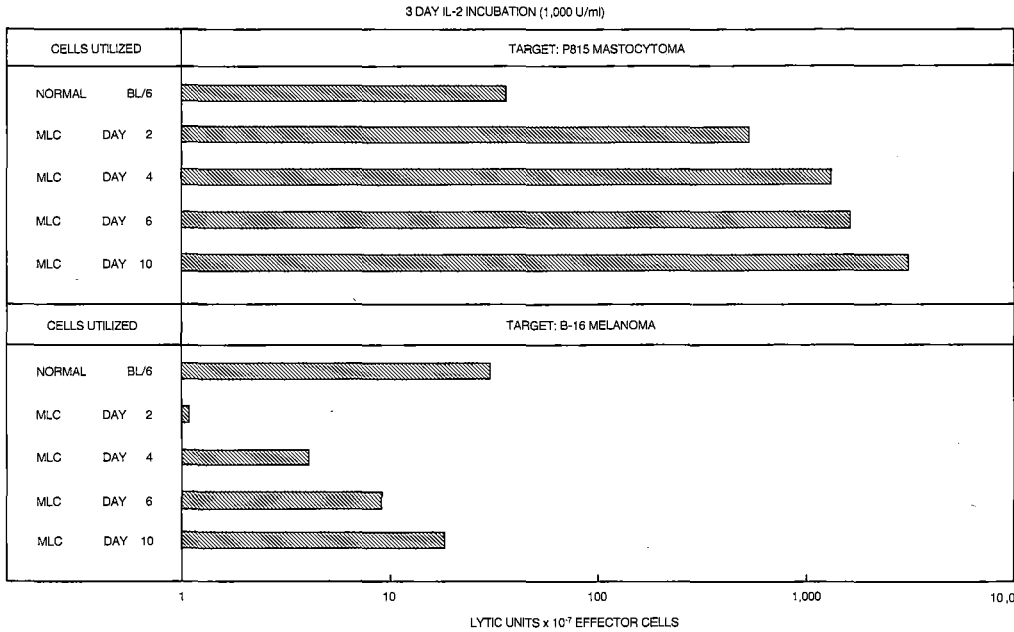

Figure 5.3 Lymphokine-activated killer (LAK) cell generation and cytolysis of allogeneic target cells by mixed lymphocyte culture (MLC) cells incubated with interleukin 2 (IL-2). BL/6 responder cells were placed in culture with DBA/2 stimulator cells and these cultures harvested 2, 4, 6, and 10 days later. Cells were counted and placed back in culture with IL-2 at 1,000 U/ml for 3 days. Incubation with IL-2 augmented the allogeneic responses. However, LAK cell killing was markedly reduced in cells harvested from MLC , especially the cells that had been in MLC for 2 days.

ponder cells from MLC, at a ratio of 1:1 or 1:10, and with IL-2 (1,000 U/ml) for three days. The cells from MLC were suppressive to the generation of LAK cells (Table 5.1 and Figure 5.4) and this suppression increased with time spent in MLC. The suppression of LAK cell induction by specific cytolytic cells from MLC (stimulator: DBA/2 splenocytes) is profound. The suppression is maximal with day 2 MLC cells and recovers thereafter. But day 10 cells from MLC still suppress LAK cell generation, compared to LAK cell generation from BL/6 splenocytes only. With P815 mastocytoma cells as stimulator cell in the MLC, suppression of LAK cell generation is retarded until day 6 compared to DBA/2 stimulated MLC cells. This suppression of LAK cell induction is maximal with day 10 MLC cells. Table 5.2 and Figure 5.5 show an increasing cytotoxicity towards the allogeneic P815 target with time spent in MLC and culture in IL-2 as compared to LAK cell

Table 5.1 Lymphokine-activated killer (LAK) cell induction by culturing BL/6 lymphocytes and day 0, 2, 4, 6, and 10 MLC cells with IL-2 (1,000 U/ml) for 3 days. Cytolysis of the B-16 melanoma target in a 16 hour ^{51}Cr release assay is expressed in lytic units / 10^7 effector cells.

TARGET B-16	**LYTIC UNITS / 10^7 EFFECTORS**			
stimulator	**DBA/2**	**DBA/2**	**P815**	**P815**
	o—o	●—●	□—□	■—■
3 day IL-2 incubation	**BL/6 : MLC 1 : 1**	**BL/6 : MLC 1 : 10**	**BL/6 : MLC 1 : 1**	**BL/6 : MLC 1 : 1**
day 0	59	42	25	36
day 2	< 1	3	26	25
day 4	< 1	5	56	24
day 6	2.5	26	7	10
day 10	10		1	4.5

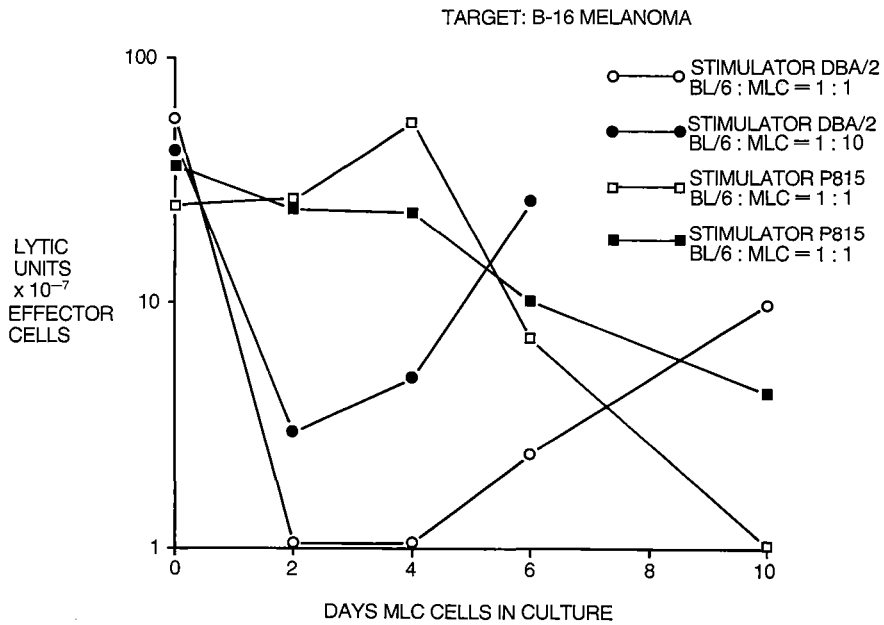

Figure 5.4 Cytolysis of B-16 melanoma cells by LAK effector cells as a function of the influence of cells taken from MLC at different time points on LAK cell generation. See Table 5.1 for time points, stimulator cell in MLC and ratio of cell mix in LAK culture.

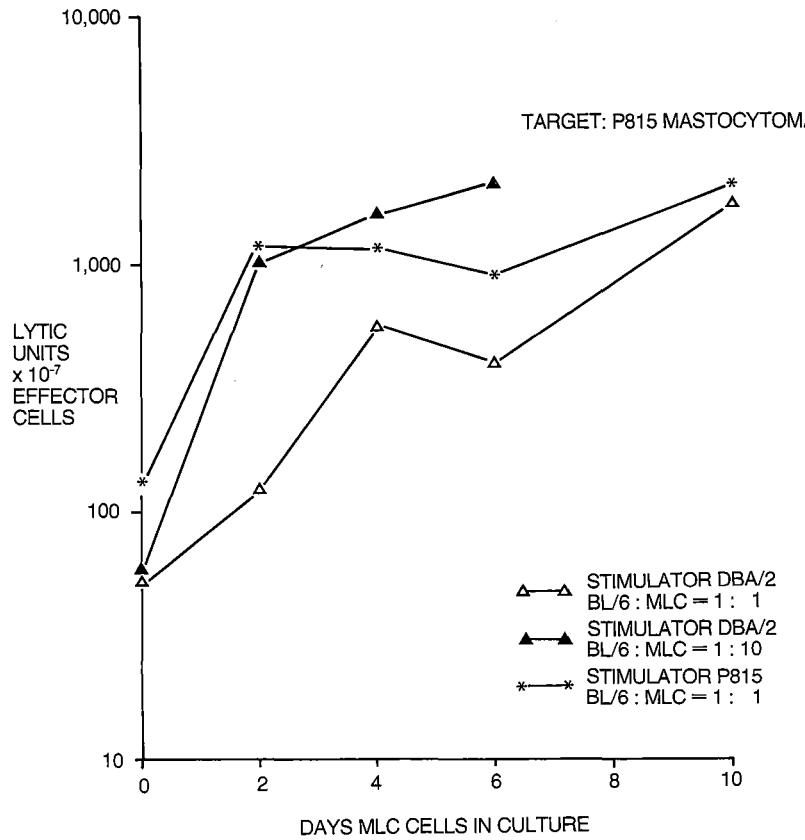

Figure 5.5 Cytolysis of P815 mastocytoma cells by LAK / CTL effector cells as a function of the influence of cells taken from MLC at different time points on LAK cell generation / IL-2 induced CTL proliferation. See Table 5.2 for time points, stimulator cell in MLC and ratio of cell mix in LAK culture.

lysis only (day 0 in MLC). DBA/2 splenocytes are less potent stimulator cells in MLC than P815 mastocytoma cells are; however a 10 fold increase of DBA/2-stimulated MLC cells in the IL-2 culture makes the cytolysis pattern approach that of P815-stimulated MLC cells.

Table 5.2 Lymphokine-activated killer (LAK) cell induction and specific cytotoxic T lymphocyte (CTL) enhancement by culturing BL/6 lymphocytes and MLC cells at various intervals with IL-2 (1,000 U/ml) for 3 days. Cytolysis of the P815 mastocytoma target in a 16 hour ^{51}Cr release assay is expressed in lytic units / 10^7 effector cells.

TARGET P815	LYTIC UNITS / 10^7 EFFECTORS		
stimulator MLC	**DBA/2**	**DBA/2**	**P815**
	Δ——Δ	**▲——▲**	***——***
3 day IL-2 incubation	**BL/6 : MLC 1 : 1**	**BL/6 : MLC 1 : 10**	**BL/6 : MLC 1 : 1**
day 0	53	59	133
day 2	125	1042	1220
day 4	588	1613	1176
day 6	400	2174	909
day 10	1852		2128

5.3.3 *Influence of MLC cells on the in vitro maintenance of LAK cells tested in a syngeneic cytotoxicity assay*

BL/6 splenocytes were incubated for three days with 1,000 U/ml IL-2. The resulting lymphokine-activated killer (LAK) cells were washed and counted. These LAK cells are put into culture again with MLC cells cultured for 2, 4, 6, and 10 days respectively (responder: BL/6 splenocyte; stimulator: P815 mastocytoma cell,

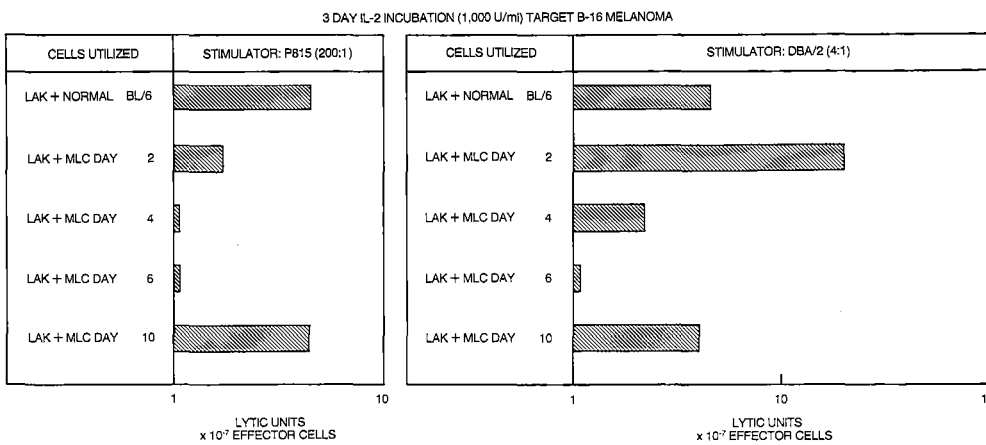

Figure 5.6 The effects of MLC cells at various intervals [responder: BL/6 splenocyte; stimulator: P815 mastocytoma cell, ratio 200:1 (**left**) and DBA/2 splenocyte, ratio 4:1 (**right**)] on LAK cell maintenance in three day culture with IL-2 (1,000 U/ml). Cytolysis in a 4 hour ^{51}Cr release assay is expressed as lytic units / 10^7 effector cells (target B-16 melanoma cells).

ratio 200:1; respectively DBA/2 splenocyte, ratio 4:1) and cultured with IL-2 (1,000 U/ml) for three days. LAK activity is tested in a ^{51}Cr release assay with the syngeneic B-16 melanoma target. Figure 5.6 shows the profound depression of LAK cell cytotoxicity by MLC cells, maximal on day 4 and 6, with P815 stimulator cells. LAK activity has returned to normal by day 10. With the DBA/2 stimulator cell there is an augmentation of LAK cell cytotoxicity with day 2 MLC cells and reduction by day 4 and 6 MLC cells.

5.3.4 *Cytotoxicity of MLC cells for BL/6 splenocytes, LAK cells and P815 tumor cells*

To rule out direct cytotoxic effects of MLC cells at different time points on BL/6 splenocytes, LAK cells, and P815 tumor cells these respective cells were

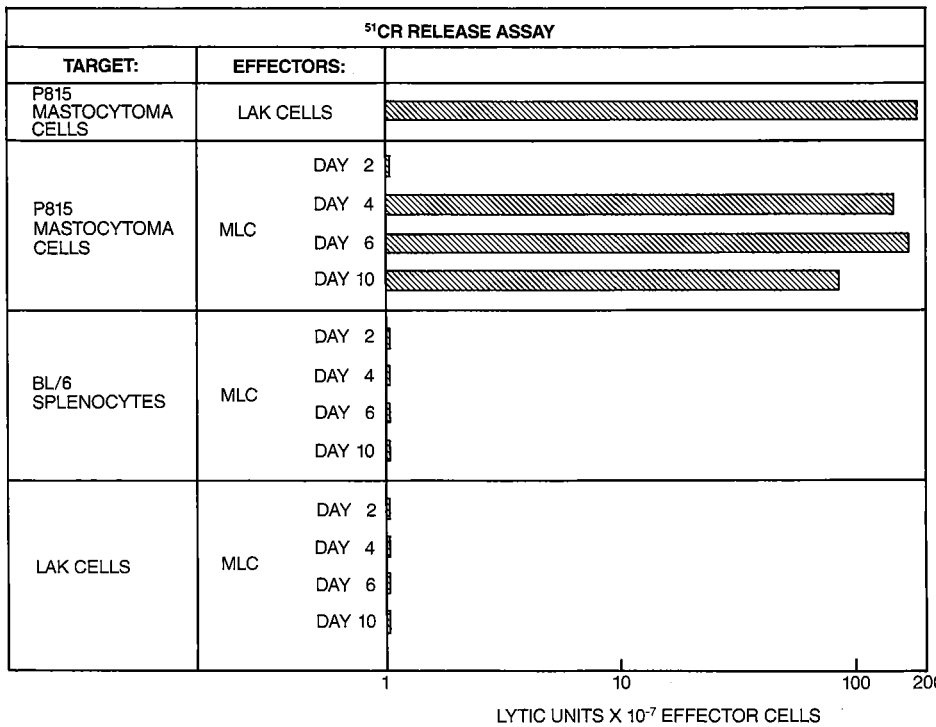

Figure 5.7 Cytoxicity of MLC cells at various time points (responder: BL/6 splenocyte; stimulator: DBA/2 splenocyte; ratio 4:1) for BL/6 splenocyte, LAK cells, and P815 tumor cells tested in a 16 hour ^{51}Cr release assay. Cytotoxicity is expressed as lytic units / 10^7 effector cells. Control: 4 hour ^{51}Cr release assay with day 3 LAK cells as effector and P815 cells as target.

labeled with ^{51}Cr and cytolysis determined in a 16 hour ^{51}Cr release assay. Day 2, 4, 6, and 10 MLC cells were the effector cells. As a positive control the cytotoxicity of day 3 LAK cells for ^{51}Cr labeled P815 mastocytoma cells was determined. Figure 5.7 shows the specific cytotoxicity of BL/6-anti-DBA/2 MLC cells for P815 mastocytoma cells, as described before (5.3.1) (Figure 5.2). No cytolysis of LAK cells or BL/6 splenocytes by any of the MLC cells was seen. Day 3 LAK cells were definite cytotoxic for P815 mastocytoma cells.

5.3.5 *Origin of the MLC cell responsible for modifying LAK cell induction, maintenance and allogeneic cytotoxicity*

Normal BL/6 lymphocytes were placed in culture with IL-2 at 1,000 U/ ml. Lymphocytes from day 6 MLC (stimulator: DBA/2 splenocytes) were added in

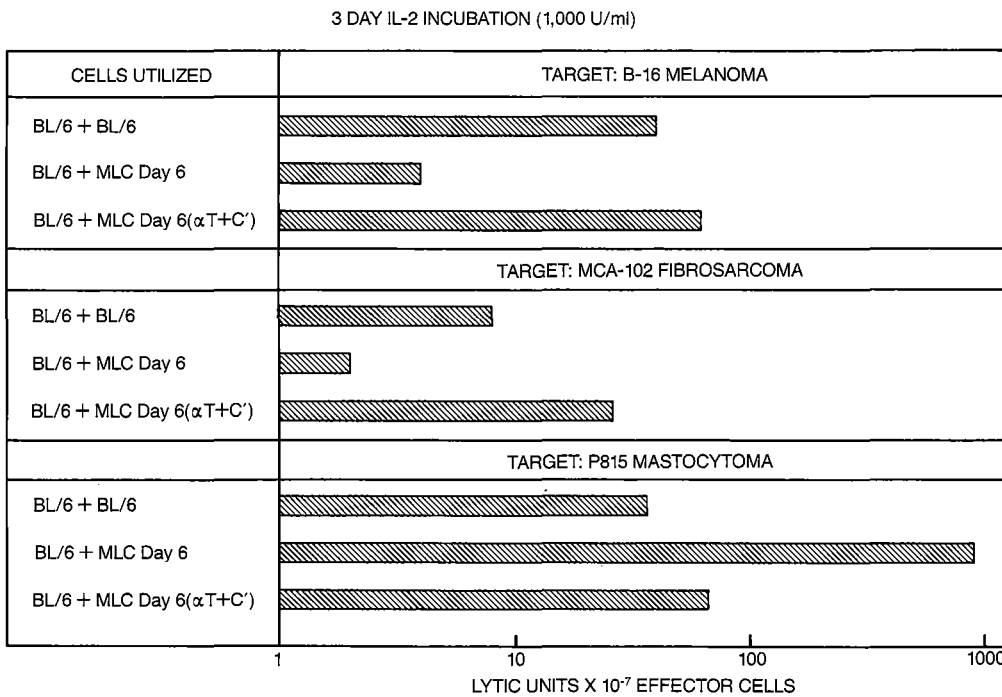

Figure 5.8 Anti-T cell serum plus complement abrogated the suppressive effects of MLC cells. BL/6 lymphocytes were placed in culture with interleukin-2 (IL-2) at 1,000 U/ml. Lymphocytes from a mixed lymphocyte culture (MLC) initiatied 6 days earlier were added in equal quantities. Some of these cells from culture were T cell depleted using anti-T cell serum plus complement (C'). T cell depletion abrogated the suppressive effects of MLC cells.

equal quantities. Alternatively, these cells were treated with anti-T cell serum plus complement. Following T cell depletion, these MLC cells were recounted and resuspended. Equal numbers of cells were added to BL/6 responder cells. Following 3 day incubation in IL-2 at 1,000 U/ml, the cell mix was harvested and the LAK cell as well as allogeneic cytotoxicity were determined. Figure 5.8 shows that the suppressive effect of MLC cells was abrogated by treatment with anti-T cell serum plus complement. This was true when the tumor target was a syngeneic B-16 melanoma or MCA-102 tumor. When cultured in the presence of IL-2 the BL/6 lymphocytes plus MLC cell mix showed markedly augmented cytotoxicity for P815 tumor cells. In the absence of IL-2 these cells would be markedly suppressive to the generation of T cell cytotoxicity toward an allogeneic target.[2] The augmentation seen by MLC cells plus IL-2 was abrogated by anti-T cell serum plus complement.

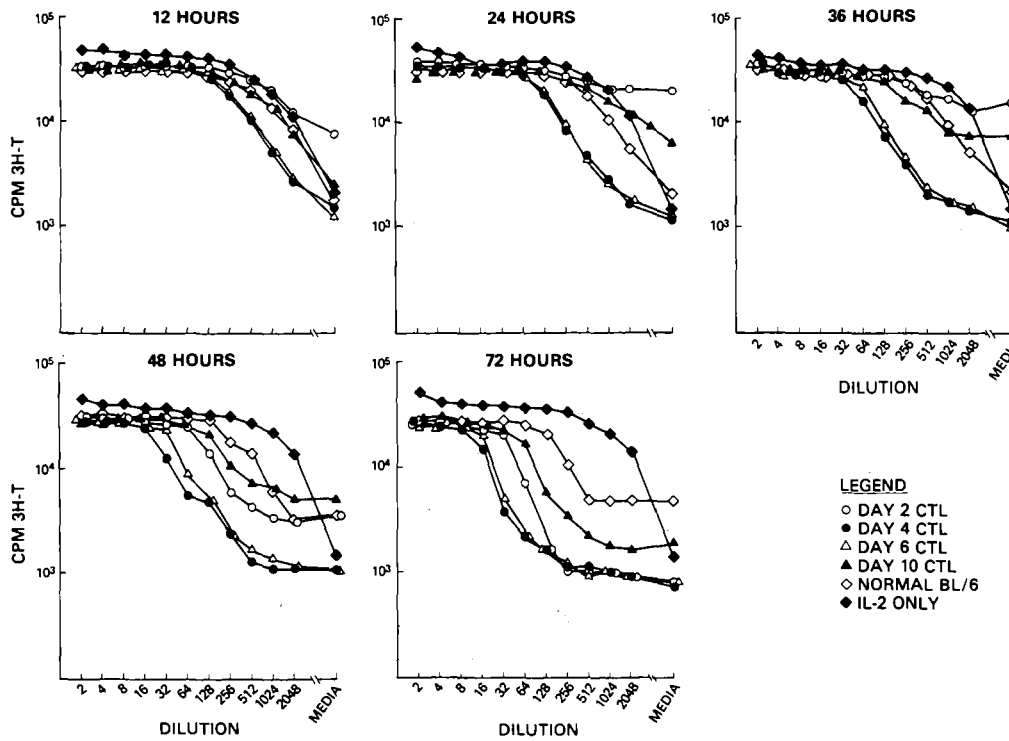

Figure 5.9 *In vitro* absorption of interleukin-2 (IL-2) by cytotoxic T lymphocytes (CTL). Cells taken from mixed lymphocyte culture at 2, 4, 6, and 10 days after initiation of culture were incubated with serially diluted IL-2 for 12, 24, 36, 48, and 72 hours. The supernatant was then titered for IL-2 activity using an IL-2-dependent cell line (M-53).

Anti-T cell serum alone or complement alone was shown to have no effect on suppression or cytotoxicity of MLC cells in culture (data not shown). The cytotoxicity of the MLC cells for a ^{51}Cr-labeled P815 target assessed immediately following depletion with anti-T cell serum plus complement was < 1 lytic unit / 10^7 effector cells. The cytotoxicity of cells not exposed to anti-T cell serum plus complement was 1,768 lytic units / 10^7 effectors. In this experiment depletion of T cell cytotoxicity with anti-T cell serum plus complement was complete (data not shown).

5.3.6 *Absorption and / or production of IL-2 by MLC cells*

MLC cells were prepared as described before (stimulator: DBA/2 splenocytes; ratio 4:1) and harvested after 2, 4, 6, and 10 days in culture. Il-2 (1,000 U/ml in 0.1 ml complete medium) was placed in the first well of a 96 well microtiter plate and serially diluted. 10^4 Lymphocytes from MLC were placed in the microtiter plate along with the serially diluted IL-2 and incubated for 12, 24, 36, 48, or 72 hours. The plates were centrifuged and supernatants transferred into wells containing the M-53 IL-2-dependent cell line. After incubation, a 4 hour pulse with [^3H]-thymidine was performed and uptake was determined. Counts per minute are shown in Figure 5.9. Immune lymphocytes from MLC after 4 or 6 days of incubation markedly reduced the titer of IL-2 in the media; this absorption of IL-2 increased with time. Cells from MLC left intact for only 2 days apparently contributed IL-2 to the media because proliferative responses of M-53 cells were seen at all dilutions tested and with media only. Normal BL/6 lymphocytes did not absorb IL-2 from media over this short incubation time.

5.3.7 *Do MLC cells produce a soluble IL-2 suppressive factor?*

Hardt[3] and Honda[4] found evidence for a soluble suppressive factor of IL-2. In order to rule out the activity of a soluble suppressive factor of IL-2, we harvested lymphocytes from MLC after 6 days. These groups of cells were incubated in 32 well plates for 24, 48, or 72 hours. The supernatants were harvested and added to serially diluted IL-2 in 96 well plates. M-53 cells were added to determine IL-2 activity. No suppression of IL-2 by supernatants from cytotoxic lymphocytes was seen (Figure 5.10). A soluble suppressor could not be shown to cause reduced proliferative effects of IL-2. These data show that in the same experiment day 6 cytotoxic T lymphocytes (CTLs) were suppressive, but supernatants from these cells showed no suppression. This experiment was repeated with day 2, 4, 6, and 10 CTL

and again no soluble suppressive factor in the supernatants could be demonstrated.

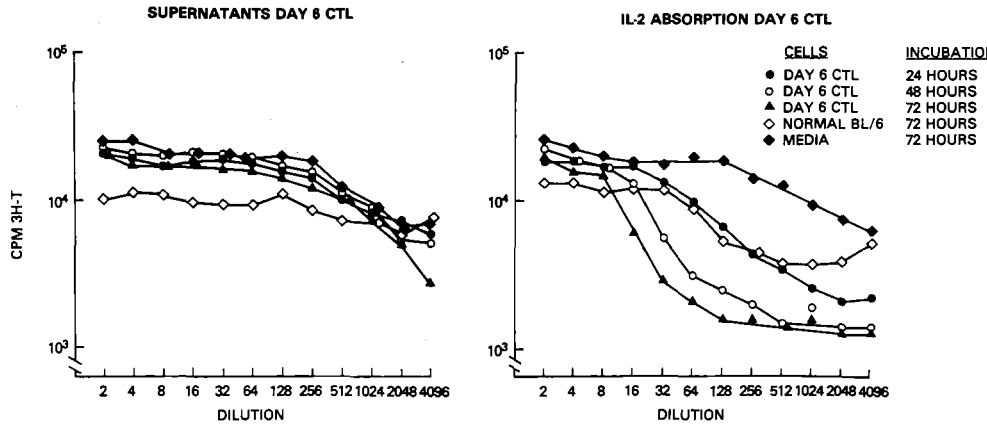

Figure 5.10 **Left:** Failure of day 6 cytotoxic T lymphocyte (CTL) supernatants to suppress interleukin 2 induced proliferation of M - 53 cells. Day 6 CTL from mixed lymphocyte culture (MLC) were incubated for 24, 48, 72 hours. The supernatants were harvested and added to IL-2, serially diluted in 96 well plates. IL-2-dependent M-53 cells were added to determine IL-2 activity.

Right: IL-2 absorption by day 6 CTL. Day 6 CTL from MLC were placed in 96 well plates along with serally diluted IL-2 for 24, 48, 72 hours. Supernatants were transferred into wells with IL-2-dependent M-53 cells to determine the IL-2 content.

5.4 Discussion

Cytotoxicity of BL/6 splenocytes challenged in a mixed lymphocyte culture by an allogeneic stimulator reaches maximal values towards the same allogeneic target in a ^{51}Chromium release assay after 4 to 6 days in culture. P815 mastocytoma cells at a ratio of 200:1 BL/6 splenocyte in MLC being more potent stimulators than DBA/2 splenocytes at a ratio of 4:1 (Figure 5.2). As others,[5 6 7 8] we found an increased cytotoxicity after incubation of the cells from MLC in IL-2 for three days. This cytotoxicity was 10 times the cytolysis of the allogeneic target by LAK cells (Figure 5.3). When the cells from MLC were mixed with normal BL/6 splenocytes and incubated with IL-2 the cytotoxicity and maximal cytolysis of the allogeneic target was similar to the cytotoxicity of MLC cells alone incubated in IL-2. Again P815 being a more potent stimulator than DBA/2 splenocytes (Table 5.2 , Figure 5.5). For the influence of MLC cells on LAK cell activity Table 5.3

gives a short summary. Induction of LAK cells from MLC cells is definitely impaired, especially on day 2, 4, and 6. Cytolysis of the syngeneic B-16 melanoma target does not even reach normal levels (Figure 5.3). Suppression of LAK cell induction from normal BL/6 splenocytes by IL-2 is most pronounced by day 2 and 4

Table 5.3 Short summary of the influence MLC cells have on LAK cell activity *in vitro*.

		'ENHANCEMENT'	'ABROGATION'
STIMULATOR IN MLC	3 DAY IL-2 INCUBATION	OF LAK CELL	ACTIVITY
DBA/2	MLC	-	DAY 2, 4, (6)
DBA/2	BL/6 + MLC	-	DAY 2, 4, (6)
P815	BL/6 + MLC	-	DAY 6, 10
DBA/2	LAK + MLC	DAY 2	DAY 4, 6
P815	LAK + MLC	-	DAY 2, 4, 6

MLC cells stimulated with DBA/2 splenocytes; while maximal suppression from MLC cells stimulated with P815 tumorcells is not reached until day 6 and 10 (Table 5.1, Figure 5.4). The maintenance of LAK cell cytotoxicity by IL-2 is impaired by MLC cells; mainly day 2, 4, and 6 with P815 stimulation in MLC (Figure 5.6 left). In contrast, day 2 MLC cells (DBA/2 stimulators) enhanced LAK cell maintenance; and day 4 and 6 suppressed LAK cells in culture (Figure 5.6 right). No direct cytotoxic effect of MLC cells for LAK cells or normal BL/6 splenocytes could be detected (Figure 5.7). The effector cell reducing LAK cell cytotoxicity must be of the T cell lineage, since specific anti-T serum plus complement treatment of allo-stimulated cells abrogated their suppressive effects, but not LAK cell induction (Figure 5.8).

We postulate that the specific cytotoxic lymphocytes elicited by the allogeneic stimulator cells in MLC absorb IL-2 with a higher affinity than LAK cell precursors and LAK cells.[9] Jacques *et al*[10] showed the induction; and Malek *et al*[11] the up-regulation of high and low affinity IL-2 membrane receptors by IL-2. The

number and affinity of IL-2 receptors on the MLC cells probably out-class those on LAK cells, although it must be a time-dependent phenomenon. Suppression develops in time and is dependent on the number and origin of the stimulator cells in the MLC (Figures 5.3, 5.4, and 5.6). Furthermore, MLC cells absorp IL-2 and this increases over time of culture; but day 2 MLC cells produce IL-2 (Figure 5.6 and 5.9).

Suppression of LAK cell generation by MLC cells is maximal on day 2 and 4 when DBA/2 splenocytes are the stimulator in MLC. This suppressive effect wears off by day 10. Surprisingly, a 1:1 ratio of cells from MLC and normal BL/6 splenocytes do suppress LAK cell generation to a greater extent than a ratio of 10:1 does. The DBA splenocyte might produce helper factors that stimulate LAK cell induction apart from being the stimulator in the MLC.[12 13] P815 mastocytoma cells probably do not produce these helper factors. Also, T-helper cells, present in the splenocyte population, will produce IL-2 locally. Furthermore, DBA/2 and the BL/6 splenocytes might be induced by IL-2 into releasing IL-2.[11] Figure 5.9 shows us this delicate balance in time: day 2 and to some extent day 10 MLC cells produce IL-2 in the media to sustain the M-53-IL-2-dependent cell line. Only after 48 and 72 hours in culture normal BL/6 splenocytes do produce some IL-2. At a ratio of 10:1 this endogeneously produced IL-2 might be sufficient to recover some of the reduced LAK activity caused by the competitive inhibition of MLC cells. In line with this observation is our finding that day 2 MLC cells (DBA/2 stimulator) enhance LAK cell maintenance (Figure 5.6)

The longer duration of the abrogation of LAK cell induction by P815 stimulator cells in the MLC (Figure 5.4) might be explained by P815 being a more potent inducer of CTLs in MLC.

Suppression of immune responses must be multifactorial: We demonstrated the suppressive effects of CTLs from MLC. The question remains whether these suppressive CTLs are equal or distinct to the suppressor T cells Ting[14] induced with IL-2. Honda *et al*[4] as well as Itoh *et al*[15] demonstrated a soluble serum suppressive factor on the induction of (lymphokine-)activated killer cells. However, we could not find an indication of a soluble suppressive factor in the supernatants of cytotoxic lymphocytes (5.3.7). Finally, in a system with proliferation inducers negative feedback signals are to be expected; Maki[16] describes 'contra-IL-2', 'a suppressor lymphokine that inhibits IL-2 activity'. Our hypothesis of IL-2 being the molecule used by a number of lymphocyte subpopulations to generate or multiply their effector functions fits in with our findings of CTLs competitively inhibiting LAK cell induction, proliferation and maintenance; as well as with the findings of others as described above. Further research should clarify the weight of this competitive

inhibition phenomenon relative to other helper (co-)factors, suppressor cells and their changing effect over time.

5.5 Summary

This chapter describes the *in vitro* influence of allogeneic challenged BL/6 splenocytes on LAK cell generation and specific cytotoxicity induced by IL-2. Cells taken from mixed lymphocyte culture and incubated for three days in IL-2 showed a reduced capability of generating LAK cells. However, their cytotoxicity toward an allo-immunogeneic target was markedly increased by three days of incubation in IL-2. Cells from MLC were markedly inhibitory to normal splenocytes in the generation of LAK cell cytotoxicity; they also interfered with the maintenance of LAK cell cytotoxicity.

A T cell was responsible for the suppressive effects on LAK cell generation, because suppression was abrogated by treatment of MLC cells with anti-T serum plus complement. The cytotoxic T cell did not lyse the LAK cell. If IL-2 was serially diluted and incubated with CTLs, the IL-2 titer was substantially reduced by 72 hours incubation. An exception should be made for day 2 and to some extent day 10 MLC cells: they produce IL-2 in the media. When supernatants from MLC were added to serially diluted IL-2, no suppression of the IL-2 induced proliferation of M-53 cells was seen. When the cells from MLC were added to serially diluted IL-2, the IL-2 was absorbed out by the MLC cells and reduced IL-2-dependent-M-53 cell proliferation was observed. This suggested that the diminished IL-2 plus LAK cell effect was not caused by a soluble suppressor factor produced by MLC cells, but by the absorption of IL-2 by CTLs.

Our hypothesis based on these *in vitro* experiments that CTLs compete with normal lymphocytes or LAK cells for IL-2 and thereby suppress LAK cell responses, is discussed.

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Chapter 6

SEQUENTIAL HARVESTING OF IMMUNE SPLEEN CELLS AND THEIR INFLUENCE ON LAK CELL GENERATION BY INTERLEUKIN 2

6.1 Introduction

Inoculation of the mouse with a cell-bound allo-antigen will set in motion a cascade of humoral and cellular immune responses. These responses will try to overcome the allo-antigenic offensive; antibodies and specific cytotoxic T lymphocytes will lyse the allogeneic cells. The defensive cellular army will withdraw, only remembering the antigenic code in order to react even faster in case of a new invasion. The induction, activation, proliferation and withdrawal will have to be coordinated with positive and negative feedback systems. Biological response modifiers are believed to be the messengers in this complicated defense system. In Chapter 5 we postulated interleukin 2 as one of the central inducing and proliferating signals and showed in a controlled *in vitro* system the competitive inhibition by cytotoxic T lymphocytes (CTL) from a mixed lymphocyte culture (MLC) of lymphokine-activated killer (LAK) cells.

In vivo the conditions are less controlable. Before investigating the influence of an on-going immune response on immunotherapy with interleukin 2 and LAK cells *in vivo*, we first studied *in vitro* the spleen cells of mice challenged with allogeneic antigens, in order to answer the following questions: 1. When do these spleen cells develop maximal cytotoxicity for the inducing allo-antigen? 2. Can these spleen cells be induced into LAK cells? 3. What is the influence of these spleen cells on LAK cell induction of normal BL/6 splenocytes?

6.2 Methods

Groups of three C57BL/6 mice were challenged intraperitoneally (i.p.) with 10^7 P815 cells 24, 21, 17, 14, 10, 7, and 4 days and for other experiments 28, 14, 7, and 4 days before sacrifice and splenectomy. After sacrifice by cervical dislocation

the spleens of three mice were harvested aseptically and prepared as described before (2.4.6). Briefly, the spleens were placed in ice cold complete medium and crushed with the hub of a syringe. Erythrocytes were lysed with ACK buffer and the remaining lymphocytes were washed in ice cold HBSS. The lymphocytes were used for ^{51}Chromium (^{51}Cr) release studies. To facilitate the discussion we will use the term 'allo-immune cells' for these spleen cells from allo-antigen challenged mice. These allo-immune cells were cultured for another 3 days with IL-2 (1,000 U/ml) in complete medium (4 x 10^6 cells/ml) and tested for LAK cell activity against the B-16 melanoma target in a 4 hour ^{51}Cr release assay (Figure 6.1). Alternatively, these allo-immune cells were mixed with normal, fresh BL/6 splenocytes in a ratio of 1:1 and cultured for three days with IL-2 (1,000 U/ml). Again their cytolytic activity was tested in a ^{51}Cr release assay with B-16 target cells. Finally these allo-immune cells were mixed with *in vitro* activated live LAK cells in

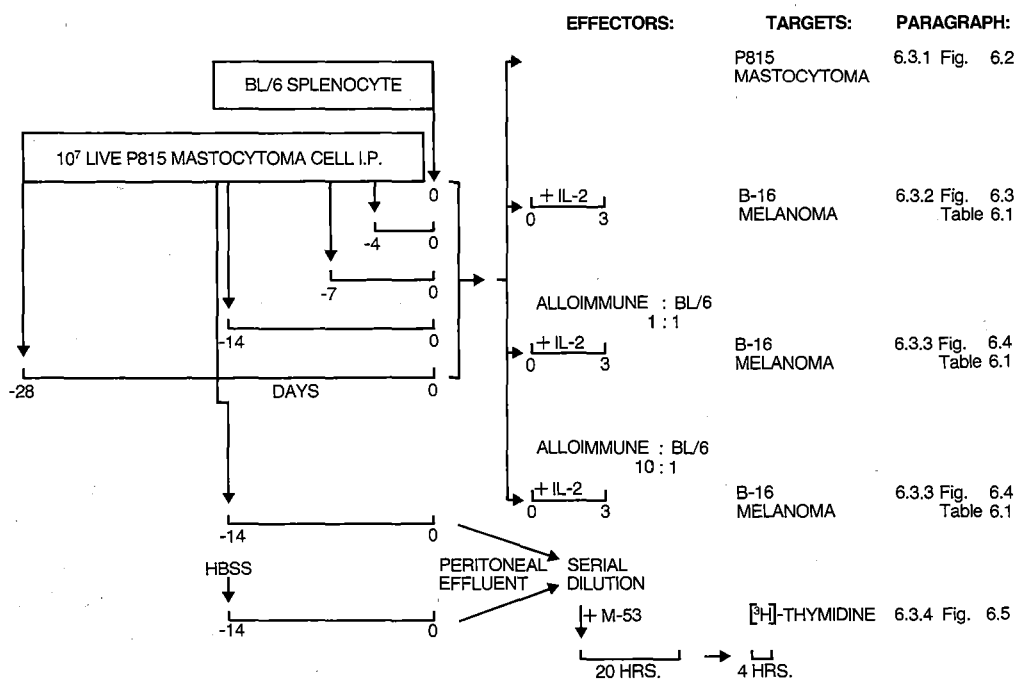

Figure 6.1 Flow chart of the experiments conducted with the spleen cells of C57BL/6 mice 48, 21, 14, 7, and 4 days after intraperitoneal allogeneic challenge with 10^7 P815 (H-2^d) mastocytoma cells. These allo-immune spleen cells were cultured with IL-2; BL/6 lymphocytes (1:1) and IL-2; or LAK cells (1:1) and IL-2. Cytotoxicity was tested in a 4 hour ^{51}Cr release assay against B-16 (H-2^b) melanoma cells.

a ratio of 1:1 and cultured for another 3 days with IL-2 (1,000 U/ml). These cells were tested against the B-16 target in a 4 hour ^{51}Cr release assay.

Experiments were done to determine the period P815 cells were still proliferating in the BL/6 abdomen (7.2.2). On day 7 and 10 after allogeneic challenge the abdomen still contained live P815 cells as determined by their appearance in 0.08% trypan blue. Upon adoptive transfer only day 7 peritoneal cells still grew out in a DBA/2 host and killed the animal. At day 10, 14, 17, and 21 P815 tumor cells did not grow in the syngeneic host and these mice survived indefinitely.

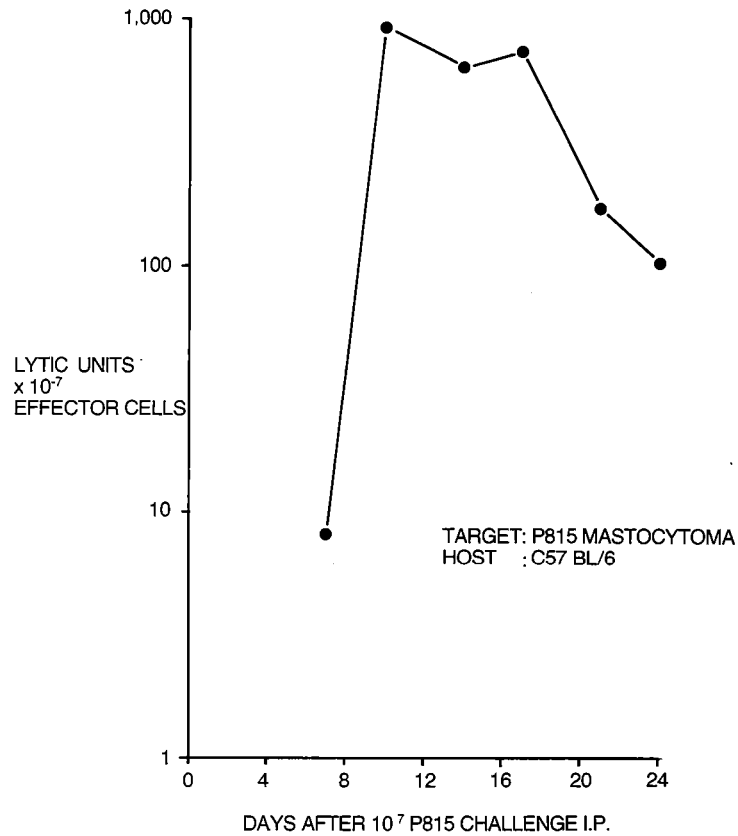

Figure 6.2 Splenic cytotoxicity in BL/6 mice after allo-antigenic challenge. BL/6 mice were challenged with 10^7 P815 cells i.p. 7, 10, 14, 17, 21, and 24 days before sacrifice and spleen harvest. On the same day 3 spleens per time point were pooled and the splenic lymphocyte cytotoxicity - expressed in lytic units per 10^7 effector cells - was tested in a 16 hour ^{51}Cr release assay against the P815 mastocytoma target.

6.3 Results

6.3.1 *Lysis of the allogeneic P815 target by BL/6 allo-immune splenocytes*

BL/6 mice were challenged 24, 21, 17, 14, 10, and 7 days before sacrifice with 10^7 P815 mastocytoma cells i.p. Mice were sacrificed on the same day; the spleens of 3 mice per time point were pooled and prepared as described before. Figure 6.2 shows the development of the cytotoxicity of the spleen cells over time towards the allogeneic P815 target. Cytotoxicity is maximal by day 10 and is at a plateau phase till day 17, to decrease gradually thereafter. Maximal cytotoxicity did not increase with a second and third challenge with 10^7 P815 cells on day 13 and 16 respectively (data not shown).

6.3.2 *LAK cell induction from BL/6 allo-immune splenocytes*

BL/6 mice were challenged i.p. with 10^7 live P815 cells 28, 14, 7 and 4 days before sacrifice. All mice were sacrificed the same day and the spleens of 3 mice per time point were pooled and lymphocytes prepared as described before. Lymphocytes were incubated for three days with IL-2 (1,000 U/ml) and their cytotoxicity was tested in a 4 hour ^{51}Cr release assay towards the B-16 melanoma target. LAK

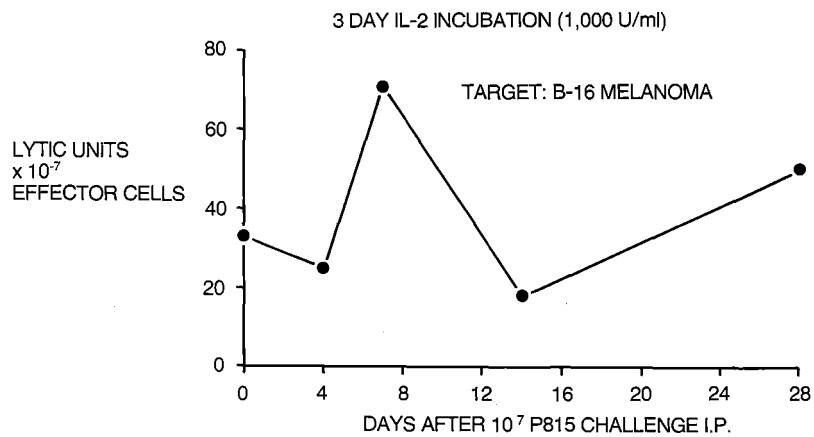

Figure 6.3 LAK cell induction from allo-immune cells. BL/6 mice were challenged with 10^7 P815 cells 4, 7, 14, and 28 days before sacrifice and spleen harvest. On the same day 3 spleens per time point were pooled and the splenic lymphocytes were put into culture with 1,000 U/ml IL-2. LAK cell cytotoxicity - expressed in lytic units per 10^7 effector cells - was tested in a 4 hour ^{51}Cr release assay against the B-16 melanoma target.

cytotoxicity was slightly depressed at day 4 and 14 and over 2 fold enhanced at day 7 compared to LAK cytotoxicity of normal (day 0) BL/6 splenocytes (Figure 6.3 and Table 6.1).

Table 6.1 Enhancement of LAK cell generation by allo-antigenic challenge *in vivo*. LAK cell cytotoxicity - expressed in lytic units per 10^7 effector cells - was tested in a 4 hour ^{51}Cr release assay against the B-16 melanoma target.

EFFECTOR CELLS: 3 day IL-2 incubation (1,000 U/ml)	TARGET CELLS: B-16 MELANOMA CELLS lytic units / 10^7 effector cells				
	day of harvest of allo-immune spleen cells				
	0	4	7	14	28
ALLO-IMMUNE CELLS	33	25	71	18	50
ALLO-IMMUNE : BL/6 SPLEEN CELLS 1 : 1	30	30	125	70	55
ALLO-IMMUNE : BL/6 SPLEEN CELLS 10 : 1	67	83	125	67	53

6.3.3 *Enhancement of LAK cell induction by adding BL/6 allo-immune splenocytes*

BL/6 mice were challenged i.p. with 10^7 live P815 cells 28, 14, 7, and 4 days before sacrifice. All mice were sacrificed the same day and the spleens of 3 mice per time point were pooled and lymphocytes prepared as described before.

Allo-immune lymphocytes and normal BL/6 splenocytes at a ratio of 1:1, and 10:1 respectively, were incubated for three days with IL-2 (1,000 U/ml) and their cytotoxicity was tested in a 4 hour ^{51}Cr release assay towards the B-16 melanoma target. LAK cell cytotoxicity of the cell mixture incubated in IL-2 was 4-, respectively 2-fold, enhanced when day 7 allo-immune spleen cells were used, compared to LAK activity of normal (day 0) BL/6 splenocytes (Figure 6.4 and Table 6.1). Allo-immune cells at a ratio of 10:1 increased LAK cell induction nearly

three fold at day 4 compared to allo-immune cells at a ratio of 1:1. Allo-immune splenocytes did not seem to inhibit LAK cell induction from fresh splenocytes.

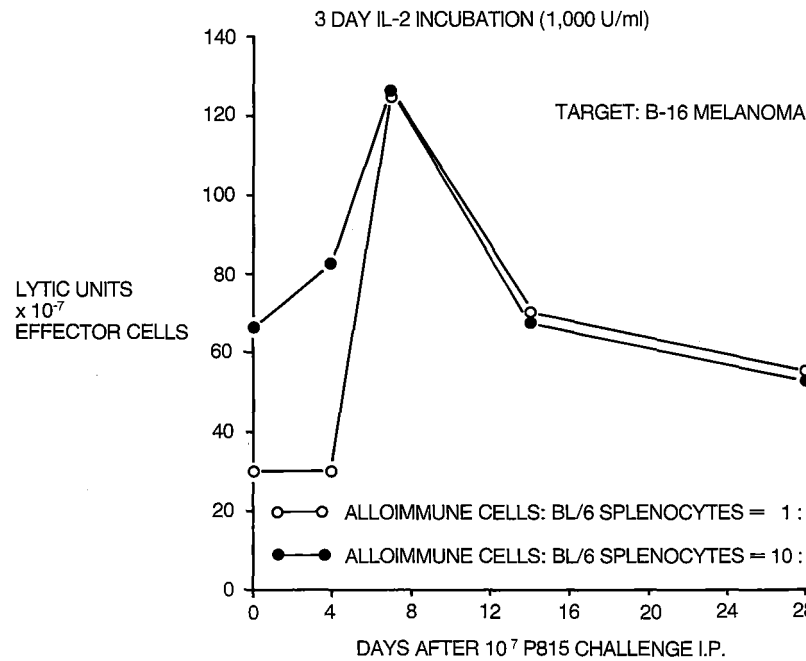

Figure 6.4 Enhancement of LAK cell induction by allo-immune cells. BL/6 mice were challenged with 10^7 P815 cells 4, 7, 14, and 28 days before sacrifice and spleen harvest. On the day of sacrifice 3 spleens per time point were pooled and the splenic lymphocytes at a ratio of 1:1, respectively 10:1 were put into culture with 1,000 U/ml IL-2. LAK cell cytotoxicity - expressed in lytic units per 10^7 effector cells - was tested in a 4 hour ^{51}Cr release assay against the B-16 melanoma target.

6.3.4 *Intraperitoneal interleukin 2 levels 14 days after intraperitoneal allogeneic challenge*

BL/6 mice were given 10^7 live P815 tumor cells intraperitoneal. Fourteen days later these animals and controlled litter mates were used to assess intraperitoneal levels of IL-2. Prior to sacrifice, normal mice (N = 5) and mice challenged with P815 tumor (N = 5) were given a peritoneal lavage with 10 ml of distilled water. The effluent from the peritoneal cavity was centrifuged to remove lymphocytes. It was then immediatley concentrated 40 times using an ultracentrifuge technique. These concentrates of peritoneal fluid were serially diluted and added to cells from an IL-2-dependent cell line (M-53). As control two different sources of

IL-2: EL-4 lymphoma derived- and recombinant IL-2 were used.

No IL-2 levels could be detected in the peritoneal exudate of mice fourteen days after challenge with the allo-antigen P815 i.p.

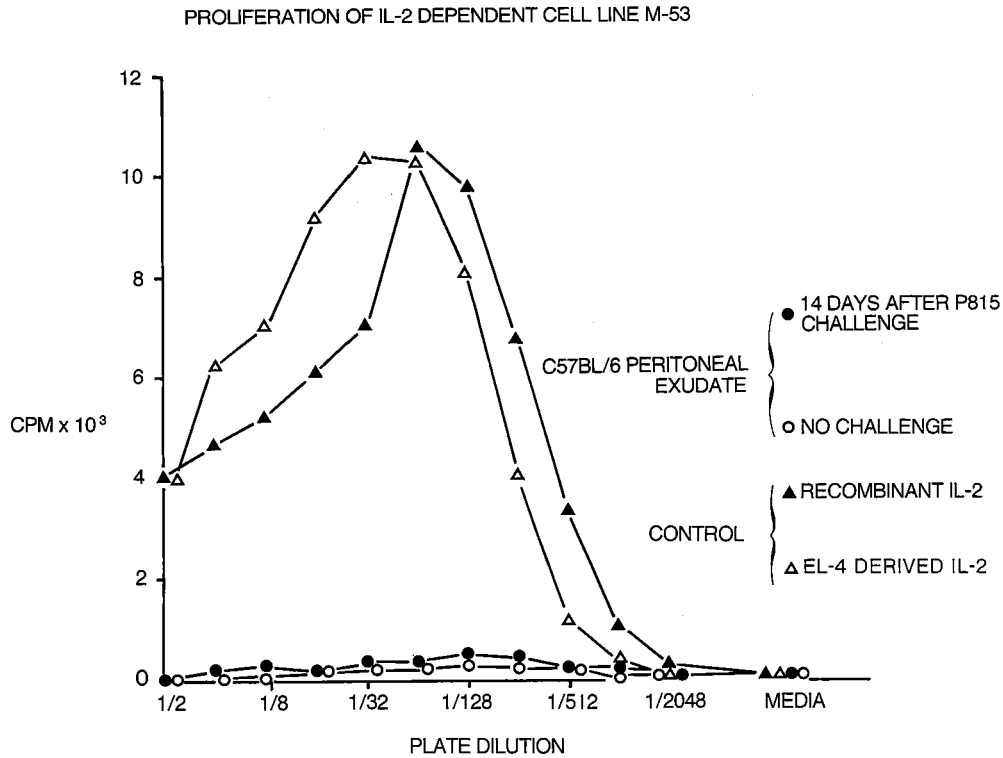

Figure 6.5 Intraperitoneal IL-2 levels 14 days after i.p. allogeneic challenge with 10^7 P815 cells in 5 mice. 5 Mice received no i.p. challenge. After peritoneal lavage the abdominal effluent was centrifuged, concentrated and added to 96 well culture plates. After serial dilution of the concentrate, 10^3 IL-2-dependent M-53 cells were added per well and cultured for 20 hours. The wells were pulsed with 2 microcurie [^{3}H]-thymidine. [^{3}H]-thymidine uptake in counts per minutes (cpm) was determined as a measure of proliferation of the M-53 cell line. EL-4 lymphoma derived- and recombinant IL-2 were used as control.

6.4 Discussion

Allogeneic cytotoxicity in the spleens of allo-antigenic stimulated mice was maximal between 10 and 17 days after i.p. challenge (Figure 6.2). Increased specific cytotoxicity of allo-immune cells after incubation with IL-2 towards the allo-

geneic challenge has been shown by many others;[1 2] so these experiments were not repeated by us. When these allo-immune cells were activated by IL-2 in a three day culture; the LAK cell activity was slightly depressed at day 4 and 14, but enhanced at day 7 (Figure 6.3). A short summary is shown in Table 6.2.

Table 6.2 Short summary of the influence 'allo-immune' cells have on LAK cell activity *in vitro*.

		'ENHANCEMENT'	'ABROGATION'
CHALLENGE IN VIVO	3 DAY IL-2 INCUBATION	OF LAK CELL ACTIVITY	
P815	ALLO-IMMUNE	DAY 7	DAY (4), 14
P815	ALLO-IMMUNE + BL/6 (1 : 1)	DAY 7	-
P815	ALLO-IMMUNE + BL/6 (10 : 1)	DAY 7	-

The same was true for LAK cell activity induced from normal BL/6 splenocytes by IL-2 and cultured together with day 7 allo-immune cells (Figure 6.4). No suppression was seen from day 14 allo-immune cells. LAK cell activity of BL/6 lymphocytes cultured concurrently with day 4 allo-immune cells at a ratio of 10:1 and IL-2 was nearly triple the LAK cell activity of BL/6 splenocytes cultured with allo-immune cells at a ratio of 1:1 and IL-2.

It should be noted that we looked at the activity and influence of allo-immune cells in the spleen and not in the abdominal cavity where the challenge had taken place. Peritoneal exudate cells were not thought suitable for these experiments since the effect of still viable P815 cells in the peritoneal exudate at day 4 and 7 could not be seperated from the effect of allo-immune cells. It should be stressed that these effects were also obtained when mice were inoculated with an irradiated challenge. The initial choice for a viable challenge was made in order to get an ongoing immune response *in vivo* (Chapter 8).

Although some factors will be different in the abdominal cavity as opposed to the spleen, stacked as it is with precursor cells, T-helper cells and committed

immune effectors, development of specific antigenic effector cells in the spleen seems to parallel their development in the abdomen.[1 3] However, Haisma[4] found elevated natural cytoxicity and antibody-dependent-cellular-cytotoxicity (ADCC) in the peritoneal exudate and not in the spleen after allogeneic tumor challenge i.p.

LAK cell induction from day 2 MLC cells is suppressed maximally (5.3.1), whereas LAK cell induction from allo-immune cells is enhanced at day 7 and suppressed at day 14. The same holds true for LAK cell induction from normal BL/6 splenocytes: abrogated by day 2 and 4 MLC cells (5.3.2) but enhanced by day 7 allo-immune cells. Peak allogeneic cytotoxicity of MLC cells starts at day 4 (5.3.1 and Figure 5.2), while allo-immune cells have their plateau phase beginning at day 10. Development of allo-immunity is slower in the living mouse than it is in the isolated cell culture. The enhancing effect of day 7 allo-immune cells seen in Figure 6.3 might coincide with an enhancing effect of day 1 MLC cells on LAK cell cytotoxicity: a time point we did not check for. A basis for this hypothesis can be found in Figure 5.9 where day 2 MLC cells produce IL-2 in their supernatants.

The stimulation of LAK cell cytotoxicity seen when day 7 allo-immune cells are cultured alone or together with BL/6 spleen cells could be explained by the endogenous IL-2 production in the spleen as well as by increased levels of LAK cell precursors and helper factors and -cells in the 'allo-immune' spleen cells. It should not be a surprise that this stimulative effect is much more pronounced and wears off much slower in the spleens of allogeneic challenged mice as opposed to the cells of MLC (Chapter 5) and the peritoneal cavity (Chapter 7): The spleen is an immune 'producing' organ. In Chapter 5 we discussed the influence of effector cells on the LAK cell induction and maintenance and in Chapter 7 we will discuss the influence of CTLs on immunotherapy in the abdomen. A higher ratio of allo-immune cells in the LAK induction culture results in a faster rising LAK cell cytotoxicity but the same peak cytotoxicity (Figure 6.1).

Again we find basis for our hypothesis that IL-2 might play a central role as inducing, proliferating and differentiating signal of immune responses. Immune responses set in motion must be stopped when the maximal response needed, is going to be reached: negative feed-back mechanisms like absorption of (excess) IL-2 will slow down the immune response. LAK cell induction from day 2 MLC cells is abrogated, while maximal cytotoxicity is reached at day 4. LAK cell generation from day 14 allo-immune cells is reduced, while peak P815 cytotoxicity is reached between day 10 and 17.

Although the IL-2 hypothesis is plausible, other suppressor mechanisms may be active as well. Proliferating suppressor T cells may slow down the immune response when the peak immune response is reached. Activation of macrophages

and other T cells may produce cytokines other than IL-2, inhibiting specific T cell precursors, but also LAK cell precursors.

It is not surprising that no IL-2 levels could be detected in the abdominal exudate at day 14. When the experiments were conducted, we expected peak endogeneous IL-2 production at the time of peak allo-antigenic cytotoxicity; while now, in view of our hypothesis, we would look for endogeneous IL-2 production at a much earlier stage of the immune response. Whether these day 14 allo-immune cells absorb more IL-2 from the media than allo-immune cells from other time points, remains to be answered.

6.5 Summary

To summarize: maximal specific cytolytic activity in the spleen is reached between 10 and 17 days after i.p. allogeneic challenge. LAK cell induction from allo-immune cells is enhanced 7 days after the P815 inoculation i.p. and reduced after 14 days. Day 7 allo-immune cells stimulate LAK cell induction from normal BL/6 splenocytes, while no real reduction was seen at any time point.

6.6 References

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Chapter 7

THE ENHANCING AND ABROGATING EFFECTS OF AN ALLOGENEIC CHALLENGE *IN VIVO* ON INTERLEUKIN 2 AND LAK CELL IMMUNOTHERAPY

7.1 Introduction

In vivo interleukin 2 (IL-2) has been used in experimental animals and humans in an attempt to augment immune responses and thereby help control cancer or an infectious disease process.[1 2 3] The *in vivo* induction of lymphokine-activated killer (LAK) cells[4] resulted in a dose dependent control of tumor load and increased survival in lung,[5] intraperitoneal,[6] subcutaneous,[1] and liver[7] inoculation models. The adoptive transfer of large numbers of *in vitro* generated LAK cells has successfully improved the immunotherapeutic effects of IL-2. Also, IL-2 can augment specific allo-immune responses both *in vitro* and *in vivo* when immune cells are exposed to adequate levels of this biological response modifier (Chapters 5 and 6).[4 8 9] In Chapter 5 we described experiments where the *in vitro* induction of LAK cells is reduced by specific-cytolytic lymphocytes. These cytotoxic T lymphocytes (CTL) induced *in vitro* might compete for IL-2, necessary for induction of LAK cells. In this chapter we show that lymphocytes activated *in vivo* by an allogeneic challenge abrogate the immunotherapeutic effect of IL-2 and LAK cells *in vivo* as well. We describe the effect of dose, timing, and route of administration of the allogeneic challenge on the IL-2 plus LAK cell immunotherapy of intraperitoneal tumor. These suppressive effects may result from competition within a microenvironment between T lymphocytes and LAK cells for IL-2. The effect of cyclophosphamide (Cy) on these cells and the competition for IL-2 is investigated.

7.2 Methods

7.2.1 *Splenic cytotoxic T lymphocyte induction by allogeneic challenge*

The development of splenic cytotoxic T lymphocytes by intraperitoneal immunization with 10^7 P815 mastocytoma cells of the BL/6 host was monitored sequentially using ^{51}Chromium (^{51}Cr) release assays with fresh P815 mastocytoma cells as target.[10] Specific CTL activity in spleen cells peaked at day 10 and stayed at a plateau phase until day 17 before it began to fall off at day 28 (Figure 7.1). Repeat experiments showed a similar pattern of immune response in mesenteric lymph

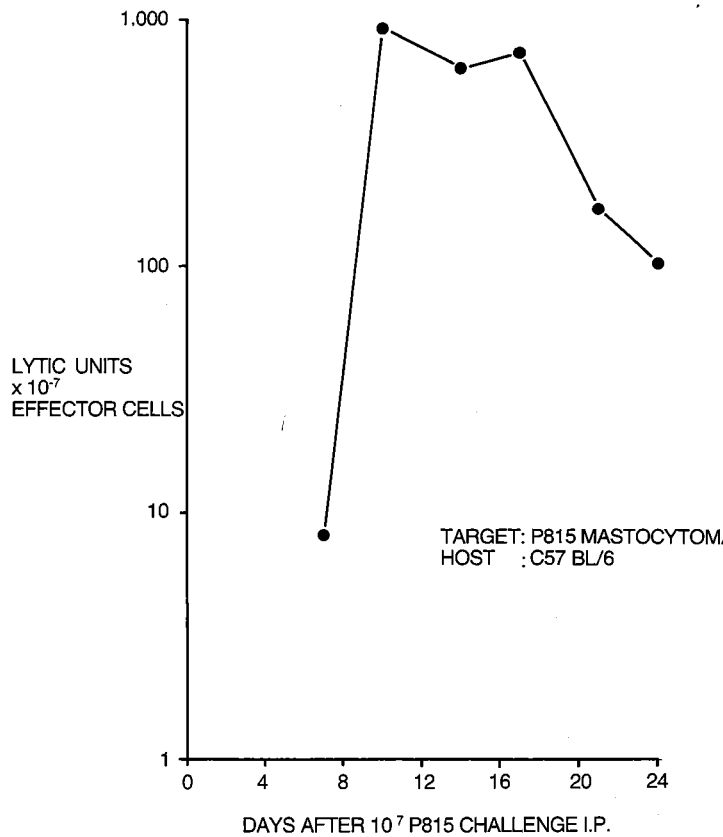

Figure 6.1 Flow chart of the experiments conducted with the spleen cells of C57BL/6 mice 48, 21, 14, 7, and 4 days after intraperitoneal allogeneic challenge with 10^7 P815 (H-2^d) mastocytoma cells. These allo-immune spleen cells were cultured with IL-2; BL/6 lymphocytes (1:1) and IL-2; or LAK cells (1:1) and IL-2. Cytotoxicity was tested in a 4 hour ^{51}Cr release assay against B-16 (H-2^b) melanoma cells.

nodes and peritoneal exudate cells. In subsequent experiments, day 14 after allogeneic challenge was chosen for tumor inoculation of the host with syngeneic MCA-105 sarcoma cells.

7.2.2 *Allogeneic and syngeneic challenge of the BL/6 host*

In all experiments, mice received syngeneic and / or allogeneic tumor cells intraperitoneally (i.p.) prior to random allocation into treatment groups. In order to rule out the effects of persistent P815 tumor on the PCI scores in the BL/6 host the following experiments were performed. On day 0, 10^7 P815 tumor cells were inoculated into BL/6 hosts. At day 7, 10, 14, 17 and 21 the peritoneal cavity of the BL/6 mouse was repeatedly lavaged with HBSS and transferred back to a DBA/2 host. The transferred solution contained live cells on day 7 and 10 as measured by 0.08% trypan blue exclusion. No syngeneic host survived transfer at day 7 because of tumor growth. At day 10, 14, 17 and 21 tumor cells did not grow in the syngeneic host and these mice survived indefinitely. Persistent P815 tumor could be ruled out as a factor causing higher PCI scores in allogeneic challenged mice.

7.2.3 *Immunotherapeutic regimen*

Treatment consisted of i.p. IL-2 and / or LAK cells. Tumor cells and LAK cells were injected i.p. suspended in 2 ml HBSS, respectively s.c. suspended in 0.5 ml HBSS. IL-2 was injected into the abdomen dissolved in 1 ml HBSS every 12 hours. Animals not receiving LAK cells or IL-2 received injections of an identical volume of HBSS. Controls had twelve mice to a group, treatment groups consisted of six mice. At the conclusion of an experiment, animals were sacrificed and the intraperitoneal tumor mass scored in a blinded fashion on a scale from 0 through 3. The score was termed the peritoneal cancer index (2.5.5).

7.3 **Results**

7.3.1 *Abrogation of the immunotherapeutic effects of IL-2 plus LAK cells by intraperitoneal pretreatment with P815 tumor cells*

Fourteen days prior to tumor inoculation, BL/6 mice were challenged i.p. with 10^7 P815 ascites tumor cells. On day 0, mice were injected i.p. 10^5 MCA-105 tumor cells in 2 ml HBSS. On day 3, mice received 10^8 LAK cells in 2 ml of HBSS i.p.; and from day 3 through day 7, mice were injected every twelve hours with 10

K units IL-2 in 1 ml HBSS i.p. Nineteen days after syngeneic tumor inoculation, mice were sacrificed; and the intraperitoneal tumor load was assessed. Experimental groups were treated with IL-2 alone or with IL-2 and LAK cells as indicated. Control animals were injected with the same volume of HBSS. The top portion of Figure 7.2 reviews the design of the experiment; the bottom shows the results in a bar diagram. Normal BL/6 mice treated with IL-2 or IL-2 plus LAK cells had reduced intraperitoneal tumor load compared to controls. Mice with an allogeneic challenge prior to syngeneic tumor injection and treated with IL-2 or IL-2 and LAK cells showed significantly increased i.p. tumor load over similarly treated, non-challenged animals. No reduction of tumor load over control mice was evident anymore. Numerous repeat experiments showed essentially the same results.

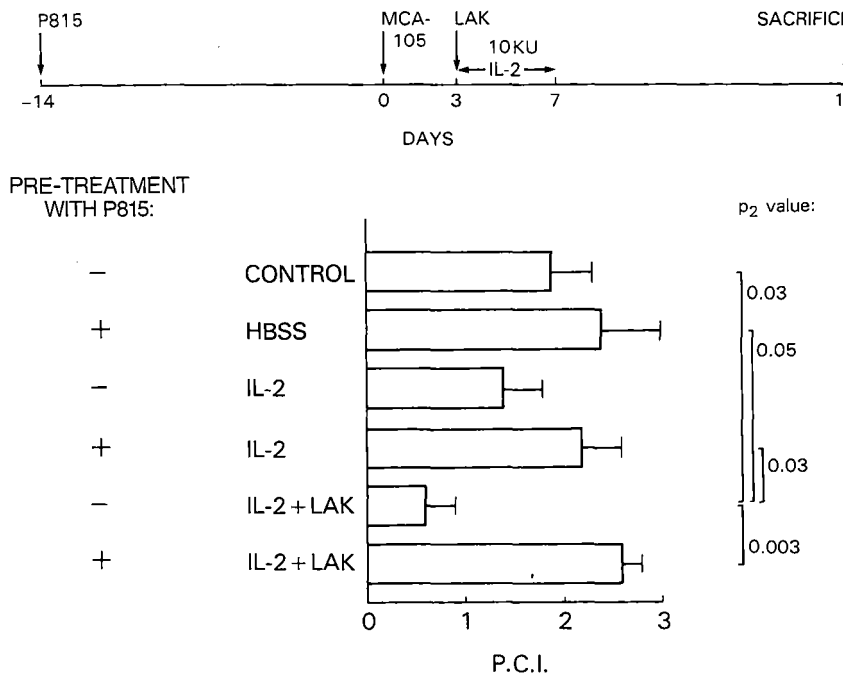

Figure 7.2 Mice were challenged i.p. with 10^7 live P815 cells fourteen days before i.p. challenge with 10^5 live MCA-105 tumor cells. On day 3 treatment groups got 10^8 LAK cells i.p. and from day 3 till day 7 10,000 units of IL-2 i.p. every twelve hours. On day 19 the PCI was scored blindly. 6 Mice in treatment groups, 12 controls. The brackets depict the two-sided p values of pairwise comparisons.

7.3.2 *Effects of increasing numbers of allogeneic P815 challenging cells*

BL/6 mice were challenged with increasing doses of allogeneic P815 tumor cells i.p. fourteen days prior to tumor inoculation. On day 0 mice received 10^5 MCA-105 tumor cells. On day 3 they were injected with 10^8 LAK cells, and from day 3 through day 7, they received 10 K units IL-2 i.p. every 12 hours. Mice were sacrificed for tumor assessment on day 19 (Figure 7.3). The beneficial effect of IL-2 and LAK cell treatment was reduced by all doses of allogeneic challenging

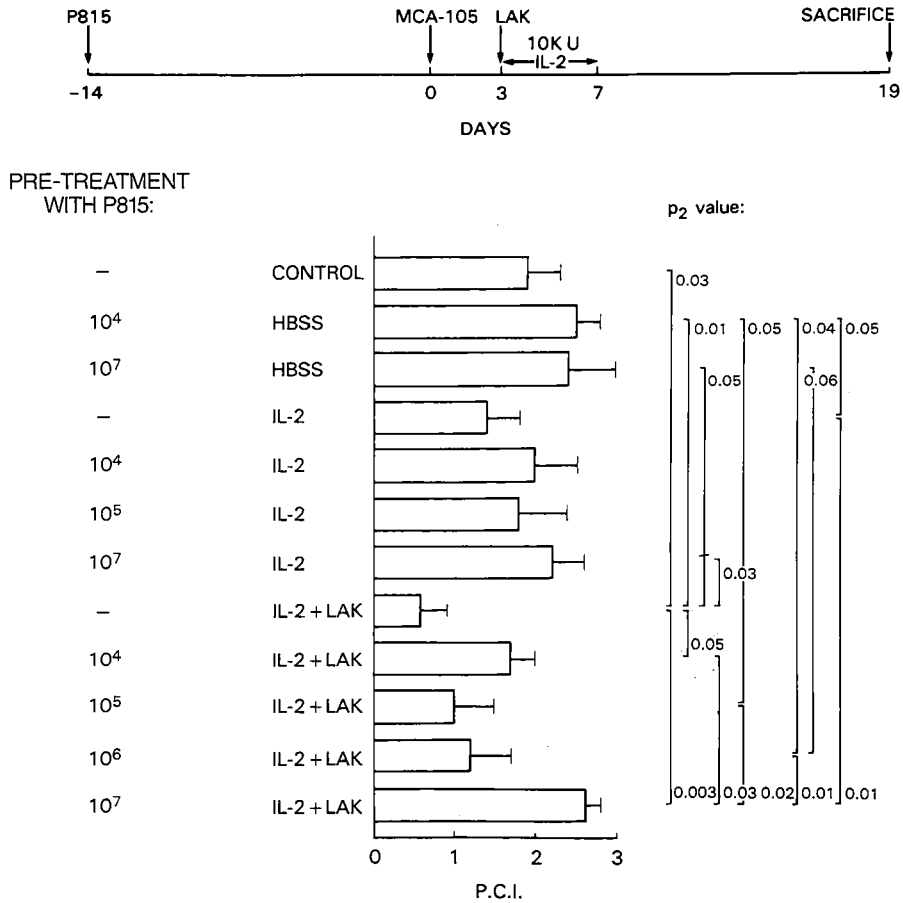

Figure 7.3 Mice were challenged i.p. with 10^4 - 10^7 live P815 cells fourteen days before i.p. challenge with 10^5 live MCA-105 tumor cells. On day 3 treatment groups (6 mice per group) got 10^8 LAK cells i.p. and from day 3 till day 7 10,000 units of IL-2 i.p. every twelve hours. On day 19 the PCI was scored blindly. 12 Mice in the control group. The brackets depict the two-sided p values of pairwise comparisons.

cells (10^4, 10^5, 10^6, 10^7 P815 cells). A trend for dose-dependency could be detected with significant differences between 10^5 and 10^6 versus 10^7 challenging cells and IL-2 and LAK cell therapy.

7.3.3 *Timing of the allogeneic tumor challenge*

BL/6 mice were challenged with 10^7 P815 ascites tumor cells either six weeks before, two weeks before, 4 days before, or at the time of inoculation with 10^5 MCA-105 tumor cells. On day 3, mice were treated with 10^8 LAK cells; and from day 3 through day 7, mice received 10 K units IL-2 every twelve hours. The PCI score was determined at day 17. In this experiment, allogeneic pretreatment 42 and 14 days before syngeneic challenge was detrimental to the immunotherapeutic effect of IL-2 and LAK cells (Figure 7.4). Allogeneic pretreatment at day -4 and at the time of syngeneic tumor inoculation did not abrogate the IL-2 and LAK cell effect, but the increased anti-tumor effect was not statistically significant.

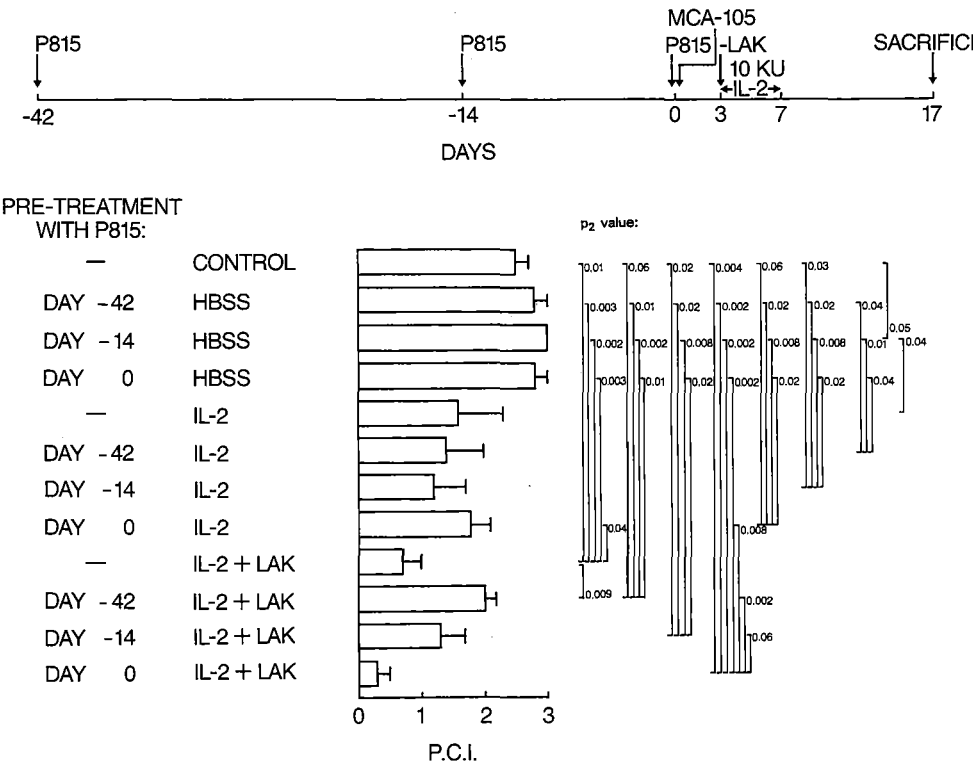

Figure 7.4 **A**

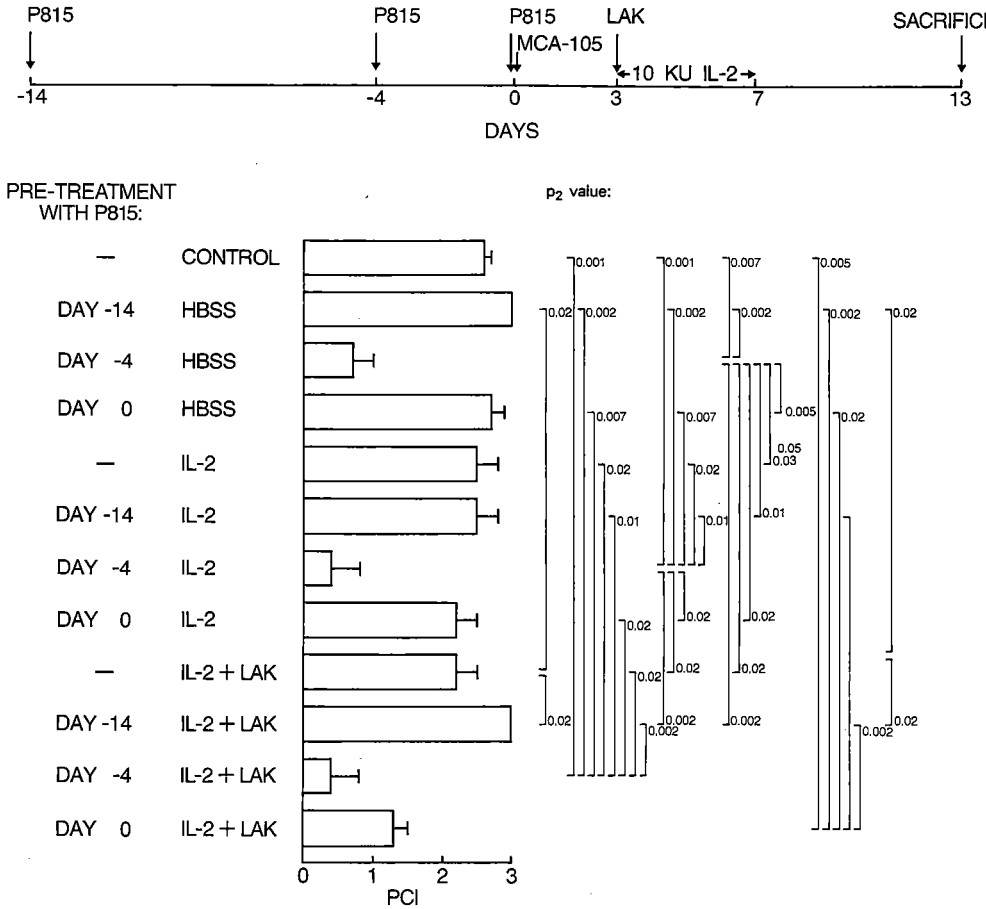

Figure 7.4 42 days before, 14 days before, and on the day (**A**), or 14 days before, 4 days before and on the day (**B**), respectively, of syngeneic tumor challenge mice were challenged i.p. with 10^7 live P815 cells. On day 0 they were injected with 10^5 live MCA-105 cells i.p. and three days later they were treated with 10^8 LAK cells i.p. and received from day 3 through day 7 10,000 units of IL-2 i.p. every twelve hours. The PCI was scored blindly on day 17. 12 Control mice and 6 mice per treatment group. Brackets depict the two-sided p values of pairwise comparisons.

7.3.4 *Live and irradiated allogeneic tumor cells*

Fourteen days prior to tumor inoculation BL/6 mice were challenged with either 10^7 P815 tumor cells or 10^7 irradiated (100 Gy) P815 tumor cells. On day 0 mice were inoculated with 10^5 MCA-105 tumor cells i.p. On day 3 through day 7 they received 25 K units IL-2 i.p. every 12 hours. Mice were sacrificed on day 16.

In this experiment a larger dose of IL-2 was used, and this IL-2 dose was effective in the absence of LAK cells so that no i.p. metastases were found at sacrifice in IL-2 treated mice. Both irradiated P815 tumor cells and non-irradiated P815 tumor cells completely and significantly reversed the IL-2 effect (Figure 7.5). With this higher dose of IL-2 (25,000 units as compared to 10,000 units in other challenge experiments) the anti-tumor effects of IL-2 plus LAK cells were maintained even though mice were challenged with allogeneic tumor cells (live and irradiated P815 cells).

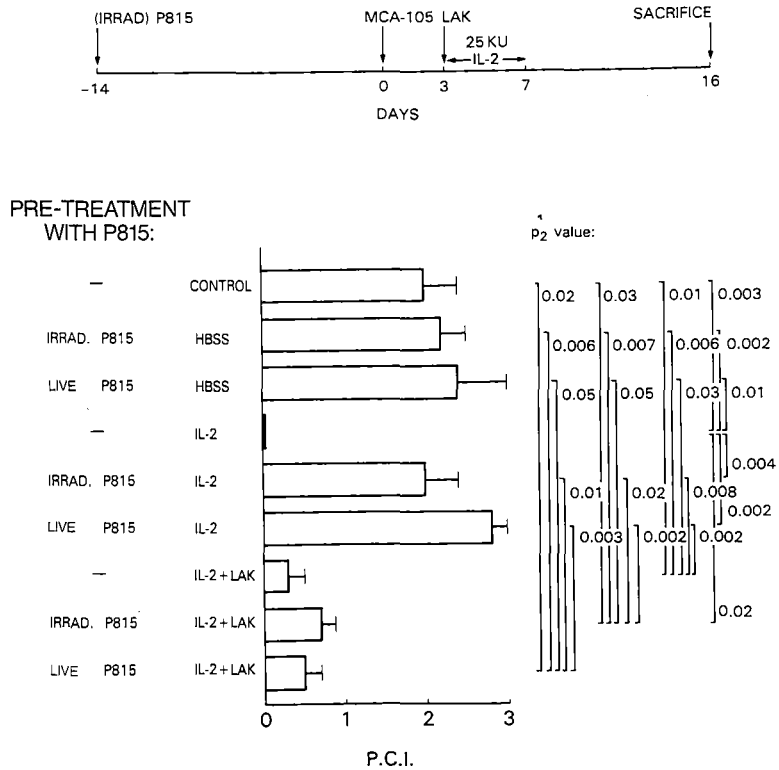

Figure 7.5 Mice were inoculated i.p. with 10^7 live or irradiated (100 Gy) P815 cells fourteen days prior to i.p. syngeneic tumor challenge with 10^5 live MCA-105 cells. On day 3 treatment groups (6 mice per group) got 10^8 LAK cells i.p. and from day 3 till day 7 25,000 units of IL-2 i.p. every twelve hours. On day 16 the PCI was scored blindly. 12 Mice in the control group. The brackets depict two-sided p values of pairwise comparisons.

7.3.5 *Subcutaneous versus intraperitoneal allogeneic challenge*

Mice were challenged with 10^7 P815 tumor cells in either the subcutaneous

or the intraperitoneal space at day -14. They received 10^5 live MCA-105 tumor cells on day 0, 10^8 LAK cells on day 3, and 10 K units IL-2 every twelve hours i.p. from day 3 through day 7. Mice were sacrificed on day 19 after tumor inoculation. Intraperitoneal allogeneic challenge reversed the beneficial effect of IL-2 plus LAK cell treatment. The same dose of P815 given s.c. did not interfere with the immunotherapeutic effects of i.p. IL-2 or IL-2 plus LAK cells on i.p. tumors (Figure 7.6). Repeat experiments showed the same pattern.

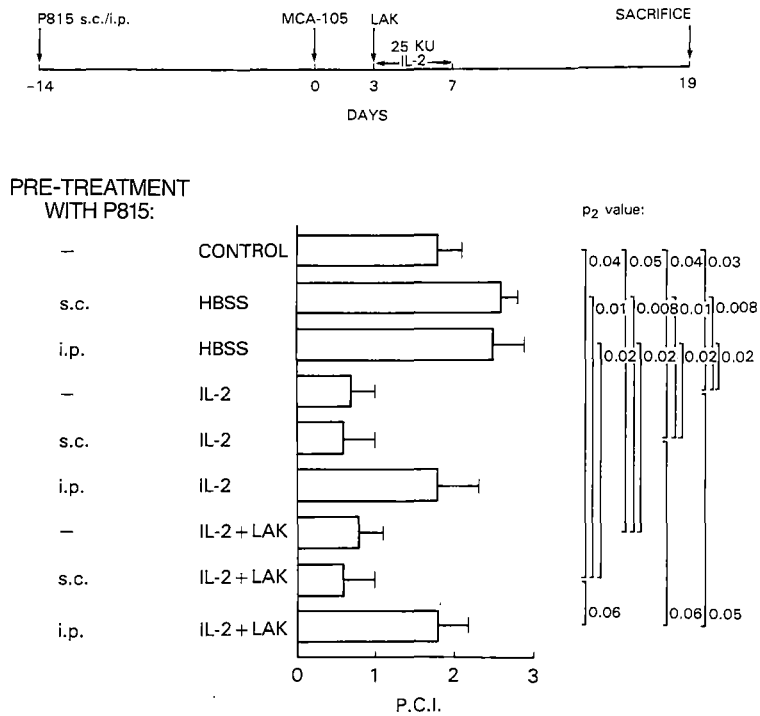

Figure 7.6 Mice were pretreated with 10^7 live P815 cells either in 0.5 ml HBSS subcutaneously or in 2 ml HBSS intraperitoneally fourteen days before i.p. challenge with 10^5 live MCA-105 cells. On day 3 treatment groups received 10^8 LAK cells and from day 3 till day 7 25,000 units of IL-2 i.p. every twelve hours. On day 19 the PCI was scored blindly. 12 Control mice and 6 mice per treatment group. The brackets depict two-sided p values of pairwise comparisons.

7.3.6 *Allogeneic challenge and high and low dose IL-2 plus LAK cell therapy*

Mice were treated in a similar fashion as described before but received either 10,000 units IL-2 or 50,000 units IL-2 every twelve hours from day 3

through day 7. The anti-tumor effects of 10,000 units of IL-2 plus LAK cells were completely lost by allogeneic pretreatment. However, this detrimental effect of allogeneic challenge could be negated by combining LAK cells with 50 K units of IL-2 every twelve hours from day 3 through day 7 ($p_2 = 0.0001$) (Figure 7.7).

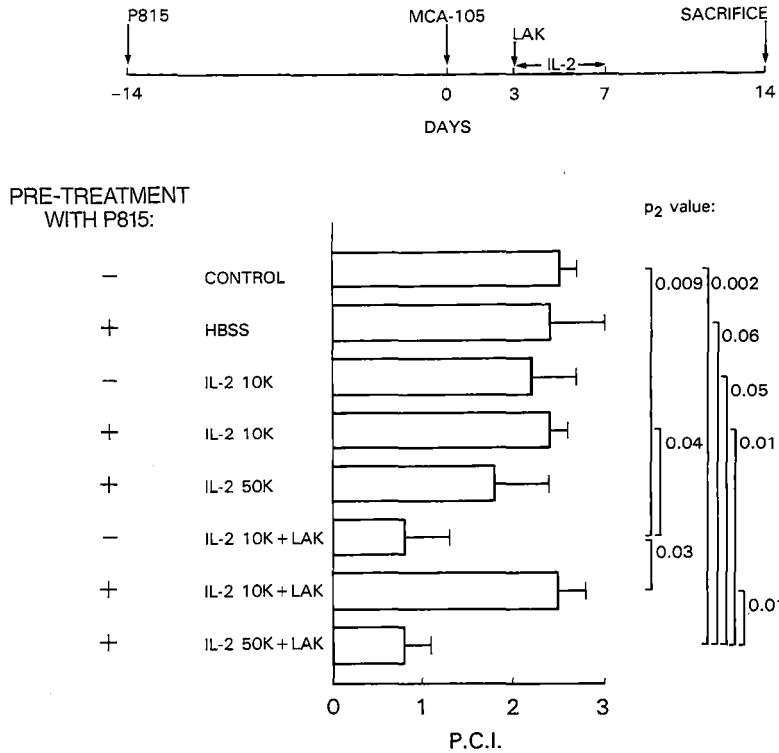

Figure 7.7 Mice were presensitized with 10^7 live P815 cells i.p. fourteen days before i.p. challenge with 10^5 live MCA-105 cells. On day 3 treatment groups received 10^8 LAK cells i.p. and from day 3 until day 7 either 10,000 or 50,000 units of IL-2 i.p. every twelve hours. On day 14 the PCI was scored blindly. 12 Control mice and 6 mice per treatment group. Brackets depict the two-sided p values of pairwise comparisons.

7.3.7 *Cyclophosphamide, allogeneic pretreatment and IL-2 plus LAK cell therapy*

In order to test the hypothesis that the specific cytolytic T cells elicited by the allogeneic challenge with P815 tumor cells are the suppressive effector of IL-2 and LAK cell immunotherapy in allogeneic pretreated mice, the following experiment was designed. As described before, mice got the allogeneic challenge on day -14. Syngeneic inoculation was given on day 0. Treatment started on day 3 with 50

mg/kg Cy i.v. followed 12 hours later by 10^8 LAK cells i.p. and 10 K units of IL-2 i.p. every twelve hours during 4 days. Due to the chemotherapeutic effect of this dose of cyclophosphamide some reduction of intraperitoneal tumor load was seen, but no synergistic or additive effect on IL-2 or IL-2 and LAK cell treatment in non-immunized mice was seen (Figure 7.8). Pretreatment of allogeneic challenged mice with Cy restored the immunotherapeutic effect of IL-2 and LAK cell therapy abrogated by the allogeneic challenge. Even a suggestion of a synergistic effect is made (PCI 2.5, 1 and 0.2; $p_2 = 0.006$, resp. $p_1 = 0.05$) (Figure 7.9).

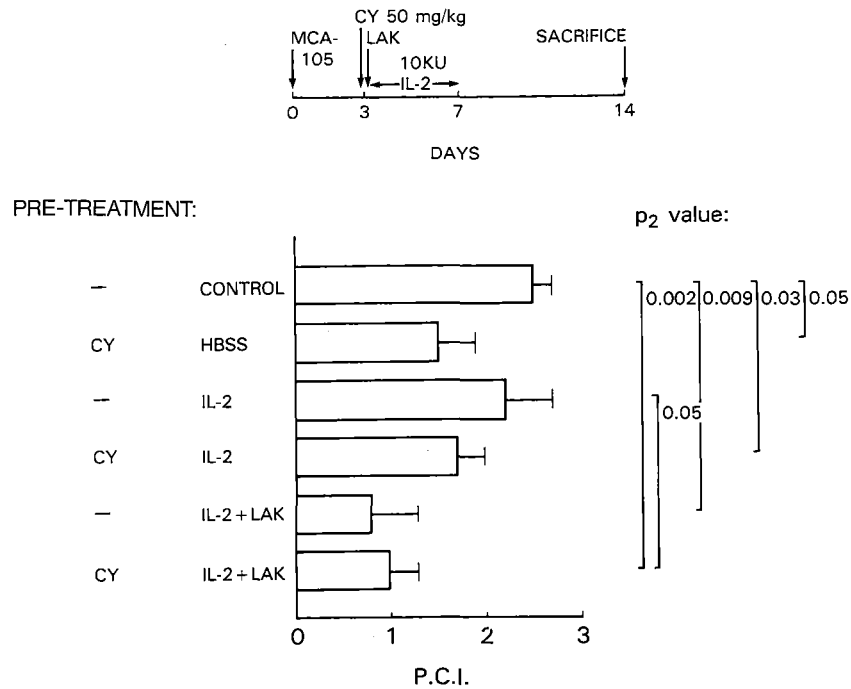

Figure 7.8 Mice were challenged i.p. with 10^5 live MCA-105 cells on day 0. On day 3 treatment groups received 50 mg/kg cyclophosphamide (Cy) i.v. and twelve hours later 10^8 LAK cells i.p. and from day 3 until day 7 10,000 units of IL-2 i.p. every twelve hours. On day 14 the PCI was scored blindly. 12 Control mice and 6 mice per treatment group. Brackets depict the two-sided p values of pairwise comparisons.

7.4 Discussion

Therapy of cancer with IL-2 has been proven effective in man[11] as well as in experimental models. IL-2 will induce LAK cells *in vivo*[12] and reduce tumor load.[1]

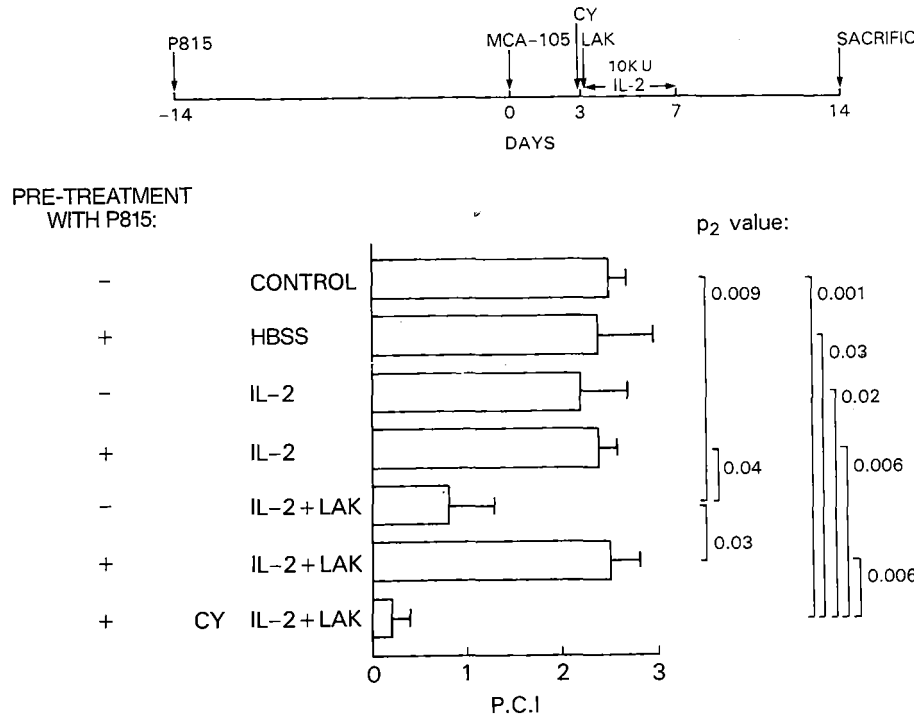

Figure 7.9 Mice were presensitized with 10^7 live P815 cells i.p. fourteen days before i.p. challenge with 10^5 live MCA-105 cells. On day 3 treatment groups received 50 mg/kg cyclophosphamide (Cy) i.v. and twelve hours later 10^8 LAK cells i.p. and from day 3 until day 7 10,000 units of IL-2 i.p. every twelve hours. On day 14 the PCI was scored blindly. 12 Control mice and 6 mice per treatment group. Brackets depict the two-sided p values of pairwise comparisons.

Aiding the immune system by injecting *in vitro* induced LAK cells boosted the immunotherapeutic effect of IL-2.[5 6 7] So far the experimentally known optimal IL-2 dose has not been used in man because of the toxic side effects of high dose IL-2.[13] Treating the symptoms of toxicity did not make it possible to raise the IL-2 dose.[11]

Exogenous IL-2 will enhance specific allo-immune responses;[8 9 14] and will reduce tumor load *in vivo*.[11] IL-2 will induce LAK cells *in vivo*[12] and *in vitro*.[15] *In vitro* induced LAK cells will lyse a broad range of tumor cells *in vitro*.[16] LAK is a phenomenon effectuated by 'NK-like'- and 'T-like'-activated cells, judged by the analysis of their phenotype (1.4.2).[17] Activated NK cells are tumoricidal for a broad range of tumor cells.[18 19] Taken together, it could be argued that IL-2 is a common pathway used by the immune system for activation and proliferation of

cell mediated defenses, be it specific or non-specific. Of course other cells and co-factors take part in this process of activation and proliferation, but will not be discussed here.

In Chapters 5 and 6 the delicate balance between enhancement and abrogation of LAK activity by cells induced by a specific allogeneic stimulus was demonstrated. In Table 7.1 we give a short summary of the effect the timing of the allogeneic challenge has on IL-2 and LAK cell immunotherapy.

Table 7.1 Short summary of the influence i.p. allogeneic challenge over time has on IL-2 and LAK cell immunotherapy *in vivo*.

CHALLENGE IN VIVO	TREATMENT	'ENHANCEMENT' OF IL-2 + LAK CELL THERAPY	'ABROGATION' OF IL-2 + LAK CELL THERAPY
P815	'STANDARD'	DAY 0, -4	DAY -14, -42

In looking for ways in which the immune system of the host could be activated to enhance the therapeutic efficacy of exogenous IL-2 and adoptively transferred LAK cells, we observed synergy between the two in the early phase of allogeneic stimulation and a near complete reversal of tumor control following allogeneic pretreatment (7.3.3). As a mechanism to explain these findings, we postulate endogenous IL-2 production in the initial phase of the allogeneic immune response and a competitive inhibition phenomenon of allo-immune cells with LAK cells for exogenously administered IL-2 in the later phase.[10 20 21]

Several observations from this chapter support the competitive inhibition hypothesis. Perhaps the most direct evidence is the simple observation that the detrimental effect of allogeneic pretreatment can be reversed by a higher IL-2 dose in combination with LAK cells. If sufficient IL-2 is provided, both CTL and LAK cell can proliferate. Second, the loco-regional nature of the suppression suggests that interaction of cells within a micro-environment may be required. Maximal competitive effects may occur when CTLs and LAK cells are adjacent to each other within the free peritoneal cavity. Greater affinity of greater numbers of IL-2 receptors on CTLs may account for their ability to steal IL-2 from the IL-2 depen-

dent LAK cell.[22 23 24] Thirdly, the requirement for the proper timing of the allogeneic challenge suggests a competition. When CTLs or T-'memory' cells are present in the peritoneal cavity at the time of LAK cell and IL-2 treatment, IL-2 is absorbed preferentially and LAK activity is reduced. When the allo-immune responses are being generated while LAK cells are present, T helper cells may produce IL-2 and cause augmentation of immunotherapeutic effects (Chapters 5, 6 and 8).[20] When a population of developed CTLs is present, they will preferentially bind IL-2, leaving less for maintenance and induction of LAK cells.

Cyclophophamide is known to 'selectively' lyse suppressor T cells at low doses. Intravenous injection of low dose Cy completely reversed the abrogation of IL-2 and LAK cell immunotherapy by a developed allo-antigenic response. Do the CTLs elicited by the allo-antigenic challenge compare to suppressor T lymphocytes? Most probably Cy is not as selectively as supposed and is reducing CTLs as well, sparing LAK precursors. This again would support our competitive inhibition hypothesis. Cyclophosphamide not only neutralized the CTLs competing for IL-2, but by lysing suppressor T lymphocytes also enhanced IL-2 plus LAK cell therapy in reducing i.p. tumor load.

In interpreting these experiments, one must note that three different doses of IL-2 given every twelve hours for four days were used. The effects of a low dose of 10,000 units of IL-2 plus LAK cells lost anti-tumor effectiveness when allogeneic pretreatment was performed. When 25,000 units of IL-2 plus LAK cells were used, allogeneic challenge decreased anti-tumor effects not significantly in one experiment (Figure 7.6), yet no reduction was seen in another (Figure 7.5). Yet, the effects of IL-2 alone were markedly reduced by allogeneic pretreatment as shown in Figure 7.5 and 7.6. It is likely that a moderate dose (25,000 units) of IL-2 given loco-regionally causes sufficient LAK cell generation within the peritoneal cavity to reduce intraperitoneal tumor growth.[12 14 25 26] When no adoptive transfer of *in vitro* induced LAK cells occurred, the allogeneic challenge reduced anti-tumor effects. When 50,000 units of IL-2 were used, the suppression usually seen with allogeneic pretreatment did not occur. Both LAK cell and CTL activity were sustained by this IL-2 level.

The allogeneic tumor challenge 6 weeks prior to syngeneic tumor inoculation also elicited an abrogation of the immunotherapeutic effect, comparable to the reversal by an established immune response. 'Memory' T cells - present in rather low quantities - are normally only capable of an immune response when activated by the specific antigen. Is it plausible to expect these cells to compete for IL-2 and reverse an expected immunotherapeutic effect? There was no renewed allo-antigeneic challenge i.p. activating the 'memory' cytotoxic T cells; nor was a prolifera-

ting allogeneic tumor absorbing IL-2. An on-going viral infection could not readily explain the phenomenon either, since the mice were checked for viruses and repeat experiments yielded similar results. Neither can the effect be explained by the simple number and affinity of the IL-2 receptors on the surface of dormant 'memory' T cells. However, when IL-2,[27] IL-2 induced 'helper' T cells[28 29] or the LAK cell itself elicit activation of the 'memory' T cells; the number of cells may increase rapidly as well as their number of IL-2 receptors and - possibly - their affinity for IL-2.[23] Others showed the augmentation of allo-immune responses by IL-2.[4 8 9 30]

Allogeneic challenge on the same day as syngeneic challenge did not abrogate the immunotherapeutic effect of IL-2 and LAK cells. Allogeneic challenge on day 0 and more explicit on day -4 before syngeneic tumor inoculation seemed to enhance the immunotherapeutic effect of IL-2 and LAK cells (Figure 7.4). It could be postulated that in a developing immune response the endogenous IL-2 production is elevated. Recognition of the antigen might trigger lymphocytes to lower their affinity for IL-2 and / or reduce the number of IL-2 receptors[22] and thus shed IL-2 in the media. The high local level of IL-2 and other lymphokines thus produced loco-regionally, might not only stimulate the development of allo-immune cells, but also the induction and maintenance of LAK cells.[31]

The allogeneic challenge in the subcutaneous compartment 14 days prior to syngeneic tumor challenge was not capable of exerting an influence on the loco-regional effects of IL-2 and / or LAK cells in this peritoneal carcinomatosis model. This is not as surprising as it may seem. First of all i.p. injected syngeneic and allogeneic cells will remain and exert their influence mainly in the peritoneal cavity.[32] IL-2 injected i.p. gaines much better and faster systemic access,[14] but still intraperitoneal levels will be much higher than for instance subcutaneous levels. Secondly, *in vivo* generation of LAK cells will for the most part take place in the loco-regional lymphoid organs.[12] Presence of a great number of allo-antigenic stimulated cells loco-regionally will thus exert a much bigger influence than a systemic immune response. Third, we did not test for peak immune response after s.c. allogeneic challenge. Subsequent experiments have shown that an ongoing allogeneic immune response to a challenge in the same subcutaneous body compartment and injected at the same time as syngeneic tumor will augment the effects of IL-2.[31] Further studies should clarify when peak allo-antigenic responses to intravenous as well as subcutaneous allogeneic challenges occur and if they do show the same initial enhancement and later competitive inhibition of loco-regional IL-2 and LAK cell immunotherapy.

This chapter shows that allo-antigenic stimulated cells through competition

with LAK cells for IL-2 abrogate the immunotherapeutic activity of IL-2 and LAK cells. However there are also instances where no reversal of the immunotherapeutic effect was seen or even enhancement might be expected. IL-2 might be the common pathway through which non-specific- as well as specific-killer activity and helper- and suppressor cell activity is induced and expanded. It does show the pivotal role the immune status of the host is playing in the delicate balance between abrogation and enhancement of immunotherapeutic effects of IL-2. The immune status of the host may be the key to the success of adoptive immunotherapy of cancer patients.[11] Indeed it will require careful planning and manipulation of the immune status of the host in order to optimize the immunotherapeutic effects of IL-2 and LAK cells. Judged by the results of this chapter the cancer bearing host would have to be depleted of suppressor T lymphocytes and competitively inhibiting CTLs with low dose cyclophosphamide. Then the immune system of the host would have to be primed by a specific or non-specific immune stimulant like *C. Parvum* or Freund's adjuvant to get maximal results of (low dose) IL-2 and LAK cell immunotherapy.

7.5 Summary

In conclusion this chapter elaborated on the infuence of an allo-antigenic stimulus on IL-2 and LAK cell therapy *in vivo*. I.p. treatment of the BL/6 host with P815 tumor cells fourteen days prior to i.p. inoculation with syngeneic tumor cells, abrogated the immunotherapeutic effects of IL-2 and LAK cell therapy. The effects of allogeneic tumor cells were dose-dependent. Abrogation of immunotherapeutic effects by i.p. allogeneic challenge with heavily irradiated tumor cells was not significantly different from the effects of non-irradiated tumor cells. Timing of the allogeneic tumor challenge was important in that allogeneic pretreatment six weeks and two weeks prior to the time of syngeneic tumor inoculation suppressed the IL-2 plus LAK cell effects. However, the pretreatment 4 days before and at the time of tumor inoculation slightly improved anti-tumor effects. When P815 tumor was inoculated subcutaneously, no change in anti-tumor effects of the IL-2 plus LAK cell therapy was seen. The detrimental effects of allogeneic pretreatment on IL-2 immunotherapy was reversed by high doses of IL-2 as well as by intravenous treatment 12 hours before syngeneic tumor inoculation with low dose cyclophosphamide.

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Chapter 8

RESPONSES TO ALLOGENEIC AND SYNGENEIC TUMOR CELL MIXTURES POTENTIATE THE IMMUNOTHERAPEUTIC EFFECTS OF INTERLEUKIN 2

8.1 Introduction

In Chapters 5, 6, and 7 we have shown the suppressive effects of specific immune-competent cells for interleukin 2 (IL-2) and lymphokine-activated killer (LAK) cell immunotherapy. The suppression could be overcome by high dose IL-2, but toxicity of IL-2 is one of the restricting factors in reaching total cures in man and murine models.[1 2] In search for synergism with the exogenously administered low dose IL-2 we looked for endogenously produced IL-2. At the time the cytolytic activity of the host spleen cells towards the challenging allogeneic cells reached the highest values, no synergism at all was seen; but instead total abrogation of IL-2 and LAK cell immunotherapy prevailed. Early-on in the immune response of spleen cells the production of endogenous IL-2 in the supernatant could be shown (5.3.6). Likewise, no abrogation of IL-2 and LAK cell therapy was seen when the immune response of the host was increasing exponentially after the allo-antigenic challenge (7.2.1; 7.3.3: day 0 and -4). Not only the endogeneous production of IL-2 will be responsible for this effect, but also other helper factors and cells. The importance of the loco-regional nature of these soluble and cellular helper factors for the potentiation of the immunotherapeutic effects of IL-2 was again shown in the following experiments. These studies suggest that lymphocytes recruited by an allogeneic challenge within tumor tissue increase LAK cell production at that site and augment the tumor destructive capabilities of exogenously administered IL-2.

8.2 Methods

Mice were injected with 10^5 MCA-105 tumor cells admixed with or without P815 mastocytoma cells intracutaneously (i.c.) in the right flank. From day 0 to day 6 mice were treated every 12 hours with 50,000 units of IL-2 dissolved in 1 ml HBSS i.p. or with an equal volume of HBSS only. This dose of IL-2 does not result in treatment related deaths from IL-2. After completion of the therapy, mice were eartagged. The key was not known to the person measuring tumors until completion of the experiment. Beginning on the tenth day after tumor inoculation, tumor growth was assessed every 3 or 4 days by measuring with calipers the largest diameter and the one perpendicular to it. The average diameter was taken as the measure of tumor size.

8.3 Synergistic effect of allogeneic and syngeneic tumor cell admixture and IL-2 therapy

8.3.1 *Tumor growth*

Groups of mice were injected intracutaneously with 10^5 MCA-105 tumor cells alone or combined with P815 tumor cells. Two different doses of P815 tumor were used: 10^5 and 10^7. The mice were eartagged and randomly distributed in their cages. For 6 days i.p. IL-2 was given at 50,000 units every 12 hours. Control mice were treated with HBSS. Tumors were measured in two dimensions twice weekly and the code was broken only at the completion of the experiment.

Figure 8.1 shows that this low dose of IL-2 alone in this intracutaneous model had no influence on the MCA-105 tumor volume.

An admixture of syngeneic and allogeneic tumor slightly reduced the rate of tumor growth at ratios of 1 to 100 (syngeneic to allogeneic tumor), but not at ratios of 1 to 1.

IL-2 treatment significantly retarded the growth rate of tumors in mice given the admixture of syngeneic and allogeneic tumor cells at ratios of 1:100 and 1:1. In the group of mice with ratio 1:100 tumor admixture IL-2 treatment did retard tumor growth, but not statistically different from the tumor admixture without IL-2 treatment. The IL-2 effects on the tumor cell admixture were significant at days 10, 14, and 17 but lost their significance at days 20 and 24 compared to mice with syngeneic tumor only treated with IL-2 (Figure 8.1 A).

At ratios of 1:1 of syngeneic and allogeneic tumor cells; the admixture was signifi-

cant different from control (MCA-105 without IL-2) at day 10, not thereafter. The groups treated with admixed tumor cells and IL-2 were the only groups significantly different at all time points from the untreated controls. Tumor growth was significantly retarded when tumor cell admixture was treated with IL-2 compared to tumor cell admixture without IL-2 treatment, except at days 20 and 24 (Figure 8.1 B). This experiment was repeated with identical results.

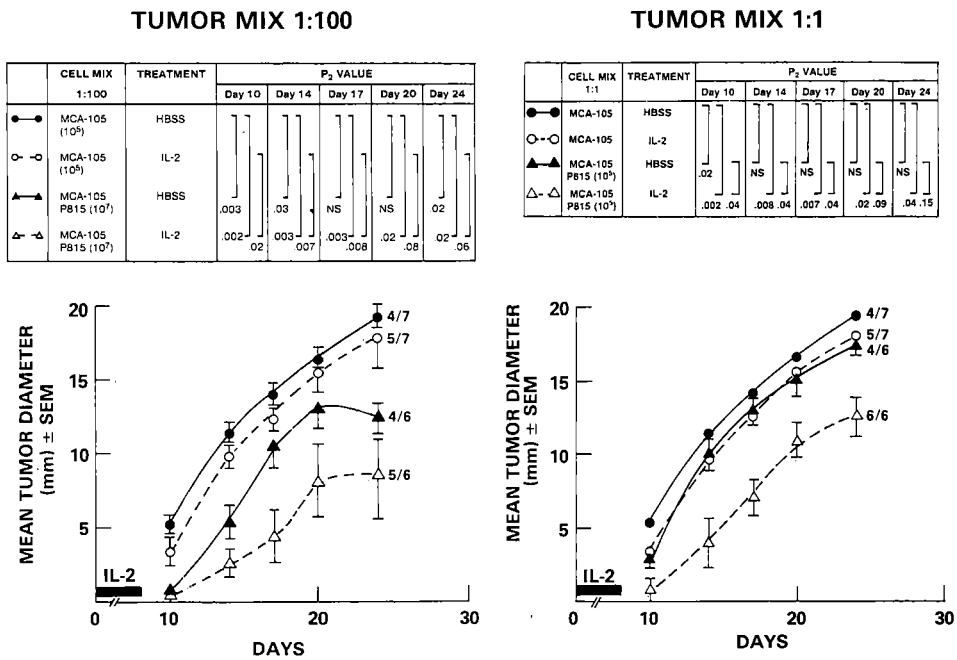

Figure 8.1 Interleukin 2 effects on intracutaneous tumor. 4 Groups of mice were given 10^5 cells of the syngeneic MCA - 105 tumor i.c. either alone or mixed with 10^7 (**A**), and 10^5 (**B**) cells, respectively, of the allogeneic tumor P815. The different groups received 2 daily injections of 50,000 units of IL-2 i.p. or were given HBSS. Noted are the number of mice still alive at day 24 out of groups of 6 or 7 mice.

A. The ratio syngeneic : allogeneic tumor was 1:100. Allogeneic tumor admixture reduced the growth of MCA-105 tumor, but not in a statistically significant fashion. When the tumor admixture was treated with IL-2, the tumor growth was significantly retarded compared to mice with syngeneic tumor-only treated with or without IL-2.

B. The ratio syngeneic : allogeneic tumor was 1:1. At this dose of allogeneic tumor, no decrease in tumor growth was noted with admixed cells alone. IL-2 given along with the tumor admixture caused a significant reduction in tumor growth.

8.3.2 *Survival*

Groups of mice were injected intracutaneously with 10^5 MCA-105 tumor cells alone or combined with P815 tumor cells. Two different doses of P815 tumor were used, 10^5 and 10^7. The mice were eartagged and randomly distributed in their cages. For 6 days i.p. IL-2 was given at 50,000 units every 12 hours. Control mice were treated with HBSS. Mice were followed for survival. Figure 8.2 shows the survival curves.

At a syngeneic to allogeneic tumor cell ratio of 1:100 there was a trend for longer survival for the mice injected with the tumor cell mixture compared to the mice that received the syngeneic tumor alone. Only when the mice with tumor cell

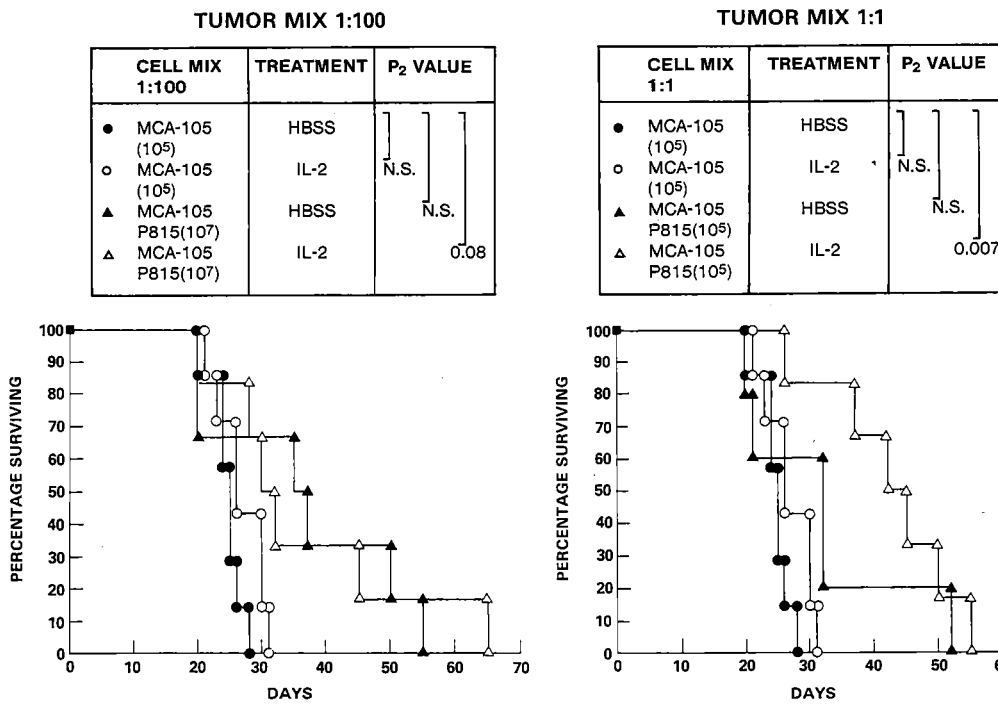

Figure 8.2 Survival of mice with intracutaneous tumors admixed with or without allogeneic tumor cells and treated with interleukin 2. Groups of mice were given 10^5 cells of the syngeneic MCA-105 tumor i.c. either alone or mixed with 10^7 **(A)**, and 10^5 **(B)** cells, respectively, of the allogeneic tumor P815. The different groups received 2 daily injections of 50,000 units of IL-2 i.p. or were given HBSS. Treatment groups consisted of 6 or 7 mice.

admixture were treated with IL-2 the increment in survival approached significance compared to mice injected with syngeneic tumor cells and without IL-2 treatment (Figure 8.2 A).

At a ratio of 1:1 there was a clear-cut and significant difference between mice receiving the tumor cell mixture and treated with IL-2 and the control group (MCA-105 alone without IL-2) (Figure 8.2 B).

8.4 Discussion

In studies to date, IL-2 alone has been shown to be of some benefit in the reduction of the size and number of tumor implants that develop following i.v. or i.p. administration of tumor. Effects on s.c. tumor deposits are seen only when maximal toxic doses of IL-2 treatment are used.[3] Cure of mice with this immunotherapeutic strategy rarely if ever occurs. This may be due to the moderate dose of IL-2 usually employed in these experiments because of the marked systemic toxicity of high levels of this drug. Adoptive transfer of LAK cells has been shown to augment the effects of exogenous IL-2 on tumor implants in the peritoneal cavity, the liver, and in the lungs. Presumably the adoptively transferred LAK cells migrate to the tumor site and IL-2-mediated expansion of these cells may consequently result in the destruction of the tumor.[4] The studies of Ettinghausen *et al*[5] may be important for understanding the immunotherapeutic effects of IL-2 used in the absence of LAK cells. He showed proliferative responses of lymphocytes in lungs and liver of mice treated with IL-2. These proliferative responses increased if LAK cells were given i.v. along with a cycle of IL-2 treatment.[6] Presumably LAK cell activation and LAK cell maintenance *in vivo* is dependent on the continuous administration of IL-2. In this manuscript, we show marked augmentation of the immunotherapeutic effects of IL-2 by promoting an immune response within the tumor mass, generated by mixing syngeneic tumor with allogeneic tumor prior to its inoculation into an experimental animal.

The potentiation of IL-2 in this model might be due to the *in situ* generation of allo-activated killer cells.[7 8 9] Cytotoxic T lymphocytes could have been generated against allo-antigens on the P815 tumor cells that could be cross-reactive with antigens expressed on the syngeneic MCA-105 cells. This phenomenon has been described by various authors.[10 11 12] This allo-immune response would then be enhanced by exogenous IL-2.[13 14] The experiments described in Chapters 5 and 7 seem to indicate that the putative cross-reactivity between alien histocompatibility antigen and tumor antigens plays no role in our model. The allo-antigenic cyto-

toxicity of cells from MLC stimulated with allogeneic cells is rising with time spent in culture; but LAK activity against the syngeneic target was reduced (Figure 4.2-3). When BL/6 mice are immunized with P815 tumor or with DBA/2 splenocytes 2 weeks prior to the inoculation of the syngeneic MCA-105 tumor, no reduction in tumor growth is seen. To the contrary: the effect of (low dose) IL-2 and IL-2 plus LAK cell treatment is abrogated (Figure 7.2-8). We favor an alternative mechanism to explain the phenomenon. LAK cells are reponsible for the immunotherapeutic effects of IL-2 against autologous tumor cells. A tumor mix of allogeneic and syngeneic tumor elicits an immune response and increases the number of LAK precursors within the tumor that may acquire LAK cytotoxic properties when stimulated with IL-2. As a matter of fact, *in vivo* allo-antigen stimulation of a host increases the generation of LAK cells from its spleen (Chapter 6). These experiments are the first to show a method for potentiating the immunotherapeutic effects of IL-2 by recruitment and activation of lymphocytes of the host to the tumor itself. The clinical implications of these experiments may be far reaching. The data suggest that a major problem with successful IL-2 immunotherapy may be overcome by recruitment of inflammatory cells to the tumor site for activation or augmentation of cytotoxic activity by exogenous IL-2. This concept would support previous experiments with up to 18-day-old established tumors.[3] These data suggested that large deposits of moderately antigenic methylcholanthrene-induced tumors are as responsive to the effects of exogenous IL-2 as are micro-metastatic deposits of tumor. One interpretation of these experiments is that inflammatory cells within large tumors were capable of responding to exogenous IL-2 to bring about the control of even a massive tumor load. Histopathologic studies of tumors treated with IL-2 and LAK cell suggest that an inflammatory cell infiltration of tumor was an integral part of the immunotherapeutic effect of IL-2.[13] The experiments reported here would further support this concept that exogenous IL-2 generates LAK cells from lymphocytes within the tumor itself to bring about tumor cytolysis.

Other data strongly suggested that IL-2 plus LAK cell immunotherapy requires cell-to-cell contact of continuously activated effector cells with tumor cells. Ottow *et al*[15] showed that the optimal route of administration of both IL-2 and LAK cells was into the body compartment that contained the tumor itself. If LAK cells were administered systemically they were largely ineffective in augmenting the effects of exogenously administered IL-2 on an intraperitoneal tumor. LAK cells are required within the peritoneal cavity in order to exert maximal effects in reducing an intraperitoneal tumor mass. Also, IL-2 was less effective when administered

systemically than when given into the peritoneal cavity.
These experiments suggest that inflammatory responses stimulated within or around a tumor may substitute for the adoptive transfer of LAK cells. If this is so, the expensive and time consuming *ex vivo* generation of LAK cells may not be necessary for IL-2 immunotherapy. Also induction of an inflammatory response within a tumor may improve the effects of tolerable doses of exogenous IL-2. This induction may be achieved by intralesional administration of agents like *C. parvum* and BCG,[16] by intra-arterial infusion of allogeneic or xenogeneic cells of organs invaded by tumor, or by embolization or irradiation of tumors.

8.5 Summary

Because of the systemic toxicity documented at doses of IL-2 required to control tumor growth, potentiation of the effects of low dose IL-2 is a necessity in effective IL-2 immunotherapy. This chapter assessed the effect of lymphocytes recruited into the tumor mass by mixing allogeneic P815 (H-2^d) tumor cells with syngeneic MCA-105 (H-2^b) tumor cells and injecting the cell mixture intracutaneously. When IL-2 alone was used to treat i.c. tumor, there was no reduction in the size of tumor implants. When allogeneic tumor was mixed with syngeneic tumor, there was a reduction in tumor size at the high dose of allogeneic tumor, but not at the low dose. Mice injected with the allogeneic and syngeneic tumor cell mixture and treated with IL-2 had significantly slower growing i.c. tumor nodules and longer survival times.

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Chapter 9

GENERAL DISCUSSION

9.1 Rationale for the conducted experiments

The armament of cancer therapy has changed dramatically with the development of new techniques in immunology. Technological breakthroughs in molecular biology and genetic engineering have provided new concepts of the molecular basis of neoplastic disease. With the discovery of oncogenes, transposons, retroviruses and peptide growth factors and the role they play in malignant transformation, better insight in the development of malignant tumors is evolving. The number of monoclonal antibodies available for diagnostic purposes and possibly therapy has increased tremendously. Finally, mediators of immune responses have been isolated, characterized and produced in large quantities and apparent homogeneity by recombinant techniques. Interleukin 2 (IL-2) has been studied extensively since it will activate and induce proliferation of T cells into so-called lymphokine-activated killer (LAK) cells. These LAK cells are cytotoxic for a broad range of fresh autologous, syngeneic, allogeneic and xenogeneic tumor cells and not for normal peripheral blood lymphocytes.[1] Combined treatment of *in vitro* activated LAK cells and exogenously administered IL-2 has been proven effective in significantly reducing tumor load and prolonging survival in lung,[2] intraperitoneal,[3] subcutaneous[4] and liver[5] inoculation models.

Rosenberg[6] obtained 22% complete and partial responses with IL-2 and LAK cell therapy in 106 patients in whom standard therapy had failed. Major morbidity and some mortality could be attributed to the high dose IL-2 therapy, given in bolus (100,000 U/kg) intravenous infusions three times daily. With the intraperitoneal route of administration[7] maximal doses of 300,000 U/kg, three times daily could be reached. Only one out of seven patients had > 50% reduction of hepatic and lung metastases; the same side effects of fluid retention and gastrointestinal toxic effects were prevailing. Indeed some were hospitalised in the intensive care, needed mechanical ventilation and vasopressor therapy (*e.g.* Dobutrex®). These authors also noted a marked increase in the number of peritoneal exudate cells and their proliferative capacity to IL-2 increased as much as 30-fold.

In looking for ways to make use of the endogenous production of IL-2 and

other helper factors, while evading known and unknown suppressor mechanisms, we tried to reduce exogenous IL-2, while enhancing the tumoricidal effects of IL-2 and LAK cell treatment. The intraperitoneal tumor model and immunotherapy strategy[8] we developed in mice, seemed best suited to accomplish these goals. The animal model aimed at a maximal cell-to-cell contact of IL-2, activated killer cells, cancer cells, and peritoneal exudate cells for maximal tumoricidal effect and maximal LAK cell proliferation *in vivo*. By introducing an allo-antigenic stimulus we and others[9] found that timing and the host immune status, locally and systemically, are important features. Direct cellular contact provides for the conditions whereby IL-2 induction of activated killer cells is enhanced.[9 10] Activated lymphocytes and tumor cells i.p. have maximal cell-to-cell contact, and are not limited by a reduced blood supply to tumors as they undergo necrosis from outgrowing their vasculature.

Another model whereby direct effector cell to tumor cell contact is established is the tumor neutralization test, referred to as the 'Winn assay'.[11] Primed lymphocytes are mixed with tumor cells and injected s.c., for measurement of tumor diameters, or injected i.p. and followed for survival.[12] In contrast to lung- (access: tail vein), liver- (access: portal vein) and dermal tumor models (access: intralesional), the peritoneal model is easily accessible for repetitive injections of activated cells, biological response modifiers and other drugs directly into the area where the tumor grows. It is important to note that the number of effector cells or concentrations of drugs may be markedly increased within the peritoneal cavity as compared to the systemic circulation.[7 13]

9.2 The visually scored peritoneal cancer index

Crucial to an experimental model is the way results are scored. The score should be objective, reproducible and easily obtainable. In Chapter 3 the first attempt is made to validate a visual method for quantitative assessment of i.p. tumorload by objective criteria. Visual scoring systems for lung implants after intravenous tumor cell injection and liver implants after injection in the spleen and subsequent splenectomy have been described by others.[14 15] The scoring systems were reliable and reproducible in judging the results of immunotherapy protocols.[15 16] Our peritoneal cancer index has proven reliable as well[3 8 17] and the more objective scoring of wet tumor mass and ^{125}IUdR uptake by the tumor legitimize the PCI.

The integrity of the groups defined by visual inspection was maintained by the objective scoring systems as demonstrated in Table 3.1. Tumor mass differed

ten fold between visually scored groups with PCI of 1 and 2; ^{125}IUdR tumor uptake differed twenty fold between these groups. The visually scored PCI groups 2 and 3 differed five fold by tumor mass and by ^{125}IUdR tumor counts. The integrity of the experimental groups was not maintained when 'total' ^{125}IUdR counts in tumor plus intestine and mesenteric fat were analyzed. The 'background' counts introduced by the ^{125}IUdR counts in intestine and mesenteric fat abrogated differences previously defined between groups 0 and 1. Also, the difference between groups 1 and 2 was only two fold and groups 2 and 3 differed only three fold. This establishes that 'total' ^{125}IUdR counts in tumor plus intestine and mesenteric fat is a less sensitive means of quantitating intraperitoneal tumor.

It should be noted that normal mice showed much higher ^{125}IUdR counts in the intestine and mesenteric fat as compared to IL-2 treated mice (found mainly in PCI groups 0 and 1). We suggest that intraperitoneal interleukin 2 causes some atrophy of the intestine or reduces bowel function in some other way by which the normal uptake of ^{125}IUdR by intestinal mucosa is suppressed. It is quite possible that cachectin / catabolin / IL-1[18] is activated as part of the activation and proliferation cascade of cytolytic cells set in motion by interleukin 2.

Judged by the mass of dissected i.p. tumor nodules, ^{125}IUdR uptake of these same tumor nodules is as good an assessment of i.p. tumor load (Figure 3.2). The same holds true for the 'total' ^{125}IUdR uptake of tumor nodules, intestine and mesenteric fat (Figure 3.3). No linear relation could be established between wet tumor mass and the PCI (Figure 3.4). Still this linear relation existed between PCI^3 and wet tumor mass (Figure 3.5) and ^{125}IUdR (Figure 3.6). It may seem somewhat surprising that the unaided eye can visually score the extent of intraperitoneal cancer as adequate as wet tumor mass or ^{125}IUdR tumor counts. Visual scoring is based mainly on perception of tumor diameters, while tumor mass is on volume ($\approx$ diameter^3). Thus it is only natural to compare PCI^3 with tumor mass and ^{125}IUdR. The three dimensional volume expansion of tumor growth is appreciated by the eye and the eye can discriminate between groups of mice.

For scoring the effect of immunotherapy on intra-abdominal tumor, the PCI performed equal to the objective scoring by wet tumor mass and ^{125}IUdR uptake of the tumor. The more simple determination of 'total' ^{125}IUdR uptake of the tumor, intestine and mesenteric fat was influenced too much by the effect the injected treatment had on intestinal mucosa and mesenteric lymph nodes.

We conclude that when simplicities of the methodologies, accuracy, and practicality of the four methods is considered, simple visual inspection is the optimal scoring system for abdominal tumor load.

9.3 Allo-antigenic competent cells and immunotherapy with IL-2 and LAK cells

The incentive for the experimental work done was to make use of the endogenous IL-2 production and possibly other endogenous helper factors for synergy with IL-2 and LAK cell therapy and thus achieving the same or better therapeutic results with less toxic - exogenous - IL-2 doses. Initially it was thought that a maximally activated immune system toward an allogenic target would be the best

Table 9.1 Summary of the effects allo-antigenic competent cells on LAK cell induction and maintenance.

STIMULATOR IN MLC OR CHALLENGE IN VIVO	3 DAY IL-2 INCUBATION OR TREATMENT	'ENHANCEMENT' OF LAK CELL OR IL-2 + LAK CELL THERAPY	'ABROGATION' OF LAK CELL ACTIVITY OR IL-2 + LAK CELL THERAPY
DBA/2	MLC	-	DAY 2, 4, (6)
DBA/2	BL/6 + MLC	-	DAY 2, 4, (6)
P815	BL/6 + MLC	-	DAY 6, 10
DBA/2	LAK + MLC	DAY 2	DAY 4, 6
P815	LAK + MLC	-	DAY 2, 4, 6
P815	ALLO-IMMUNE	DAY 7	DAY (4), 14
P815	ALLO-IMMUNE + BL/6 (1 : 1)	DAY 7	-
P815	ALLO-IMMUNE + BL/6 (10 : 1)	DAY 7	-
P815	'STANDARD'	DAY 0, -4	DAY -14, -42

moment for the IL-2 and LAK cell treatment. We showed that the specific-immune effectors at that time point were detrimental to LAK cell induction and maintenance *in vivo* and *in vitro*. Table 9.1 summarizes the effects allo-antigenic competent cells have on LAK cell induction and maintenance *in vitro* and *in vivo* (Tables 5.3, 6.2, and 7.1).

We postulate that these specific cytotoxic lymphocytes absorb IL-2 with a higher affinity than LAK cells and their precursors.[19] Certainly, the immunotherapeutic effect of IL-2 administration may be profoundly changed if *in vivo* generation of LAK cells or the *in vivo* maintenance of *in vitro* generated LAK cells is not achieved because of too low IL-2 levels. Similar changes in the *in vivo* activity of IL-2 may be expected for other stimuli of T cell responses. One would expect that an infectious or inflammatory process might profoundly affect the host's ability to respond to IL-2 treatment or IL-2 plus LAK cell treatments.

The competitive inhibition CTLs can exert on the responses furnished by biological response modifiers may help explain other immunologic phenomena in patients with cancer. Allogeneic blood transfusions prolong the survival of organ allografts[20 21] and seem to have a detrimental effect on survival of cancer patients undergoing a surgical procedure.[22 23 24 25] Likewise, sepsis after operative resection of colon carcinoma seems to be associated with poor prognosis.[26] Endogenous IL-2 may be 'consumed' by lymphocytes responding to allo-antigens or lymphocytes with high affinity IL-2 receptors. Thus induction and proliferation of natural killer (NK), endogenous LAK cells and natural cytotoxic (NC) cells may be impaired and with it the immune surveillance of proliferating cancer - primary or metastatic - cells.

Immunotherapy with biological response modifiers may depend on too high a dose before effect is seen and thus toxic levels will be reached.[6 7] In some instances immunotherapeutic effects may not be seen unless cells that will compete for immune modulators are eliminated from the host (Chapter 7).[27 28 29]

In all systems examined there was an enhancing effect of allogeneic competent cells on LAK cell induction and maintenance, besides the suppressive effect just discussed. Day 2 MLC cells produce IL-2 in the media; day 7 allo-immune cells have augmented LAK activity when stimulated by IL-2 and enhance LAK induction from normal BL/6 lymphocytes; and allogeneic challenge at day -4 enhances the IL-2 and LAK cell treatment. The kinetics of the immune responses *in vivo* and *in vitro* are different; but during the period the immune response increases exponentially there was enhancement of LAK induction; while maximal immune responses exerted suppression.

9.4 Abrogation of suppression of IL-2 and LAK cell immunotherapy

The competitive inhibition phenomenon of LAK cell induction and maintenance by CTLs can be overcome by high dose IL-2. We have shown that the same effect can be achieved by 'selectively' killing 'suppressor' cells with low dose cyclophosphamide. This effect cannot only be attributed to killing selectively the CTLs elicited by the allogeneic challenge; suppressor T cells induced by the on-going immune response will be lysed as well. Apparently LAK cell precursors are saved.

Others too, have reported the beneficial effect of combining immunotherapy with (low dose) chemotherapy.[30 31 32 33] The question of the mechanism by which this chemotherapy works, remains. Does the chemotherapeutic agent act solely on suppressor T cells themselves and the co-suppressive factors they produce;[34 35] does it also interfere directly with the tumor-induced immuno-suppression by reducing the tumor bulk itself;[36 37] does it act solely by reducing competitively-inhibiting-CTLs; or does it make the tumor more sensitive for immunotherapy? CTL competition with LAK cells for IL-2 is not the only known suppressant of immunotherapy. Several phenomena that could lead to insufficient IL-2 production or absorption in patients with malignancies have been proposed and / or demonstrated, including poor immunogenicity of the tumor itself, the presence of circulating or secreted soluble supppressors of IL-2, the augmentation of monocyte / macrophage or suppressor T lymphocyte-mediated suppression, the effects of therapy, and the adsorption of IL-2 by tumor cells.[9 37 38 39]

9.5 Enhancement of IL-2 and LAK cell therapy by modulating local host immune factors

The on-going immune response in the abdomen was detrimental to IL-2 and LAK cell therapy when the i.p. allogeneic challenge was given 14 days before syngeneic tumor injection. No abrogation of IL-2 and LAK cell therapy was seen at day 0 (of allogeneic challenge), and at day -4 there was a suggestion of enhancement. Our *in vitro* work suggests the enhancement is due to production of IL-2 and attraction and priming of immune competent cells in the abdomen. It is plausible that monocytes / macrophages and cytokines other than IL-2 play a role in the loco-regional enhancing effect of IL-2 and LAK cell therapy.[40] This loco-regional enhancing effect may produce cytokines locally that may have an advantagous systemic effect.[37] No ready exchange exists between i.v. injected lymphocytes and the abdomen or vice versa.[41 42] These patterns of lymphocyte migration may be even more restrictive when IL-2-generated lymphocytes were used.[43]

Confirmation of these hypotheses was found in the dermal mixing experiments (Chapter 8). The addition of allogeneic tumor cells to the syngeneic challenge resulted in a clinically evident, initial fast growing, oedematous injection site as opposed to the non-mixed syngeneic challenge. Apparantly there was a quick recruitment of immune competent, inflammatory responsive cells. Question is to the nature of these cells: are they comparable to the tumor infiltrating lymphocytes (TIL) described by Rosenberg?[35] These TIL cells have potent cytolytic capacity when activated by IL-2: the necessary IL-2 dose could be reduced to 10%. Fact is that IL-2 is a potent immunotherapeutic tool, even when administered in a different compartment.

9.6 Future developments

Although cure rates for some cancers have increased, for most cancers no progress has been reported. Trust in the technological capacities of mankind made people in the Western World believe every problem could be tackled within reasonable time. Since World War II medicine and medical technology has come a long way in the Wealthy West. Only one major - deadly - threat was still menacing us: Cancer. And a new one has arrived: AIDS. Only second to these unreasonable high expectations of mankind is the commercial interest farmaceutical companies have in new therapies for cancer and AIDS. Not to mention the financial interest the researcher has in publicity on new therapeutic accomplishments because of research funding. These reasons lay at the basis of the media up-roar around new treatment modalities like interferon and interleukin 2. When the first excitement has cleared, the second thoughts are prevailing: Nothing has changed.[44 45 46 47 48] New accomplishments are no universal remedies for cancer; they are pieces in the jigsaw-puzzle: sometimes essential pieces. Interleukin 2 may be such an essential piece; further experiments *in vivo* and *in vitro* have to determine the final place IL-2 will have in cancer treatment. So far it is clear we do have a powerful tool to induce omnipotent killer cells, although we do not know enough about the side effects of IL-2. Immunotherapy is here to stay.[49] The more and better we get to know the biological response modifiers, the better we can manipulate the immune system. Still the immune system remains a surveillance system of living creatures: a tremendous capacity to check and balance our system. It might not be expected that an uncontrolled growth that has gone on for a certain period will be brought under control just by augmenting the cytotoxic capacity of our immune system with some biological response modifiers. Immunotherapy will find its place next to and as an adjuvant to surgery, radiotherapy, and chemotherapy. 'Debulking' of primary or

metastatic cancer will be essential,[37] but either modality will have its adverse and sometimes beneficial effects on one of the other modalities and the general health of the patient.[50]

Cancer therapy of the future might look as follows: The cancer-bearing patient might be primed pre-operatively with chemotherapy and biological response modifiers, of which IL-2 will be the most important. The aim will be to restore the general immune status of the patient and to overcome the immunosuppression of surgery and anaesthesia; resulting in less take of circulating tumor cells and in elimination of dormant micro-metastases. At the operation enough material for culture in IL-2 should be obtained. The resulting activated TIL cells will be expanded and adoptive transfer performed. This transfer should take place through selectively placed catheters with run-off in the primary tumor bed and the most probable organs of distant metastases. Prior to transfer and after systemic reduction of suppressor cells with low dose chemotherapy the area should be properly primed by a loco-regional perfusion with a specific or non-specific immune stimulant like an irradiated allogeneic vaccine, *C. parvum* or Freund's adjuvant. It remains to be seen if monoclonal antibody treatment will be specific enough to boost the LAK cell precursor by wiping out the majority of cells with suppressive activities and without being of detriment to the patient. Finally, the transfer of *in vitro* expanded and activated LAK and / or TIL cells will be accompanied by continuous or frequent bolus administration of IL-2 i.p. and / or i.v. Combination with gamma-interferon, tumor necrosis factor and others could be imagined.

The experiments described in this thesis show the pivotal role the immune status of the host plays in the delicate balance between abrogation and enhancement of the immunotherapeutic effects of IL-2. When we have assessed the immune status of the patient as well as the immunological properties of the tumor, we can make a treatment plan. The immunological effects of anaesthesia, surgery,[51] blood transfusion,[24] chemo- and radiotherapy should be taken into consideration. Multimodality and probably multi-compartimental approaches will mark the new era of cancer therapy.

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Chapter 10

SUMMARY

Cancer therapy with surgery, chemo- and radiotherapy alone or in combination has not - overall - increased survival time dramatically in the last 20 years. The effects of immunotherapy have been disappointing and the few positive findings were difficult to reproduce until recombinant DNA technology and the discovery of the biological response modifiers have boosted the interest in immunotherapy: advances in cancer cure and survival may be at the horizon. Interleukin 2 (IL-2) and lymphokine-activated killer (LAK) cell immunotherapy so far seemed the most promising, but the systemic and toxic side effects were dose-limiting. The host immune status might be a two-edged sword: it has an enhancing and an abrogating capacity. This thesis will discuss the interaction between IL-2 plus LAK cell therapy and the host immune status and its effect on cancer control.

Chapter 1 gives an introduction into the different parts composing the immune system as well as the non-specific nature of tumor-associated antigens. Very seldom tumors do express specific antigens that are amenable to specific immunotherapy. Non-specific cytotoxic cells can be activated by different biological response modifiers, one of them being IL-2. This IL-2-activated LAK cell is cytotoxic for a broad range of tumor cells and blast cells, but not for normal, differentiated cells. The nature and phenotype of the precursor and effector LAK cell is discussed. Since both may be 'T-like' and 'NK-like', the term LAK cell is used to describe a phenomenon, not a specific cell.

In view of the serious systemic toxicity of IL-2 and the advantages of a compartmental approach to cancer therapy, a loco-regional, intraperitoneal (i.p.) solid cancer model was developed in C57BL/6 (BL/6) mice. Chapter 3 gives a validation of the developed - subjective - visual scoring method through - objective - determination of wet tumor mass and ^{125}IUdR uptake of i.p. tumor nodules in mice. The more simple assessment of 'total' ^{125}IUdR uptake of tumor, intestine and mesenteric fat is influenced too much by the effect the therapy has on mesenteric lymph nodes and intestinal mucosa, to be of value in assessing intraperitoneal tumor load. The simple quantitative visual scoring of i.p. tumor load in 4 groups - named the peritoneal cancer index (PCI) - is easy, simple, accurate and practical.

Also, the effect of immunotherapy of tumor bearing mice upon intraperitoneal solid cancer nodules can be judged equally effective by objective wet tumor mass and ^{125}IUdR uptake by these nodules; as by subjective visual scoring of the PCI. 'Total' ^{125}IUdR uptake is affected by the tumor load, but injecting the mice as well as the treatment modality is confounding the ^{125}IUdR uptake. Although a simple and objective scoring method, 'total' ^{125}IUdR counts are unreliable for scoring the effect of intraperitoneal immunotherapy (Chapter 4).

The influence the (manipulated) host immune system has on the outcome of loco-regional immunotherapy is discussed in Chapters 5 through 8.

Chapter 5 shows the effect of *in vitro* challenged syngeneic (BL/6) splenocytes - mixed lymphocyte culture (MLC) - on IL-2 activation, LAK cell generation, and LAK cell maintenance. Although the specific allogeneic cytotoxicity is increasing over time and after IL-2 activation, these MLC cells are markedly inhibitory to the LAK cell maintenance as well as LAK cell induction from normal BL/6 spleen cells by IL-2. The effector cell of this suppression was a T cell. Lysis of the LAK cell or the target cell by the MLC cell could not be detected. After a short MLC culture time IL-2 levels could be detected in the media; while after longer culture times IL-2 was absorbed when supplied. There was no evidence for a soluble IL-2 suppressive factor in the supernatants. We postulate that MLC cells produce IL-2 in the initial phase and absorb IL-2 with a higher affinity than LAK cells and their precursors after longer culture times in MLC.

The spleen cells of mice injected i.p. with allogeneic cells showed a maximal specific cytotoxicity towards the challenging cells when harvested 10 to 17 days after the i.p. injection. When harvested 7 days after the i.p. challenge, these so called 'allo-immune' spleen cells have an increased LAK activity when cultured with IL-2 and have the a favorable effect on LAK cell induction from normal BL/6 splenocytes. In Chapter 6 we did not see any real reduction in LAK cell induction and activity by allo-immune cells harvested longer than 7 days after the i.p. challenge. May be we did not check enough time points; or the harvested spleens contained other helper factors and / or cells neccessary in the LAK cell activation process to make up for the absorption of IL-2 by the CTL.

In vivo the i.p. allogeneic challenge may enhance immunotherapy with IL-2 and LAK cells when given 0 - 4 days before syngeneic tumor challenge. When injected i.p. 14 or 42 days before syngeneic i.p. tumor injection a definite abrogation of IL-2 immunotherapy was seen. Chapter 7 shows that this effect is dependent of the dose of the allogeneic challenge and independent of the viability of the challenging allogeneic cells. The kinetics of a subcutaneous allogeneic challenge

may be different: it did not affect IL-2 and LAK cell immunotherapy. Since the negative effect of allogeneic pretreatment could be overcome by high dose IL-2, we postulate that CTL competitively inhibit LAK cell induction through preferential consumption or binding of IL-2. In the early phase of CTL induction IL-2 is produced endogeneously which can explain the synergistic effect on tumor load reduction by IL-2 and LAK cell immunotherapy. The abrogation of IL-2 and LAK cell immunotherapy can also be overcome by 'selectively' reducing the suppressive CTL cell population with low dose cyclophosphamide i.v. The improved therapeutic results of this combined chemo- and immunotherapy have evident clinical implications.

In Chapter 8 we demonstrate the synergistic effect of lymphocytes recruited to the tumor area by mixing allogeneic and syngeneic tumor cells before i.c. injection and treating the mice with IL-2 injections i.p. during 5 days. Not only was the tumor growth rate significantly retarded, also the survival of mice increased significantly.

Chapter 9 places the role of IL-2 in immunotherapy and immune responses into perspective. We postulate that IL-2 is produced endogeneously in the early phase of the specific immune response and is absorbed preferentially by activated CTL and 'memory' T cells in competition with LAK cells. The kinetics of immune responses are different in MLC and *in vivo* depending upon the body compartment or organ. High dose IL-2 therapy could overcome the detrimental effect of allogeneic pretreatment. The same effect could be ascertained by low dose cyclophosphamide-chemotherapeutic pretreatment followed by non-toxic IL-2 doses.

The implications for cancer treatment in the future is discussed: after assessment of the host' immune status an individualized multi-compartmental and multi-modality treatment plan should be carefully planned and instituted.

Chapter 11

SAMENVATTING

Genezing van kanker door chirurgische excisie, chemotherapie en / of bestraling is in het algemeen de laatste 20 jaar niet dramatisch verbeterd. Tot nu toe is het effect van immunotherapie teleurstellend geweest en bovendien zijn de enkele positieve resultaten moeilijk reproduceerbaar geweest. Recombinant DNA technologie en de ontdekking van stoffen die het haematologische systeem activeren en cel differentiatie induceren - de 'biological response modifiers' - , hebben onderzoek naar nieuwe mogelijkheden van immunotherapie enorm gestimuleerd: doorbraken in de kanker genezing lijken in het verschiet te liggen. Tot nu toe lijkt immunotherapie met interleukine 2 (IL-2) en door lymfokine (ge)activeerde lymfocyten - de 'lymphokine-activated killer' (LAK) cellen - het meest veelbelovend. Tot op heden echter beperkten de toxische bijwerkingen de voor een maximaal effect noodzakelijk geachte dosis. De immuunstatus van de tumordrager zou hierbij de rol van een twee-snijdend zwaard kunnen hebben: het effect van de toegediende immuuntherapie kan zowel versterkt als afgezwakt worden. Dit proefschrift gaat in op de interactie tussen enerzijds IL-2 en LAK cell immunotherapie en anderzijds de immuunstatus van de gastheer en het effect van deze interactie op de kanker genezing. Het onderzoek werd verricht in weefselkweek en bij muizen.

Hoofdstuk 1 geeft een kort overzicht van de verschillende onderdelen van het immuun apparaat, alsmede van de a-specifieke aard van de tumor-geassocieërde antigenen. Slechts zelden hebben tumoren specifieke antigenen waartegen specifieke immunotherapie opgewekt kan worden. Niet-specifiek cytotoxische cellen kunnen geactiveerd worden door verschillende 'biological response modifiers', onder andere IL-2. Deze door IL-2 geactiveerde LAK cel is cytotoxisch voor een breed scala van tumor cellen en blast cellen, maar niet voor normaal gedifferentieerde cellen. De oorsprong en het fenotype van de voorloper en van de LAK cel effector worden besproken. Daar beide zowel van het 'T-type' als van het 'NK-type' kunnen zijn, wordt de term LAK cel gebruikt om een fenomeen aan te geven, niet om een specifieke cel te kenschetsen.

Gezien de ernstige toxische bijwerkingen van IL-2 en de voordelen van een

compartimentele kanker therapie, werd een loco-regionaal, intraperitoneaal, solide tumor model in de C57BL/6 (BL/6) muis ontwikkeld. Hoofdstuk 3 onderbouwt de ontwikkelde, visuele - subjectieve - peritoneale kanker index (PCI) met de - objectieve - bepaling van de natte tumor massa en de ^{125}IUdR opname van de tumoren. De simpele bepaling van de 'totale' ^{125}IUdR opname van tumor, darm en mesenterium wordt teveel beinvloed door het effect van de therapie op de mesenteriale lymfklieren en de darm mucosa om op een adequate wijze de intraperitoneale tumor last te quantificeren. Geconcludeerd wordt dat de PCI een eenvoudige, makkelijke, accurate manier is om de intraperitoneale tumor last in 4 groepen in te delen.

Het effect van immunotherapie van de tumor dragende muis op de solide intraperitoneale tumoren wordt even goed gequantificeerd door bepaling van de natte tumor massa, de ^{125}IUdR opname door de tumoren, als door de visuele PCI. 'Totale' ^{125}IUdR opname wordt mede bepaald door de tumor last. Het feit dat de muizen met i.p. injecties behandeld werden, versluierde het aandeel van de tumoren teveel om de 'totale' ^{125}IUdR methode hanteerbaar te doen zijn.

De invloed van de (gemanipuleerde) immuunstatus van de gastheer op het resultaat van loco-regionale immunotherapie wordt besproken in de Hoofdstukken 5 tot en met 8.

Hoofdstuk 5 laat het effect van - in een 'mixed lymphocyte culture' (MLC) - allogeen, geprikkelde syngene (BL/6) milt cellen op de IL-2 activatie, LAK cel generatie, en het onderhouden van de LAK cel zien. Hoewel de specifiek allogene cytotoxiciteit in de tijd en onder invloed van IL-2 toeneemt, remmen de MLC cellen het onderhoud van de LAK cel activiteit en de inductie van de LAK cel uit BL/6 milt cellen door IL-2. Dit effect wordt geëffectueerd door een T cel. Er kon geen toxisch effect van de MLC cel voor de LAK cel of de doel-cel in de *in vitro* cytotoxiciteits test aangetoond worden. Na korte MLC incubatie werd IL-2 in de kweek waargenomen. Na langere incubatie werd IL-2 echter gebonden indien aangeboden. Er kon geen oplosbare IL-2-onderdrukkende factor aangetoond worden. Wij postuleren dat de MLC cellen IL-2 produceren in de vroege inductie fase, maar in de latere fase IL-2 met een hogere affiniteit binden dan LAK cel voorlopers en LAK cellen.

De milt cellen van muizen die met allogene cellen i.p. geinjecteerd waren, lieten na 10 tot 17 dagen een maximale cytotoxiciteit voor deze allogene cellen zien. Deze zogenaamde 'allo-immuun' milt cellen lieten na 7 dagen een verhoogde LAK activiteit zien indien met IL-2 gekweekt. Hetzelfde effect werd gezien wanneer deze 'allo-immuun' cellen tezamen met BL/6 milt cellen en IL-2 gekweekt werden.

In de in Hoofdstuk 6 beschreven experimenten kon geen significante reductie van LAK cel inductie en activiteit door 'allo-immuun' cellen aangetoond worden. Misschien werd niet frequent genoeg naar het effect van 'allo-immuun' cellen op LAK cel inductie gekeken, of bevatten de milten andere helper cellen en factoren die nodig zijn in het LAK cel inductie en activatie proces om op die wijze de absorptie van IL-2 door de cytotoxische T lymfocyten (CTL) te compenseren.

In vivo kunnen de i.p. geinjecteerde allogene cellen het immunotherapeutische effect van IL-2 en LAK cellen versterken indien 0 - 4 dagen voor de syngene tumor inoculatie gegeven. Als de allogene prikkel 14 tot 42 dagen van tevoren wordt gegeven dan werd een volledig afbreken van de IL-2 en LAK cel therapie waargenomen. Hoofdstuk 7 laat zien dat dit effect dosis afhankelijk is van de allogene prikkel, maar onafhankelijk van de levensvatbaarheid van de allogene cellen. De kinetiek van de immuun reactie na een subcutane allogene prikkel is waarschijnlijk anders: er werd geen effect op de IL-2 en LAK cel therapie gezien. Daar het negatieve effect van allogene voorbehandeling overwonnen kon worden door hoge doses IL-2, postuleren we dat CTL de LAK cel inductie competitief remt door IL-2 met een hogere affiniteit te binden. Waarschijnlijk wordt in de vroege fase van CTL inductie endogeen IL-2 geproduceerd; hiermee kan het synergistische effect van allogene voorbehandeling met het tumor verlagende effect van IL-2 en LAK cel immunotherapie verklaard worden. Het negatieve effect van allogene voorbehandeling 14 dagen voor syngene tumor injectie kan ook teniet gedaan worden door 'selectieve ' vermindering van de suppressor cellen en de suppressieve CTLs met lage doseringen cyclofosfamide i.v. De verbeterde therapeutische resultaten van deze gecombineerde chemo- en immunotherapie hebben duidelijke klinische implicaties.

In Hoofdstuk 8 worden experimenten besproken die het potentiërende effect van allogeen reactieve lymfocyten ter plaatse van de intracutane (i.c.) syngene tumor laten zien. De muizen werden met een allo- en syngene tumor mix i.c. geinjecteerd en met IL-2 i.p. behandeld. De combinatie behandeling liet een significant vertraagde tumor groei en verlengde overleving zien.

Hoodstuk 9 plaatst de rol van IL-2 in immunotherapie en de immuun response in perspectief. Wij postuleren dat IL-2 in de vroege fase van de specifieke immuun response endogeen geproduceerd wordt en dat IL-2 in de latere fase door geactiveerde CTL en 'memory'-CTL cellen met een hogere affiniteit dan LAK cellen gebonden wordt. De kinetiek van de immuun response is verschillend in MLC en *in vivo* afhankelijk van lichaams compartiment en orgaan. Hoge doseringen IL-2 kunnen het afbrekende effect van allogene voorbehandeling

overwinnen. Hetzelfde effect kan bereikt worden door lage dosis cyclofosfamide-chemotherapeutische voorbehandeling gecombineerd met niet-toxische doses IL-2.

De implicaties voor kanker therapie in de toekomst worden besproken: nadat de immuun status van de gastheer geinventariseerd is, kan er een geindividualiseerd multi-compartimenteel behandelingsplan waarin chirurgische excisie, chemotherapie, bestraling en immunotherapie gecombineerd worden, opgesteld en uitgevoerd worden.

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CURRICULUM VITAE

The author was born in June, 1951 in Amersfoort, the Netherlands. After Montessori Elementary School, he attended the Rijks hbs B in Amersfoort and graduated in 1968. The following year he was an exchange student at East Detroit High School, U. S. A. from where he graduated with honours. In 1969 he started his medical studies at Leiden University, the Netherlands. During this time he was a student- assistant in Anesthesiology (Prof. Dr. Joh. Spierdijk) and spent half a year in a Family Practice in Reinbeck, Iowa, U. S. A. (Dr. W. H. Verduyn). He passed the E. C. F. M. G. examination in 1976. These years he was active in sports as a member of the rowing team, coach and committee member of the K. S. R. V. 'Njord'. After certification as a medical doctor in june 1976, the author spent a year as a resident in Surgery (Dr. J. van Lochem,† Dr. H. Reynders, P. H. A. M. Delfgaauw and A. N. Berkhout) and Gynecology and Obstetrics (Dr. H. van Opstall and J. Kropveld), Queen Juliana Hospital, Hengelo, the Netherlands. The following months he was trained at the Dutch Reformed Mission Institute 'Hendrik Kraemer' and attended the course in Tropical Medicine and Hygiene at the Royal Tropical Institute, Amsterdam, the Netherlands. From 1978 till 1980 he was employed by the Eglise Evangélique de Cameroun to work at the Protestant Hospitals of Bonaberi, Ndoungué and Foumban, Cameroun. Before starting his surgical training (Prof. Dr. H. van Houten† and Prof. Dr. J. Jeekel) at the Academic Hospital 'Dijkzigt', Rotterdam, the Netherlands in 1981; he worked as a company doctor at Marmul Field, Oman and started investigations in colon carcinogenesis at the Laboratory for Experimental Surgery (Prof. Dr. D. L. Westbroek†), Rotterdam. The experiments described in this thesis were executed during a year spent as Visiting Fellow in Colo-Rectal Surgery (Prof. P. H. Sugarbaker, M. D.) at the Surgery Branch (S. A. Rosenberg, M. D., Ph. D.), National Cancer Institute, Bethesda, U. S. A. July 1st 1987 the author was registered as a General Surgeon and is currently employed at the Academic Hospital 'Dijkzigt', Rotterdam with a special interest in Surgical Oncology and Traumatology.