1990

The T Cell-mediated Response To A Syngeneic Tumour: Precytotoxic T Lymphocyte Frequency Measured By Limiting Dilution Assay

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THE T CELL MEDIATED RESPONSE TO A SYNGENEIC TUMOUR: PRE-CYTOTOXIC T LYMPHOCYTE FREQUENCY MEASURED BY LIMITING DILUTION ASSAY

by

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Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

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The University of Western Ontario
London, Ontario
January 1990

ISBN 0-315-55274-3

ABSTRACT

The T cell-mediated response to a syngeneic tumour has been studied using the DBA\2-P815X mouse tumour model. If one hopes to intervene to change (improve) the immune response of a cancer patient to their tumour, it is necessary to understand the role(s) of the cells involved in that response.

The potential magnitude of a cytotoxic T lymphocyte (CTL) response depends, in part, upon the precursor or pCTL frequency. A limiting dilution assay (LDA) has been developed and used to measure the frequency of pCTL's in the animal both prior to tumour implantation and during the growth of the tumour. As well, it indicates whether or not suppressor cells, capable of down-regulating the immune response to the tumour, are present.

With the DBA\2-P815X syngeneic tumour model, there is an increase in pCTL frequency in the spleens of tumour-bearing mice two weeks after tumour implantation. Also, a correlation between pCTL frequency and tumour size, and between the presence of suppressor cells and tumour size, has been found. When pCTL frequencies are measured in normal animals, prior to tumour implantation, there is a correlation of that frequency with tumour progression after implantation. Higher frequencies correlate with smaller tumour sizes.

These results suggest that the frequency of precursors of cytotoxic T lymphocytes, specific for a syngeneic tumour, and the presence or absence of suppressor cells, may influence the growth of that tumour.

ACKNOWLEDGEMENTS

This thesis is dedicated to Linda, Liane, Sara, and Garth. Especially to Linda - thanks for everything!

I thank Dr. Nick Sinclair and Dr. Pat Taylor for their guidance and help and encouragement throughout this project and especially Nick for giving me the opportunity.

I am grateful to Mrs. Jean Oliver for her assistance in the laboratory and to Dr. Alan Groom for his advice and encouragement in entering the program.

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NOMENCLATURE

Ab	antibody
ADCC	antibody dependent cell-mediated cytotoxicity
Ag	antigen
ANOVA	analysis of variance
APC	antigen presenting cell
B cell	the lymphocyte which forms antibodies
C	complement
CMI	cell mediated immunity
ConA	concanavalin A -- a lectin which stimulates T cells
concomitant immunity	an effective response to a tumour implanted at a second site, even though the initial tumour implant continues to grow (ref.338)
CTL	cytotoxic T lymphocyte
Do	the reciprocal of the final slope of the radiation survival (or response curve): the dose required to reduce the number of surviving cells to 37%
dth	delayed type hypersensitivity
Effector cells (Effectors)	see Methods section II-1 for an operational definition
FCS	fetal calf serum
Fc receptor	a receptor for the Fc portion of antibody
Filler cells (Fillers)	irradiated (20 gray) spleen cells added to a culture (Methods section II-4)
Helper cells (Helpers)	spleen cells treated with anti-Ly-2 Ab plus complement
HLA	histocompatibility locus A
^{3}HTdR	^{3}H-thymidine
IFN	interferon

IL-	interleukin-
K cell	killer cell
LAF	leukocyte activation factor
LAK	lymphokine activated killer cell
LDA	limiting dilution assay
LGL	large granular leukocyte
ln	lymph node
LU	lytic unit
Ly-	lymphocyte antigen-
2-me	2-mercaptoethanol
MHC	major histocompatibility complex
MLC	mixed lymphocyte culture
MoAb	monoclonal antibody
M-W-U test	Mann-Whitney-U test (statistics)
NK cell	natural killer cell
PBL	peripheral blood leukocyte
pCTL	pre-cytotoxic T lymphocyte
PHA	phytohemagglutinin a lectin which can stimulate T cells
PMA	phorbol myristate acetate
PMN	polymorphonuclear leukocyte
Responder cells (Responders)	see Methods section II-1 for an operational definition
SD	standard deviation (statistics)
SEM	standard error of the mean (statistics)
SN2°MLC	supernatant from a mixed lymphocyte culture which has been stimulated for a second time
SNK test	Student-Neumann-Keuls test (statistics)

Stimulator cells (Stimulators)	see Methods section II-1 for an operational definition
T cell	thymus derived lymphocyte
TAA	tumour associated antigen
TAL	tumour associated lymphoid cell
Target cells (Targets)	see Methods section II-1 for an operational definition
TASA	tumour associated surface antigen
TATA	tumour associated transplantation antigen
Tc	cytotoxic T cell (CTL)
TCGF	T cell growth factor
Th	T helper cell
Thy-1	antigen found on the surface of T cells
TIL	Tumour infiltrating lymphocyte
TNP	trinitrophenol
Ts	T suppressor cell
TSA	tumour specific antigen

CHAPTER I INTRODUCTION AND REVIEW

I-1 GENERAL INTRODUCTION

We are studying the cell-mediated immune response to a syngeneic tumour, using a mouse tumour model. If one hopes to intervene to change (i.e. improve) the immune response of a cancer patient to their tumour, it is necessary to understand the role(s) of the cells involved in that response and how those cells might be manipulated.

One of a number of different possible responses to a tumour involves the cytotoxic T (or thymus-derived) lymphocyte which is capable of killing a target cell. This response may be important in immune surveillance against very early tumours but it is more likely to be important in controlling metastases and in the response to the primary tumour in some cancers. Therefore it is important to understand the T cell response to syngeneic tumours.

Studies of the T cell-mediated response to syngeneic tumours have usually involved testing the *in vitro* cytotoxicity after either *in vivo* or *in vitro* sensitization to tumour cells and testing the *in vivo* cytotoxicity with adoptive transfer types of experiments. Activity of T lymphocytes has been measured, using various methods, either by the killing of tumour cells, or by cytostasis assays in which division of the cells is halted.

When a pre-cytotoxic T lymphocyte (pCTL) is stimulated by presentation of an antigen on a cell and provided with necessary growth conditions and factors, it undergoes differentiation and division. Most of the studies to date are of the fully differentiated cytotoxic T

lymphocyte. Since the pCTL is the cell that is initially stimulated and the production of CTL's results from the division of the pCTL, one of the important factors in the response to a tumour is the frequency of the pCTL's in the various lymphoid organs of the tumour bearing host. A limiting dilution assay (LDA) allows us to calculate the frequency of pCTL's present in various lymphoid cell populations. We can also measure the intensity of the immune response, using a lytic unit (LU) determination, in vitro. This assay provides a quantitative estimate of the amount of cytotoxicity produced by the stimulation of a given number of pre-cytotoxic T lymphocytes.

Since these two assays can be performed when no tumour is present or at various times after implantation of the tumour, they allow us to follow and study the time-course of the T-cell-mediated immune response to a syngeneic tumour throughout the course of the tumour growth. One purpose of this thesis is to present results from a study of such a time-course of events. I will show how the T cell response to a syngeneic tumour, measured with the LDA and LU activity assays, changes during the course of tumour growth. Results concerning the influence of T-helper (Th) cells or factors, which increase the T cell response, and T-suppressor (Ts) cells, which suppress the immune response, will be presented. Another purpose of the thesis is to determine whether a correlation exists between the pCTL frequency in a normal animal, before a tumour has been implanted, and the subsequent growth of the tumour and eventual outcome to the animal. If the T cell-mediated response to a tumour influences the growth of that tumour, and because one important determinant of the strength of a T cell response is the frequency of precursors, then one might expect there to be a correlation between pCTL

frequency and tumour growth.

In the following review, the immune response to a tumour will be discussed. A discussion of cell types, other than CTL's, is included in order to show how the type of response studied in this thesis relates to the many other possible responses. Following that is a detailed description of the T cell and its possible interactions with other factors and cells.

I-2 THESIS LAYOUT

Chapter I gives an introduction to the immune response and particularly the immune response to a tumour. The potential involvement of cells other than T cells is described, in the appendix, in order to show that many cell types may play a role and that the T cells, described in this thesis, are not the only players in the game. T cells and their role in an anti-tumour response are described as well as assays used to measure their activity.

Chapter II contains the methods for the research. The quantitation of cell killing by the chromium release assay and the measurement of pCTL frequency by the LDA is described.

Chapter III includes the results of the optimization of the system. Since the DBA/2 anti-P815 response is a weak syngeneic one (thus similar to spontaneous human tumours), the cytotoxicity measured is low. Special efforts have to be made to optimize all conditions so that a peak response occurs and can be measured. This is necessary also for a LDA determination of precursor frequency where the presence of one pre-

cytotoxic cell in a culture well must be detectable. Also included in Chapter III is a characterization of the cells involved as far as antigenic markers and radiation sensitivity is concerned. A comparison of this syngeneic system to a stronger allogeneic response is also made.

Chapter IV contains the results of a study of the time course of response after tumour injection. This was done by measuring pCTL frequencies in normal mice and also in those which had been injected with tumours at some time previously. We also determined the correlation between tumour size and pCTL frequencies in the spleens and between tumour size and the presence of suppressor cells. Furthermore, we studied a correlation between pCTL frequency in normal animals and what happens in those same animals after subsequent tumour implantation. Lastly, we include the anatomical locations of pCTL's in tumour-bearing mice.

Chapter V contains a discussion of the results as well as some conclusions.

Because many of the terms used in this thesis may be unfamiliar to someone not involved in immunologically related research, a glossary of terms and abbreviations is included on pages xii-xiv.

I-3 REVIEW

I-3-1 Immune Response In General

The immune response in mammals can be divided into two broad categories - adaptive and innate (213). Innate immunity is the first

line of defence against infectious agents and is carried out by blood monocytes (macrophages in the circulation) and polymorphonuclear leukocytes which phagocytize and destroy those agents. Another part of the innate response is the natural killer (NK) cell which can recognize and destroy virally infected cells and thus prevent the spread of a viral infection.

Adaptive immunity concerns specific responses by lymphocytes to foreign agents. The term 'memory' is used to describe a system where subsequent challenges by the same agent usually invoke a stronger, more effective response. Adaptive immune responses fall into two classes - humoral and cell-mediated immunity (125,213).

Humoral immunity results in the production of specific antibodies (Ab), by plasma cells which have transformed from B lymphocytes in response to antigens or immunogens. After these antibodies have combined with the foreign agent, the result can be its destruction. Antibody synthesis and release usually occurs in the tissues of the immune system.

In cell-mediated immunity (CMI), T lymphocytes (or T cells) specifically recognize foreign antigens and destroy the agent. Direct cell-cell contact between a cytotoxic T cell (CTL or Tc) and the foreign agent is required. Cell-mediated immunity defends the host against parasites including viruses and some bacteria that can live within cells. Like NK cells, T cells can recognize virally infected cells and kill them. As well as acting in graft rejection or transplantation immunity, some T cells can recognize whatever it is on the cell surface that marks a tumour cell as being different from a normal cell and kill that tumour cell.

The different cell types in the immune system do not function in isolation but rather through interactions with other cells and/or factors produced by other cells.

This thesis examines a T-cell-mediated immune response against a tumour. Therefore it deals with the cell-mediated type of adaptive immunity.

I-3-2 Immune Response To Tumours

Recent general reviews have described the immune response to tumours (9,209,234,252,294). Immunological responses to a tumour may be divided into non-specific (or non-adaptive) and specific (or adaptive) immunity (175,278); each category containing different cell types. Adaptive immunity is acquired and is mediated by B and T lymphocytes, and K cells (ADCC) when the specific component is supplied by humoral antibody (175,209). Non-adaptive immunity is mediated by 'null' cells - comprising haemopoietic precursors, NK and K (ADCC) cells (175).

A major question in tumour immunology today concerns the relative contribution of specific and non-specific mechanisms. Which part of the immune response plays a dominant role in the development of resistance to tumour growth (209,278)?

A wide variety of human tumours have been shown to be capable of promoting a measurable immune response under certain conditions (45,89,128,234,252,273). In many human tumour patients, an immune response does occur (45,294). Activated lymphocytes have been isolated

from the tumour and from draining lymph nodes of nasopharyngeal tumour patients (74). It is possible to generate cytotoxicity against autologous tumour cells with mixed lymphocytes and tumour cell cultures (128,279). An indication of response is the presence, in many human and experimental animal tumours, of host cells infiltrating the tumour cell mass (74,213). Although there seems to be no simple overall relationship between host cell infiltration and a favourable prognosis (213), there appears to be a correlation in some cases (278).

Concomitant immunity is described as an effective response to a tumour implanted at a second site, even though the initial tumour implant continues to grow (68). The demonstration of concomitant immunity in an animal tumour model is strong evidence that the immune system is responding, even though the response may not eliminate the primary tumour (68,125,252).

An immune response by a patient to a tumour can often be measured (128,236). In one study, approximately one-third of cancer patients, at surgery, exhibited PBL or LN cell cytotoxicity directed against fresh autologous tumour cells (213).

Foley was one of the first researchers to demonstrate tumour-associated transplantation immunity in an animal tumour model (68). He injected three spontaneous and six chemically induced tumour cell lines into mice. After the tumours had reached a certain size, he removed them and implanted fresh tumour cells. The chemically induced tumour cell lines were rejected by most of the mice: they had become immune. However, against the spontaneous lines, the mice were unable to produce an effective response. He concluded that tumour cell lines can stimulate an immune response but there appear to be differences among

the various tumours, according to their origins, in their ability to elicit the response. It has since been found that the effectiveness of different tumour cells in eliciting an immune response ranges from zero to pronounced and that the response cannot be easily classified according to tumour type (209,288).

Most experimental animal tumour models are rather artificial in that tumours are experimentally induced (by chemicals, viruses, radiation etc.) and have been transplanted into the animals. Sometimes they elicit a stronger immune response than tumours that arise spontaneously, such as those occurring in humans.

While the response to a tumour is often beneficial to the organism, it is possible for protection of the tumour to occur. With Moloney-virus-induced animal sarcomas, host cell infiltration frequently is associated with regression (213). There is a correlation of general immunocompetence with tumour size and prognosis in patients with cancer of the head and neck (294). However it has been observed that the immune system can suppress (or down-regulate) an effective response to a tumour (252). Blazar et al. (16) found that tumour-associated lymphoid (TAL) cells stimulated the growth of tumour cells in five out of seven experiments. However, these same lymphoid cells, when combined with macrophages isolated from the spleen cell population, inhibited tumour cell growth. This demonstrates the variable effect that cells, derived from the immune system, may have on tumour cells.

Stutman (252) has outlined three levels of tumour-host interactions. There may be early recognition of a malignant change in situ and a reaction to it. If the response is effective in eliminating the tumour, there may be no evidence of such an occurrence. The term

immunological surveillance has been used to describe this recognition and elimination of tumour cells before a recognizable tumour has formed. The second level comprises the complex immunological response of the host to a developing tumour once it reaches a certain critical mass. One difference between the immune response to a tumour and to other diseases is that the tumour bearing host must cope with a constantly increasing source of antigen as the tumour mass increases (9). Immunological mechanisms affecting the metastatic spread of the tumour make up the third level.

Cell types taking part in an anti-tumour response include various lymphocyte subsets (128,213,273,278) and mononuclear phagocytes (213). Macrophages may be important in combination with lymphocytes (16). In some assays, it is difficult to distinguish between an NK-like cell and T cell response to fresh autologous tumour cells, especially when a growth factor (ie. IL-2) is added or produced. Various cell types, other than the cytotoxic T lymphocyte, which may be involved in a response to a tumour will be described more fully in the appendix.

I-3-2-1: Immune Surveillance

Immune surveillance is a term that refers to the recognition of, and effective response to, tumour cells in the body. There are generally considered to be two forms. Neoplastic cells may arise occasionally and be detected and eliminated by one of the immune system effector cell types. Thus, a nascent tumour may be eliminated by one or more components of the natural or induced effector mechanisms and there

will be no evidence that this has actually occurred. Even if the transformed cells are not eliminated, immune surveillance may act to impede tumour growth (102). Supposition that this form of immune surveillance exists is based primarily on evidence that immunosuppression can lead to increased tumour incidence (102). The incidence of cancer (primarily skin cancers and lymphomas) is 11 fold higher in immunosuppressed kidney transplant patients than in the general population (125).

When tumour cells are artificially introduced into an animal, the response to the tumour can be considered to be a form of immunosurveillance. Differences in such a system that set it apart from what is normally considered to be immunosurveillance include a much larger initial tumour burden (many cells or a cell mass instead of a single cell), and the fact that in most cases, the tumour has been artificially induced originally.

A second form of immunosurveillance may act to eliminate or inhibit potentially metastatic cells, released by the primary tumour into the circulation and responsible for the generation of metastases. There is evidence for the ability of one immune system cell type, the natural killer cell, to eliminate metastatic tumour cells and inhibit the spread of tumours (103).

Possibly, immune surveillance is active only against certain types of tumours. Those that are inducible or arise spontaneously only when the immune system is compromised may normally be controlled effectively by immune surveillance. Examples might be tumours induced by oncogenic viruses (288). Inducible or spontaneous tumours which arise with no evident impairment of the immune system may not be controlled by

surveillance.

The NK cell may participate in tumour surveillance. Although nude mice are deficient in their T cell response, they are not more susceptible to cancer than normal mice and this may be due to the presence of high levels of NK cells (102,125). NK cells have also been implicated in surveillance against metastatic spread of cancer (103,104,126,127,209, 213,252,278,292).

Lymphokine activated killer cells (LAK) may be an expression of immune surveillance (88) and could be important against NK-resistant solid tumour cells (87). Although the participation of macrophages in immune surveillance is not proven, they appear to be important in the destruction of tumours once that process has been initiated by other cells (245). The frequency of DNA virus-induced tumours in T cell-deficient mice is greater than in normal animals; showing that T cells may also have a role in immune surveillance, at least in some circumstances (102,213).

Herberman (102) has raised the possibility of several effector cells participating sequentially. Because of spontaneous reactivity and rapid activation, NK cells and macrophages may be the initial defence. If these natural effector cells are not completely sufficient, then immune T cells (or LAK cells?) may be induced to complete the elimination of tumour cells. It should be emphasized that since there appears to be little direct evidence of such a response, immune surveillance against primary tumours is largely a hypothesis.

I-3-2-2: Tumour Antigens

Antigens on tumour cells can be categorized into three classes (208,213). These class designations are not to be confused with classes of MHC antigens. Class I antigens are absolutely restricted to a single tumour. They cannot be detected on any other tumour or normal cell type. Class II antigens are found on some tumours of related origin in different individuals and also on some normal cell types. Class III antigens are expressed on a wide range of normal and malignant tissue. They are not restricted in any way to tumour cells. Problems with defining tumour antigens include the fact that, although they may be found on tumour cells and may seem to be unique to those cells, they may represent antigens not found in the normal counterpart of the tissue of origin, but found in another tissue. Or, they may be differentiation antigens found normally (perhaps rarely) in early developmental stages but not on mature, differentiated tissue.

Tumour-associated antigens (TAA) and tumour associated surface antigens (TASA) usually are of Class II type. They are associated with, but not restricted to, tumour cells (167,234). Examples are alpha-fetoprotein and carcinoembryonic antigen (CEA) which are normally found only on foetal and not on adult tissue. Alpha-fetoprotein is associated with hepatocellular carcinomas while CEA is a foetal colon cell surface glycoprotein often found on intestinal adenocarcinoma as well as on some cancers from other sites. When cells from patient tumours have been compared with those same cells grown *in vitro*, major differences in TAA's have not usually been found (253).

Tumour-associated transplantation antigens (TATA) refer to antigens

which can be detected by transplantation rejection experiments; ie. an immune response directed toward these determinants can result in tumour rejection in an immunized host (234).

Tumour-specific antigens (TSA) and tumour specific transplantation antigens (TSTA) are defined in similar ways as the TAA's and TATA's except that these are tumour unique or Class I antigens not found on other tumour or normal cell types (167). Because of the difficulty in testing whether an Ab to a TSA will react with any other tumour or normal cell type, one cannot be sure that an antigen is truly tumour specific (253). Some feel that there is very little experimental evidence for the occurrence of TSA's on spontaneous human tumours (220,302).

Evidence suggests that experimental tumours induced by the same chemical or by irradiation or by physical agents do not share common antigens (9,125,234). This contrasts with virally induced tumours where TATA's produced by the same virus will cross-react (9,213). This cross-reaction probably occurs because of the expression of similar viral components on the cell surface. Antigens produced by tumours induced by different viruses do not appear to cross-react. Generally, spontaneous animal tumours produce immunologically weak TATA's (or TAA's) (209).

As a tumour grows, there is an increasing source of antigen and therefore the presence of circulating Ag in the blood can provide a marker for tumour burden (9). There is evidence that antigen expression can vary from cell to cell within a tumour, or between the primary and a metastasis; with antigen expression being dependent on cell cycle, proximity to blood supply, or even clonal differences (9,71,253). Clonal differences in antigen expression have been found in melanoma

lines isolated from patients (208).

Although human cells normally express HLA Class I antigens, some tumour cells do not express any or express them at low levels (278). It has been postulated that the loss of Class I MHC antigens from mouse tumour cells could lead to escape from CTL recognition and destruction since these antigens are normally required for recognition by T lymphocytes (84).

The DBA/2 P815X murine tumour model used in the research for this thesis employs a mastocytoma cell line induced by a methylcholanthrene injection. This has resulted in a tumour specific (or associated?) transplantation antigen which produces a "weak" immune response. Al-Rammahy et al. (2,3), have shown that it is difficult to raise a significant level of transplantation immunity in syngeneic DBA mice by either removing early growing tumours or by attempted immunization with irradiated tumour cells. Uyttenhove et al. (270) have also characterized the P815X as a weakly antigenic line. They were unable to immunize mice with irradiated tumour cells.

Al-Rammahy has produced antibody raised to a 70 kD TAA on P815X cells and showed that this antibody can block <u>in vitro</u> killing of tumour cells by syngeneic cytotoxic lymphocytes (2). As a further demonstration that P815X cell possess possibly unique tumour antigens, two CTL clones have been reported which are directed against a TATA on those cells (2). However, Sulitzeanu has noted that in dealing with cell-mediated immune responses, the specificity of antigens is very difficult to establish (253).

Therefore, the chemically induced P815X tumour model has been characterized as a weakly immunogenic cell line expressing tumour-

associated (or specific?) transplantation antigens.

1-3-2-3: The Immune Response During Tumour Growth - Possible Reasons For Change

The ability of the immune system to respond to tumour cells may change as a tumour progresses. This change in the capacity to respond has been studied in a number of animal model systems.

A model system has been described in which virally or chemically induced syngeneic solid tumours were grown in mice (8,10,298). At various stages of tumour growth, host cells were recovered by lavage from the peritoneal cavity and tested *in vitro* in a colony growth inhibition assay against the tumour cells. The Effector/Target cell ratio was quite large (100-200/1). During the initial phase of tumour growth (days 7-10), the peritoneal cells significantly inhibited (although sometimes slightly) the colony formation of plated tumour cells. Depending on the tumour cell line, the inhibition of colony formation ranged from 17 to 95%. This inhibitory capacity disappeared when the tumours became larger. When the tumours were surgically removed, the inhibitory capacity of the host's peritoneal cells reappeared with time. The authors showed that sera from tumour bearing mice could suppress the activity of the peritoneal cells in the *in vitro* colony inhibition assay. Possible reasons presented for this suppression were circulating tumour antigens or antigen-antibody complexes which would presumably react with antigen receptors on the peritoneal cells to block killing of the tumour cells.

Leclerc and Levy studied cell-mediated immunity in a tumour bearing animal (138). They employed a ^{51}chromium release assay in which spleen or lymph node cells from animals with either MSV-induced sarcomas or transplanted syngeneic lymphomas, grown subcutaneously, were tested for their ability to kill the chromium-labelled tumour cells in vitro. When young (15 day) animals were injected with Moloney sarcoma virus (MSV), the tumour progressed until they died. Older animals, injected at 30 days, produced tumours which usually developed and then regressed.

In both "progressors" and "regressors", cytotoxic lymphoid cells were detected during tumour growth but disappeared after 13 to 15 days in progressors and much more slowly in mice that rejected the tumour. Therefore, the response to a growing tumour was the development of cells, in both the spleen and lymph nodes, which were cytotoxic to the tumour. Then these cells disappeared; the rate of disappearance correlating with whether the tumour continued to grow or regressed.

The authors found no blocking factors in the sera of mice with progressing tumours that could explain the lack of an effective response. They also noted that when the transplanted lymphomas were grown in the peritoneal cavity, there was no detectable cytotoxicity of the lymphoid cells.

In a different system, it has been shown that ascitic tumours can elicit a measurable response in the lymphoid cell population (15). Significant levels of specific cell-mediated cytotoxicity to P815Y mastocytoma cells were found in the host cells within the peritoneal cell population at 8 to 16 days after tumour inoculation. Host cells were isolated by STAPUT (sedimentation at unit gravity), and cytotoxicity was measured against ^{51} Cr labelled tumour target cells in

vitro at an E:T ratio of 100:1. These effector cells were T lymphocytes and were less cytotoxic from mice 16 days after tumour injection than 8 days after.

The authors also found that day 16 tumour cells were less susceptible to lysis and they hypothesized that tumour cells from progressing tumour "lose" surface TAA's and therefore are not as easily recognized and killed by cytotoxic T lymphocytes. They found no significant quantities of blocking antibodies or factors in the ascitic fluid.

The functional activity of intratumoural T lymphocytes has also been correlated with the stage of tumour growth (78). Purified suspensions of T lymphocytes, obtained from within the tumour by enzymatic disaggregation, were tested 11 and 13 days after the induction of progressing or regressing Moloney sarcomas. The cells were tested for their ability to lyse ^{51}Cr-labelled tumour target cells. At day 11, there was no difference in the ability of the T cells to lyse tumour cells. By day 13, the T cells from regressing tumours were much more cytotoxic than those from progressing tumours and were more active than those from a regressing tumour at day 11. Therefore the functional activity of intratumoural T cells was shown to be correlated with the stage of tumour growth and with whether the tumour would progress and kill the animal or regress.

The authors could not determine why T lymphocytes from a progressing tumour lost activity in the assay. They also noted that T lymphocytes from regional lymph nodes draining progressing tumours reached peak levels of cytotoxic activity 2 to 4 days before those from lymph nodes near to regressing tumours.

In a series of experiments, North and Bursuker showed that the progressive growth of the Meth A fibrosarcoma in a syngeneic mouse results in the generation and then subsequent loss of concomitant immunity (26,191). That is, an animal with a progressive tumour was able to inhibit the growth of a second implant of the same tumour cells 6 or 9 days after the original tumour implant. By day 16, concomitant immunity was no longer present. This immunity was associated with Ly-1^-2^+ T lymphocytes which could adoptively immunize against an established tumour in a T cell-deficient mouse. These effector cells appeared between days 3 and 6 after tumour implantation, peaked on day 9 and then were quickly lost by day 12.

They were also able to show that suppressor T cells (Ly-1^+2^-) arose after day 6 in animals with progressing tumours. At day 9 or later, suppressor T cells from tumour bearing mice could inhibit the regression of tumours caused by adoptive transfer of T lymphocytes from immunized mice. Suppressor activity increased from days 9 through 15.

They hypothesized that as the tumour grows and reaches a critical size, the generation of Ly-1^-2^+ effector T cells occurs and reaches a peak on day 9. As the tumour gets larger, Ly-1^+2^- suppressor T cells then down regulate the production of the effector T cells. The authors equate activity with numbers of effector or suppressor cells and not with differences in activity per cell. The authors note reports in the literature of suppressor cells of both the Ly-1^-2^+ and Ly-1^+2^- phenotypes (191). They present the possibility that Ly-1^+2^- suppressor cells are inducer suppressors that aid in the development of Ly-1^-2^+ suppressor T cells.

In these experiments and others with DBA/2 mice and the P815X

mastocytoma and P388 lymphoma tumours, they show that T cells, which can adoptively transfer immunity, and T cells which can suppress it, are specific for the particular tumour cell line. Specific suppressor T cells have also been demonstrated, by adoptive transfer, in other syngeneic tumour models (136).

In experiments where the Meth A tumours were excised at different times, it was shown that the dominant immune state of the animal towards the tumour cells depends on the time of excision (26,191). If it is early, then there is a prolonged immunity to the challenge of new tumour cells. If it is later, when the suppressor T cells have started to down-regulate the effector cells, then the animal remains unresponsive to a new challenge and retains the suppressor T cell population. Therefore in this system, an immune response occurs to a progressively growing tumour, reaches a peak, and then is suppressed by the immune system itself.

These studies show that the measurable immune response to a tumour can change dramatically while the tumour is growing. Because of the possible influence of helper or suppressor cells (or factors), it is difficult to ascertain what is happening with the CTL population during tumour growth and whether T cells can alter the outcome of a tumour. The variable immune response can depend on whether a tumour is a progressing or a regressing one. An important question is whether this variable immune response is a result of, or a cause of the possible differences in outcome.

Cells (CTL's) have been found which can inhibit the growth of cancer cells and other cells have been found which can apparently suppress this inhibition. Is the suppression acting on the numbers of

precursors of cytotoxic T lymphocytes (pCTL's) present, or on the activation of these precursors? That is, is there suppression because very few pCTL's are present in mice with progressing tumours?

The response to a tumour has been equated with the number of effector cells present and the effectiveness per cell has not been addressed. Neither has the influence of precursor frequency been considered. Is the number of cells potentially capable of responding to the tumour important in its progress?

The research presented in this thesis is an attempt to provide answers to these questions.

I-3-3 T Cells Involved In The Immune Response To Tumours

This thesis presents a study of T lymphocytes, a cell type that has long been accorded a role in the immune response against tumours. T-lymphocytes isolated from some patients are cytotoxic to tumour cells from that same patient, or at least have shown the potential to be stimulated to become cytotoxic. When freshly isolated tumour cells are used as targets in short-term cytotoxicity assays, about thirty percent of patients are shown to have cytotoxic T lymphocytes in their blood, as well as in lymph nodes draining the tumour site (278). When lymphocytes are cultured with autologous tumour biopsy cells, the resulting cytotoxic cells are specific for those tumour cells (273). The cytotoxicity is mediated by T lymphocytes which recognize antigens on the target cells. There is also evidence of T lymphocyte infiltration in a variety of different types of tumour (278).

These cytotoxic or activatable T cells appear to be important in the immune response to some tumours (209). Patients with advanced head and neck cancers have been shown to be deficient in T lymphocyte numbers and those deficiencies are frequently detected in those with early disease (294). Activation of T cells can occur in tumour patients as shown by the response of these cells to IL-2 without other stimulation (278,294). As well, these studies demonstrate that the response can be positive (stimulation of Tc or Th cells) or negative (induction of Ts cells). Various subsets of T lymphocytes may therefore play roles as cytotoxic cells, which under certain conditions can kill tumour cells, or as helper or suppressor cells to regulate the response of CTL's and other cell types to the growth of a tumour.

Each T cell is able to recognize a single antigenic determinant due to the uniqueness of the receptors on its surface (9,100). As will be shown in this thesis, there is a very limited number of T cells capable of responding to a particular cancer cell antigen.

Although T cells may be important in the immune response to tumours, their primary role is probably to protect the host against parasites such as viruses (and some bacteria) that can live within cells (83,100). CTL's with appropriate receptors, and after stimulation (activation), can eliminate virally infected cells. Inflammatory Th1 cells can activate macrophages to destroy mycobacteria inhabiting vesicles within the host macrophages (18). Since the mycobacteria are resident within the host cells, they are protected against an antibody-mediated immune response. An effective T cell response is the primary way that the body fights a viral infection (83).

I-3-3-1: a) Morphology And Abundance

Unactivated T and B lymphocytes are morphologically identical. Normal lymphocytes are small and are produced at a rate of approximately 10^9 per day in an adult human. The average body load is about 10^{12} total lymphoid cells with lymphoid tissue comprising approximately 2% of the total body mass. Of the circulating white blood cells, around 20% are lymphoid (most of these are T cells - B cells accounting for 5-15% of circulating lymphocytes), with the majority of the white blood cells being polymorphonuclear cells (213).

The average lifespan of circulating lymphocytes in the human is over four years and some may continue to circulate for 20 years (125). T cells normally only leave the blood when they are in post-capillary venules of lymphoid tissue. The exception is when T cells are responding to a localized antigenic stimulus.

In the human circulation, approximately 65-80% of T cells have a morphology characteristic of small lymphocytes. Once activated, T cells undergo differentiation and division to form larger blast cells. Ts and Tc blasts have a granular cytoplasmic morphology.

Cytotoxic T lymphocytes are characteristically medium sized lymphoblasts, but under certain culture conditions, T cells can form large, granulated and vacuolated cells with a morphology termed LGL or large granular lymphocyte (290). Therefore, the morphology of T cells can vary greatly from typical small unactivated lymphocytes to large blast forms with granular and vacuolated cytoplasm.

b) Where Are They Found? - Spleen, LN, Thymus, Peripheral Blood

As previously mentioned, about 20% of white cells circulating in the blood (PBL or peripheral blood lymphocytes) are T cells (213).

Cell-mediated immunity, of which T cells are a major component, can occur anywhere within the body. Precursors of T cells originate in the bone marrow and reach a functional maturity in the thymus, hence the origin of the name 'T' (for thymus-derived) cell (125).

The thymus is composed of two major anatomical areas, the cortex and the medulla. Almost all cortical thymocytes are immature while most of the thymocytes in the medulla are mature, immunocompetent cells. Indeed, it is in the thymus where T cells are thought to undergo the process leading to immunocompetence (31).

Lymph nodes act as filters in that most bacteria entering the LN are trapped. Lymph nodes contain a paracortical area called the thymus or T-dependent area where T lymphocytes are found. The response at LN's tends to be localized and involvement of draining lymph nodes is a major factor in determining cancer stage in humans.

The spleen and particularly the white pulp plays a major role in the immune response against blood-borne antigens that enter the circulation (125). Large numbers of splenic lymphocytes are easily obtained by animal model researchers interested in a convenient source of T cells for study. The T lymphocytes found in the T-dependent areas of peripheral lymphoid tissues (peripheral blood, spleen and lymph nodes) are functionally mature and immunocompetent.

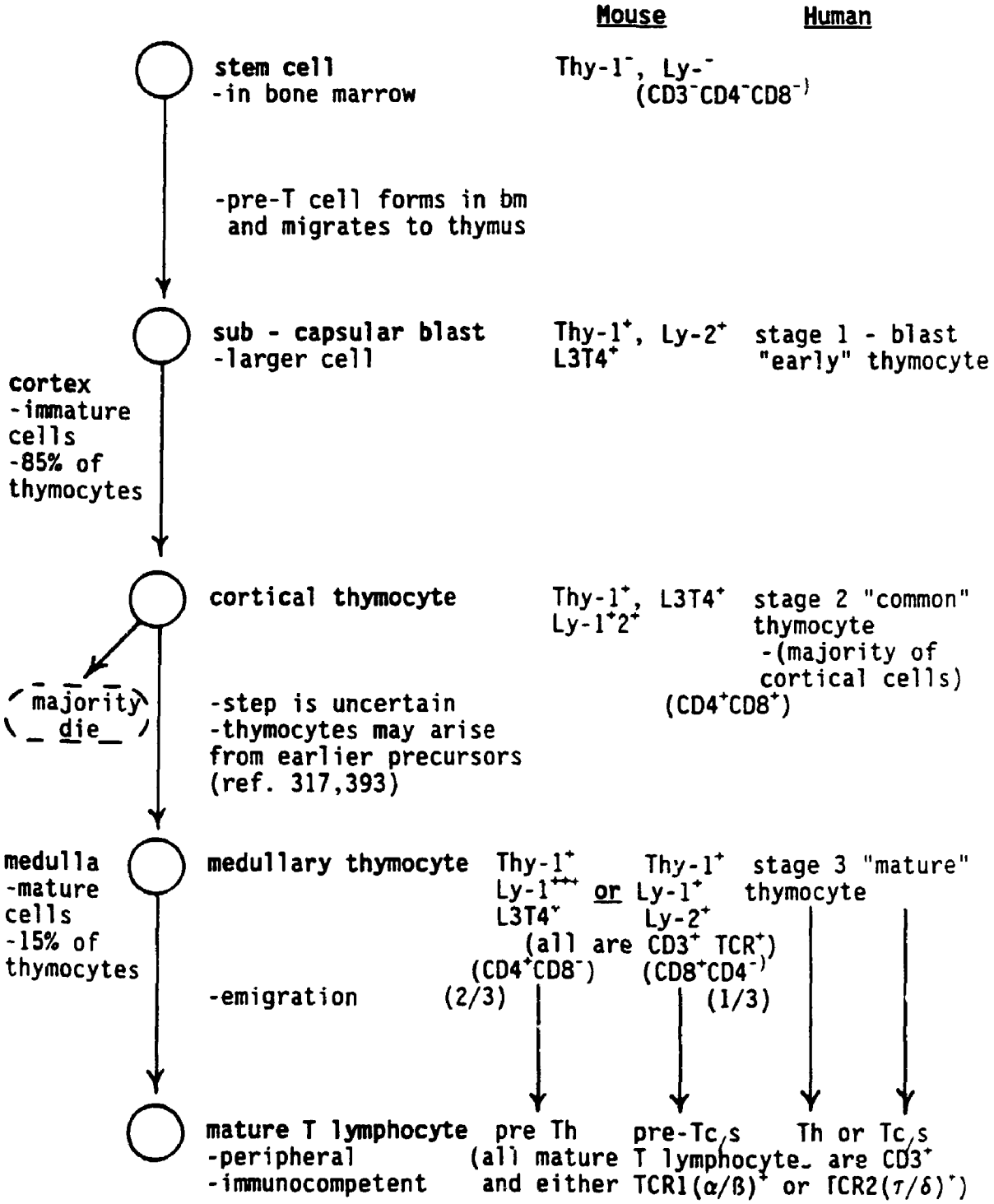
c) Thymic T Cells - Differentiation And Maturation

The scheme presented in figure I-1 for thymic T cell differentiation and maturation is taken from a number of reviews (4,9,32,97,125,153, 156,213,223,224,275) and particularly that of Ceredig et al. (31) who present a model based on a consensus of published papers of other researchers. There are alternate suggested schemes (230,244), but this model seems to fit well with the results from a variety of laboratories.

The thymus continually receives small numbers of primitive precursor or stem cells from the bone marrow. Once there, the cells undergo considerable proliferation and differentiation to form immunocompetent T lymphocytes which then emigrate into the blood circulation (32). It is thought that within the thymus, the genetic rearrangement occurs that is necessary for the generation of clonally diverse antigen receptors (31,156,244). As well as various subpopulations of thymocytes, APC's (dendritic cells and mature macrophages) and other supporting cell types (epithelial and epidermal) are found. These other cells may assist in the differentiation and maturation of the thymocytes and may be responsible for conferring MHC restriction on the developing thymocytes (221,224,244).

The thymus is divided into three main anatomical regions and this compartmentalization may reflect functional variations (31,224). In the peripheral subcapsular region are many large blast cells and most of the proliferation in the thymus occurs here. The cortex contains the largest population of thymocytes. The medulla contains medium size cells and most emigrating cells appear to leave the thymus from here.

Figure I-1 Thymic T Cells - Differentiation And Maturation



Most measurable thymocyte function occurs amid medullary-type cells. Since medullary thymocytes and peripheral T lymphocytes are similar phenotypically, cells found in this region are termed "mature" thymocytes (32). Differentiation into functionally mature cells occurs before exit from the thymus. Therefore, there is no evidence for the need for major maturation steps to take place after exiting the thymus (222,230).

The T cell receptor (TCR) in peripheral T cells is made up of heterodimers of polymorphic protein chains termed α and ß (TCR2 - the majority of peripheral T cells), or of chains γ and δ (TCR1). The T cell receptor is always associated with a complex of proteins called CD3. The CD4 (L3T4) and CD8 (Ly2/3) complexes are other structures also involved in T cell interactions.

In the thymus, $CD3^-CD4^-CD8^-$ cells appear to be the precursors for $CD4^+CD8^+$, $CD4^-CD8^+$, and $CD4^+CD8^-$ cells (97,156). Here also occurs the rearrangement and expression of genes coding for the $\alpha/ß$ and γ/δ TCR heterodimers. Although the expression of TCR1 bearing cells occurs first in fetal animals, those cells do not appear to be the direct precursors of $TCR2^+$ cells (97,156).

After leaving the thymus, cells migrate to T-dependent areas of the peripheral lymphoid system. In the mouse, peripheral T cells are Tc/s ($Ly\text{-}2^+$, $L3T4^-$) or Th ($Ly\text{-}2^-$, $L3T4^+$) and these populations are mutually exclusive. The Th cells can be further divided into Th1/inflammatory cells which produce IL-2 and IFN-γ and are responsible for delayed type hypersensitivity, and Th2/helper cells which produce IL-4 and IL-5 and are important for B cell functions (18,177). All thymocytes and peripheral T lymphocytes express Ly-1 Ag. Ly-1 is also

expressed on some B lymphocytes and is not a T lineage-specific marker (31). Approximately 2/3 of the emigrating thymocytes have markers consistent with Th cells, while the remaining 1/3 appear to be pre-Tc/s cells.

That the thymus is important for the normal development of T cells is shown by experiments with congenitally athymic "nude" mice. These mice, when old, have some cells which are Thy-1^+, Ly-1^+2^+ and which can generate CTL's and produce IL-2 in vitro. The ratio of Thy-1^+ cells in normal versus nude mouse spleens is 50:1 and for pCTL's (lectin-assisted assay), the ratio is 460:1 (37). There are also differences in the phenotypic markers on the T cells in nude mice (154). Therefore, although it is not necessary for mature, functional T cells to have been processed through the thymus, it is certainly important for the normal development of T cell-mediated immunity.

Environmental factors other than those experienced in the thymus may be important in T cell differentiation and in the selection of antigen receptor specificity. Allospecific CTL clones can develop syngeneic target cytotoxicity under appropriate culture conditions (39). These cells develop an autoreactive cytotoxicity which leads to annihilation of the clones. Thus differentiation has taken place influenced by environmental factors outside the thymus. It should be remembered that this occurs in vitro under somewhat artificial conditions.

Rapid changes in vitro in specificity within a single clone of cytotoxic effector cells has been noted (210,291). The fact that specificity changes can occur in clones of CTL's argues against the clonal selection theory. These results demonstrate the possibility that

the environment outside the thymus, perhaps even in vivo, can influence and change the CTL specificity. This suggests that T cells learn to recognize Ag in the MHC context of the environment in which they develop and not simply in the context of the MHC of the T cells themselves (210). One possible means for the rapid changes in specificity that can be induced would be by somatic mutation of the genes responsible for the T cell Ag receptor (210).

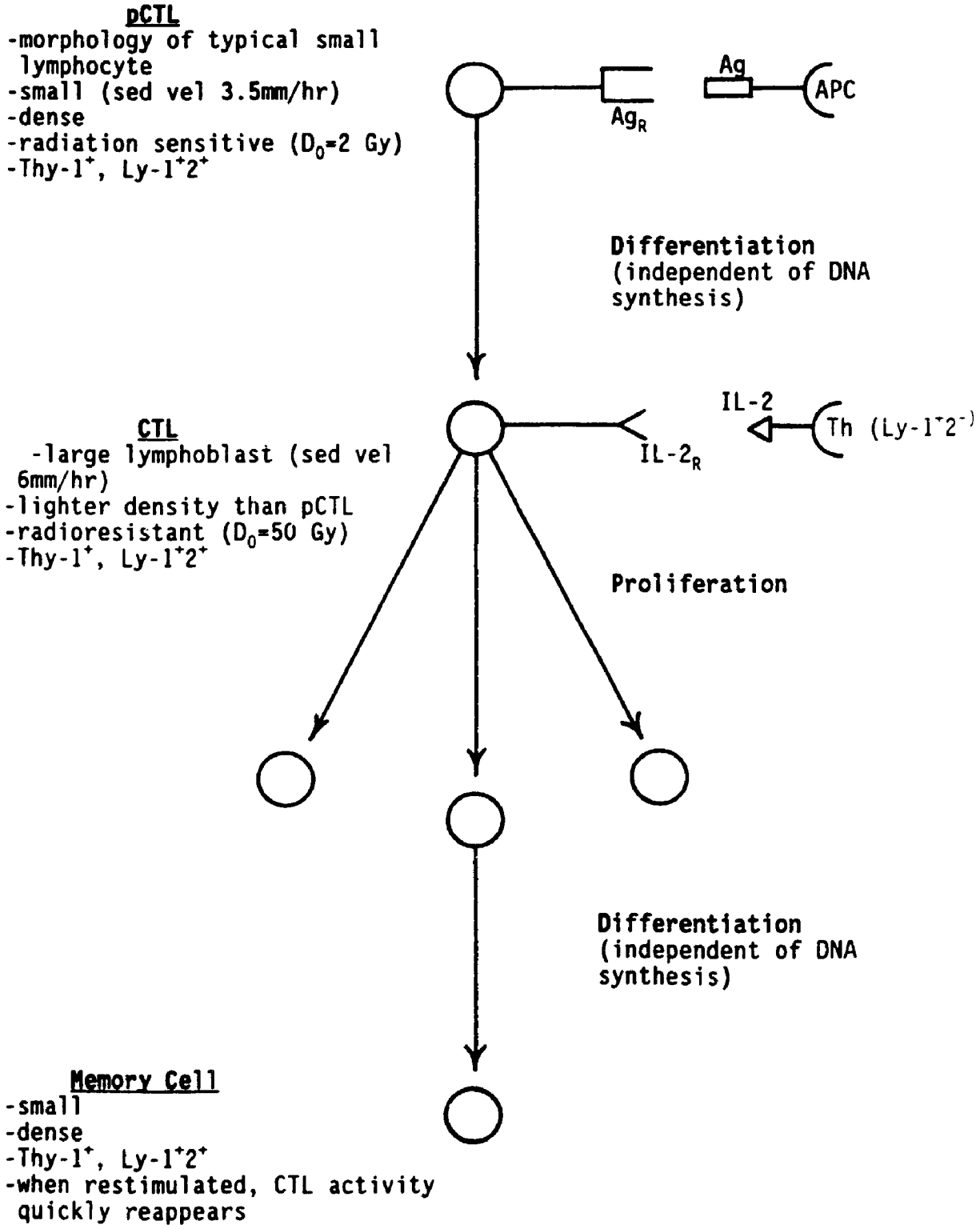
d) Activation

It has long been recognized that pCTL's undergo physical changes in the development of functional CTL's (231). These changes are presented in figure I-2 (based on 153,231). Acuto and Reinherz (1) have outlined 5 stages that T cells go through during activation. These stages appear to be similar whether the response is to allografts or to syngeneic tumour antigens.

Stage 1. On the resting pCTL, a mature and fully competent T cell, there are few or no IL-2 receptors and the cell has its maximum number of Ag receptors (1). The pCTL is measured operationally by its ability to differentiate into active CTL's capable of lysing appropriate targets: hence the term pCTL. They are small, dense, highly radiosensitive and have the morphology of typical small lymphocytes. Surface antigens include Thy-1, Ly-1 and Ly-2 (153).

Stage 2. The second stage occurs when antigen, in association with appropriate MHC molecules, on the surface of APC's such as macrophages, dendritic cells or B cells, is presented to T cells (9,52,125). It is not necessary for responder cells to "see" tumour cells themselves,

Figure I-2 T Cell Activation



since tumour antigens on the surface of APC's can at least induce proliferative responses in Ly-1$^+$2$^-$ (probably Th) T lymphocytes. Ts induction may occur as a result of released tumour antigen that is not processed by I-A$^+$ APC's (52). It has been demonstrated however, that APC's are not required for Ag stimulation of pCTL's. Allogeneic stimulator cells are a sufficient Ag source (63,64).

Stage 3. The number of surface Ag receptors then decreases and the expression of surface IL-2 receptors is induced. Antigen stimulation has been shown to regulate the expression of high-affinity IL-2 receptors in a cytolytic T lymphocyte clone (149). These receptors are maintained for several days.

Stage 4. At the same time, either IL-2 is produced endogenously, secreted and bound (1), or IL-2, produced by Ly-1$^+$2$^-$ Th cells, is then bound to the Il-2 receptors.

Stage 5. Once sufficient IL-2 - IL-2-receptor complexes have formed (and been processed?), then DNA synthesis and cell division occurs (1). CTL's, after being stimulated by Ag, can undergo at least 6 doublings in response to IL-2 (149). It is not necessary for Ag to be present during this proliferation period.

Finally, without continued antigen stimulation, the antigen receptor complex is reexpressed and IL-2 receptors are reduced in number and the cell reverts to a resting state (1,149). The cell will not then respond to IL-2 alone.

One hypothesis of T cell activation states that activation of pCTL's to mature, functional CTL's requires only two signals - specific antigen and IL-2. Antigen stimulates the surface expression of IL-2 receptors and then subsequent growth is dependent only on the presence

of IL-2 (149). This model would support the idea that antigen-activated cells are already fully functional, even in the absence of DNA synthesis and cell division (153). Also, since expression of IL-2 receptors (and subsequent division in response to the presence of IL-2) would be dependent on a particular antigen, immunological specificity would be maintained (1).

Experiments have shown that the cycle from pCTL to CTL to "memory" pCTL to CTL etc. can be repeated, perhaps indefinitely. Therefore, the quantitative differences between primary and secondary (after a second stimulation) generation of CTL's in MLC's may be due simply to differences in pCTL frequencies (153).

With purified Ly-2$^+$ mouse spleen cells, neither differentiation factors nor accessory cells are required for complete allogeneic CTL activation (63,64). Therefore APC's are not a requirement. This contrasts with the activation of Th cells which appear to require interaction with Ia$^+$ accessory cells.

It has been shown that even when Th cells are activated to an IL-2 receptor positive stage, most must be further (a second time) stimulated with the original antigen before they are able to secrete IL-2 (114,165). When they reach the IL-2 receptor-positive stage, they can proliferate in response to IL-2 alone but cannot produce IL-2 themselves, until a second Ag-dependent stimulation occurs.

In contrast to the two signal hypothesis, others have suggested that at least three signals are required for induction of CTL's from resting precursors (112), Hardt et al. (95) hypothesized the interaction of RIF (IL-2 - Receptor Inducing Factor) and IL-2 to produce clonal growth followed by CTDF (Cytotoxic I cell Differentiation Factor)

to produce CTL differentiation. However, the evidence indicating only two signals (Ag and IL-2) are required for T cell activation of purified Ly-2$^+$ spleen cells appears convincing (63,64).

e) Markers - Ag determinants
- Ly determinants (and comparable human markers)
- T cell receptors
- MHC products and restriction

There is a large variety of marker molecules found on the surface of T cells (31,106,213,225). Figure I-3 lists some of the better studied ones. They are generally glycoproteins that are exposed on the surface of cells. Since MoAb's have been raised to most of them, subclasses of T cells may be identified and selected, either for or against, by the use of antibodies directed against these various markers. For most, there are equivalent human and mouse determinants. Some of there markers are involved in the sensitization and response of T cells to either a "foreign" transplanted cell or to antigens, which may be involved in self recognition and cooperation (or suppression), on a syngeneic cell.

T cells generally recognize only Ag's present on surfaces of other cells in the context of cell-surface molecules encoded by MHC genes (134). Since T cells have dual specificity for Ag and allele-specific determinants of MHC molecules, antigen recognition is considered to be "MHC restricted". The mechanism of this dual specificity (for both Ag and MHC encoded molecule) is not known (134). Two possibilities are that Ag and MHC molecules are recognized separately by two different

Figure I-3 List Of T Cell Markers

Mouse	Human
Thy-1 -pan T cell marker	**T11** (=CD2=Leu5) -pan T cell marker -SRBC receptor
Ly-1 (=CD5) -all T cells (variable) -formerly considered to be a marker for Th as well as some precursor populations	**T1** (=CD5=Leu1) -all T cells (variable)
-cells respond to Ag in association with Class II MHC molecules	
Ly-2 (=CD8) **Ly-3** -marker for $Tc_{/}s$ (cytotoxic/suppressor) cell -"sees" antigen in association with Class I MHC molecules	**T8** (=CD8=Leu2a) **Leu2b**
L3T4 (=CD4) -marker for Th -"sees" antigen in association with Class II MHC (Ia) molecules	**T4** (=CD4=Leu3a)
TCR -T cell receptor -encodes antigen specificity and MHC restriction -a polymorphic heterodimer of two chains: either α/β(TCR2) or γ/δ(TCR1)	**TCR**
CD3 -always found associated with TCR -a complex of at least 4 non-polymorphic glycoproteins	**CD3**(=T3=Leu4)
IL-2 Receptor -receptor for IL-2	**Tac** (=CD25) -receptor for IL-2

note: CD = cluster designation

receptors or that one receptor recognizes both Ag and MHC product together. This latter explanation has become more widely accepted.

It is known that a number of accessory molecules play important roles in T cell antigen recognition and activation (134). As well, the phenotype of T cell subsets, such as those cytotoxic for syngeneic tumours, have been studied in a number of laboratories. Shiku et al. (227), by treating immune mouse lymphocytes with antibodies against specific markers, showed that cytotoxic cells were Thy-1$^+$ (therefore T cells), Ly-1$^+$ and Ly-2/3$^+$.

Many of these markers are considered to be members of the Immunoglobulin Gene Superfamily. A gene superfamily has been described as a set of multigene families and single copy genes related by sequence (134). Although this relationship implies a common ancestry, members of a gene superfamily are not necessarily related in function.

Thy-1. Thy-1 in mice and T11 in man (the srbc receptor) are both pan - T cell markers, found on all peripheral T cells. These two markers have no structural homology. Thy-1, a 25 kD glycoprotein, has two allelic forms (Thy-1.1 and Thy-1.2) (125). Thy-1 (or molecules closely associated with it), may function in T lymphocyte triggering (155).

Ly-1 (CD5). Earlier research seemed to show that the Ly-1 marker (originally called Lyt-1) could be used to divide T cells into helpers (Th) and cytotoxic or suppressor cells (Tc/Ts) since Ly-1 could be demonstrated on Th cells but not on Tc cells. It is now recognized, however, that all mouse T cells (including thymocytes) express Ly-1 and all human T cells express T1 (31,139,158). Since the marker is also present on some antigen presenting B lymphocytes, it cannot be

considered a T-lineage specific marker (31,125). Ly-1 is present at relatively low levels on Tc and Ts cells and this may be the reason why it was not detected in some experiments (125,139).

Ly-2/3 (CD8). The Ly-2/3 and T8 molecules have been used to distinguish T cytotoxic/suppressor cells (Ly-2/3$^+$) from T helper cells (Ly-2/3$^-$). In combination with another marker specific for Th cells (L3T4 = CD4), mouse peripheral T cells can be divided into:

Tc - Ly-2$^+$, L3T4$^-$ and

Th - Ly-2$^-$, L3T4$^+$ (31,115,200).

It is now understood that Ly-2/3$^+$ and T8$^+$ cells recognize Ag in association with Class I products of the MHC (4,60,158,167,283). Mouse CTL's, which normally recognize Class I MHC antigens, are predominately Ly-2$^+$. The CD8 structure recognizes non-polymorphic parts of Class I MHC determinants and probably serves to increase the binding or probability of interaction between the TCR and the Ag/MHC complex (60).

Experiments suggest that Ly-2/3 and T8 (and T4) may be important for cytotoxic function (1,180,283). A correlation between Ag density on target cells and Ly-2/3 function has been found suggesting that the role of the molecule may be to strengthen the number of binding sites and to facilitate interactions that lead to lysis (152,229).

L3T4 (CD4). Helper/inducer T cells with the L3T4 (mouse) or T4 (man) markers recognize Ag in association with Class II MHC products (4,60,158,167,213,283). T4 doesn't appear to be necessary for T cell activation and may act to stabilize cell-cell contacts necessary for efficient cytolysis (1). By binding to invariant parts of MHC Class II molecules, it may increase the avidity of the T cell for its target (60). T4$^+$ clones which are not helper/inducer cells but rather are

cytotoxic, are directed at MHC Class II Ag's.

IL-2 Receptor (CD25). Receptors for interleukin-2 are found on activated T cells (hence the name Tac for human IL-2 receptor), as well as on B cells and macrophages.

TCR (T cell Antigen Receptor). The T cell receptor (Ti in humans) is a disulfide-linked polymorphic heterodimer that encodes antigen specificity and MHC restriction. All of the Ag receptors on a particular T cell are identical. Each of the two subunits has variable portions that confer specificity as well as portions that are constant between T cells (1,44,134,167,182). The two chains are either α/β (TCR2) or γ/δ (TCR1). Most mature T cells have the α/β proteins (109,196). If they are also $CD8^+CD4^-$ they recognize Ag in conjunction with MHC Class I determinants. If $CD4^+CD8^-$, they recognize Ag together with MHC Class II (Ia) molecules (156,289).

$TCR1^+$ $CD4^-CD8^-$ cells are found in the thymus and periphery (196) and may be mainly specific for Class I MHC products not normally found on most somatic cells (109). These TCR1 bearing cells predominate in the epithelia, but represent only 1 to 2% of the cells found in the lymphoid organs (109), Both $TCR1^+$ T cells and NK cells are responsive to IL-2 and activation of both types occurs under similar conditions (285).

The T cell Ag receptor is called a clonotypic structure since it is specific and distinct for different cell clones (283). Estimates of T cell receptor diversity indicates a possibility of about 10^7 (134) to 10^9 (163) different T cell Ag receptors.

CD3 (T3). CD3 (T3) is a pan T cell marker associated with the T cell receptor (283). It is a complex of at least four non-polymorphic

glycoproteins (81). It may be important in the signal transduction for T cell activation. Cross-linking the TCR/CD3 complex leads to T cell activation (289). Possibly MHC/Ag can cross-link the TCR/CD3 complex with either CD4 or CD8 to activate T cells (60). Thus a "quaternary complex" of MHC Class I or II, Ag, TCR/CD3, and CD4 or CD8 would be formed. Alternatively CD4 and CD8 might simply bind to invariant MHC portions to increase the binding of the T cell and target cell (60).

MHC Products And Restriction. In order for a T cell (receptor) to interact effectively with an antigen, that antigen must be presented together with a product of the major histocompatibility complex (MHC). An individual T cell has a single specificity. Since there is a requirement for the dual recognition of Ag and a MHC product, T cells are said to be MHC restricted. The MHC gene complex produces a number of peptides, divided into classes, which are associated with the cell surface.

Generally, Tc cells respond to antigens "seen" in association with Class I molecules while Th cells "see" antigen in conjunction with Class II molecules (113). Thus the activity of Tc cells is restricted by the Class I MHC antigens on the cells with which they interact. Likewise the interaction between Th and APC's is MHC Class II restricted.

Although Tc cells were generally thought to be Ly-1^-2^+ while Th cells were thought to be $Ly1^+2^-$, it is now recognized that the Ly surface phenotype correlates with the class of MHC molecule recognized and not with the function of the cell (83,113,255). That is, T cells (Tc or Th) which are restricted to Class I molecules are Ly-2^+ while those (Tc or Th) restricted to class II molecules, are Ly-2^-. Th cells which are $Ly2^+$

have been shown to generate helper activity against Class I Ag's while CTL's directed against Class II Ag's are Ly-2$^-$ (83,113).

There are non-MHC antigens that can also serve as part of whatever is "seen" by T cells. These include minor histocompatibility antigens, foreign proteins, haptens coupled to cell-surface components, certain tumour antigens, and antigens expressed on the surface of virally infected cells (113). The mechanism of MHC restriction is not known, but it is probably by selection and not learning (83).

The MHC is a group of genes that codes for a large number of glycoproteins associated with the cell surface. In mice, the complex is called H-2 (H for histocompatibility antigen) and in humans it is called HLA (for human leukocyte antigen) (167). In the mouse, over thirty histocompatibility genes have been designated H-1, H-2,---etc. (113,125,167). The MHC genes code for three classes of products of which Classes I and II are major histocompatibility antigens while Class III is not.

Class I MHC molecules, together with foreign Ag, are recognized by CTL's. They are the principal targets during graft rejection; targets of both CTL's and Ab (83,182). They act as cell surface recognition molecules which can be identified by the cytotoxic T cells. Note that CTL's can recognize allogeneic MHC molecules without the need for self MHC recognition.

The control of tumour growth in syngeneic mice may depend on the expression of Class I MHC antigens as well as tumour antigens on the tumour cells (284). With a murine fibrosarcoma line that lacks MHC H-2K antigens, it was found that when these antigens are expressed de novo, tumourgenicity is reduced and metastases do not occur.

MHC Class II determinants are integral membrane glycoproteins found on some (B cells, some activated T cells, APC's) of the cells actively involved in the immune system (167). In conjunction with antigen, Class II molecules are required for activation of Th cells (83). Helper and inducer T cells are normally restricted by MHC Ag's of Class II (182). Since Th cells recognize Ag on macrophages and B cells in association with H-2I antigens, these markers act as recognition signals (or restriction elements) between APC's and the responding lymphocytes (66,213).

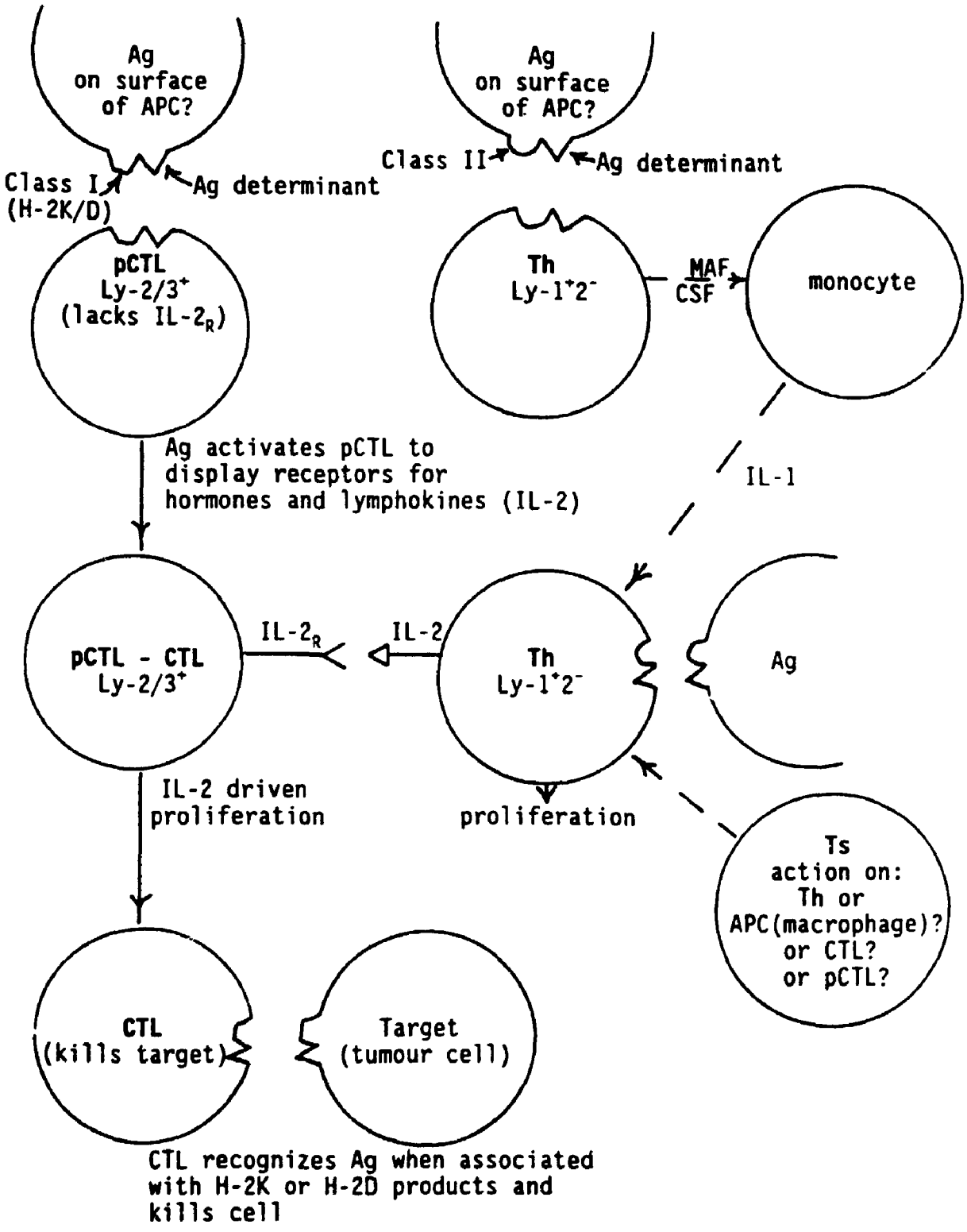
I-3-3-2: a) T Cell Subclasses - Interactions

The production of T cells capable of killing tumour cell targets involves the interaction of Tc, Th, Ts, APC's and tumour antigens (figure I-4; based on 100,213). APC's (ie macrophages, Langerhans cells in the skin, B cells) present antigen, in association with MHC products, to lymphoid cells. This presentation can occur in lymph nodes draining a tumour site.

APC's, expressing MHC Class II molecules, internalize and process Ag prior to its presentation to T lymphocytes. They also secrete growth-differentiation molecules, such as IL-1, which activate T cells. IL-1, produced by macrophages, is an important component (although it may not be essential), in the activation of T cells during Ag presentation. It induces IL-2 receptors and stimulates IL-2 production (268).

Tc. Cytotoxic T lymphocytes, responsible for killing tumour cell

Figure I-4 T Cell Subclasses: Interactions



targets, recognize a complex composed of an antigenic determinant together with Class I MHC molecules (167,268). CTL's are necessary for the regression of a murine sarcoma virus induced tumour growing in B6 mice (201). Prior to activation, pre-cytotoxic T lymphocytes (pCTL's) are small to medium size. Cells capable of lysing tumour target cells have been shown to be large and blast-like (201). In response to Con A and in the presence of IL-2, an Ly-2$^+$ cell can proliferate to produce a clone size of several hundred cells (135).

Although Tc's are responsible for the lysis of the tumour cell target, they are aided in their development by T helper cells. The optimal generation of Ly-2$^+$ CTL's requires the presence of L3T4$^+$ Th cells (or soluble helper factors). During the generation of Ly-2$^+$ CTL's to a syngeneic tumour expressing only Class I MHC products, Ia$^+$ accessory cells are required to process Ag and present it to Class II restricted Th's (121). (The addition of exogenous helper factors can replace the requirement for the Ia$^+$ macrophage). Although for primed T cells, triggering seems to depend only on the cross-linking of the TCR molecule, for normal resting T cells, there appears to be a requirement for viable APC's (249). It appears that many cell types (both Ia$^+$ and Ia$^-$) can act as APC's for CD8$^+$ cells, at least in allogeneic responses. The Ia$^-$ P815 tumour line can present antigen effectively to stimulate division of Class I different CD8$^+$ cells (249). Therefore, the production of CTL's to a syngeneic tumour can include the cellular cooperation of up to three different cell types (as well as require the presence of the tumour). However soluble factors can replace the need for Th's and perhaps APC's.

Th. Helper T cells produce lymphokines which act to recruit and

activate T and B lymphocytes, NK cells and macrophages (9,268). These lymphokines include: IL-2 (TCGF) which induces proliferation of activated CTL's (213) as well as acting on B and NK cells; IFNgamma which acts on macrophages and also increases NK cell activity (195); IL-4, which, in addition to having many B cell effects, is a T cell growth factor (177,195,233,256) and also acts to increase MHC Class II (Ia) expression on macrophages and B cells - thereby enhancing their ability to present Ag to T cells (177,195,256); IL-5, a factor in T cell differentiation (256) and possibly playing a role in induction of cytotoxic T lymphocytes (195,233); and IL-6 - involved in the development of immature thymocytes (295). These factors all tend to increase or help an immune response. Th cells can also stimulate the differentiation of Ts cells which can in turn suppress the production of chemical mediators by Th cells (9).

Th's produce factors which serve to attract, immobilize and activate macrophages at the site of an immune response, thus forming part of an inflammatory reaction. Macrophages have surface receptors for Th produced lymphokines and when activated, become highly tumouricidal (268).

Th cells play a role in the activation of pCTL's to become active killer cells. There is strong evidence that IL-2, produced by Th cells, is the lymphokine responsible for CTL helper activity (100). Il-2 is a potent stimulator of T cell proliferation. Various experiments suggest that IL-2 is the mediator of T cell proliferation found in MLC and ConA supernatants (100). T helper cells can often be replaced by SN2°MLC or by IL-2 (63,64).

The APC's serve as substrate for the recognition of Ag by Th cells

(268). The Ag is presented together with Class II MHC molecules and it is in this context that Th cells recognize Ag (6,47,268). Class II restricted T helper cells appear to require interaction with Ia^+ antigen presenting accessory cells (63,64), especially if the response is to a syngeneic tumour expressing only Class I MHC products (121). B cells, as well as macrophages, can present Ag to Th cells. Studies have shown that most clones of Th cells respond equally well to activated B cells or macrophages when these act as APC's (47). The differentiation state of B cells is important in their ability to present antigen effectively.

Recent studies with cloned Th cell lines show that these cells can be divided according to factors produced and to expressed receptors (18,177). Th1/inflammatory cells, probably responsible for delayed type hypersensitivity (dth) reactions, produce IL-2 and IFN-γ as well as other factors. Th2/helper cells, important in B cell functions, produce interleukins 4 and 5 but not IL-2 or IFN-γ.

Ts. It has been recognized that tumour bearing animals can have cells that negatively regulate or inhibit the immune response to a tumour. These may be T suppressor cells (Ts) and may be an important reason why an apparently immunocompetent animal fails to mount an effective immune response to an antigenic tumour (192).

Ts cells have been found in the spleens of mice with progressing tumours (72) and in the blood of humans with various malignancies (278). They may be tumour specific (70,72,234) or nonspecific (278). Other types of cells (including macrophages, B lymphocytes, and null cells) may also act to suppress the immune response to cancer (209,214,234,274,278).

Levy et al., using the P815/DBA/2 model, have shown that suppressor

cells occur in the spleens and thymuses of mice during the late stages of tumour growth (257,258). These suppressor T cells (Thy-1$^+$) are specific for P815 tumour cells (259). In other tumour models, they found non-specific macrophage suppressor cells in the spleens of tumour bearers (203).

Several ways in which Ts cells could act have been suggested by Simon et al. (237): - elimination or inactivation of stimulator (helper) cells, - inactivation of pCTL's, - inhibition of clonal expansion of activated pCTL's by depletion of lymphokines such as TCGF.

Levy noted that the effect of the suppressor cells in the P815/DBA/2 system might be on the helper cell (144). Others have shown that the Ts produced later in the immune response acts by blocking the Th pathway (167).

Sinclair et al. have shown, in an allogeneic cell mediated response, that suppressor cells act primarily through an anti-responder cell effect (238,240). Frost et al. (70) found that the suppressor T cells in their syngeneic tumour model system appeared to impair the precursor rather than mature cytotoxic cells. MacDonald (201) also showed that while suppressor T cells were not inhibitory to effector cells in a ^{51}Cr release assay, they did block generation of CTL's. Conversely, another study has shown that large Ts cells can suppress the activity of small cytotoxic cells during the effector phase of CMI (219).

Dye and North have demonstrated the presence of Ts cells in tumour bearing mice. In order to show adoptive T cell-mediated regression of established tumours, it was necessary to use tumour bearing recipients that had been made T cell deficient by thymectomy and gamma irradiation

(56). This T cell inhibition was necessary to deplete the animal of Ts cells which would otherwise act to suppress the anti-tumour response. They showed that these mice have a tumour induced state of T cell mediated immunosuppression at day fifteen in animals with progressively growing tumours. The results indicate that the suppressor cells inhibit the replication of passively transferred immune cells. They also showed that it was possible to inhibit T cell mediated (adoptive) regression of tumours by prior infusion of splenic T cells from T cell intact, tumour bearing donors.

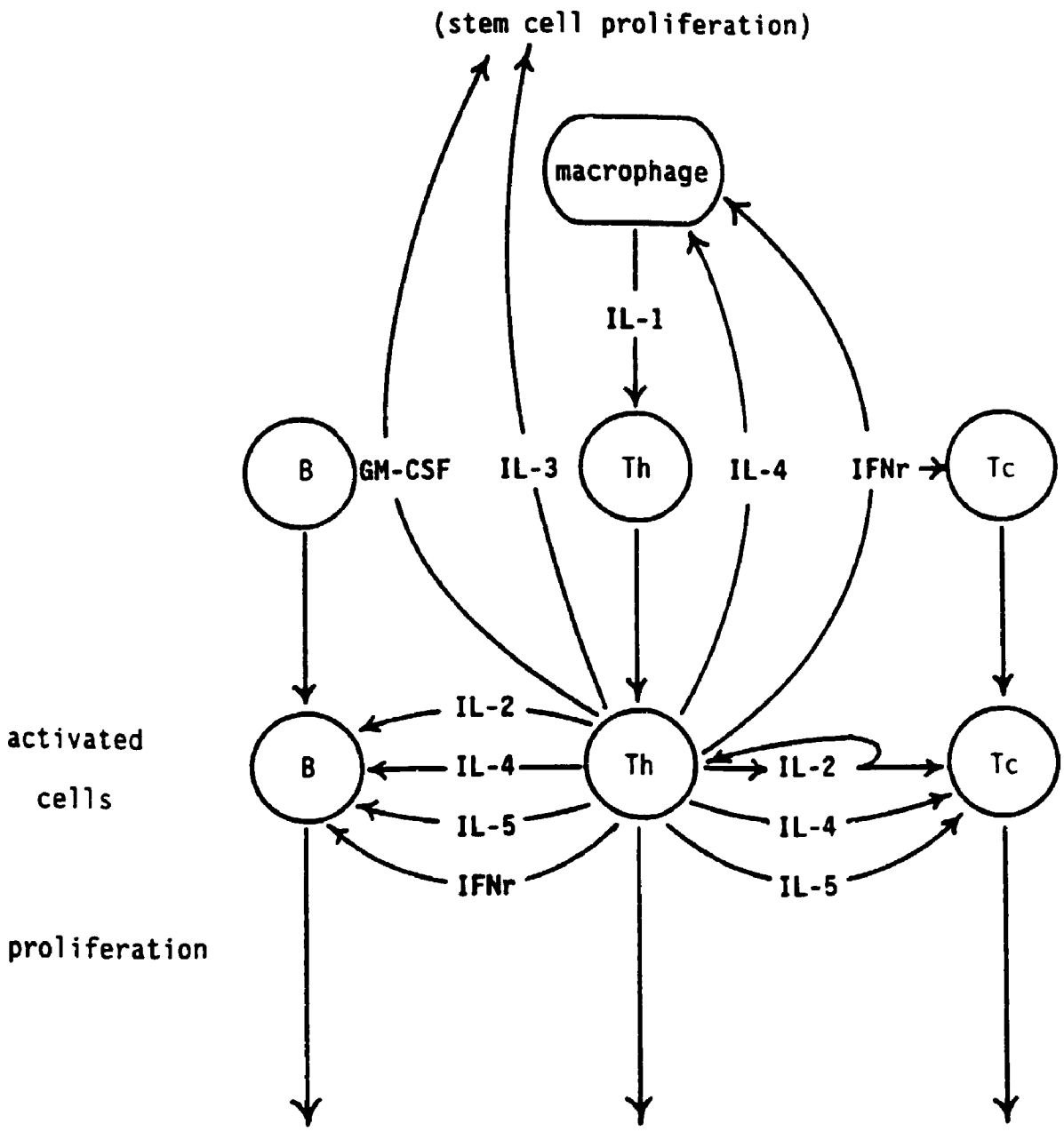
Because Ts and Tc cells share several characteristics (i.e. expression of Ly-2/3), some researchers feel that a clear separation of Ts cells from Tc cells has not been achieved (212,237). Suppressor cells may serve to regulate unwanted clones of lymphoid cells in autoimmunity or leukemia (67).

Ts cell induction may occur as a result of released tumour antigen that is not processed by I-A$^+$ APC's (52).

b) Lymphokines

Macrophages process Ag and present it to T cells in conjunction with MHC molecules (MHC restriction). Th cells, after activation by specific antigen and stimulation with interleukin-1 (IL-1; produced by macrophages), make and secrete interleukin-2 (IL-2) (176,231), (figure I-5; based on 76,195). The term interleukin is used because these substances are produced by, and act on, mainly lymphocytes (50). (However note that IL-1 is produced by, and IL-4 acts on, macrophages).

Figure I-5 Some Lymphokines Produced By Th Cells



Concerning Tc cells, the specific, MHC restricted, binding of Ag to T cells is the first signal for T cell activation and results in IL-2 receptor expression. The so-called second signal for T cell proliferation is the binding of IL-2 to IL-2 receptors on an antigen-activated T cell. The resulting progeny are antigen specific and, with IL-2 receptors, will proliferate if IL-2 is provided.

Interleukins 4 through 7 have many and varied effects on B cells, as well as some effects on T cells (178,195,256). Lymphokines are produced by, and act on, different cells types and are not confined to single functions (195).

IL-1. IL-1, a hydrophobic polypeptide (MW = 15kD), is produced primarily by activated macrophages (50,76) as well as by other cell types (50,195) including keratinocytes (85). Originally called Leukocyte Activating Factor (LAF), it was first described by Gery in 1971 (194). It may actually represent a family of polypeptides (50).

The key role of IL-1 appears to be in stimulating or triggering the production and release of IL-2 by Ag reactive Th lymphocytes (76,85,194,209). However it does not appear to be necessary or obligatory for the release of IL-2 (209). As well as being involved in B cell responses (76) and increasing NK activity (195), it acts as a chemoattractant for neutrophils, monocytes, B, and T cells <u>in vitro</u> (50). IL-1 appears to account for the generally observed requirement for macrophages in a variety of immunological assays (108) and its administration may be able to overcome selective deficiencies in APC function (85).

IL-2. Interleukin-2 (IL-2) or T cell growth factor (TCGF) was first described by Morgan et al. in 1976 (176). It is a hydrophobic

glycoprotein with a MW of about 15 kD (50,76,86,108). It readily forms dimers and mouse IL-2 has an apparent MW of 30 kD on gel filtration (76,86,198). Other terms for IL-2 or TCGF include: thymocyte mitogenic factor, thymocyte stimulating factor, co-stimulator, killer cell helper factor, and secondary cytotoxic T cell inducing factor (108,197), T cell maturation/stimulating factor, and TRF or T-cell replacing factor (195). IL-2, produced by activated T lymphocytes (76), is found in medium conditioned by T cell mitogen-stimulated lymphocytes (79). It is now recognized that Th cells can be divided into Th1/inflammatory and Th2/helper cells (18,177). IL-2 is produced by Th1 and not by Th2 cells (18,177,195).

IL-2 is a growth factor for T lymphocytes with helper, suppressor or cytotoxic function (50,76,85,209), particularly those expressing high-affinity receptors (50,86,176). Proliferation and generation of CTL activity by lectin-stimulated Ly-2$^+$ cells has been shown to be IL-2 dose-dependent (63). Marginal CTL responses against syngeneic tumours may be limited not by antigen presentation but by an insufficient IL-2 signal (174). IL-2 has been presented as being the only molecule capable of promoting DNA duplication and cell division in T cells (245). When IL-2 is removed from cultures of proliferating human T lymphocytes, the cells enter a non-dividing (G_0) cell cycle phase (13). It is also a growth factor for B lymphocytes (76,150,195). High affinity IL-2 receptors are expressed on activated B cells and IL-2 may promote B cell proliferation and differentiation and may induce immunoglobulin secretion and increase IL-2 receptor expression (86).

IL-2 enhances the tumouricidal activity of T cells and LGL's (50) and is required for long term culture of NK cells (108). It also

appears to serve as a lysis inducing signal (287). It potentiates the release of important lymphokines such as IFNgamma (85,86,176) and B cell growth factor (BCGF; 76).

Gilles (79) has developed a test for TCGF based on tritiated thymidine incorporation by a continuous murine tumour-specific cytotoxic T cell line (CTLL). In this line, DNA replication (measured by tritiated thymidine uptake) and cell division relies on the presence of IL-2.

IL-2 Receptors (Tac in Humans). On activated T cells, IL-2 binds to specific membrane receptors that are not present on resting cells and which appear soon after T cell activation (50,86). The sustained proliferation of T cells <u>in vitro</u> depends on the presence of membrane IL-2 receptors as well as IL-2 availability (50). There are high and low affinity IL-2 receptors (86,150). The high affinity receptors appear to mediate growth in response to IL-2. The high affinity receptors are composed of a 55 kD α chain (which binds anti-Tac Ab) together with a 75 kD ß chain (246,247). Both the α and ß chains can, by themselves, bind IL-2 at a lower affinity.

IL-3. IL-3, a glycoprotein, is a Th cell ($ly\text{-}1^+2^-$) (both Th1 and Th2; (177)) product which acts as a growth factor for hematopoietic cells of multiple lineages (76,195,211). It can induce colony formation of stem cells (76). It is involved in the regulation of early T cell differentiation (85,108) and may be responsible for a broad range of factors and activities, mostly involving colony stimulation or blast formation of immature pluripotent cells (76).

It may be required for the growth of certain non-lymphoid cell lines (85). It can support the growth and differentiation of progenitor

cells for the monocyte, granulocyte, erythrocyte and megakaryocyte lineages and also sustain growth of pluripotent precursor cells (195,211). It is important to note that these effects may be mediated by secondary cells responding to IL-3.

IL-4. Produced by activated Th2 cells, IL-4 is a growth factor for T cells (177,195,233,256), and induces cytotoxic T lymphocyte activity (195). It is important in B cell functions and other names include B-cell growth factor (BCGF) and B cell stimulating factor-1 (BSF-1). The factor increases MHC Class II (Ia) expression and antigen presenting ability of mouse macrophages and B cells (177,195,256).

IL-4 can replace IL-2 as the only factor necessary in the growth and differentiation of cytotoxic precursors into effector cells (256). It can generate LAK activity in mouse splenocytes as well as increase LAK activity in mouse cells co-cultured with IL-2 (178). In contrast, IL-4 prevents IL-2 activation of human LAK precursors; but once the cells are activated by IL-2, IL-4 increases LAK activity (116).

IL-5. IL-5 is produced by Th2 cells and, in addition to having many effects on B cells, plays a role in T cell differentiation (195,256). It is involved in the induction of cytotoxic T lymphocytes, possibly through the enhancement of IL-2 receptor expression by T cells (233). Other names include T replacement factor (TRF - important in T cell dependent B cell responses) and B cell growth factor II (BCGF-II) (195,256).

IL-6. Produced by T cells as well as by other cell types, IL-6 increases the proliferation of PMA (phorbol myristate acetate) and IL-4 stimulated thymocytes (195). In combination with IFN-γ and IL-4, it appears to be a co-factor for the differentiation and development of

immature thymocytes (295). Other names include T cell activation factor (TAF) and cytolytic T cell differentiation factor (CDF).

IL-7. IL-7 is a growth factor for early lymphoid cells of both B and T cell lineages (178). It affects the proliferation of early ($CD4^-$ $CD8^-$) resting thymocytes, but not mature T cells. It may act by causing IL-2 production and IL-2 receptor expression (178).

IFN-γ. Produced by T cells (Th1) and NK cells, IFN-γ has many B cell effects (195). It also increases Ia expression of macrophages (177,195,256), thereby increasing Ag presentation capability. It may also enhance differentiation of pCTL's (256).

GM-CSF. Another factor produced by Ly-1^+2^- Th cells (Th1 and Th2; 177) and which is distinct from IL-3 is GM-CSF (177,198,211). This is a colony stimulating factor for granulocytes and macrophages.

c) Helper And Suppressor Factors

Various helper and suppressor factors which can affect the immune response to a tumour have been described. An antigen specific helper factor which enhances the <u>in vitro</u> generation of tumour specific CTL's has been isolated by Levy et al. from the spleens and thymuses of DBA/2 mice bearing P815 syngeneic mastocytoma tumours injected eight days previously (123,124). The factor enhances the level of cytotoxicity generated but not the number of precursors triggered. Other researchers have also described various helper factors which can enhance the generation of cell-mediated killing of syngeneic tumours (73,267). There are also results suggesting that clones of activated T cells can

produce more than one type of helper factor. After Con A stimulation, a T cell hybridoma produced both TCGF and GM-CSF (42,43).

As well as helper factors, Levy et al. have studied suppressor factors isolated from cells from tumour bearing mice (250,251,260). The tumour-specific factor will suppress the *in vitro* generation of syngeneic cells cytotoxic for P815 targets when it is added to culture vessels within the first thirty hours of culture. The thymuses in mice bearing small P815 tumours have high numbers of suppressor cells in this system and the factor is obtained from a sonicated cell extract. The suppressive effect was not large; the cytotoxicity measured by a ^{51}Cr release assay being reduced by about 50% (260). When injected into DBA/2J mice, the factor specifically enhanced the growth of P815 tumour cells (251). Other factors, also able to suppress an immune response to a tumour, have been described (99).

d) How Do T Cells Kill? The Interaction With Target Cells

The destruction of a target cell by a CTL can be divided into three stages (209). The first stage requires specific recognition and attachment to the target cell. Then, the target cell membrane is lethally damaged. During the third stage, lysis occurs and the presence of the cytotoxic cell is not required.

Lysis requires energy (glucose) and occurs from a single meeting and is dependent on a viable effector cell (100). Protein, RNA and DNA synthesis are not required nor do effector cells have to proliferate.

The first stage, requiring specific recognition and attachment to

the target cell, is dependent on the engagement of the T cell's Ag receptor (100). In most cases, the CTL and target cell share MHC gene products. Adhesion is a metabolically active process, inhibited by low temperatures and by metabolic inhibitors.

The second stage entails the lethal damage of the target cell membrane (209). For this, direct cell-cell contact is required (100,209). Henney and Gillis (100) have offered three possibilities for the lytic event: a) a soluble mediator, b) intercytoplasmic connections, c) an enzyme system of the effector cell membrane.

Candidates for soluble mediators include perforin, granzymes, lymphotoxin and tumour necrosis factor (TNF). Perforin, also called cytolysin and pore-forming protein (PFP), is found in storage granules in some CTL and NK cell lines tha have been cultured in IL-2 (18,299). Upon binding of the cytotoxic cell to a target cell, degranulation occurs and perforin is released into the intercellular space, monomers bind to the target cell membrane and assemble complement-like pores. The osmotic imbalance causes water to leak into the target cell, through the pores, while small ions leak out. As a result, the cell swells and dies.

Other proteases, called granzymes, may be responsible for the lytic activity of CTL's (110). The appearance of granules containing perforin and granzymes A, B and C has been correlated with the lytic activity of lymph nodes cells stimulated <u>in vitro</u> with leukagglutinin and IL-2 (75). Levels reach a maximum on day 5 of culture and decline by day 7. Since L3T4$^+$ cells contain as much granzyme A as activated Ly-2$^+$ cells, but are not cytotoxic, this material is probably not involved in the lytic event (75).

Other mechanisms, including DNA degradation, may be more important (299). There is considerable evidence against a perforin or perforin-like molecule being the primary, normal lytic method of CTL's (40,41). Suicide by the target cell may be induced by the transfer of an inducer molecule from the cytotoxic cell (82).

The third stage of lysis does not require the presence of the cytotoxic cell (209). The membrane rupture causes the cell to die. Lesions appear to arise by polymerization of monomeric precursors. These form tubular complexes which perforate the target cell membrane. There is no evidence that a target cell which has received a "hit" can recover. After delivering a lethal hit, an effector cell can then interact with other targets (11). In experiments where CTL's are nonspecifically bound to target cells by agglutinins, such as Con A or PHA, lysis will occur (100).

Podach (202) has asserted that lysis is associated with the formation, on the target cells, of lesions caused by cytoplasmic granules, composed of protein, from the CTL's. The granules are neither specific nor are they H-2 restricted. Target cell lines have been shown to vary in sensitivity to lysis by similar concentrations of granules (202).

I-3-3-3: The Demonstration That T Cells Can Be Effective Against Tumours

A large amount of research in recent years has shown that T cells can be effective against tumours; at least in some tumour model systems

and perhaps after certain procedures have been performed. Some procedures are often necessary to remove or reduce a suppressor cell population which acts to reduce, or suppress completely an effective response against a syngeneic tumour.

Small and Trainin (243) showed that in three murine tumour model systems, there were two (probably T) lymphocyte populations with conflicting activities within sensitized spleens. When tested in a Winn assay (293), a population of larger cells enhanced tumour growth while a population of smaller cells retarded . When the two cell types were used together, enhanced tumour growth occurred, indicating that the larger cells may have been suppressor cells.

Also using a Winn assay, Shimizu and Shen found that T cells (Thy-1$^+$) from immunized mice could prevent specific progressive tumour growth when injected together with tumour cells into untreated syngeneic animals (228). LeClerc and Cantor found that two types of T cells participate in mediating the response to Moloney leukemia virus / murine sarcoma virus-induced (MLV/MSV-induced) tumours (137). These tumours usually regress after an initial growth period. An Ly-2/3$^+$ T cell from "progressor" mice prevents the growth of Molony leukemia virus-induced (MLV-induced) lymphomas; a reaction directly related to CTL activity. Ly-1$^+$ T cells from "regressor" mice prevent MSV-induced tumour (sarcoma) growth in normal mice.

Another indication that T cells may be active against tumours is that they have been found infiltrating solid murine neoplasms and have been shown to be cytotoxic to those tumour cells (21,300). In MSV-induced tumours, the concentration of pCTL's on a per T cell basis is five to ten times higher than in the spleen or peripheral blood (21).

Although they did not determine which T cell subset is responsible for the effect, Ezaki and Marbrook showed that a reduction in growth of a hybridoma tumour is due to a T cell-mediated immune response (65).

T cells are responsible for the rejection of MDAY-D2 tumours (179). With this system, whole-body irradiation is required to reduce suppression of the anti-tumour response. The authors noted the likelihood that Th cells are a necessary component for tumour regression in treated mice. The effectiveness of cells used for immunotherapy did not appear to be related to the CTL activity of the cell preparation. However an important question is whether the effectiveness might be related to pCTL frequency. That is, there might be very little measurable cytolytic activity but if a large number of precursors are available and can be stimulated to produce many CTL progeny, the resulting anti-tumour response might be large and effective.

Mills and Paetkau have described experiments with the same tumour model system (DBA/2J anti-P815) used in the research for this thesis. They produced CTL's against syngeneic P815 tumour cells from DBA/2J spleen cells cultured with IL-2 (co-stimulator) and irradiated P815 cells (173,174,199).

When the stimulated cells are injected IP, two hours after ^{125}I-labelled tumour cells, killing of the tumour cells can be detected. An indication that a radiosensitive host suppressor cell may play a role in this system was that mice irradiated (6 Gy) two days before the tumour cells and CTL's were given, cleared the labelled tumour cells more rapidly.

More cytolytic activity was generated from spleen cells of P815 tumour bearing mice than from spleen cells of normal mice (174,199).

The authors noted that CTL's generated from tumour bearing mice did not lyse normal DBA mouse cells. They also found that a peak response occurred when spleen cells were used at around day twenty of tumour growth. Following that time, there was a decline in responsiveness of the cells.

These experiments indicate that lymphoid cells from cancer bearing mice can be stimulated in vitro to produce cells capable of killing the tumour cells. A change in the response to the growing tumour is indicated by the presence of a peak response when cells from mice twenty days after tumour implantation were used. The response with normal mice was minimal, followed by an increasing response to day twenty, after which the responsiveness of the cells decreased. The authors feel that responsiveness is related to pCTL frequency. However without performing an assay for pCTL frequency, such as a limiting dilution assay (LDA), it is impossible to distinguish between decreased pCTL frequency, perhaps in normal mice and after the peak at day twenty, and the increasing influence of a putative suppressor cell population. By careful analysis, employing a measure of pCTL frequency, one might be able to distinguish between these two possibilities.

North, Mills and Dye have shown that with the DBA/P815 system, T cells can be made to mount an effective response to the P815 tumour (57,58,170,171,187,193). They have also demonstrated the important role that suppressor cells play in this model.

P815 cells injected intradermally into normal animals produce progressing tumours and a suppressor cell population after fifteen days. Normal animals injected with P815 cells together with C. parvum in order to stimulate the immune response, produce tumours which grow for nine to

ten days and then regress. From this second set of animals, immune or sensitized T cells are obtained which, when injected into animals treated to abolish a potential suppressor cell population, cause tumour regression. Suppressor cells from animals with progressing tumours can abrogate this effective immunotherapy.

In another system, North has shown that Ly-2$^+$ cells generated in the spleen of a tumour bearing animal are capable, in an adoptive transfer experiment, of causing regression of an established tumour in irradiated recipients but not in normal recipients (185,186). He relates the increase in activity to a peak on day six of tumour growth to the numbers of T cells involved. If the frequency of these pCTL's is measurable in a LDA, one should be able to determine whether the frequency of pCTL's changes during the course of tumour growth, rising to a peak and then declining, or whether the pCTL frequency remains relatively constant and other factors, such as suppressor cells, might affect the overall apparent activity. This determination would depend on the CTL's also being pCTL's or on the pCTL itself being the important cell. In fact, Mills and North note the importance and benefits of using a LDA to answer questions like these in their research (171).

Another of the pieces of evidence that CTL's are required for the anti-tumour effect, is that the regression is preceded by a peak production of CTL's in the draining lymph nodes (170). These cells are cytotoxic in a ^{51}Cr release assay and are effective in vivo in a Winn assay (57).

I-3-3-4: The Radiation Sensitivity Of T Cells

Radiation can have a powerful effect on the T lymphocyte population. Yarilin et al. measured the number of Thy-1$^+$ T cells remaining two days after 20 Gy of whole body irradiation in CBA mice (297). T cells were considerably depleted with only 2-4% of the original numbers remaining in the spleen, thymus and lymph nodes. Total lymphoid irradiation (TLI), used in Hodgkin's disease treatment, has been shown to be a powerful immunosuppressive agent in patients (92).

The state of lymphocyte activation can be a significant determinant of irradiation consequences (148). Murine thymocytes and peripheral lymphocytes were tested *in vitro* in a dye exclusion assay and doses as low as 0.2 Gy caused some lymphocytes to die rapidly. More peripheral lymphocytes died at lower doses than did thymocytes. T and B lymphocytes were killed at equal rates while mitogen-activated T and B cells were not killed as quickly.

A good method of measuring the response of pCTL's to radiation is by using a LDA to measure the change in frequency of pCTL's as a function of dose. Measured in this manner, allogeneic pCTL's have shown a Do of 1.65 Gy (254), while non specific pCTL's yielded a Do of 1.20 Gy (266). (The Do value equals the reciprocal of the final slope of the radiation survival curve and this equals the dose required to reduce the number of surviving cells to 37%). In the first experiment, a Ly-1$^+$ Th cell population gave a Do of about 0.85 Gy; showing these helper cells to be much more radiosensitive than the pCTL's. The influence of the radiosensitivity of Th cells on the pCTL frequency determination could be removed by replacing the helper cell activity with IL-2 (254).

A similar relationship in response between Th cells and pCTL's was shown in experiments where 5.0 Gy of whole body irradiation had no effect on the increase in pCTL frequency when it was measured in a secondary response, while Th's were impaired by this radiation dose (113).

Mills et al., in experiments with P815 tumour cells and DBA/2 mice, showed that cytolytic lymphocytes are effective in killing ascites tumour cells (173). Irradiation of these CTL's with 25 Gy did not diminish the efficacy of these cells in increasing tumour clearance or survival times. Therefore, CTL's in this system are very radiation resistant.

Suppressor cell function has been removed in several tumour systems by giving the mice 4 to 6 Gy of whole body radiation (48,173,183,187). While five Gy of whole body irradiation, 24 hours prior to harvest, was enough to prevent suppression of adoptive immunotherapy to a growing murine tumour, 2.5 Gy had little effect (184). In vivo splenic irradiation, at a dose of 4.0 Gy, can eradicate suppressor T (Thy-1$^+$) cells and cause the regression and inhibition of an established tumour in up to 50% of tested animals (62).

Therefore, Th cells appear to be the most radiosensitive, followed by pCTL's and pre-Ts cells with about the same sensitivity. Fully activated CTL's appear to be very radioresistant.

I-3-4 Assay Techniques For The T Cell Response

I-3-4-1: The Development Of The ^{51}Cr Release Microcytotoxicity Assay

The release of radioactive chromium (^{51}Cr) has been used to study the killing of labelled target cells. In this procedure, target cells are incubated with radioactive ^{51}Cr and incorporate the label. If the cell is then killed, the membrane lyses and the chromium is released into the supernatant where it can be measured.

Sanderson measured Ab-mediated cytotoxicity against allogeneic lymphocytes by this method (217). He used the following expression to quantitate cytotoxicity:

$$P = \frac{(S - S_0)}{(S_M - S_0)} \times 100$$

where: P = percentage of cells lysed

S = supernatant count after lysis

S_0 = supernatant count before lysis

S_M = supernatant count in 100% lysis tube

When 100% of the cells were lysed, 70-80% of incorporated label was released to the supernatant. We also use Sanderson's method of measuring cell killing and quantitating chromium release. We express ^{51}Cr release as percent specific chromium release which is similar to his P value or percentage of cells lysed.

Brunner et al. were the first to use ^{51}Cr-labelled target cells to test for cell-mediated cytotoxicity in an immune response (22,23). They sensitized C57Bl mice with an IP injection of DBA/2 mastocytoma P815-X2

cells and studied the allogeneic response. Lymphocytes from various organs were harvested and tested for the ability to lyse ^{51}Cr-labelled P815 target cells. They showed that the system is technically simple and provides accurate, reproducible and quantitative results. It was also noted that cytotoxicity generated against syngeneic tumour cells is weak compared with the allogeneic system (23).

After studying other tumour cell systems, Brunner et al. concluded that tumour cells can vary considerably in sensitivity to lysis and are not generally more sensitive than normal cells (22,23). The P815 mastocytoma appears to be a particularly good target cell since it takes up label readily and is somewhat more sensitive to cell-mediated lysis by sensitized lymphocytes than other cell lines.

Cerottini and Brunner showed that lymphoid cells from mice immunized with allogeneic cells could destroy (in vitro) target cells bearing the alloantigens of the donor strain (33). They used ^{51}Cr-labelled target cells at various ratios of Effector / Target cells in order to produce chromium release curves. From the plot of percent lysis versus Effector / Target cell ratio, the lymphoid cell / target cell ratio necessary to obtain 33% lysis was found. The number of lymphoid cells corresponding to this ratio was arbitrarily defined as one lytic unit (or LU-33). They noted that the number of LU-33's present in 10^6 lymphoid cells or in the lymphoid organ could then be calculated. This was the first use of the chromium release curve to determine a lytic unit value; a measure of the degree of cytotoxicity of the effector cells. It allowed one to assign a single value to the killing demonstrated by a complete chromium release curve.

In a move to an in vitro sensitization, Wunderlich and Canty were

able to stimulate lymphoid cells and then to test for cytotoxicity in a ^{51}Cr release assay (296). The percent lysis measured in a three hour assay was low (about 9%) but significant.

Wagner and Feldman described another system in which lymphoid cells are sensitized by an *in vitro* stimulation by allogeneic cells and then tested by ^{51}Cr release (280). In order to maximize the response, the system was optimized for various parameters including the number of stimulator cells, culture time, and the Effector / Target cell ratio used in the lysis assay. A comparison of cells stimulated *in vivo* and *in vitro* showed almost identical cytotoxicity when expressed as the rate of lysis or as total lytic activity.

Another step in the development of the ^{51}Cr release assay system was to test it with mouse lymphocytes which had been immunized *in vitro* against syngeneic tumours (281). Using responder cells from two different mouse genotypes, a syngeneic tumour response was compared to that to an allogeneic tumour. Results were plotted as percent lysis versus log of the Effector / Target cell ratio. Response curves were obtained for each condition and compared by considering the Effector / Target cell ratio when 50% lysis was reached. By taking the ratio of these two values, the authors estimated that the response to an allogeneic tumour was about thirty times as strong as the response to a syngeneic tumour stimulation.

Although these results indicate that the allogeneic response is much stronger than the response to a syngeneic tumour, in order to make a fair comparison, the authors should have used the same responding cells. They did however, come to several important conclusions. Tumour immunity can be generated *in vitro* against syngeneic tumour cells and

measured quantitatively by a ^{51}Cr release assay. Also, there are quantitative differences between *in vitro* cytotoxicity responses directed against normal transplantation antigens and those directed against strictly tumour associated-antigens.

MacDonald et al., employing sensitized cells and a ^{51}Cr assay, plotted specific cytotoxicity against Responder / Target cell ratios and obtained sigmoid curves with a central linear portion (159). The slopes of the titration curves were shown to be independent of the cell concentration and source of the effector cells and proved to be quite reproducible in separate experiments. This was interpreted as suggesting that individual effector cells kill the target cells with the same efficiency in the different cases.

Miller and Dunkly showed that the chromium is released from lysed target cells for up to a further 1.5 hours at 37 degrees C after killing has been stopped (168). Therefore, in a short term assay, the amount of chromium released at any particular time, is an unc restimate of the actual number of target cells killed. In a long term assay (i.e. 24 hours), the relative amount of ^{51}Cr released is a truer indication of the number of target cells lysed.

With short term assays, we have shown that killing of target cells as indicated by ^{51}Cr release can be correlated with killing as indicated by the inability to form a colony *in vitro* (140,157). This is demonstrated by adding EDTA to prevent any further cell - cell contacts (and therefore killing) and allowing time for ^{51}Cr to be released from the already dying cells. The specific amount of released ^{51}Cr, measured in this manner, is closely correlated to the fraction of dead cells as measured in a clonogenic assay.

1-3-4-2: The Limiting Dilution Assay (LDA)

The Limiting Dilution Assay can be used to enumerate certain cells, including precursors of cytotoxic T lymphocytes, involved in an immune response (142,166,169). One can also use it to study the properties of single clones of specific cell types.

Under appropriate conditions, a pCTL will be stimulated to differentiate and divide to form cytotoxic lymphocytes capable of killing tumour target cells. If the number of pCTL's per culture well is such that some wells will have no pCTL's while others will have one or more; and if the presence of one or more pCTL's in a well can be detected by the production, after a culture period, of cytotoxic cells, then the distribution of positive (or negative) wells can be used to determine the pCTL frequency in the total cell population. The LDA measures the frequency of pCTL's in the Responder cell population.

There are three main requirements for a valid assay (169):

1) The number of pCTL's must be reduced, by diluting the Responder cells put into a set of culture wells, until there is no response in most of the wells.

2) Cultures without pCTL's must be distinguishable from those with one or more pCTL's. Under optimal conditions, one pCTL in a culture is sufficient to produce enough CTL's that a cytotoxic response can be detected. It has been estimated that one pCTL can produce from 2^6 to 2^{10} CTL progeny under appropriate conditions *in vitro* (135,153). By making the culture and assay conditions as optimal as possible, as described in Chapter III, an attempt is made to satisfy this second requirement.

3) Because the pCTL must be the limiting cell, it must be the least frequent required cell type in the suspension being diluted. To the Responder cell population are added either Helper spleen cells or SN2°MLC which substitutes for the Helper cells.

Theory

The theoretical basis of the LDA can be explained as follows (169). Assume we are interested in measuring the frequency of precursors of cytotoxic T lymphocytes (pCTL) which can be activated to kill a tumour cell target and that culture conditions are sufficient so that the progeny of a single pCTL can be detected. Let the frequency of pCTL's in a suspension of cells be F. Let all other cells and factors required for pCTL activation and differentiation be present at higher frequencies (i.e. in excess). Let the total number of cells be N. In the total population of N cells, only a few of them are capable of actually responding and this number is given by N X F or NF If we set up replicate cultures with various numbers of N, there are three conditions of interest:

1) If NF >> 1, (i.e. many pCTL's in each culture) then all the cultures will produce cytotoxic cells (i.e. be positive).

2) If NF << 1, then the probability of having even one pCTL in a culture will be small and very few cultures will produce CTL's.

3) If NF is approximately equal to one, then some cultures will contain one or more pCTL's and be positive while others will contain none and be negative.

The Poisson distribution allows one to predict the probability Pn of obtaining n responding cells in a culture as a function of N, the total number of cells and F - the frequency of cells capable of

responding.

$$Pn(N,F) = \frac{(NF)^n e^{-NF}}{n!}$$

Experimentally we will not attempt to distinguish between positive cultures with varying numbers of precursor cells. We will simply distinguish between positive cultures, those with one or more precursor cells, and negative cultures, those without any precursors. Following that, the fraction of non-responding cultures will be determined as a function of the total number of cells cultured.

The probability of obtaining a negative culture is obtained from the n = 0 term of the Poisson distribution.

$$P_0(N,F) = \frac{(NF)^0 e^{-NF}}{0!} = e^{-NF}$$

Assume the probability of obtaining a negative culture to be equal to the frequency of negative cultures obtained experimentally. At a frequency of non-responding cultures of 1/e, the frequency of cells capable of responding will be equal to 1/N. If we plot the log of the frequency of non-responding cultures versus the total number of cells, we should obtain a straight line and be able to determine the frequency of pCTL's in the cell population. It has been shown that obtaining a straight line "single hit curve" indicates the presence of a single limiting cell type (143,282).

Early Use Of The LDA

Groves et al. studied the immune response of mice responding to

srbc's (sheep red blood cells) as an antigen (90). Using diffusion chambers containing spleen cells and srbc's and implanted into the peritoneal cavities of irradiated mice, they performed nine experiments with a total of 231 samples and combined the results into one LDA curve. They estimated that there was one response unit per $2.25X10^6$ cells giving a frequency of 0.44 units per 10^6 cells. They called the response unit an antibody forming unit and not an antibody forming cell since it was not clear that the limiting cell was a pre-Ab forming cell.

Other studies of the immune response to heterologous red cells have produced frequencies of pre-Ab forming cells of 5.5 per 10^6 cells (141), and 16.7 per 10^6 cells (205) in different mouse systems.

Measurement Of pCTL Frequencies In Allogeneic Responses

LDA's have been used to estimate the frequency of pCTL's capable of responding to antigen on cell surfaces. These cells, when stimulated under the proper conditions, will produce cells capable of lysing target cells with antigenic determinants shared with the stimulator cells.

Skinner and Marbrook measured the frequency of cells in CBA mouse spleens capable of responding to (CBA X DBA)F1 spleen cells (242). The production of cytotoxic T lymphocytes was studied by the killing of ^{51}Cr-labelled P815 mastocytoma cells. The frequency of pCTL's to the allogenic stimulation was estimated to be 620 per 10^6 nucleated cells. In later research with the same system, Marbrook et al. found the frequency to be about 500 per 10^6 cells (162).

In studying various allogenic murine systems, Teh et al. found frequencies of 1160, 1290, and 2080 pCTL's per 10^6 cells (263.265). They also estimated the average clone size of CTL's to be 1040 after seven days of culture (263). This would indicate that about ten cell

doublings had occurred.

An early report by Miller et al. indicated that approximately 20 % of pCTL's from the lymph nodes of H-2^b mice are capable of responding to H-2^d alloantigens (169). One possible explanation for this high value is the presentation of self antigen by the foreign MHC and the subsequent activation of a large proportion of pCTL's. In a later paper, Miller reports that up to several percent of total pCTL's are activated to make CTL's specific for sensitizing antigen (166).

Measurement Of pCTL Frequencies In Responses To Syngeneic Tumours

By the use of an LDA to measure pCTL frequencies, several groups have studied the pCTL response to a syngeneic tumour. Brunner et al. have measured the precursor frequency of cytolytic T lymphocytes reactive against syngeneic Moloney sarcoma - leukemia virus complex-induced (MSV-MLV-induced) tumour cells (20,21,24,25). When a tumour homogenate containing the MSV-MLV complex is injected i.m. into a suitable mouse, a tumour, induced by the viral complex, appears and grows. It should be noted that whole, viable cells are not required for the formation of a tumour: thus this system is somewhat different from a normal syngeneic tumour model where viable, intact cells must be used. After about two weeks, the tumour regresses and spleens from these animals are called regressor spleens. The autnors note that this is a highly immunogenic tumour that always regresses spontaneously. In one study of two different mouse strains and tumour cell combinations, they found precursor frequencies of 755 (±59;SEM) and 1730(±270;SEM) per 10^6 cells (20). It was noted that the frequency of pCTL's reactive against MSV-MLV-associated Ag's in normal spleens is at least an order of magnitude lower than in regressor spleens.

In other studies, the frequency of precursors in the spleens of normal animals was compared to that in regressor animals 15 days after the implantation of the tumour homogenate (24). In normal animals the frequency was 135 per 10^6 cells and in regressor animals it was 2085 per 10^6 cells, a difference of 15 fold.

Brunner et al. were able to demonstrate different precursor frequencies in various anatomical locations of mice at the onset of tumour regression, 10 days after injection (21). They found precursor frequencies in spleens to be 1240 (±190;SEM) per 10^6 cells, in PBL to be 2320 (±420;SEM) per 10^6 cells , and in tumour infiltrates to be 12,100 (±1700;SEM) per 10^6 cells. The results suggest a selective accumulation of tumour-specific pCTL's in tumour tissue. Does this indicate an effective anti-tumour immune response? Recall that all of the tumours did regress. Therefore the results of a LDA may be useful as an indication of a potentially effective immune response to a tumour.

Several studies have dealt with the determination of the frequency of pCTL's to syngeneic tumours which will continue to grow and are not the 100% regressing type of tumour studied by Brunner et al.. In two different syngeneic tumour model systems, Teh et al. found the precursor frequency in spleen cells from normal C57Bl/6 mice to be 132 per 10^6 cells and 231 (162-329;95% confidence limits) per 10^6 cells (264).

Kilburn et al. published the results of one experiment in which they determined the pCTL frequency in the spleen cells of normal animals responding to P815 stimulator cells <u>in vitro</u> (123). The frequency was found to be 719 (462-1118;95%confidence limits) per 10^6 cells. In further experiments Hancock and Kilburn found the frequency in the same system to be 3350 pCTL (2060-5440;95% confidence limits) per 10^6 spleen

cells (93).

Uede et al. reported experiments in which T lymphocytes, isolated from transplanted tumour tissue, exhibited strong cytotoxicity against syngeneic gliosarcoma T-9 cells (267). Spleen cells from the same rats failed to show cytotoxicity and could be induced to demonstrate lytic activity only after a two day incubation in the presence of mitomycin-C-treated T-9 cells plus supernatant from spleen cells stimulated with Con-A (most likely IL-2 is the active factor). The results suggested that the spleens contained pCTL's but the authors could not explain the apparent lack of effector cells in the spleens when initially assayed with the tumour target cells.

If a LDA had been performed, perhaps this question could have been answered. Perhaps the pCTL's in the spleen are indeed CTL's as well, but they are so few that they can produce no detectable cytotoxicity. Only after the pCTL/CTL's had been expanded in culture, did lytic activity become apparent.

I-4 SPECIFIC INTRODUCTION

Using a mouse tumour model, we are studying the T cell-mediated immune response to a syngeneic tumour. Since a T cell response may participate in controlling metastases and in the response to the primary tumour in some cancers, it is important to understand this response in order to able to manipulate it and change (i.e. improve) the eventual result of the disease.

When a cytotoxic T lymphocyte precursor (pCTL) is stimulated by

the presentation of an antigen of a tumour cell, and provided with the necessary growth conditions and factors, it undergoes differentiation and division to produce many cytotoxic cells capable of killing the tumour cells. One of the important factors in the response to a tumour is the frequency of the anti-tumour pCTL's in the host.

We can use a microcytotoxicity assay, the ^{51}Cr release assay, to assess the lytic activity of cytotoxic T lymphocytes. With a limiting dilution assay (LDA), we can measure the pCTL frequency. These two assays allow us to follow and study the time-course of the T cell-mediated immune response to a syngeneic tumour throughout the course of the tumour growth.

I-4-1 Objectives

1) To develop and optimize procedures in order to be able to perform a LDA to measure pCTL frequencies.

Syngeneic immune responses are generally weaker than allogeneic ones (151) and the DBA/2-P815X model has been described as a weakly antigenic system producing a relatively poor immune response (23,270). Therefore, if one is to study the response in such a system, it is necessary to optimize conditions in order to achieve the maximum measurable response.

With optimal responses, one can perform a LDA to measure pCTL frequencies, a very important determinant of the outcome of an immune response. A LDA will enable us to follow the response of an animal as the tumour grows. If suppressor cells are present in animals with

tumours, the LDA should help us to separate the effects of pCTL frequency, CTL production and suppressor cells. Mills and Paetkau, using the same tumour model, have postulated that the responsiveness of lymphoid cells taken from tumour bearing mice is related to pCTL frequency (173,174,199). Without performing an assay for pCTL frequency, it is difficult to distinguish between reduced pCTL frequency and the presence of a suppressor cell population when only overall responsiveness is considered. By careful analysis with a LDA, we should be able to distinguish between these two possibilities.

2) To obtain a time course of response during tumour growth.

In previous work in this laboratory, it was found that cells from normal animals could not be stimulated in vitro to produce cytotoxic cells, while those from tumour-bearing animals, two weeks after injection, could be. These results indicate a change as a function of tumour growth.

Others have also shown variable responses in lymphoid cells from animals as a tumour progresses (8,10,15,26,78, 138,174,191,199,298). The variable response to these tumours has generally been equated to the number of effector cells and not the precursor frequency. One question to be answered is whether the number of cells potentially capable of responding to a tumour is important to, or can be correlated with, its progress. This can be determined by using a LDA to measure pCTL numbers as the tumour progresses.

3) To determine the influence of suppressor cells on the time course of response to a tumour.

In early studies of tumour growth, when a T cell response was looked for in animal models and in human cancer patients, a significant

and consistent response was not found. This was partly due to the role of suppressor cells being poorly understood. Levy et al., using the DBA/2-P815 model, have shown that suppressor cells occur in the spleens and thymuses of mice during the late stages of tumour growth (257,258).

If one is to study the time course of response to a tumour, and if there is suppression of the response when potential cytotoxicity of T cells is measured, then the LDA can be used to distinguish between the effect of reduced precursor frequency and suppressor cell activity.

4) To determine whether a correlation exists between pCTL frequency in normal animals and, after a subsequent injection, the growth of a tumour.

Are some animals innately resistant to the growth of tumour cells? I am not aware of any studies that have been done to correlate the potential response to a tumour in a normal animal and what happens after a subsequent implantation of a tumour. By measuring pCTL frequencies in animals, and then following the growth of a tumour, this type of study can be performed in this system.

5) To determine the anatomical location of pCTL's

The spleen is a convenient (for the researcher) source of lymphocytes for experimental use. Peripheral blood, obtained by cardiac puncture, can also be a source of lymphocytes for pCTL frequency determinations. Since the thymus participates in T cell development, and lymph nodes are often involved in the immune response to a tumour, pCTL frequencies in these organs may be important.

I-4-2 The DBA/2-P815X Tumour Model

The DBA/2-P815X murine system is a model of a syngeneic tumour that has been widely studied (2,3,12,17,23,26, 53,57,58,123,144, 151,170,171,173,174, 183,187,191,193,199, 241,251,257,258,259, 260,269,270,271,286). The P815 mastocytoma tumour was induced when the skin of a DBA/2 castrated male mouse was painted with the carcinogen methylcholanthrene (53). Eleven months later, tissue from a tumour mass over the sternum was transplanted to other mice and this was the origin of the line. After three passages, an ascites line was established and the tumour can be grown as an ascites or as a solid tumour. The tumour appears to be a poorly differentiated mast cell (53).

T cells can be made to mount an effective immune response to the tumour (57,58,170,171,187,193). These authors, and others, have shown the important role that suppressor cells play in this model (173). Others have shown that suppressor T cells (Thy-1$^-$) occur in the spleens and thymuses of mice during the late stages of tumour growth (257,258,259).

The P815 mastocytoma appears to be a good target for cell-mediated cytolysis determined by ^{51}Cr release since it incorporates the label readily and is sensitive to cell-mediated lysis (22,23,239). In our laboratory, ^{51}Cr-labelled P815 cells have been used as targets in cytotoxicity assays of an allogeneic cell-mediated immune response (239). In these experiments ^{51}Cr release curves were produced by testing serial dilutions of culture equivalents of cytotoxic cells against labelled target cells.

The P815 mastocytoma has been described as being weakly antigenic

or immunogenic (2,3,12,23,270). The immune response in this system, as measured by cytotoxicity assays, is much weaker than an allogeneic response (23). The tumour does express tumour-associated antigens, as shown by two CTL clones which are directed against a tumour-associated transplantation antigen (TATA) (271). Antibodies to the tumour do not appear to be produced in the syngeneic host (12).

Another factor making the P815 cell a good target for studying the T cell mediated immune response is that it appears to be relatively resistant to lysis by NK cells (120,122). As well, DBA mouse spleen cells immunized against P815 mastocytoma kill only the mastocytoma cells and not normal cells from DBA animals (151,174). It was shown that the normal cells could serve as targets and be lysed by immunized allogeneic lymphocytes (151). Others have shown the cytotoxic cells to be tumour specific. They would lyse P815 cells but not other H-2d syngeneic tumours (P388, L5178Y) nor an allogeneic H-2b tumour (EL-4) (57).

In this laboratory, it has been shown that spleen cells from DBA/2 mice bearing the P815X tumour can be stimulated <u>in vitro</u> by mastocytoma cells to produce T cells cytotoxic to the P815X cells (241). These cytotoxic cells do not kill allogeneic AKR tumour cells or EL4 lymphomas. When cells from normal animals were tested as responders, no cytotoxicity was produced, indicating a change in responsiveness of the cells as a function of tumour implantation. As well, when an attempt was made to stimulate cells <u>in vitro</u> in the absence of tumour cells, no cytotoxicity occurred, indicating the development of lytic cells was in response to the tumour cells. When cells from tumour-bearing animals were tested directly in a ^{51}Cr release assay, no lysis of the target cells occurred.

CHAPTER II METHODS

II-1 Introduction

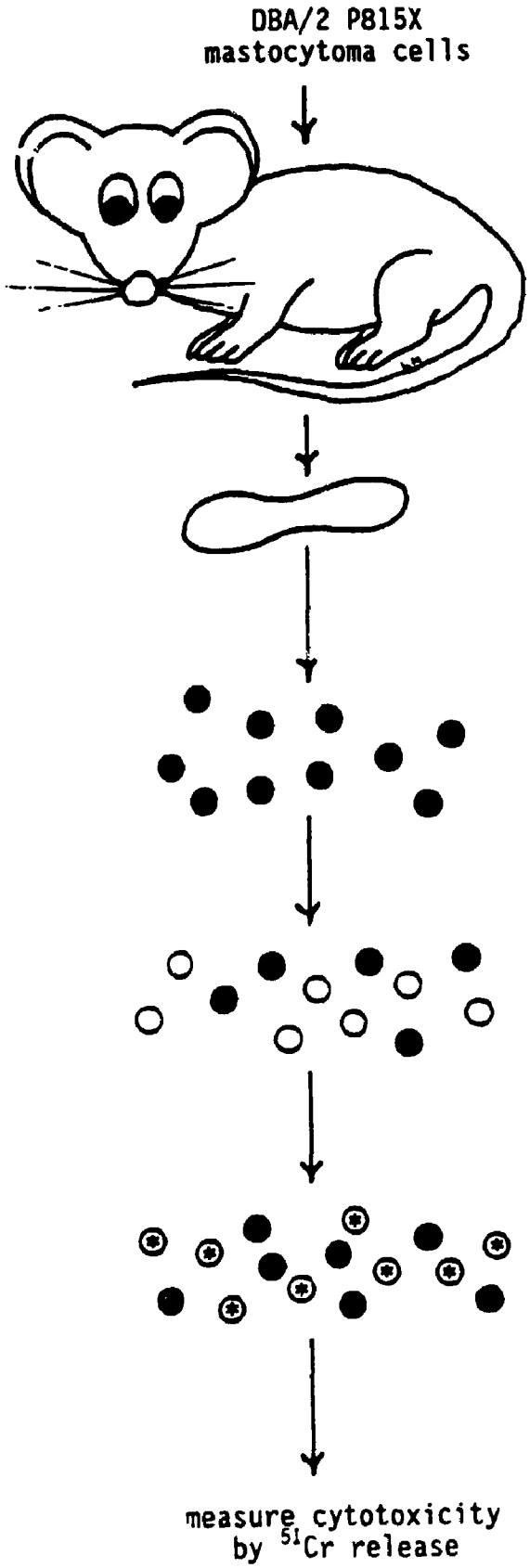
T-cell-mediated cytotoxicity against a syngeneic tumour was studied using the DBA/2-P815X murine tumour model. It has been shown in this laboratory that spleen cells from DBA/2 mice bearing the P815X tumour can be stimulated *in vitro*, by the tumour cells, to produce T cells cytotoxic to the P815X mastocytoma (241). The protocol to generate and test for the cytolytic cells is presented in figure II-1.

Live (trypan blue excluding) DBA/2 P815X mastocytoma cells were injected into DBA/2 mice, and, after a period of tumour growth, spleens were excised and a single cell suspension was made. These spleen cells (termed 'Responder' cells) were then cultured with irradiated P815X tumour cells ('Stimulators') in order to produce cells (called 'Effectors') which would then be tested in a cytotoxicity assay against ^{51}Cr-labelled P815X Target cells. If cells cytotoxic to the targets are produced, then the labelled cells are lysed and ^{51}Cr is released into the supernatant. By measuring the amount of radioactive chromium in the supernatant, we can determine whether cytolytic cells are present and also measure the lytic activity of these cells.

In the following sections, I will describe procedures for culturing and assaying cells and also methods to quantitate the pCTL frequency and the killing of tumour cells.

Chapter III will present the results of experiments done to improve the methodology of the system. Many of the details of the standard methods have been established by determining optimal conditions as

Figure II-1 **PROTOCOL**



presented in Chapter III-I.

II-2 Lymphocytes

Lymphocytes from mice were a source of Responder and Helper cells. The DBA/2 female mice used in these studies were obtained from Jackson Laboratories, Bar Harbor, Maine USA and maintained ad libitum on water and commercial food. They were six to eight weeks old when received and were housed in the University of Western Ontario animal research facility for at least two weeks before being used. When entered into the experimental protocol, animals were two to four months old. It has been reported that the P815 tumour does not show any difference in behavior when grown in mice of either sex (53).

Depending on the experiment, lymphocytes were obtained from either tumour bearing or normal animal spleens. After the animal had been killed by cervical dislocation, the spleen was removed aseptically and put into complete culture medium containing 20% FCS (fetal calf serum). The spleens were then disaggregated using a loose-fitting tissue homogenizer, and the mixture was allowed to settle for ten minutes, during which any remaining solid lumps of tissue settled out. The supernatant was decanted, and the cells washed three times (250g for 5 minutes) in complete medium to remove dead cells and debris. The cells were then counted with a hemocytometer and a microscope using Trypan blue exclusion as an indicator for viable cells. About 10^8 viable cells per spleen were usually obtained.

As another source of Responder cells, peripheral blood lymphocytes

(PBL's) were obtained by cardiac puncture from mice under ether anesthesia. In preparation for cardiac puncture, mice were swabbed with 70% ethanol and 0.1 ml of serum free medium containing 10 units per ml of heparin was drawn through the needle (BD, 1/2in, 26 gauge) into the 1.0 ml sterile disposable syringe (BD). Afterwards, the blood obtained (0.2-0.3ml) was dispersed into five ml of serum free medium containing 10 units per ml of heparin.

The diluted blood was layered onto 4 ml of a Ficoll-Hypaque preparation made by mixing 12 parts of 12% Ficoll (Ficoll 400, Pharmacia Fine Chemicals, Uppsala, Sweden) with 5 parts of 32.8% Hypaque (Hypaque sodium 50%, Winthrop Laboratories, Aurora, Ontario). A final density of 1.0871 was measured using a specific gravity bottle and analytical balance (pyknometry). The nucleated and red cells were separated by centrifugation at 700 g for 20 minutes at room temperature. Nucleated cells were collected from the medium and Ficoll-Hypaque interface, washed three times (1000 g, 20 minutes) and counted. About $4X10^6$ nucleated cells were obtained from 1 ml of blood and there were fewer red than white cells in the final suspension.

To test for antigenic determinants, populations of lymphocytes with specific determinants were lysed by incubation with the appropriate antibody plus complement. Cells, at a concentration of 10^7 per ml, were incubated at 4° C for one hour with the antibody in phosphate buffered saline according to the supplier's specifications (for anti-Ly-1.1 and anti-Ly-2.1: monoclonal antibody in ascites fluid, New England Nuclear, Boston, MA). The cells were then washed in PBS (phosphate buffered saline), a 1:12 dilution of complement (Cedarlane, M-Rabbit-Low-Tox) was added and the cells were incubated at 37°C for one hour. During the

incubation with complement, the cells with attached antibody were lysed. After being washed three times in complete medium and counted, the remaining cells were made up to appropriate concentrations.

Helper cells, a source of a factor necessary for T cell division, were obtained by treating spleen cells with anti-Ly-2 Ab plus complement in the above manner. This procedure inactivated any pre-cytotoxic T lymphocytes present in the spleen cell population.

II-3 Tumour Cells And Tumour Measurement

The P815X mastocytoma tumour cell line was originally obtained by our laboratory from Dr. R. A. Phillips, Ontario Cancer Institute, Toronto Ontario. The cells were passaged weekly as an intraperitoneal ascites tumour in DBA/2 mice. Tumour cells for propagation and experimentation were obtained by sterile aspiration from the peritoneal cavity of mice injected (i.p.) seven days previously with $2\text{-}3X10^6$ cells. Before use, the cells were washed three times in serum-free medium and counted.

Solid tumours were also a source for Stimulator and Target cells. To obtain single cell suspensions, solid tumours were disaggregated, as follows, using an enzyme cocktail (107).

After a tumour had been removed from the mouse aseptically, it was minced with scissors and forceps into pieces approximately 1 mm or less in diameter. Twenty ml of an enzyme cocktail of DNAse (0.01%; Sigma Chemical Company), Pronase (0.025%; Calbiochem - Behring Corp.) and Collagenase (0.025%; Sigma) in PBS (phosphate buffered saline) was added

to 0.1 g of the minced tumour in a fluted trypsinization flask containing a magnetic stirring bar. After the mixture had been stirred at low speed for 1/2 hour at 37°C, complete medium (RPMI-1640 containing FCS) was added to arrest the action of the enzymes. By shaking this mixture vigorously for sixty seconds, the viable cell yield was increased substantially. The suspension was then filtered through several layers of sterile cotton padding to remove undissociated cells. After three washings and counting, the cells were ready for use.

Solid tumours were produced in DBA/2 mice by injecting $2X10^6$ P815X cells in 0.1 ml of serum-free medium subcutaneously into the upper dorsal shoulder region of anaesthetized (ether) mice. After about seven days, solid tumours could be palpated at the site of injection.

Tumour growth was measured by two different methods. If the animal was killed, the tumour was excised and its mass measured. If the animal was to be kept alive, then the tumour volume was measured with calipers. Assuming the tumour to be approximated by a hemi-ellipsoid, the volume (V) was estimated as:

$$V = 0.5(4/3\pi)(L/2)(W/2)H$$

$$= 0.52L \cdot W \cdot H$$

where: L = length along the long axis

W = width orthogonal to the long axis

H = height above the normal curvature of the back

An attempt was made to allow for the skin thickness by subtracting an estimate of it from the measurements.

II-4 Culture System

After lymphocytes (as Responders) and P815X tumour cells (as Stimulators) have been combined in the culture system, the pre-cytotoxic cells in the Responder population may differentiate and divide to become cytotoxic (capable of killing the tumour cells).

Responder cells from tumour-bearing or from normal animals were cultured with lethally irradiated (30 gray = 3000 rad) tumour Stimulator cells. Cells were irradiated in a dilute suspension at room temperature to eliminate the radiobiological oxygen effect. The radiation source was a ^{60}Co Gammacell 220 (AECL; Ottawa, Canada) with a dose rate of 34 gray per minute.

The medium normally used for cultures and assays was RPMI-1640 (Grand Island Biological Company (GIBCO), New York) with the addition of 20% fetal calf serum (FCS; also from GIBCO) and ten units each per ml of penicillin and of streptomycin. This constituted "complete" medium. Additions to the culture system included $5X10^{-5}$ M 2-mercaptoethanol (2-me) and to the assay cultures - 10 mM HEPES buffer (to maintain the proper pH).

The micro-culture and assay systems used sterile Limbro/Titertek microtitration multi-well plates (Flow Laboratories Inc., 1760 Meyerside Drive, Mississauga, Ontario) covered with lids from Falcon Plastics (Micro Test II Tissue Culture Plate Covers). The plates had 96 round-bottomed wells: each about 1.0 cm deep and 0.7 cm in diameter.

Typically $3X10^5$ nucleated spleen cells in 0.2 ml of medium were cultured, in each well, together with $3X10^3$ irradiated P815X cells which were added in 0.02 ml of medium. If supernatant from twice stimulated

mixed lymphocyte cultures (SN2°MLC) was added, it contributed 0.1 ml of the total volume. If the Responder cell population was less than $3X10^5$ cells, irradiated filler cells (spleen cells; 20 gray) from normal animals were added to bring the total cell number up to $3X10^5$. After the cells in each well had been mixed, cultures were placed for five days, at 37°C, in a water-jacketed incubator, with a 5% CO_2-in-air atmosphere.

SN2°MLC was added to the culture medium in some experimental protocols. This was the supernatant of lymphocyte cultures which had twice been stimulated with allogeneic cells. CBA spleen cells ($2.5X10^7$) and irradiated (10 gray) DBA spleen cells ($2.5X10^7$), in 10 ml of medium with 10% FCS and $5X10^{-5}$ M 2-me, were incubated for 10-14 days in upright flasks (Falcon, 30 ml tissue culture flasks). After the remaining cells had been harvested, washed and counted, 10^7 cells were incubated with $4X10^7$ fresh, irradiated DBA spleen cells in 10 ml of medium (containing 10 mM HEPES buffer) in flasks laid horizontally. After a further twenty-four hours, the supernatant was collected and passed through a 0.2 micrometre pore size sterile filter. Then the resulting SN2°MLC was tested for its ability to support cytotoxic cell production in vitro. The effectiveness of the test SN2°MLC in promoting cytotoxic cell production was compared with normal medium and with a SN2°MLC known to promote a good response.

II-5 Chromium Release Assay System

Following the culturing of Responder and Stimulator cells, the ^{51}Cr release assay system was used to test for the presence of cells

cytotoxic to chromium-labelled tumour cells. After the Responders and Stimulators had been cultured for an appropriate time (usually five days), the remaining cells were harvested from the culture wells by gentle pipetting, washed once (250 g in a centrifuge for 5 min.) in complete medium, and counted.

These Effector cells were then incubated with ^{51}Cr-labelled Target cells. During the assay culture period, the lysed target cells release the ^{51}Cr into the supernatant. The amount of radioactive label released is directly dependent on the number of cells lysed.

Because P815X tumour cells incorporated the label easily and released it when killed, they were ideal for Target cells. For labelling, $5X10^5$ to $5X10^6$ P815X cells (washed 3 times and counted) in 0.5 ml of complete medium were incubated with 0.05 ml of ^{51}Cr solution at 37° C. The chromium, in the form of sodium chromate, (25-300 microcuries per 0.05 ml; specific activity 25-400 microcuries per microgram) was obtained from the Radiochemical Centre, Amersham, England. A 15 ml tube containing the label and cells was placed in a humidified incubator, with a 5% CO_2 in air atmosphere, for one hour during which the tube was shaken every fifteen minutes. After this, the cells were washed three times (250 g), thereby removing the unbound ^{51}Cr (about 80% of that added). Approximately 10% of the cells were lost in the three washings.

Dilutions of the Effector cells were made in 0.2 ml of complete medium containing 10 mM HEPES buffer in the microwells. Then, 10^3 ^{51}Cr-labelled Target cells were added to each microwell, the cells were mixed, and the assay cultures were placed in an incubator.

After incubating from four to twenty-four hours, the plates were

centrifuged at 500 g for 10 minutes and 0.1 ml of supernatant was removed for counting in a glass counting tube in a well-type gamma counter (Searle Analytic Inc., model 1185). From these counts, the amount of cell killing could be measured.

II-6 Quantitation Of Cell Killing. Percent Specific Radioactive Chromium Release

The amount of ^{51}Cr released from the lysed tumour Target cells into the supernatant during the assay culture period, was determined in a well-type gamma counter. The radioactivity, expressed as counts per minute (CPM), was converted to percent specific chromium release which is defined as:

$$\text{percent specific chromium release} = \frac{\text{(experimental value - spontaneous release)}}{\text{(total release - spontaneous release)}} \times 100$$

The spontaneous release was determined by incubating the Target cells in the absence of Effector cells, and the total release by incubating the Target cells in 0.2 ml of 5% Isoterge - a detergent that totally lyses cells. Others have found no significant difference between the spontaneous release calculated from target cells alone, or from target cells incubated with normal allogeneic spleen cells (23,30).

To obtain a mean percent specific ^{51}Cr release, the contents of four wells were assayed independently and the results analyzed. These values were determined at a specific Effector / Target cell ratio or culture dilution.

II-7 Chromium Release Curve And Lytic Unit Calculation

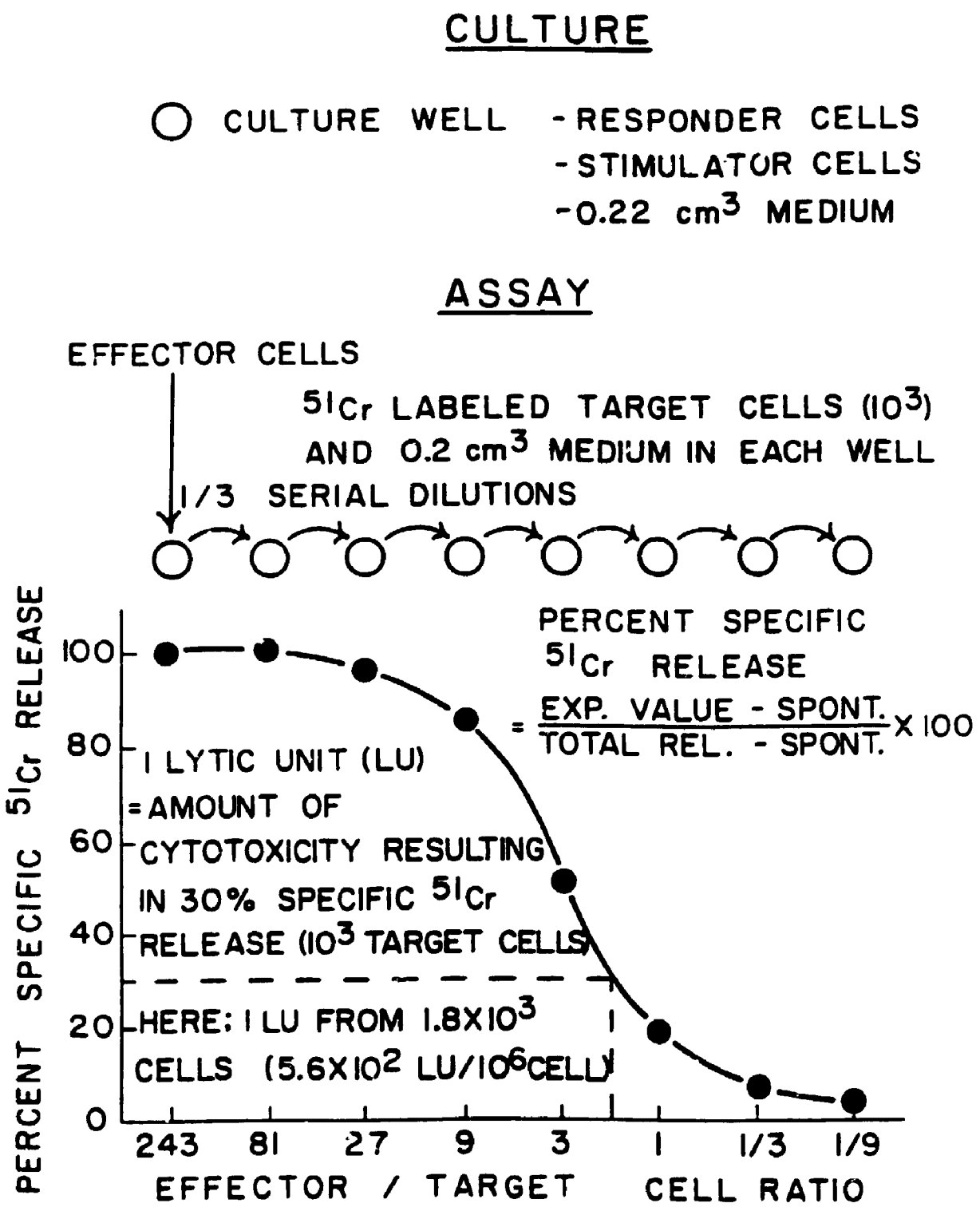
Another method of quantitating the relative number of cytotoxic cells is to measure ^{51}Cr release as a function of the ratio of Effector to Target cells, keeping the target cell number constant. The results are similar to measuring chromium release as a function of either Effector cell number, or of culture dilution.

A plot of percent specific chromium release versus Effector / Target cell ratio yields a chromium release curve from which we calculate a lytic unit (LU). A lytic unit, in our experiments, is defined as the amount of cytotoxicity resulting in 30% specific chromium release from 10^3 target cells. (Another interpretation is that a LU is the number of cells required to produce 30% specific chromium release).

Cells from a pool of 16 to 24 culture wells were collected, washed and then diluted serially. At each dilution, quadruplicate assay cultures were set up with a known Effector / Target (E/T) cell ratio. Depending on the dilution, these ratios varied from 243:1 to 1:9. The mean percent specific chromium release was calculated for each E/T cell ratio and then plotted against the logarithm of the ratio to produce a chromium release curve (fig. II-2). At low E/T cell ratios there was little killing and at high ratios, the killing could approach 100% if cytotoxic cells were present.

The number of Effector cells required to produce a lytic unit is determined by finding the intersection of the 30% specific ^{51}Cr release level and the experimental curve and then calculating how many Effector cells are required to produce that level of cytotoxicity (fig. II-2). In practice, points on the curve are joined by straight line segments,

Figure II-2 Protocol: chromium release curve



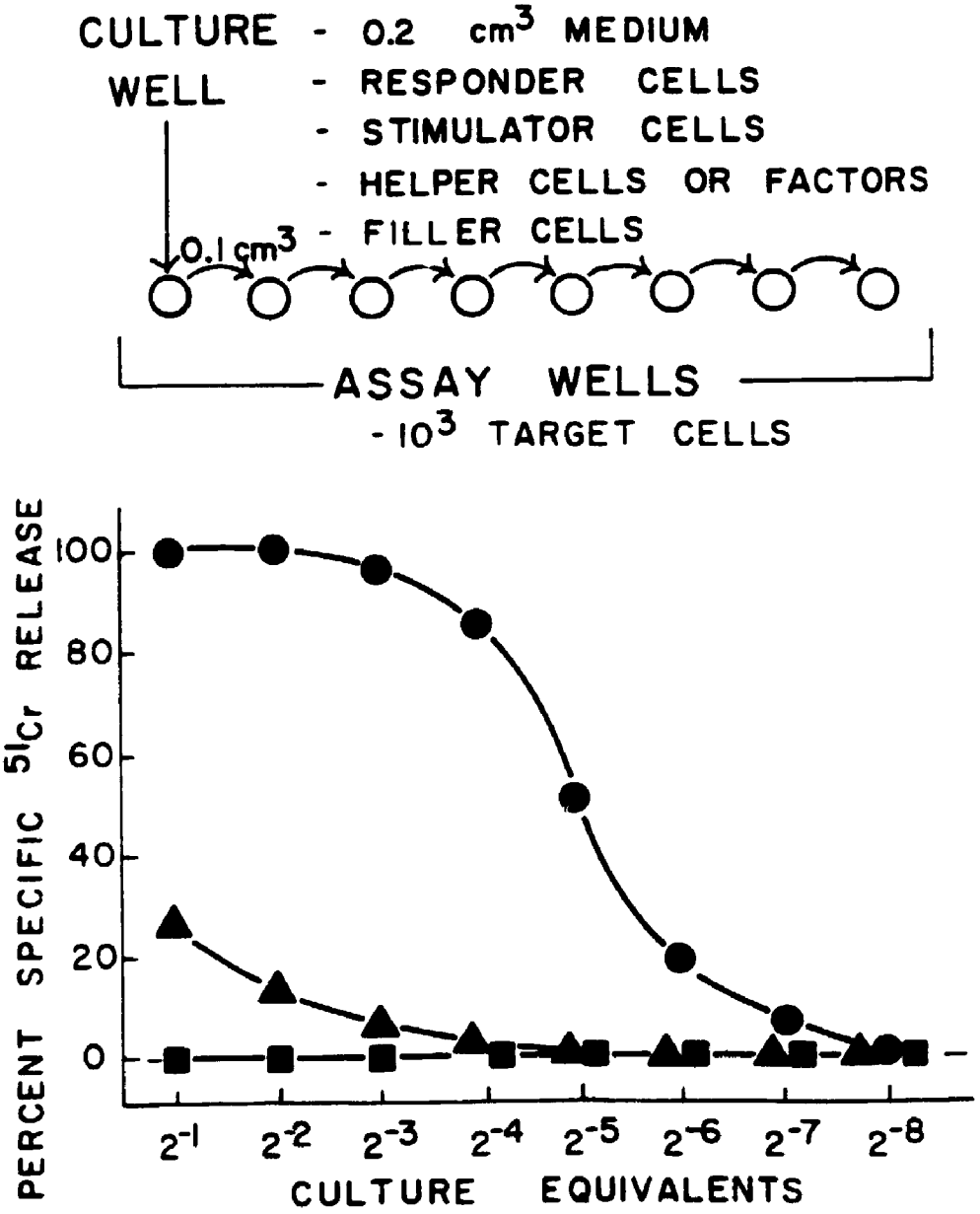
without the use of curve-fitting techniques. Then, the number of lytic units produced by 10^6 Effector cells (LU's per 10^6 cells) is calculated for each curve. Thus, groups of Effector cells are compared quantitatively by the number of LU's per 10^6 Effector cells (or per culture). It is also possible to quantify the number of lytic units per 10^6 Responder cells in the original cultures containing Responder and Stimulator cells.

II-8 Limiting Dilution Assay (LDA): Measurement Of pCTL Frequency

LDA's were performed by the following procedure. From 24 to 64 replicate cultures at 3 to 5 different Responder cell dilutions were set up. If SN2°MLC is used, as it was in almost all of the LDA's, then filler cells (normal spleen cells irradiated with 20 gray) are added to bring the total Responder plus filler cell population up to $3X10^5$ cells. Otherwise, Helper cells are added. These additions will provide a non-limiting quantity of any other cell type needed (or a substitute), without adding pCTL's (which are radiosensitive). Each culture well also contained $3X10^3$ Stimulator cells.

After the culture period, the contents of each culture well was serially diluted two to eight times (fig. II-3). Chromium release curves were obtained to ensure that cultures would be scored as either positive or negative without ambiguity. Assay medium and 10^3 labelled Target cells were then added to each assay well and the contents mixed. After twenty-four hours incubation, 0.1 ml of the supernatant was taken from each assay well, and the percent specific chromium release value

Figure II-3 Protocol: limiting dilution assay (LDA)



Responder cells are cultured together with Stimulator cells, Helper cells or SN2°MLC, Filler cells and medium. After the culture period, the contents of each well are serially diluted and 10^3 Target cells are added to each assay well.

- ● - many CTL's present in the culture well after the culture period
- ▲ - relatively few CTL's present in the culture well - this would be recorded as a positive culture
- ■ - no CTL's present in the culture well - this would be recorded as a negative culture well containing no pCTL's originally

determined. This was plotted against the culture dilution for each assay well (fig. II-3).

Each culture was scored as either positive or negative. For each Responder cell number, the proportion of non-responding cultures was determined.

When Responder cells are cultured, the pCTL's are randomly distributed in the wells and this distribution follows Poisson statistics. The probability (Pn) of finding n responding cells in a culture well is given by:

$$Pn(N,F) = \frac{(NF)^n e^{-NF}}{n!}$$

where: N = total number of cells in a culture

F = frequency of responding cells (pCTL's)

And the probability of no responding cells in a culture is:

$$Po(N,F) = e^{-NF}$$

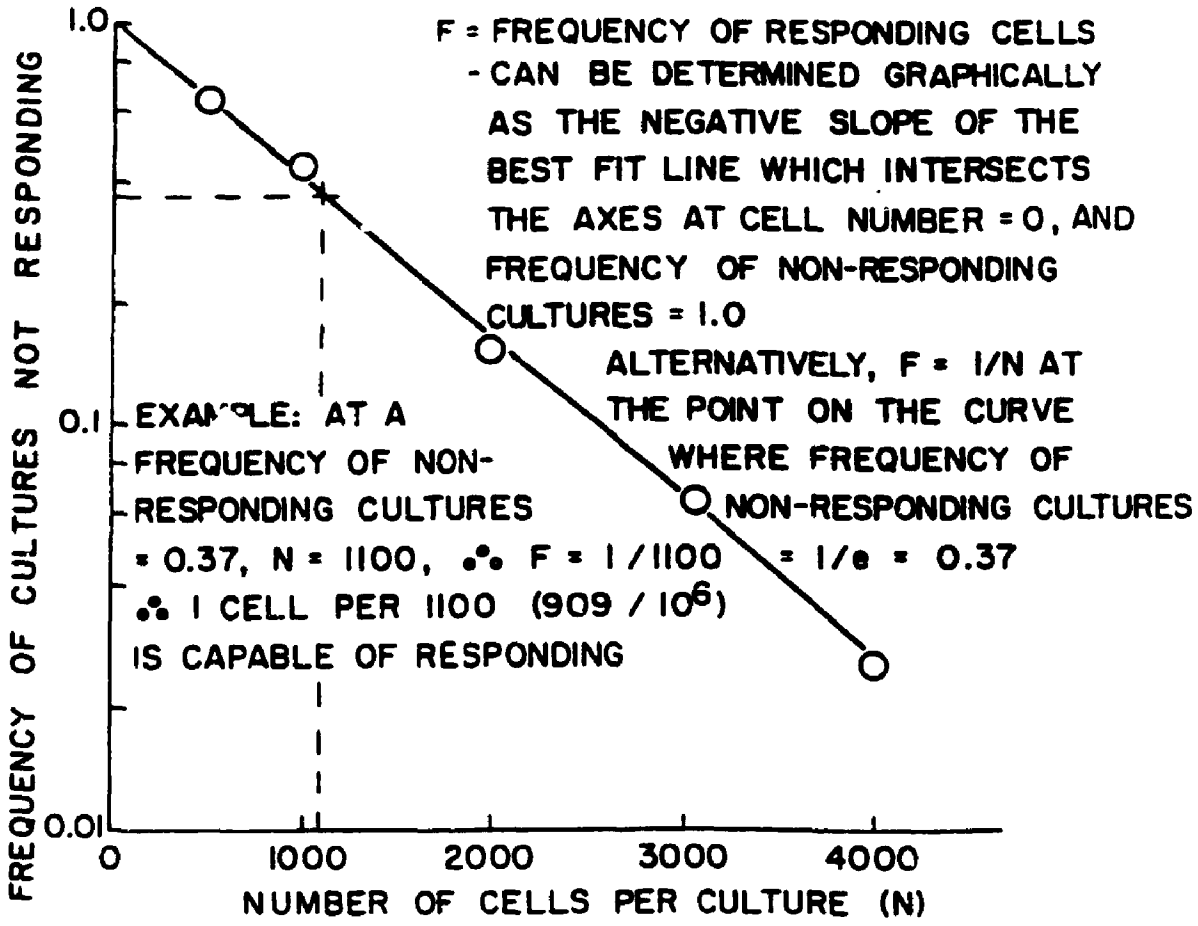
Po(N,F) is approximated by the frequency of non-responding cultures. From a plot of log(frequency of non-responding cultures) versus cell number (fig. II-4), F can be determined graphically as the negative slope of the best fit line to the data which intersects the axes at a value of cell number = 0, and a frequency of non-responding cultures = 1.0. Alternatively, F = 1/N at the point on the curve where frequency of non-responding cultures = 1/e = 0.37.

$$\text{fraction of non-responding cultures} = Po(N,F) = e^{-NF}$$

$$\text{therefore} \quad F = \frac{-\ln(\text{fraction of non-responding cultures})}{N}$$

$$\text{and} \quad \text{slope} = \frac{\ln(\text{fraction of non-responding cultures})}{N}$$

Figure II-4 Limiting dilution assay theory - an example



PROBABILITY (Pn) OF FINDING n RESPONDING CELLS IN A CULTURE WELL IS GIVEN BY:

$$Pn(N,F) = \frac{(NF)^n e^{-NF}}{n!}$$

N = NUMBER OF CELLS IN A CULTURE
F = FREQUENCY OF RESPONDING CELLS (pCTLs)
PROBABILITY OF NO RESPONDING CELLS IN A CULTURE IS $Po(N,F) = e^{-NF}$ AND IS APPROXIMATED BY THE FREQUENCY OF NON-RESPONDING CULTURES

The frequency of pCTL's, as well as the 95% confidence limits was calculated by the method of Porter and Berry (204), using a program kindly provided by Dr. R. G. Miller, Ontario Cancer Institute, Toronto Ontario (166). Also, a χ^2 value to test for the goodness of fit of the data to a straight line (an assumption of a Poisson distribution), was calculated by the program. Frequencies are expressed as number of pCTL's per 10^6 Responder cells along with 95% confidence limits. All pCTL frequencies presented in this thesis had values of χ^2 which showed that the data fit a straight line with at least a 95% probability (i.e. $P < 0.05$).

CHAPTER III CHARACTERIZATION OF THE SYSTEM

III-1 OPTIMIZATION OF THE SYSTEM

A micro-culture and assay system was developed for the study of the production of cytotoxic cells against the P815X tumour. Previous studies in this laboratory employed Marbrook cultures which required 10^7 Responder cells per culture (161,241). Since experiments were planned using limited numbers of Responder cells, a new micro-culture system was set up. The development of this system involved the testing of a number of parameters including: 1) cell numbers, 2) culture conditions, and 3) time courses of cultures and assays, all of which had to be optimized.

III-1-1 Cells

Cell Numbers

The numbers of three different populations of cells (Responders, Stimulators and Targets) were varied systematically to obtain the greatest cytotoxic response to syngeneic tumour cells.

In the absence of Helper cells and SN2°MLC, Stimulator and Target cell numbers were tested in the range 10^3 to $3X10^4$ and Responder cell numbers in the range $3X10^5$ to $3X10^6$. The optimal numbers are as follows: Responder cells - 10^6, Stimulator cells - $3X10^3$ and Target cells - 10^3 (fig. III-1). Although these values were obtained for a five day

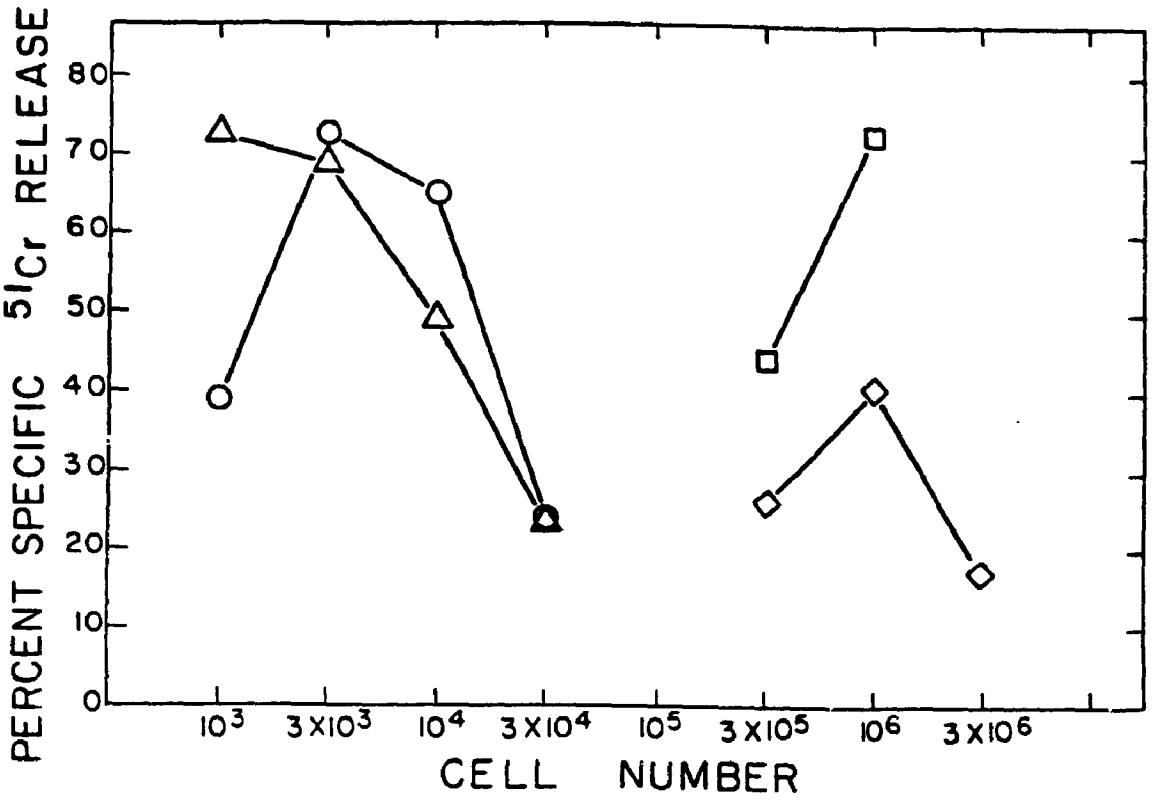


Figure III-1 Optimization of cell number

	VARY	KEEP CONSTANT
○ -	Stimulator cell number	10^6 Responder cells 10^3 Target cells
△ -	Target cell number	10^6 Responder cells $3X10^3$ Stimulator cells
◇ □ -	Responder cell number (two experiments)	$3X10^3$ Stimulator cells 10^3 Target cells

culture conditions:
- 5 day culture period and 4 hour assay
- no SN2°MLC or Helper cells

culture and a four hour assay, they were not unique to these conditions since the same values were optimal for a six day culture with a four hour assay and also for five day cultures with a twenty hour assay (data not presented).

With the addition of SN2°MLC to the cultures, the optimum Responder cell number becomes $3X10^5$, while the other optimum cell numbers remain the same. When Helper cells were used instead of SN2°MLC, the total Responder plus Helper cell population was usually kept at $3X10^5$.

When the number of lytic units produced per culture well was plotted as a function of Responder cell number, the highest response was seen with $2X10^6$ cells instead of 10^6 when lytic units per 10^6 Effector cells were used (fig. III-2). Figure III-2 demonstrates that an increase in lytic units produced per culture well is not necessarily accompanied by an increase in lytic units produced per 10^6 cells. This can occur because there is not a linear correlation between the number of lytic units produced and the number of Responder cells in a culture well. Therefore optimum conditions may vary depending on whether one is concerned about the response on a per cell basis (Responders) or on a per culture basis. The relationship between Responder cell number and lytic unit production is presented in figure III-2. Below a threshold Responder cell number, no cytotoxicity was observed while at higher numbers, lytic unit production increased to a maximum and then decreased.

When various numbers of Effector cells were assayed with specified numbers of Target cells, chromium release curves were obtained (figure III-3a). These curves are presented as percent specific chromium release (or cytotoxicity) versus culture equivalent (or Effector /

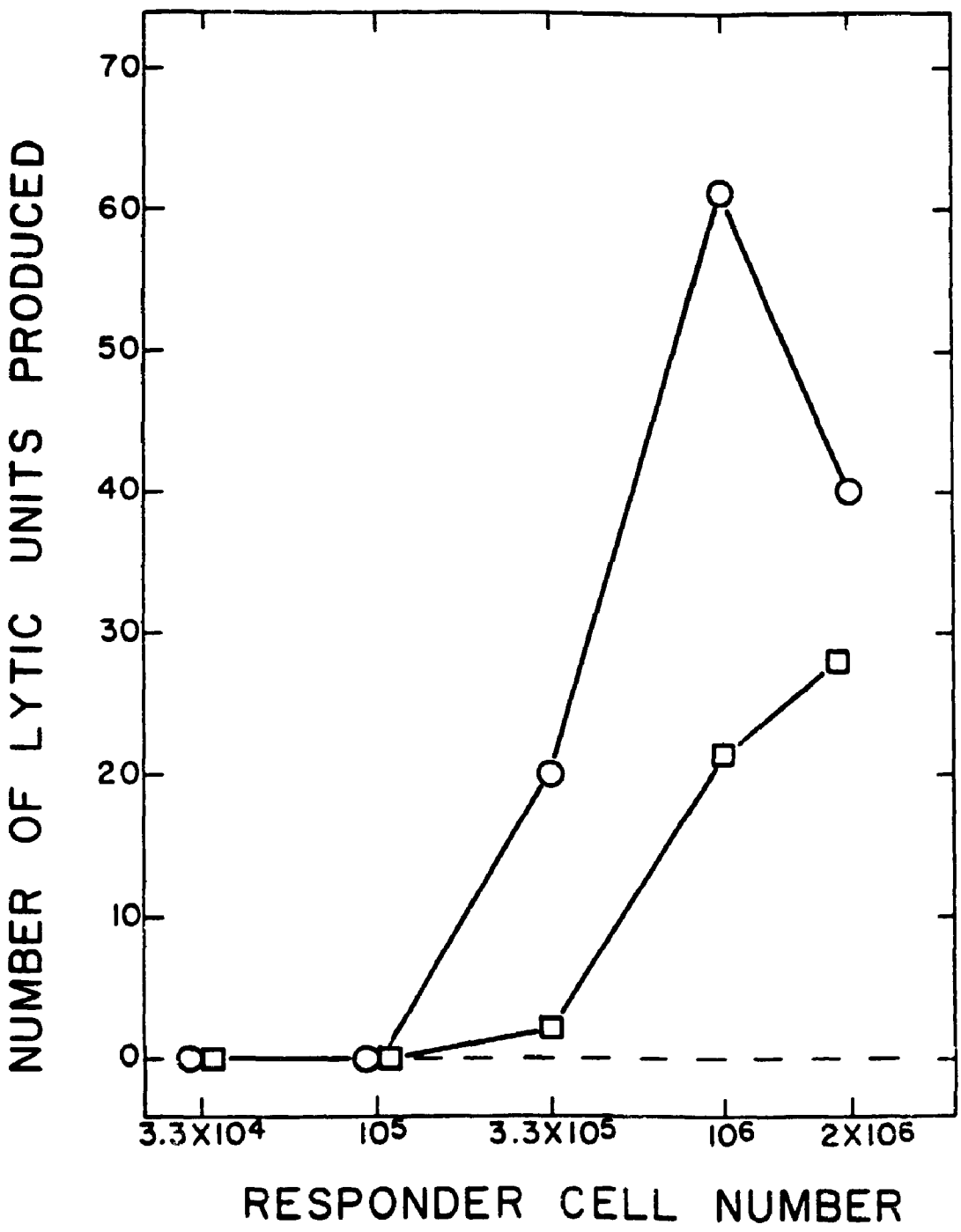


Figure III-2 Lytic activity (lytic units produced) as a function of Responder cell number

○ - lytic units per 10^6 cells
□ - lytic units per well

culture conditions:
- $3X10^3$ Stimulator cells and 10^3 Target cells
- no SN2°MLC or Helper cells

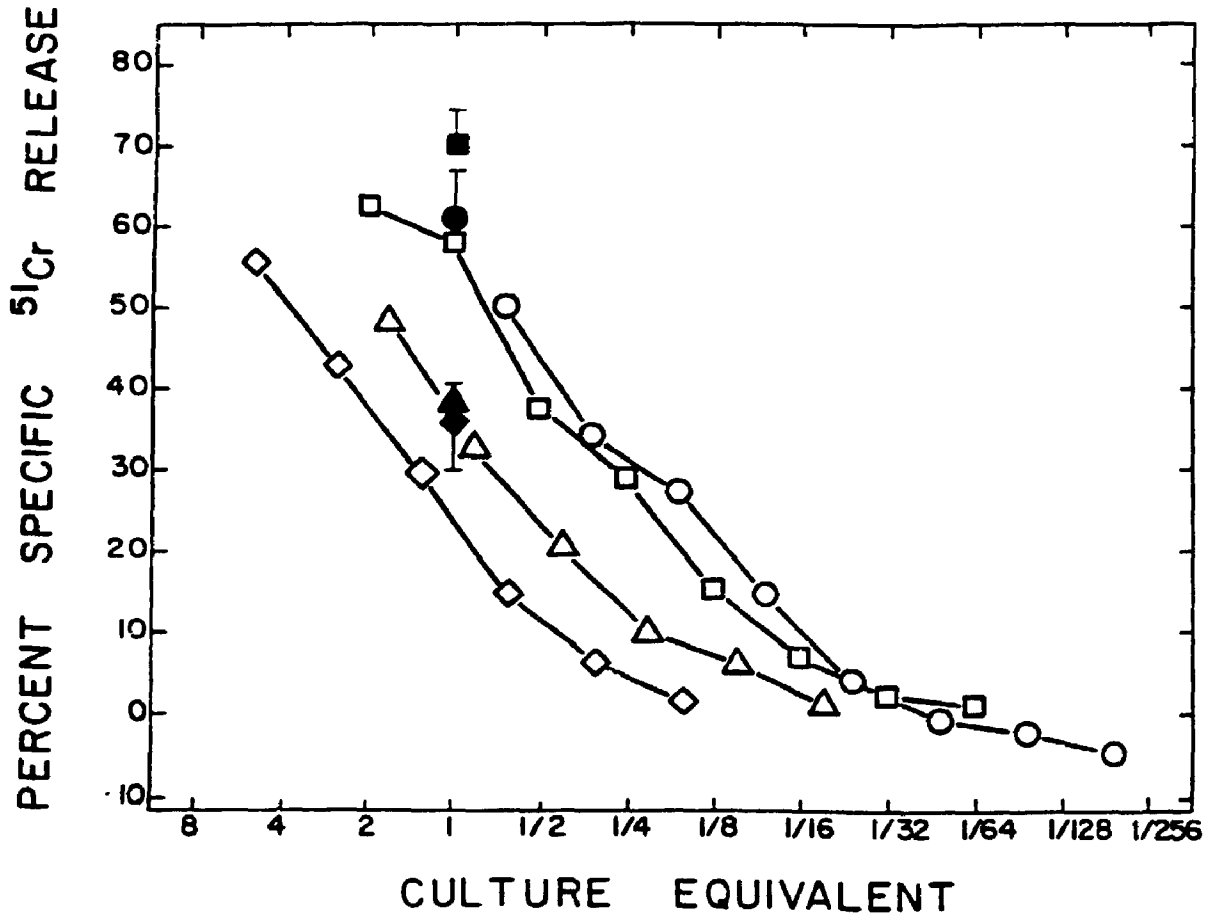
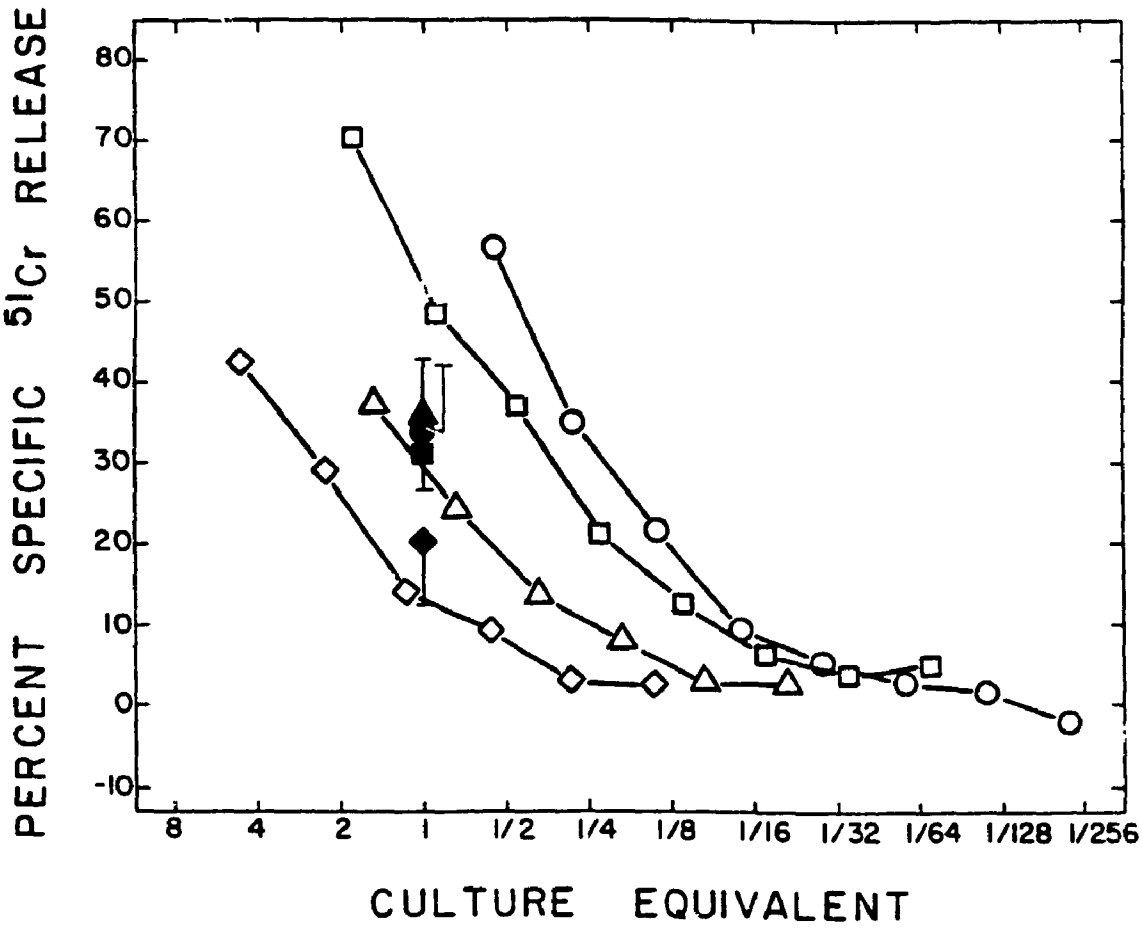


Figure III-3a, b The use of chromium release curves compared to measuring cytotoxicity as ^{51}Cr release at a single Effector / Target cell ratio (or culture equivalent)

a) 10^6 Responder cells and $3X10^3$ Stimulator cells
- ○ - 10^3 Targets; 23.2 LU produced per 10^6 Effector cells
- □ - $3X10^3$ Targets; 18.9 LU produced per 10^6 Effector cells
- △ - 10^4 Targets; 7.2 LU produced per 10^6 Effector cells
- ◇ - $3X10^4$ Targets: 4.1 LU produced per 10^6 Effector cells

The closed symbols (±1SEM) represent the activity measured, at a single Effector / Target cell ratio, by adding target cells directly to the culture wells. The single points for 10^3 Targets (●) and $3X10^3$ Targets (■) are not significantly different (P=0.20); neither are the points for 10^4 Targets (▲) and $3X10^4$ Targets (◆) (P>0.50).
culture conditions:
- no SN2°MLC or Helper cells



b) 10^6 Responder cells and 10^3 Stimulator cells

○ - 10^3 Targets; 20.2 LU produced per 10^6 Effector cells
□○ - $3X10^3$ Targets; 14.0 LU produced per 10^6 Effector cells
△□ - 10^4 Targets; 4.7 LU produced per 10^6 Effector cells
◇ - $3X10^4$ Targets; 2.0 LU produced per 10^6 Effector cells

The closed symbols (±1SEM) represent the activity measured, at a single Effector / Target cell ratio, by adding Target cells directly to the culture wells. There is no significant difference between these points ($P>0.25$; $F=0.95$).

culture conditions:
- no SN2°MLC or Helper cells

Target cell ratio if the Effector cells are counted). At low culture equivalents, there is no cytotoxicity; as the culture equivalent increases, so does the killing.

With cytotoxicity presented in this manner, a curve displaced towards lower Effector / Target cell ratios (or culture equivalents) demonstrates more killing, since, for a given chromium release (say 30%), fewer Effector cells are required to kill the same number of Target cells. The set of curves presented in figure III-3 further demonstrates that 10^3 Target cells produces more specific ^{51}Cr release than $3X10^3$, 10^4 or $3X10^4$ labelled targets. At low Target cell numbers, fewer cells have to be killed to achieve the same percent specific chromium release.

The Use Of Chromium Release Curves To Assess Cytotoxicity

In the optimization of the system, complete curves were usually produced to test the effects of changing various parameters. This was necessary when using lytic unit values to compare results since our calculation of a lytic unit required a chromium release curve that reached the 30% specific release value.

The resulting curves in figures III-3a and -3b were obtained by collecting the cells from the cultures, diluting serially the Effector cells and then adding Target cells to each well. When Target cells were simply added to the culture wells, without making any dilutions, single points, instead of complete curves were obtained (figure III-3b). The relationship of these values (single points) to each other was sometimes dissimilar to that shown by the complete curves.

With 10^5 Stimulator cells, there were no significant differences

($F = 0.95$; $P > 0.25$) in percent specific chromium release when 10^3 to $3X10^4$ Target cells were added directly to the culture wells and yet, the complete chromium release curves showed quite different cytotoxicities (fig. III-3b). This result provides a further reason (other than being able to measure cytotoxicity by lytic units) to produce these curves when searching for optimal conditions.

Reincorporation Of Chromium: Negative Percent Specific ^{51}Cr Release Values

It is possible to have negative percent specific chromium release values (figures III-3a and -3b). This probably results from the uptake, by Effector cells, of radioactive chromium released by lysed Target cells. This will lead to less chromium in the supernatant than when Target cells alone are present: a negative specific chromium release value can result. This phenomenon is more pronounced for longer assay times and especially for low cytotoxicity; others have failed to find evidence of reutilization of ^{51}Cr, at least in a 6 hour assay (30). Although calculations of absolute lytic unit values may be slightly affected, the LDA results are not altered.

Effect Of Separation Of Lymphocytes From Red Cells

Normally, spleens were taken as the source of Responder cells for the **in vitro** cultures. Because the presence of red cells in the spleen population might influence the results, Ficoll-Hypaque separated spleen cells were tested as Responders. Also Ficoll-Hypaque separation of peripheral blood lymphocytes from red cells is necessary when PBL's are to be used as Responder cells. With $3X10^5$ Responder cells per culture

well, separated spleen cells produced 86.8 LU per culture well while unseparated cells produced 116.2 LU. With 10^5 Responders, the values were 32.3 for separated and 29.3 LU for unseparated cells. The Ficoll-Hypaque separation procedure did not affect the response of given numbers of cultured spleen cells so that the presence of red cells from the spleens did not likely change the response.

The Source Of Tumour Cells: Ascites Versus Solid Tumours

It was determined whether cells disaggregated from a solid tumour would serve as Stimulators and Targets. The use of cells, (both host and tumour), from a solid human tumour would be a logical extension of the culture and assay system worked out for this mouse tumour model.

Solid P815X tumours which had been grown for two weeks in DBA/2 mice were disaggregated with an enzyme cocktail which had been shown in other systems to be very effective in producing a single cell suspension of viable cells (107). With two populations of Effector cells, obtained by culturing 10^6 Responder cells with either 10^4 or $3X10^3$ Stimulator cells (from ascites), and three Target cell numbers, six comparisons were made of target cells from either solid tumours (incubated <u>in vitro</u> for 4 hours) or from ascites.

Although the mean percent specific chromium release was slightly lower for the solid tumour cell targets (26.4% vs.32.5%), a paired t-test showed no significant difference between the two targets ($0.50 > P > 0.20$).

In a subsequent experiment, both ascites and cells from solid tumours were tested as Stimulators and as Targets, with and without a period of time in culture (table III-1a). Taking Responder cells from

		TARGETS					
		Ascites		Solid Tumour 0 hours		Solid Tumour 4.5 hours	
STIMULATORS	cell #	10^3	$3X10^3$	10^3	$3X10^3$	10^3	$3X10^3$
Ascites	$3X10^3$	57.9 ±5.9	39.3 ±2.6	27.2 ±5.7	36.3 ±8.9	38.3 ±0.7	24.8 ±2.6
	10^4	52.8 ±3.3	38.1 ±5.4	5.4 ±11.1	32.9 ±10.1	36.9 ±5.6	29.4 ±2.9
Solid Tumour 0 hours	$3X10^3$	61.1 ±4.0	35.9 ±2.3	18.2 ±9.2	53.2 ±8.0	42.5 ±5.9	29.0 ±2.7
	10^4	62.0 ±4.4	37.0 ±4.4	46.3 ±12.1	39.7 ±18.7	39.0 ±3.2	35.3 ±3.6
Solid Tumour 24 hours	$3X10^3$	25.7 ±5.4	22.8 ±1.8	22.5 ±11.3	37.5 ±12.3	20.1 ±8.6	8.2 ±0.8
	10^4	27.8 ±4.0	28.2 ±2.6	8.5 ±10.7	19.4 ±10.1	10.0 ±1.6	8.7 ±0.6

Table III-1a Effect Of Tumour-Cell Source On Lytic Activity

- values are mean percent specific ^{51}Cr release (±1SEM) for four culture wells
- the 0 and 24 hours for Stimulators, and 0 and 4.5 hours for Targets, refer to time in culture prior to use
- Responder cells are from mice injected two weeks previously

	df	SUMS OF SQUARES	MEAN SQUARES	F VALUE	P VALUE	
TarType	2	5379.755	2689.878	12.753	P<0.0005	SIG
TarNum	1	239.734	239.734	1.137	P>0.25	
StimType	2	11783.476	5891.738	27.934	P<0.0005	SIG
StimNum	1	206.401	206.401	0.979	P>0.25	
TarType X TarNum	2	5852.240	2926.120	13.873	P<0.0005	SIG
TarType X StimType	4	1655.593	413.898	1.962	.10<P<.25	
TarType X StimNum	2	410.513	205.257	0.973	P>0.25	
TarNum X StimType	2	401.130	200.565	0.951	P>0.25	
TarNum X StimNum	1	4.551	4.551	0.022	P>0.25	
StimType X StimNum	2	574.208	287.104	1.361	P>0.25	
TarType X TarNum X StimType	4	863.461	215.865	1.023	P>0.25	
TarType X TarNum StimNum	2	492.381	246.190	1.167	P>0.25	
TarType X StimType X StimNum	4	905.747	226.437	1.074	P>0.25	
TarNum X StimType X StimNum	2	635.093	317.547	1.506	.10<P<.25	
TarType X TarNum X StimType X StimNum	4	1232.682	308.170	1.461	.10<P<.25	
ERROR	106	22357.373	210.919			
TOTAL	143	52994.338				

Table III-1b Statistical Analysis Of Data Presented In Table III-1a

- TarType = Target type
- TarNum = Target Number
- StimType = Stimulator Type
- StimNum = Stimulator Number
- SIG = significant

mice injected two weeks previously, three different Stimulator and three different Target conditions and two cell numbers for each were tested. A four-way ANOVA showed that the cell killing with the three target types was different ($P < 0.0005$), and that the three Stimulator types were also different ($P < 0.0005$), (table III-1b). The only significant interaction was between Target type and Target number ($P < 0.005$).

Fresh solid tumour target cells (0.01 CPM per cell) did not incorporate the label as readily as ascites (0.08 CPM) or solid tumour cells which had been incubated for 4.5 hours (0.19 CPM). The spontaneous release from fresh solid tumour cells (27%) was higher than from either ascites (8%) or solid tumour cells incubated for 4.5 hours (15%), indicating either that fresh tumour cells are more fragile or perhaps there is a cytotoxic cell in the tumour cell population.

The significant ($P<0.005$) interaction between Target type and Target number may also indicate the presence of some cytotoxic cells in the freshly isolated tumour cell population. This significant interaction term occurs because the ^{51}Cr release increases with Target cell number for solid tumour Targets with no incubation (Table III-1a). With other target types, the ^{51}Cr release decreases with increasing Target number. The decrease in cytotoxicity with increasing Target number for the ascites cells may indicate the absence of cytotoxic cells. If cytotoxic or pre-cytotoxic cells were present in the Stimulator cell population, they would have been inactivated by the radiation dose.

The results indicate cells from solid tumours can be used as either Stimulators or Targets although perhaps not as effectively as ascites. With cells from solid tumours as Targets, an in vitro incubation of 4.5

hours decreased the variability in ^{51}Cr release (table III-1). As Stimulators, the solid tumour cells with no incubation, produced a greater response than they did after 24 hours in culture.

In summary, it has been possible to obtain optimal cell numbers for Responder cells (10^6, ($3X10^5$ with SN2°MLC)), Stimulator cells ($3X10^3$), and Target cells (10^3). On a per nucleated cell basis, the response was as great with either unseparated spleen cells or Ficoll-Hypaque separated cells. The use of complete chromium release curves, rather than single points, allows the measurement of cytotoxicity based on combining data at various culture equivalents (i.e. calculation of lytic units) and should yield more consistent results.

III-1-2 Culture Conditions

In order to obtain the maximum response from the cells in vitro, various culture changes and additions were tested. These included: type of medium, addition of 2-mercaptoethanol, feeding with supplementary FCS, Filler cells, Helper cells and SN2°MLC.

Type Of Medium

Various culture media (Minimum Essential Medium (MEM - Eagle), CMRL 1066 and RPMI 1640) were tested for their ability to support the production and testing of cytotoxic cells in the culture and assay systems. As media in the cultures, RPMI 1640 gave a 60% greater lytic unit production compared with MEM (an average of 5.41 versus 3.37 LU/10^6 cells) (fig. III-4). In the assay, there was no difference between the

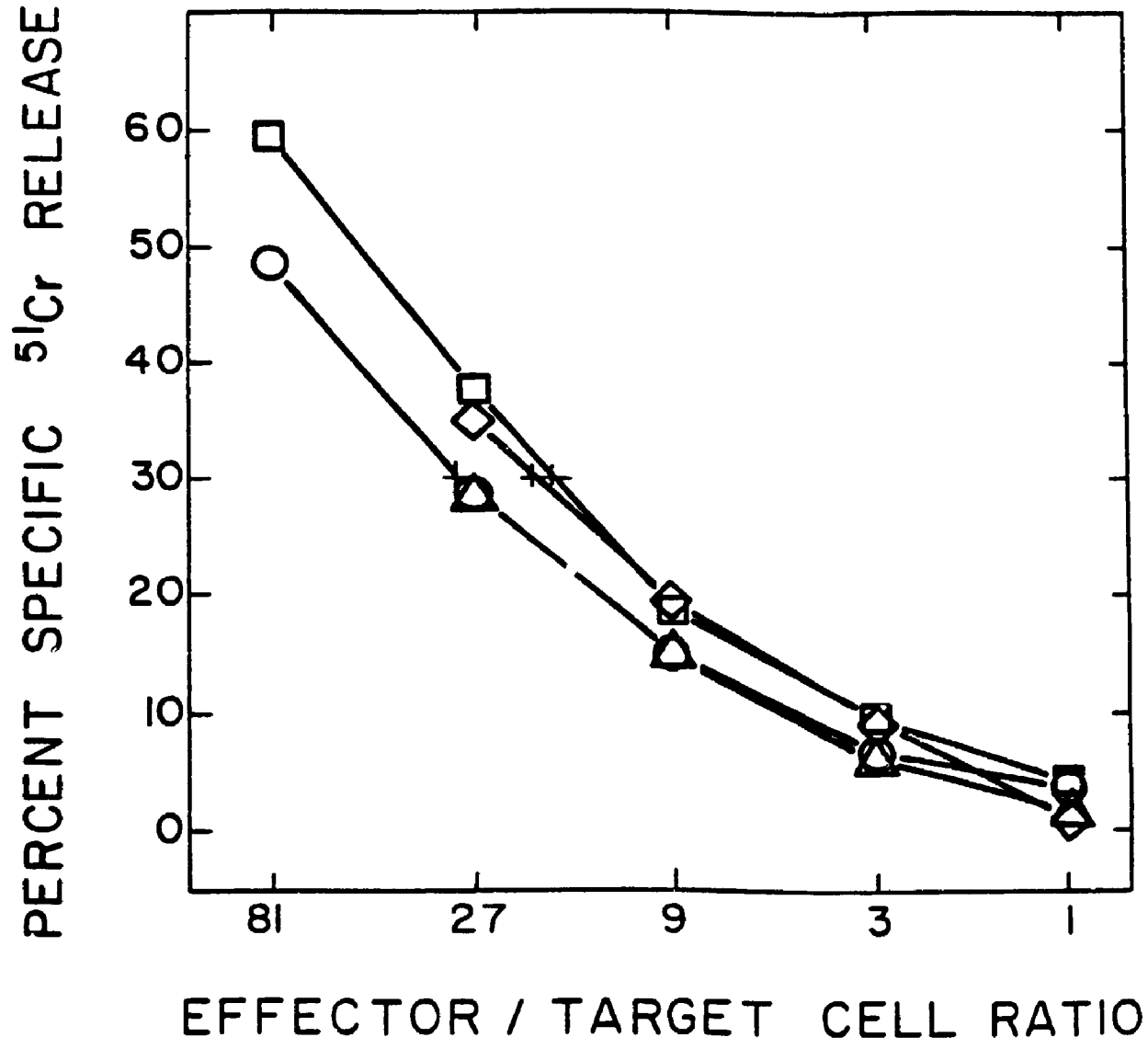


Figure III-4 Optimization of culture medium

	CULTURE	ASSAY	LU / 10^6 cells
□ -	RPMI 1640	CMRL 1066	5.67
◇ -	RPMI 1640	RPMI 1640	5.15
○ -	MEM	CMRL 1066	3.37
△ -	MEM	RPMI 1640	3.37

culture conditions:
- 10^4 Target cells
- no SN2°MLC or Helper cells

use of CMRL 1066 and RPMI 1640 (fig. III-4), nor between RPMI 1640 and MEM (data not shown).

Addition Of 2-Mercaptoethanol

It has been observed that when 2-mercaptoethanol (2-me) is added to the culture medium in a mixed lymphocyte culture (MLC), a stronger response occurs. When 2-me was present, the production of cytotoxicity was increased considerably (table III-2) with the largest response occurring at a dose of $5X10^{-5}$ M. The assay medium did not contain 2-me.

Feeding With Supplementary FCS

By feeding the cultures with FCS or with irradiated filler cells (20 gray), an attempt was made to increase the cytotoxic response of a given number of Responder cells. With no SN2°MLC or Helper cells present, the addition of FCS in either single (20 microliters) or multiple doses (4X10 microliters), had no positive effect on the response. While the response without additional FCS was 9.7 to 11.7 LU per 10^6 Effector cells, single additions produced 7.5 to 8.4 LU and multiple additions produced 4.7 LU per 10^6 cells.

Filler Cells

When heavily irradiated filler cells from the spleens of DBA/2 mice with or without tumours, or from CBA, AKR or C57Bl mice were added to the culture wells, there was no change in the resulting cytotoxicity. This lack of a filler cell effect was irrespective of the number of Responder cells present. In spite of this result, irradiated (20 gray) filler cells from normal DBA/2 animals were added to each culture to

2 - ME CONCENTRATION	RESPONDER CELL NUMBER	LYTIC UNITS PER 10^6 EFFECTOR CELLS	LYTIC UNITS PER MICROWELL
0 M	10^6 $3X10^5$ 10^5	625.0 77.5 0.0	101.3 7.5 0.0
$5X10^{-5}$ M	10^6 $3X10^5$ 10^5 $3X10^4$	194.8 1189.0 90.9 0.0	34.7 136.7 8.3 0.0
$5X10^{-4}$ M	10^6 $3X10^5$ 10^5 $3X10^4$	250.0 239.8 60.0 0.0	39.8 26.4 2.5 0.0
$5X10^{-3}$ M	10^6 $3X10^5$ 10^5 $3X10^4$	0.0 0.0 0.0 0.0	0.0 0.0 0.0 0.0

Table III-2 Addition Of 2-ME To The Culture Medium

- ME = mercaptoethanol
- M = molar

bring the total cell population in each well up to $3X10^5$, to maintain a reasonably consistent cellular environment from group to group and from experiment to experiment.

Helper Cells

Helper cells or SN2°MLC might increase the cytotoxic activity of the Responder cells. A subclass of T cells, helper cells (Th), is known to increase the response of a pCTL to an antigen and they can be distinguished from pCTL's by their surface markers. As a subclass of T cells, they have the Thy-1 surface antigen and are generally thought to have the Ly-1 marker as well. Because the pCTL's have the Ly-2 marker while the Helper cells do not, treatment of a mixed population of cells with antibody to the Ly-2 antigen (anti-Ly-2 Ab) plus complement will lyse the pre-cytotoxic cells, leaving the Helper cells intact.

Helper cells, produced from spleens of normal and tumour - bearing mice in this manner, were tested in the culture system (fig. III-5). The addition of approximately 10^5 Helpers to the $3X10^4$ or 10^5 Responder cells in culture increased the CTL response without contributing any additional pCTL's ($3X10^5$ Helper cells, alone, gave no response).

SN2°MLC

The addition of SN2°MLC to the Responders and Stimulators in vitro had a marked effect on the cultured cells. SN2°MLC is supernatant obtained from mixed lymphocyte cultures (MLC) after a second stimulation. Supernatant from a CBA anti-DBA/2 cell MLC boosted the response of 10^5 Responder cells from 0.5 to 149.3 LU per culture well

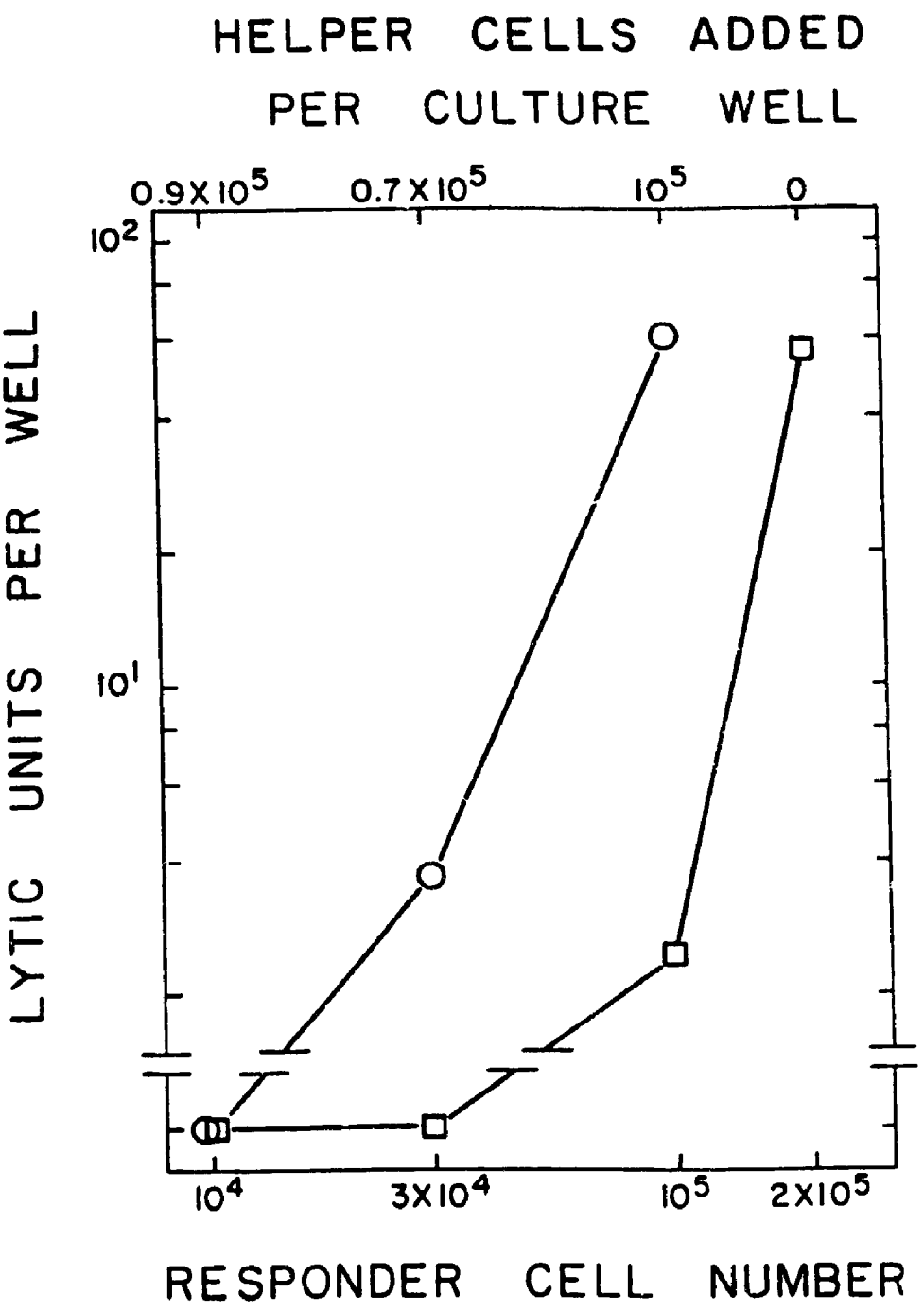


Figure III-5 Addition of Helper cells to the culture medium

The effect of the addition of anti- Ly-2.1 treated Helper cells from tumour-bearing animals to cultures.
○ - with Helper cells
□ - without Helper cells
note: $3X10^5$ Helper cells alone gave no response
culture conditions:
- filler cells were added for a total of $3X10^5$ cells per culture well
- points below the break in the Y axis have zero magnitude

while the DBA/2 anti-CBA supernatant increased the response from 0.5 to only 19.2 LU per well (table III-3). Other experiments confirmed that the CBA anti-DBA/2 supernatant was superior to that produced in a DBA/2 anti-CBA MLC and therefore it was decided to use CBA anti-DBA/2 SN2°MLC in subsequent experiments. Others have noted that CBA mice produce a stronger response to alloantigens than do DBA mice. Perhaps the strong response is related to the effect of Th cells - presumably responsible for the production of the active factor(s) in the SN2°MLC. Also the SN2°MLC was more effective, than the numbers of Helper cells tested, in increasing the cytotoxic response of DBA/2 spleen cells to syngeneic tumour cells.

Adding SN2°MLC to cultures increased the cytotoxic response of cells from both tumour-bearing and normal animals (fig. III-6). With the improvements to the culture and assay systems, which are described in this chapter, and especially with the addition of SN2°MLC, cytotoxic activity was routinely demonstrated using the spleen cells of normal animals, although this activity was generally much lower than with cells from a tumour-bearing animal two weeks after tumour injection (fig. III-6). Because SN2°MLC gave higher responses than Helper cells when added to up to 10^5 Responder cells in culture and since it could be stored for long times (years) at -70°C, and then used in several experiments, large batches (500 ml) of SN2°MLC were produced, tested and stored frozen until needed.

SN2°MLC - Is The Active Component IL-2?

In order to determine whether the active component of SN2°MLC might be interleukin-2 (IL-2), the results of a proliferation assay which

RESPONDER CELL NUMBER	SN2°MLC (0.1 ml/well)	LU PER 10^6 EFFECTOR CELLS	LU PER CULTURE WELL
$3X10^5$	none	176.8	20.0
10^5	none	(17.6)	(0.5)
10^5	50% DBA anti-CBA	448.4	19.2
10^5	25% DBA anti-CBA	111.9	4.8
10^5	12.5% DBA anti-CBA	44.5	2.3
10^5	50% CBA anti-DBA	2362.5	149.3
10^5	25% CBA anti-DBA	2158.5	123.9
10^5	12.5% CBA anti-DBA	959.3	57.9

Table III-3 Addition Of SN2°MLC To The Culture Medium

- the Effector cells are pooled from 24 culture wells
- (brackets) indicate an estimated number
- LU = lytic units

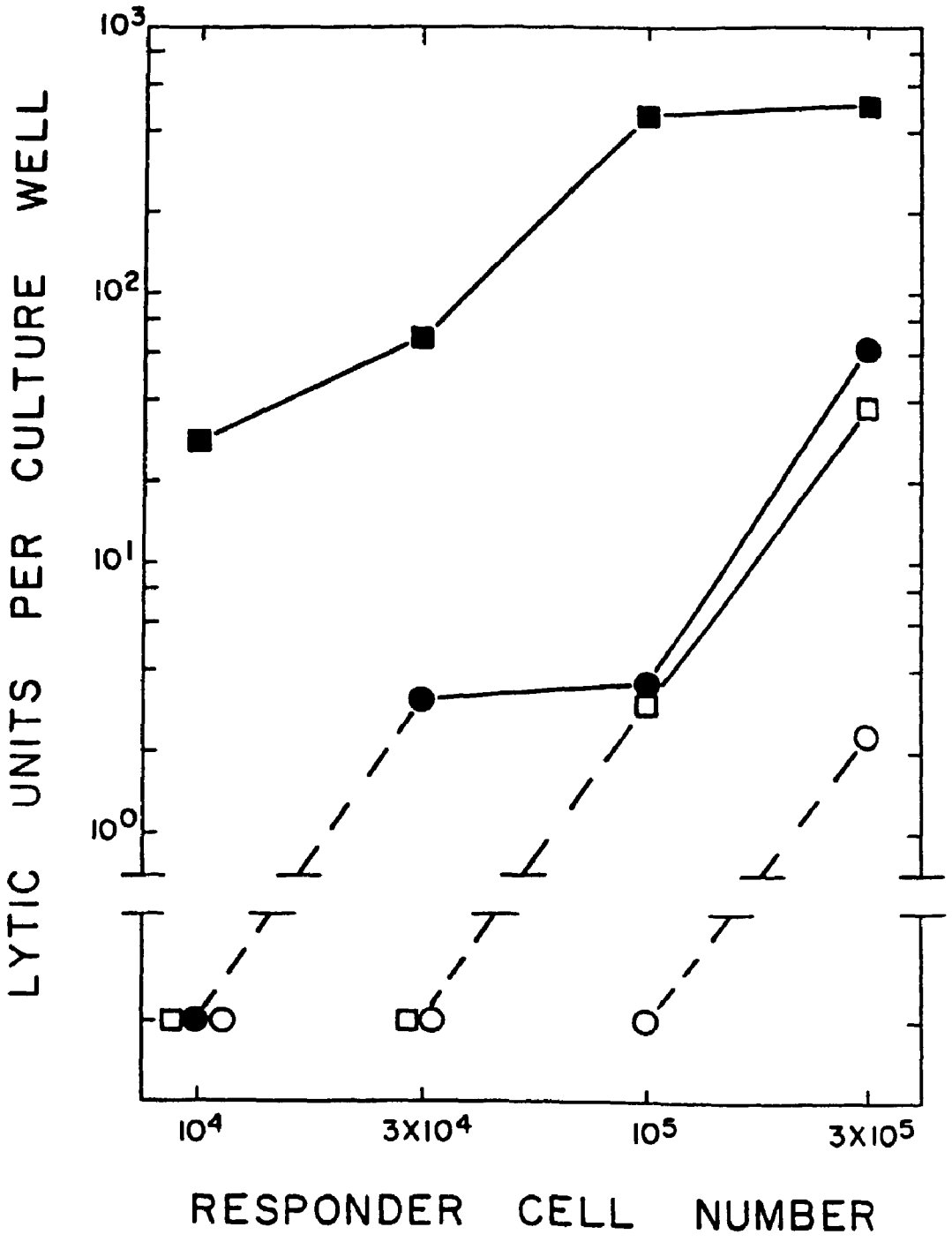


Figure III-6 SN2°MLC increases the response of cells from both normal and tumour-bearing animals

■ - Responder cells from tumour-bearing animals; with SN2°MLC
□ - Responder cells from tumour-bearing animals; without SN2°MLC
● - Responder cells from normal animals; with SN2°MLC
○ - Responder cells from normal animals; without SN2°MLC
- points below the break in the Y axis have zero magnitude

measured the IL-2 content of various SN2°MLC's were compared to other experiments which tested LU production, using those same supernatants. An IL-2-dependent cell line (CTLL or Cytotoxic Lymphoid Line) (77,79) was obtained from Keith Cousins, University Hospital, London Ontario. Cultures were set up containing: 1) $5X10^4$ CTLL cells, 2) complete medium and 3) 25% of either SN2°MLC or IL-2 (Human T-cell Growth Factor (TCGF) from Cellular Products Inc., 688 Main St., Buffalo, NY, 14202).

After being washed in IL-2 free medium and then cultured for eighteen hours, the cells were pulsed for six hours with 2 microcuries of ^{3}HTdR (^{3}H-Thymidine) per well. Cells were collected on filter paper, washed and the ^{3}HTdR content determined. The mean counts per minute for triplicate runs are given in table III-4. A 1/10 dilution of IL-2 gave an 80 fold increase in the uptake of ^{3}HTdR over background (medium alone). Assuming that the assay tests for the presence of IL-2, the results of the various supernatants indicated that they contained IL-2. Some gave higher counts than the dilutions tested of the IL-2 standard.

The ^{3}HTdR uptake using some of those supernatants was compared with the number of lytic units produced with $5X10^4$ Responder cells in previous experiments. Supernatants which had not been tested with $5X10^4$ Responder cells were not included in this analysis. Numbers of Lytic Units produced per culture well were plotted versus the IL-2 content (CPM of ^{3}HTdR above background) (fig. III-7). Regression analysis showed an excellent correlation between Lytic Unit production per well and the IL-2 content of the supernatant ($r^2 = 0.982$, $0.002 > P > 0.001$). When no SN2°MLC was added, there was no lytic activity and the CPM's, in the assay, was the background level. The conclusion is that the active factor in the SN2°MLC is probably IL-2.

FACTOR ADDED TO CULTURE	^{3}HTdR UPTAKE (CPM ±SEM; n=3)
medium control	$7.57 \pm 1.08 \times 10^2$
SN - 8	$12.11 \pm 0.39 \times 10^4$
SN - 12	$9.77 \pm 0.38 \times 10^4$
SN - 6	$2.24 \pm 0.43 \times 10^4$
SN - 6-2	$6.24 \pm 0.20 \times 10^4$
SN - 7-2	$1.70 \pm 0.16 \times 10^4$
SN - 17	$8.15 \pm 0.38 \times 10^3$
SN - 4-2	$3.99 \pm 0.27 \times 10^3$
IL-2 (1/2)	$7.40 \pm 4.14 \times 10^3$
IL-2 (1/10)	$6.07 \pm 0.16 \times 10^4$
IL-2 ($1/10^2$)	$7.64 \pm 1.09 \times 10^4$
IL-2 ($1/10^3$)	$1.98 \pm 0.17 \times 10^3$
IL-2 ($1/10^4$)	$5.87 \pm 0.65 \times 10^2$

Table III-4 An Assay For IL-2 In SN2°MLC

- SN = SN2°MLC
- the supernatant number denotes various batches
- (fractions) indicate dilutions of IL-2

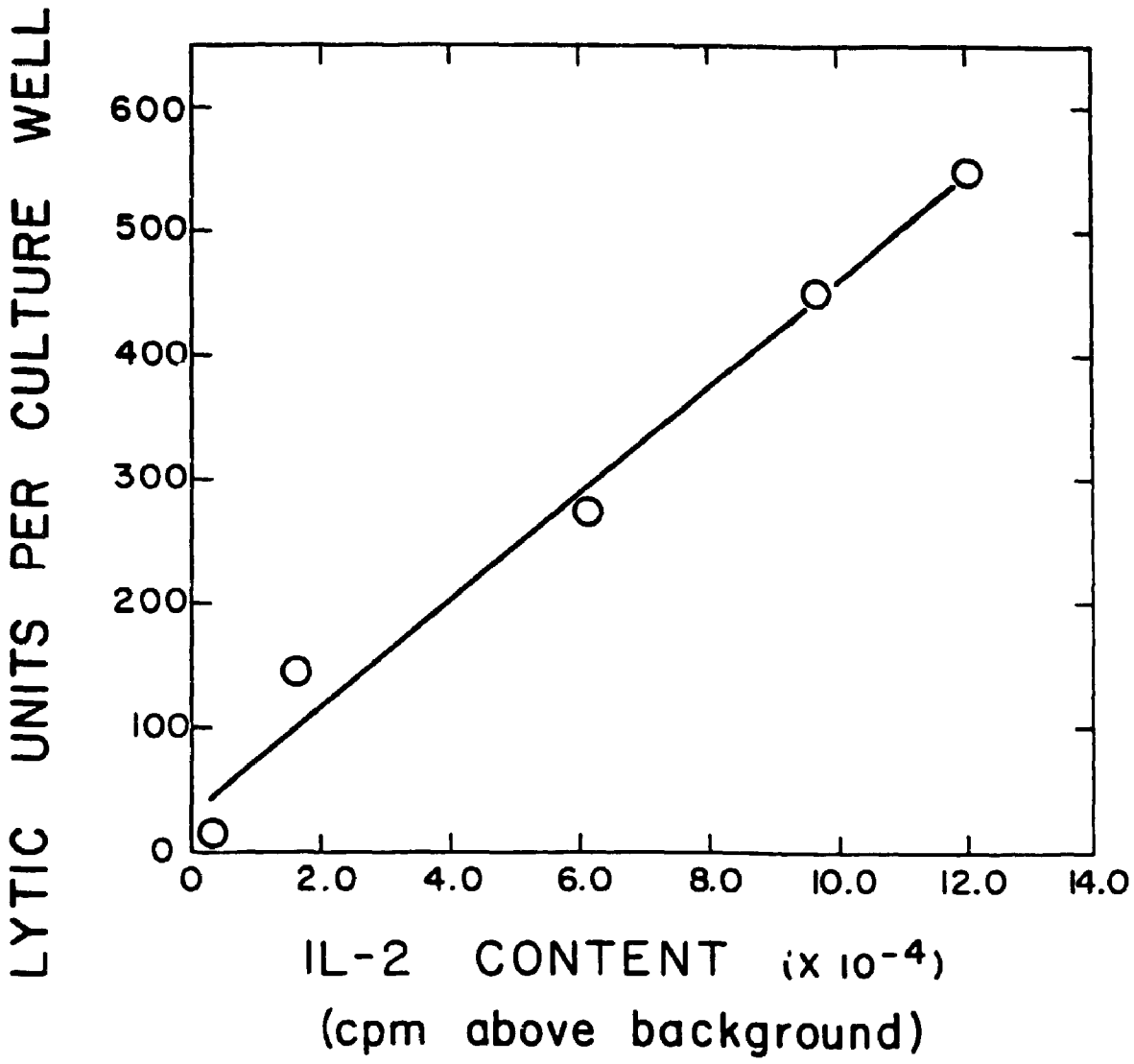


Figure III-7 Lytic unit production versus IL-2 content for various SN2°MLC's

- each symbol represents a different SN2°MLC
- the regression line is the least squares best fit; correlation coefficient r = 0.991; SEr = 0.077; t = 12.87 ($0.001 < P < 0.002$)
- when no SN2°MLC was added, there was no response

culture conditions:
- $5X10^4$ Responder cells per culture well

Shape Of The Culture Wells

Multi-well culture plates were obtained with differently shaped microwell bottoms. Some were tested with flat, V-shaped, and round bottoms during the culture phase. Although the differences were not significant ($F = 4.46$, $0.10 > P > 0.05$), the round bottomed microwells gave slightly higher chromium release (68.8; ±4.5%,SEM), compared with V-shaped (51.2; ±3.9%,SEM) and flat wells (56.6; ±4.9%, SEM).

Mixing Of Cells Before The ^{51}Cr Release Assay Period

Before the cultures were placed in the incubator, the cells were thoroughly mixed by pipetting the contents of each well several times. It was determined whether mixing of the cells before or after the assay period had any effect on the amount of killing and subsequent chromium release.

Using either $3X10^3$ or 10^4 Stimulators with 10^6 Responder cells, and, after the culture period, 10^3 or $3X10^3$ Targets, mixing prior to the assay increased the specific ^{51}Cr release from 66.1% (±5.6;SEM) to 76.9% (±2.7;SEM). Mixing afterwards (69.7%; ±4.5%) was not different from the control while mixing before and after (76.2%; ±1.7%) was the same as mixing only before.

Test Of An Alternate Supernatant Harvesting System For The Chromium Release Assay

Normally 0.1 ml of the assay culture volume of 0.22 ml was taken for a ^{51}Cr content determination. If the ^{51}Cr concentration was not uniform throughout the supernatant, then taking only part of the supernatant might lead to erroneous results. A supernatant harvesting

system was tested in which all of the liquid was absorbed in a cotton plug while a filter paper prevented the cells in the microwell bottom from being drawn with the supernatant into the plug. There was no difference in the cytotoxic activity measured using the two supernatant recovery methods and for reasons of economy, the procedure of taking 0.1 ml of the supernatant volume for radioactivity testing was employed. Such a harvesting procedure might be useful if very low levels of radioactivity were present. Taking all the supernatant might then help to differentiate between positive results and background.

In summary, RPMI 1640, with the addition of $5X10^{-5}$ M 2-me, is the medium of choice for cultures. SN2°MLC is effective in increasing the response of Responder cells to the presence of tumour cells. Round bottom microwell plates were more effective than V-shaped or flat ones and mixing of the cells prior to the assay culture period increased the amount of ^{51}Cr released.

III-1-3 Length Of The Culture And Assay Period

When cultures for the production of cytotoxic cells from the interaction of Responders and Stimulators were incubated for four to seven days, the highest response was observed at five or six days (figure III-8a). Five days was chosen as the standard culture period, although for a few experiments six was used.

Assay culture periods of one to fifty hours were tested for various Responder and Stimulator cells combinations after the initial five day incubation (fig. III-8b). The maximal lytic unit production occurred at

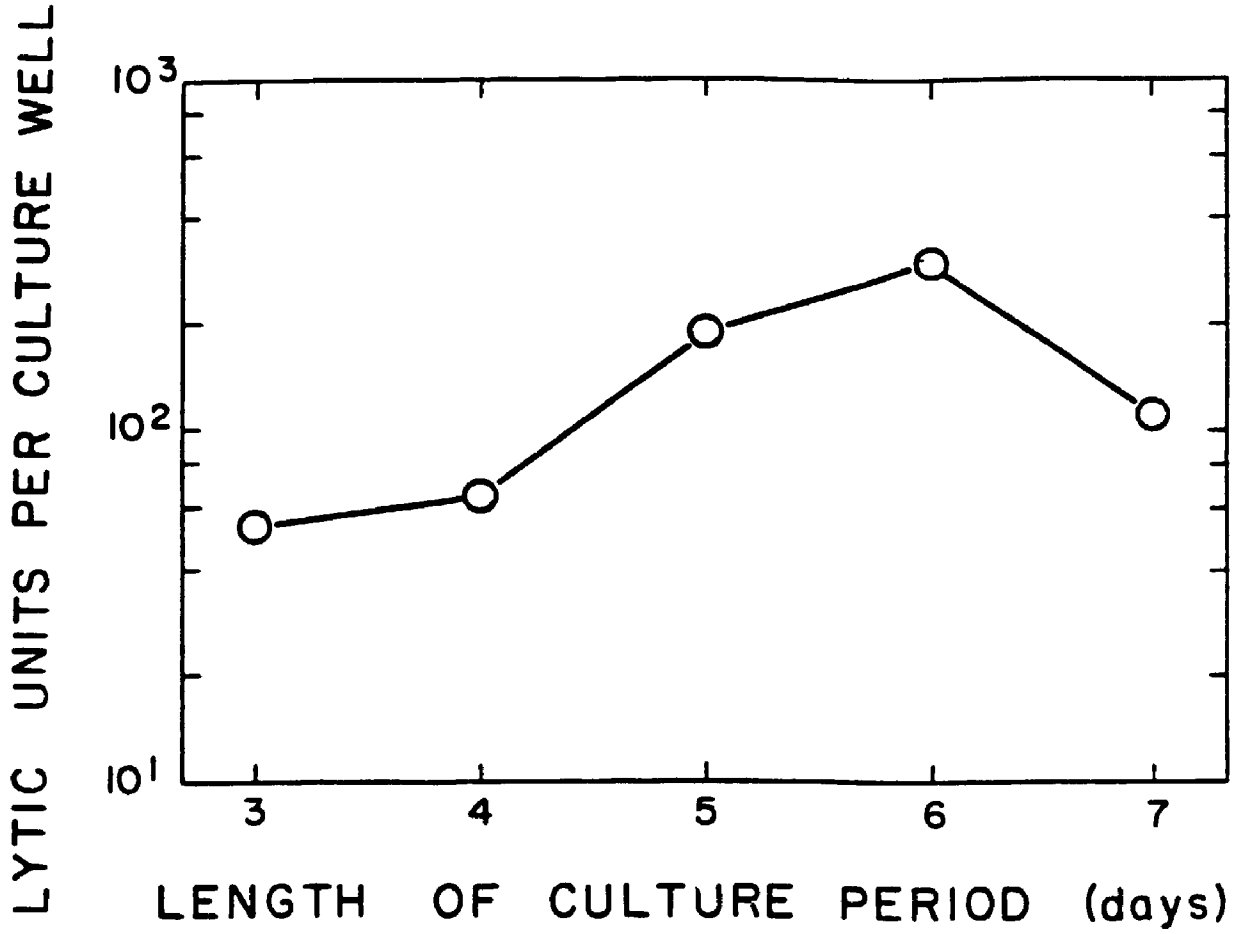


Figure III-8a Lytic unit production as a function of the culture period

culture conditions:
- 10^6 Responder cells and $3X10^3$ Stimulator cells
- no SN2°MLC present
note: in another experiment with 10^6 Responder cells and $3X10^3$ Stimulator cells and 10^3 Target cells, the percent specific ^{51}Cr release decreased from 72.5% on day 5 to 57% on day 6 in a 4 hour assay

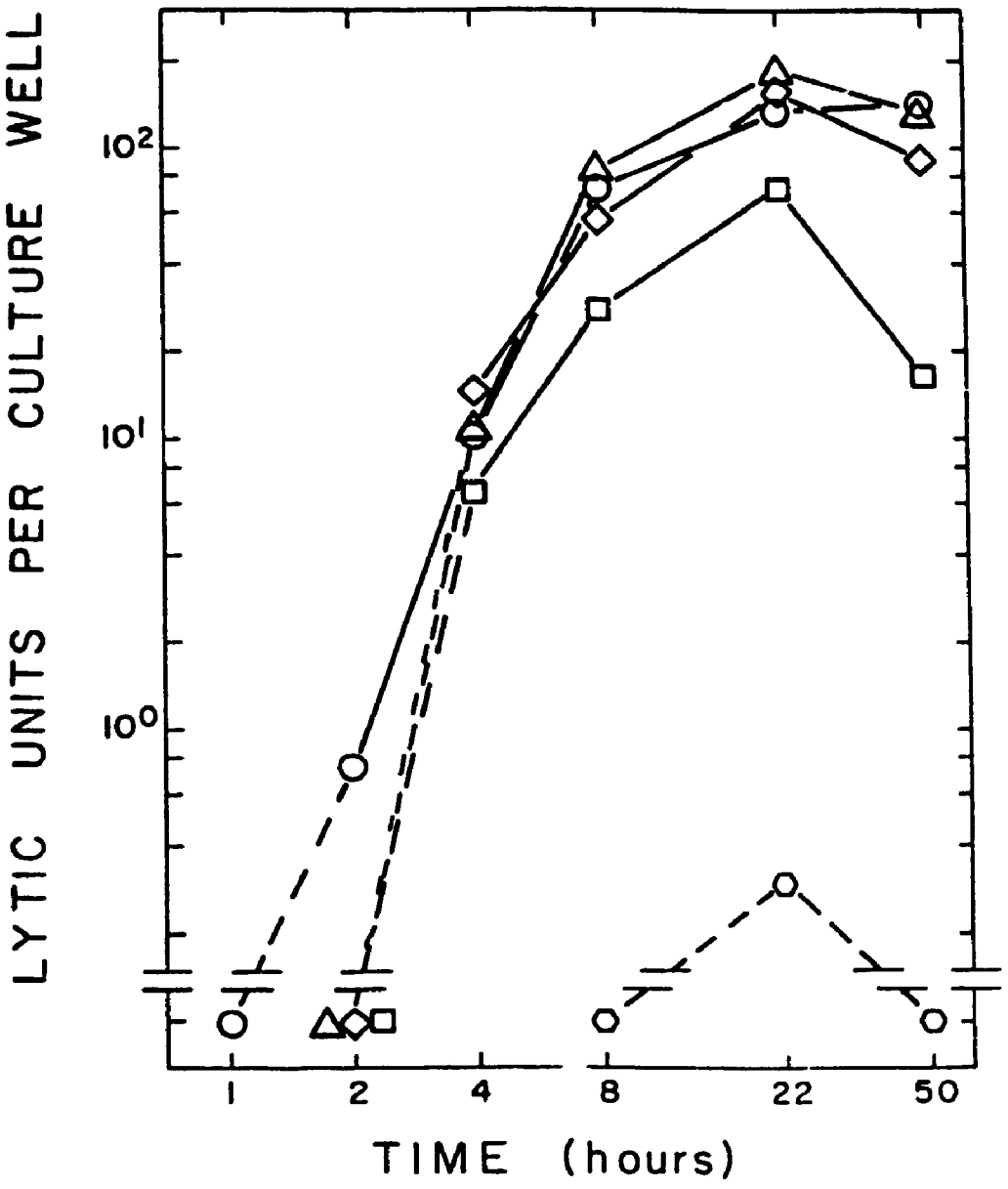


Figure III-8b Lytic unit production as a function of the assay culture period

- ○ - 10^5 Responder cells and $3X10^3$ Stimulator cells; (with 10^5 Responder cells and 10^4 Stimulator cells - no response)
- ◇ - $3X10^5$ Responder cells and $3X10^3$ Stimulator cells
- □ - $3X10^5$ Responder cells and 10^4 Stimulator cells
- ○ - 10^6 Responder cells and $3X10^3$ Stimulator cells
- △ - 10^6 Responder cells and 10^4 Stimulator cells

culture conditions:
- no SN2°MLC or Helper cells
note: log scale on time (x) axis
- points below the break in the Y axis have xero magnitude

twenty-two hours. With minimal response such as with 10^5 Responders and $3X10^3$ Stimulators per culture, the only positive response was after a twenty-two hour assay period. It appears that, with low responses especially, a twenty-four hour assay could produce measurable cytotoxicity where a four hour assay might not. In experiments with SN2°MLC in the culture medium, the lytic unit production was higher at twenty-four hours than at four or forty-eight hours (data not shown).

III-2 CHARACTERIZATION OF THE CELLS INVOLVED IN THE PRODUCTION OF CELL-MEDIATED CYTOTOXICITY AGAINST A SYNGENEIC TUMOUR

In this section are reported the results of experiments which characterize the cells involved in the production of cytotoxicity against a syngeneic tumour. These are compared to the results for an allogeneic system (i.e. cells of one mouse strain reacting against those of another). These experiments fall into three categories: 1) a study of the antigenic determinants on the cells, 2) a comparison of the syngeneic system to an allogeneic system, 3) a study of the radiation sensitivity of the cells involved.

III-2-1 Antigenic Determinants On pCTL's, CTL's and Helper Cells

In the study of Sinclair et al. (241), it appeared that the pCTL's and CTL's were Ly-1, Ly-2 and Thy-1 positive (abbreviated Ly-1^+,2^+ and Thy-1^+). Incubation of these cells, first with antibodies specific for

Ly-1, Ly-2 or Thy-1 and then with complement would prevent a response. Since Helper cells or SN2°MLC are important in the response, those experiments were repeated to determine whether the pCTL's or the Helper cells (or both) had been inactivated by the antibody plus complement treatment.

Helper cells were produced by treating spleen cell populations with anti-Ly-2 Ab plus complement (see methods). Since this treatment prevented the production of CTL's and yet produced cells capable of helping to increase the response of non-treated cells, Helper cells were shown to be Ly-2$^-$.

To further characterize the Helper cell population, cells were treated with anti-Thy-1, anti-T or anti-Ly-1 Ab plus complement. Following this, $2.7X10^5$ Helper cells were incubated with $3X10^4$ Responder cells in culture and subsequently a chromium release assay was carried out (table III-5).

In five out of six experiments, treatment with anti-Ly-2 Ab plus complement eliminated the CTL production in the Helper cell population, although these cells were still capable of boosting the response of $3X10^4$ Responder cells. In two out of four experiments, anti-Ly-1 treated 'helper' cells increased the response , although not as much as the anti-Ly-2 treated cells. In the other two experiments, anti-Ly-1 treated cells gave no help. Anti-Thy-1 and anti-T treated cells gave some help in only one out of three and one out of two experiments respectively.

There are some problems with this analysis. The various treatments of the Helper cells were not tested in each experiment; treatment with anti-Ly-2 Ab plus complement did not eliminate the pCTL's in experiment

	CONTROLS (no Helper cells)		TREATMENT OF HELPER CELLS (use $3X10^4$ Resp and $2.7X10^5$ Helpers)*			
EXPT #	$3X10^{5**}$ Resp	$3X10^4$ Resp	anti-Thy-1	anti-T	anti-Ly-1	anti-Ly-2
1	2.5	0.0	0.0	nd	0.0	3.7 (0.0)
2	82.4	0.0	nd	nd	6.5 (0.0)	44.9 (0.0)
3	42.0	0.0	1.9 (0.0)	3.1 (0.0)	2.1 (0.0)	5.3 (0.0)
4	3.8	0.0	0.0	0.0	0.0	53.6 (31.1)
5	9.1	0.0	nd	nd	nd	2.3 (0.0)
6	17.8	0.0	nd	nd	nd	1.7 (0.0)

Table III-5 Ly Determinants On Helper Cells

- values given are lytic units per well
- (in brackets) - activity of treated cells alone
  - if not indicated, treated cells alone gave no response
- nd = not done
- Resp = Responder cells

* - expt. 1 had $0.7X10^5$ Helper cells
expts. 4 and 5 had $1.7X10^5$ Helper cells

** - expt. 1 had 10^5 Responder cells
expts. 4 and 5 had $2X10^5$ Responder cells

- expts. 1 and 4 - Helper cells were from tumour-bearing animals

4; and, there was some help from anti-Ly-1, anti-T and anti-Thy-1 treated Helper cells in some of the experiments. There is, however, an overall indication of the presence of a Ly-1^+, Ly-2^- population of helper cells.

It appears therefore that the major population of Helper cells in the system consists of Ly-1^+,2^- and T^+, Thy-1^+ cells; placing them in the Th subclass of T cells. Having determined some of the antigenic determinants on the Helper cells, experiments were performed to reveal the Ly determinants on pCTL's. Because there was no CTL production when the total Responder cell population was treated with anti-Ly-2 Ab plus complement, and since Helper cells survive this treatment, the pCTL's were shown to be Ly-2^+.

The results of an experiment which demonstrates that the pCTL's are Ly-1^+ are summarized in table III-6. Although there was a low lytic unit production in the experiment, sufficient help was available from anti-Ly-2 treated Helper cells to produce a positive response from 10^5 control Responder cells. There was no response when anti-Ly-1 treated Responder cells plus anti-Ly-2 treated Helper cells were cultured together; thus demonstrating that the pCTL's are Ly-1^+,2^+ T cells.

Therefore it has been shown that the major population of Helper cells in this system are Ly-1^+,2^- and that the pCTL's are Ly-1^+,2^+ and that they are both T cells.

III-2-2 Comparison With An Allogeneic System

Along with the syngeneic tumour system, an allogeneic system, the

CELL NUMBER	TREATMENT	ACTIVITY (LU/well)
$3X10^5$	control cells	8.9
10^5	control cells	0.0
$3X10^5$	anti - Ly-1	0.0
$3X10^5$	anti - Ly-2	0.0
10^5 plus $2X10^5$	anti - Ly-1 plus anti - Ly-2	0.0
10^5 plus $2X10^5$	control cells plus anti - Ly-2	5.2

Table III-6 Ly Determinants On pCTL's

- known: pCTL's are Ly-2^+
  - because Helper cells are Ly-2^- and there is no response when the total cell population is treated with anti-Ly-2 Ab plus complement
- results: pCTL's are Ly-1^+
  - because 10^5 anti-Ly-1 treated Responder cells plus $2X10^5$ anti-Ly-2 treated Helper cells produced no activity
- conclusion: pCTL's appear to be Ly-1^+,2^+
- treated cells are incubated with complement after the Ab

CBA anti-DBA/2 mouse MLC was studied. This comparison was done to confirm that the procedures developed for the syngeneic LDA were reasonable. pCTL frequencies had already been determined for the allogeneic CBA anti-(CBA X DBA/2)F1 response (162,242). After irradiated DBA/2 spleen cell Stimulators had been cultured with CBA spleen cell Responders, cytotoxicity was tested against ^{51}Cr labelled P815X cells which carry the same H-2 determinants as DBA/2 cells.

As in the syngeneic tumour system, there was an optimal range of Stimulator cell number for the maximum production of cytotoxic cells at a given Responder cell Number (fig. III-9). Comparing the two systems, lytic activity was obtained with as few as 10^4 Responders in the allogeneic system. In the syngeneic system, 10^4 Responder cells did not produce lytic activity unless SN2°MLC or Helper cells were present. Also the optimal number of Stimulator cells ($3X10^5$ or 10^6) was much higher in the allogeneic than in the syngeneic system ($3X10^3$).

In experiments with the same allogeneic cell combination (CBA anti-DBA/2), Sinclair et al. found that the optimum anti-DBA/2 mastocytoma cell response occurred with 100 fold fewer P815 Stimulator cells than DBA/2 Stimulator cells (239). This 100 fold difference in the optimal number of Stimulator cells may be related to the relative size of the cells; the P815 cell is much larger than DBA/2 spleen cells and therefore would present a larger surface. Since the target cell is the P815X mastocytoma, perhaps the CBA cells are also responding to tumour associated, as well as MHC, antigens. Another possible explanation is that irradiated P815X cells remain in the culture, and stimulate longer, than do irradiated DBA/2 cells. Sinclair et al. found the optimal dose of irradiated DBA/2 spleen Stimulator cells was ten fold higher compared

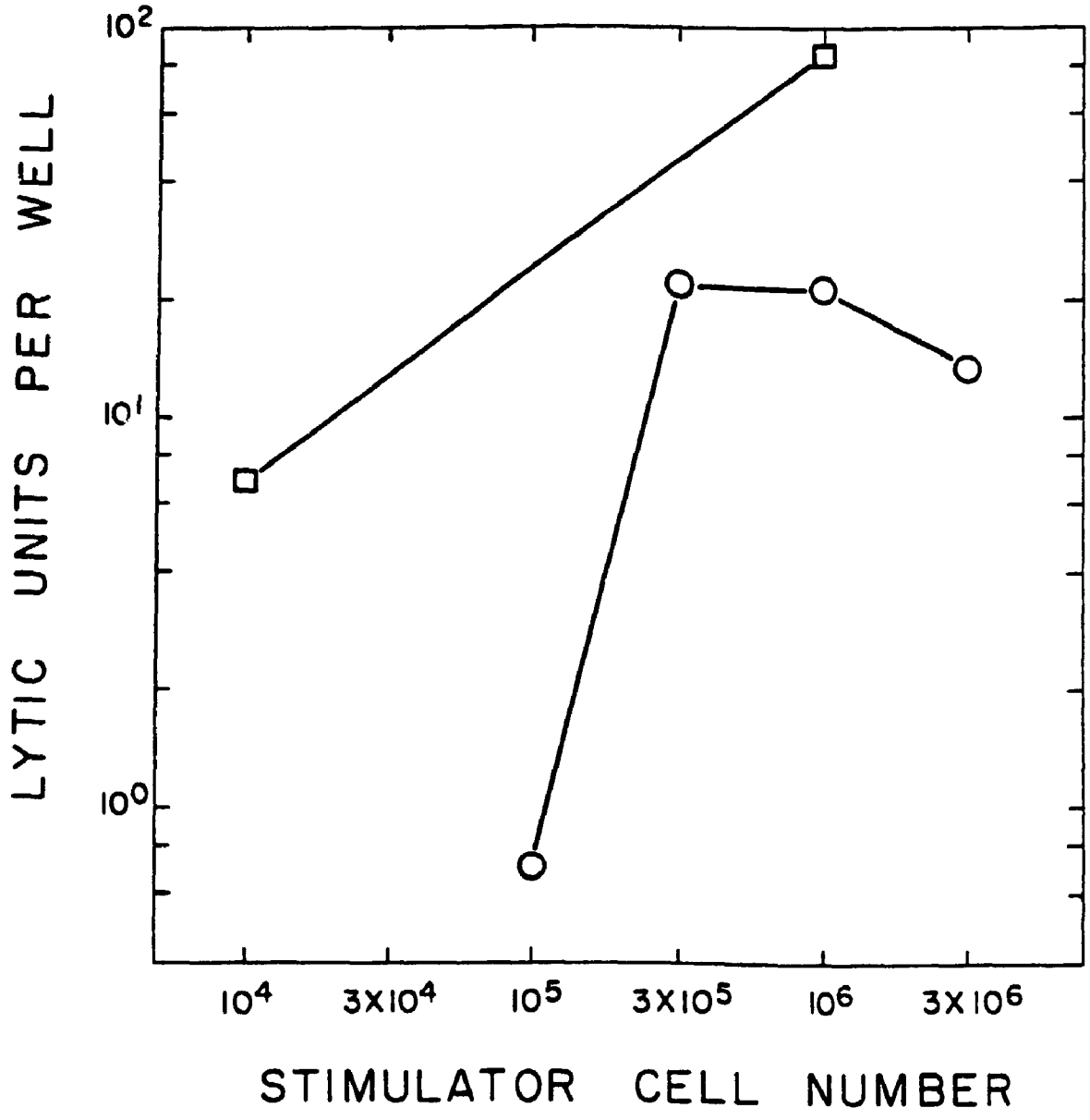


Figure III-9 Allogeneic system (CBA anti-DBA): optimal Stimulator cell number

- the open squares (□) and circles (○) represent the results from two separate experiments
culture conditions:
- 10^5 Responder cells

to unirradiated cells (239).

In a study of the Helper cells, CBA spleen cells were treated with anti-Ly-2 Ab plus complement. There was an increase in lytic activity with the addition of sufficient numbers of Helper cells to various numbers of Responder cells to bring the total cell number up to 10^5 (Figure III-10). In a further study, CBA spleen cells were incubated with anti-Thy-1, anti-Ly-1 or anti-Ly-2 Ab plus complement before being tested as Helpers. The anti-Ly-2.1 plus complement treated Helper cells gave sufficient help so that lytic activity was obtained with $3X10^3$ Responder cells (table III-7). With 10^4 Responder cells, these Helper cells produced 31.4 LU per culture well while anti-Thy-1 and anti-Ly-1.1 treated cells gave much less help (2.6 and 4.3 LU per well respectively) and produced no lytic activity when cultured with $3X10^3$ Responders. Therefore, a major population of helper cells in the allogeneic system (as in the syngeneic tumour model) appears to be $Ly\text{-}1^+,2^-$ T helper cells.

Using a limiting dilution assay, the minimum frequency of precursors in CBA spleens to DBA/2 spleen cell antigens was determined. The relatively high frequency obtained (484 per 10^6 spleen cells, 95% confidence limits 373-629, $X^2 = 3.93$) was in agreement with the lytic activity from pooled cultures of low Responder cell numbers. This frequency agrees well with determinations of approximately 500 and 620 per 10^6 cells for CBA spleen cells responding to (CBA X DBA/2)F1 spleen cells <u>in vitro</u> (162,242).

It may seem contradictory that an average of $3X10^2$ Responders per culture produced a measurable lytic activity (figure III-10), and yet the frequency of pCTL's was approximately 500 per 10^6 spleen cells or

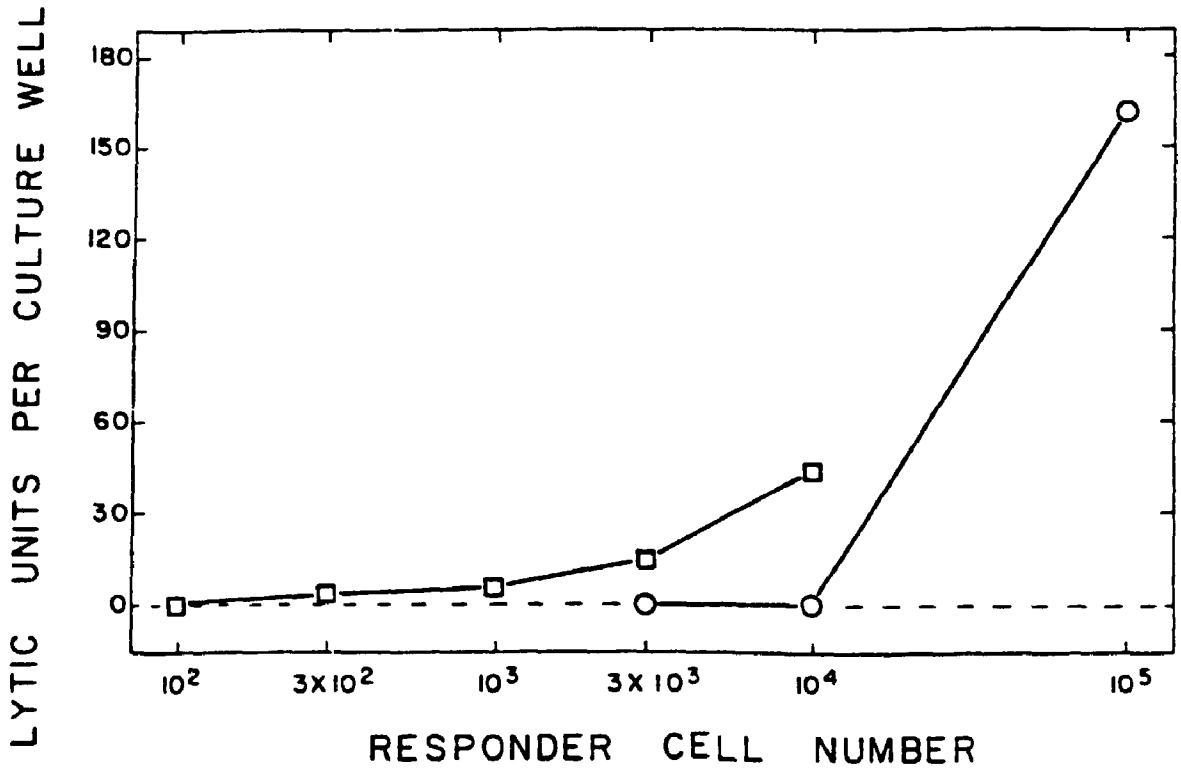


Figure III-10 Allogeneic system; LU production as a function of Responder cell number; the effect of addition of Helper cells

□ - with Helper cells - sufficient Helper cells were added to bring the total cell number to 10^5

○ - no added Helper cells

note: 10^5 Helper cells alone gave no response

CELL NUMBER	CONTROL (Responder cells alone)	TREATMENT OF HELPER CELLS		
		anti-Thy-1	anti-Ly-2.1	anti-Ly-1.1
10^5 treated cells alone		0.0	0.0	0.0
10^4 Resp plus $9X10^4$ treated cells	0.0	2.6	31.4	4.3
$3X10^3$ Resp plus $9.7X10^4$ treated cells	0.0	0.0	19.8	0.0

Table III-7 Antigenic Determinants On Helper Cells In The Allogeneic System

- values expressed as Lytic Units per microwell
- 10^5 Responder cells produced 106.5 LU per well
- cells are treated with Antibody plus complement
- Resp = Responder cells

1 per 2000. For the chromium release curves, the contents of 24 culture wells were routinely pooled and thus, at $3X10^2$ Responders per well, the response was actually measured in $7.2X10^3$ total Responder cells. When lytic activity is expressed as average number of lytic units per culture well, the calculation is based on the pooled population of cells and not on measuring the lytic units produced from each culture well separately and then determining a mean. At a frequency of 500 per 10^6, there should be an average of 3.6 precursors in the twenty-four wells, and it is the cytotoxicity produced from the stimulation of these 3.6 precursors that is being assayed for in the lytic unit measurement. Therefore the results of the pooled cultures showing activity with as few as $3X10^2$ Responder cells per culture and the LDA showing 1 pCTL in 2000 are not in disagreement.

III-2-3 Radiation Sensitivity

The radiation sensitivity of the pCTL's, Helper and Effector cells was determined in order to further characterize the cells.

Radiation dose-response curves were obtained for Responder cells irradiated *in vitro* prior to the culture period and for Effector cells irradiated prior to the assay culture period. The response was measured as lytic units produced per 10^6 Effector cells or per culture well.

Radiation doses up to 6.0 gray, just prior to the assay period, had little or no effect on Effector cells (fig. III-11). For Responder cells, a dose of 6.0 gray reduced lytic activity to a level of 0.5%. After a dose of 4.0 gray, the lytic unit production was reduced to

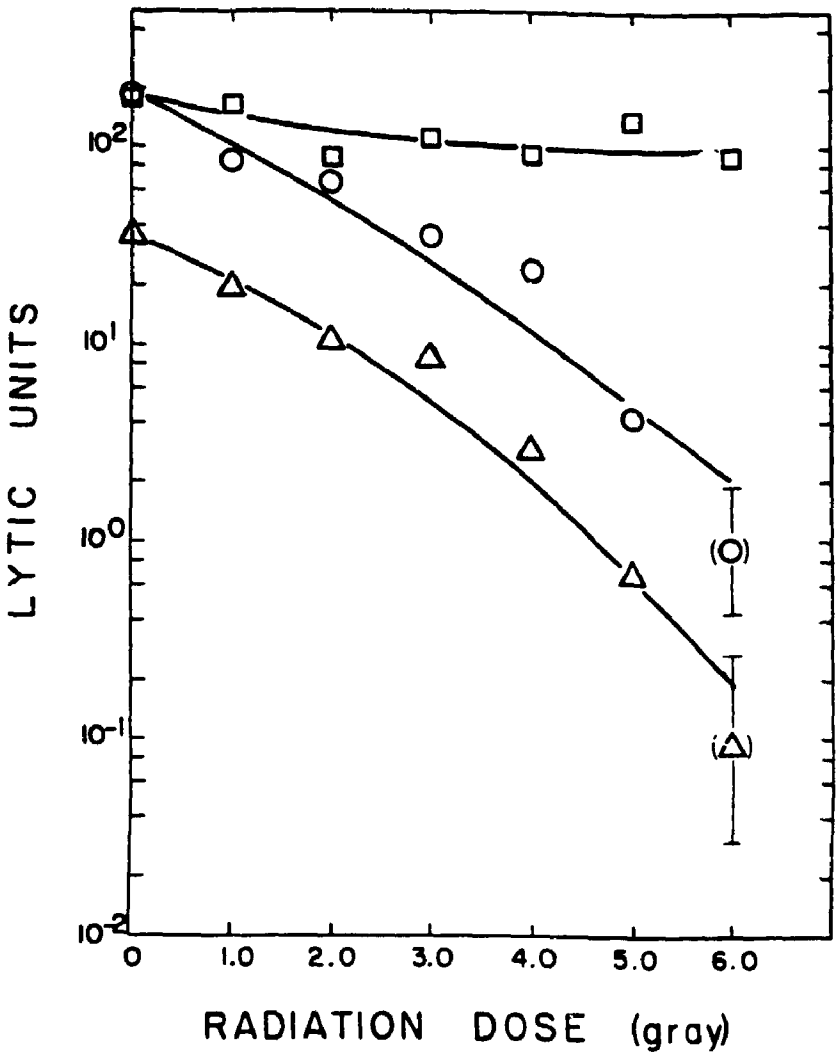


Figure III-11 Radiation sensitivity of Effector and Responder cells

Responder cells irradiated pre-culture
○ - lytic units produced per 10^6 cells
△ - lytic units produced per culture well
(brackets) and error bars indicate estimates and their ranges
Effector cells irradiated pre-assay
□ - lytic units produced per 10^6 cells
- the curves are the best fit lines to the expression $S = e^{-(\alpha D + \beta D^2)}$
where: S = surviving fraction, D = radiation dose
note: this is not a survival curve, but rather a radiation response curve
- the effect on cytotoxic cells (Effector cells) does not incorporate the ability to survive (usually tested by the capability of reproduction)
culture conditions:
- 10^6 Responder cells per culture
- $3X10^3$ Stimulator cells per culture

approximately 5.7% of that of unirradiated cells. This level was similar to that of an experiment in which a whole-body in vivo dose of 4.0 gray was given to live mice and then Responder cells were obtained from the spleens. There, the lytic units produced per culture decreased from 10.3 for unirradiated mice to 0.3 (to a level of 2.9%). After a dose of 8.0 gray, there was no detectable activity. Irradiation experiments involving live mice were carried out with a ^{137}Cs Gammacell 20 (AECL, Ottawa, Canada) at a dose rate of 1.5 gray per minute.

It should be noted that these curves do not represent classical cell survival curves but rather the ability of the cells to respond and produce lytic activity after irradiation. They measure the survival of a function. The curves fitted to the data in figure III-11 derived from the survival expression:

$$S = e^{-(\alpha D + \beta D^2)}$$

where: S = surviving fraction

D = radiation dose

This expression (called the linear-quadratic or α,β model) has been found to fit dose-response curves well over at least the first couple of logs of survival (34,35,51,107,130).

Because all of the cells capable of responding had been irradiated in these experiments, it was impossible to determine whether an effect on pCTL's or possibly on the Helper cells was responsible for the shape of the curve. In an experiment to answer this question, 10^5 Responders were irradiated and then cultured with $2X10^5$ Helper cells (treated with anti-Ly-2 Ab plus complement): Figure III-12 shows the resulting dose-response curve.

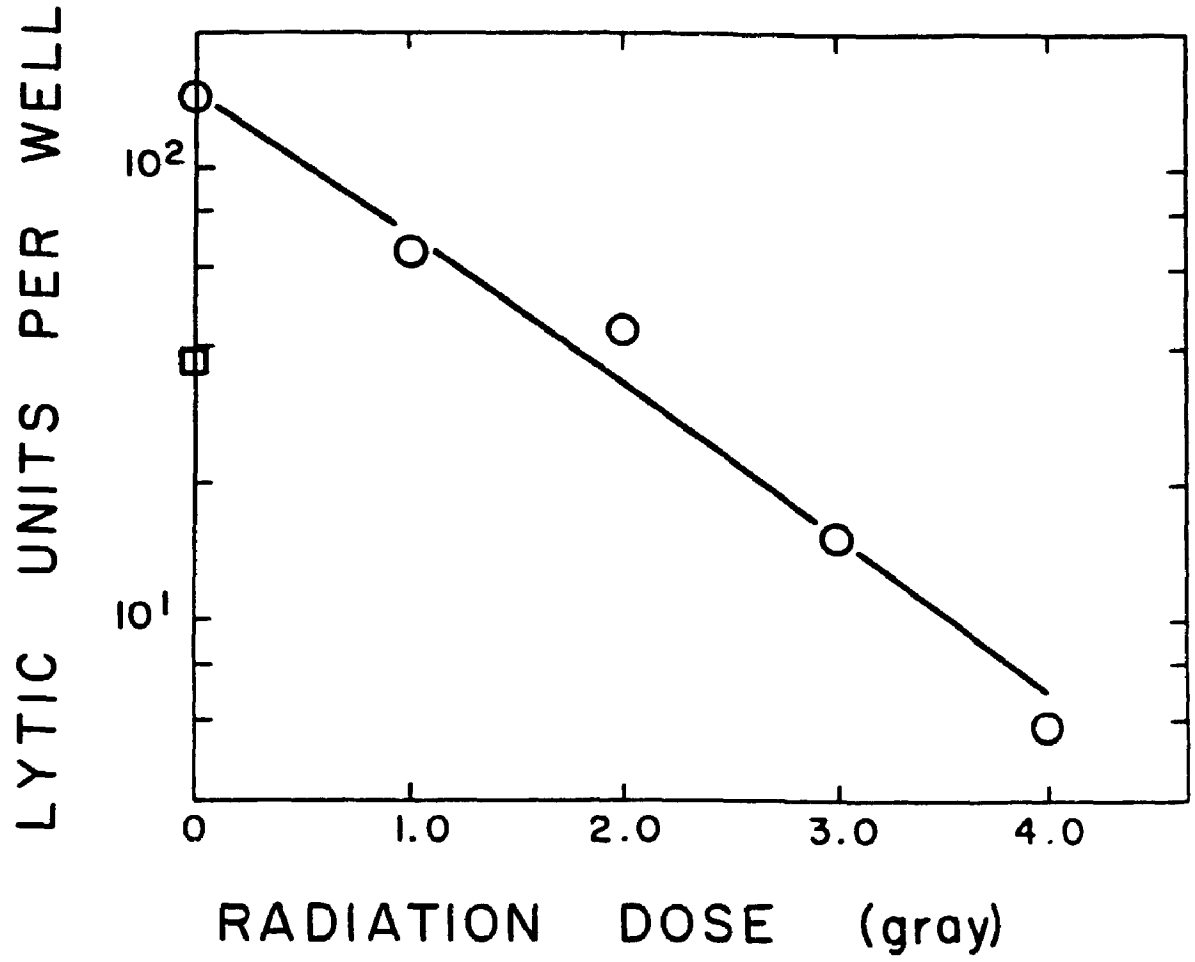


Figure III-12 Radiation sensitivity of 10^5 Responder cells with the addition of $2X10^5$ unirradiated Helper cells per culture well

○ - radiation response of 10^5 Responder cells together with $2X10^5$ Helper cells per culture well

□ - 10^5 Responder cells alone

- with $2X10^5$ Helper cells alone, there was no response
- the curve represents the least squares best fit to the expression

$$S = e^{-(\alpha D + \beta D^2)}$$

where: S = surviving fraction and D = radiation dose

- with this curve, $\alpha = 0.69$, $\beta = 0.017$

There were sufficient Helper cells in all cultures to increase the response of 10^5 unirradiated Responder cells from 37 to 142 LU per culture. Since Helper cells alone produced no lytic activity, the dose response curve was due to the survival of pCTL's alone. In this experiment, the activity after 4.0 gray was 5.6% of that of unirradiated cells - very similar to the 5.7% when the total Responder cell population was irradiated and no Helper cells were used (figure III-11).

To a limited extent, the radiation response of the Helper cells was determined also. When 10^5 Responders were cultured with $2X10^5$ Helper cells, the Helper cells increased the Responder cell activity from 37 to 143 lytic units per well while producing no lytic activity themselves. After a dose of 4.0 gray to the Helper cells, the activity was still 95 LU per well, indicating that the helpers are relatively radiation resistant.

In an attempt to extend the response curve for Helper cells, doses up to 12.0 gray were given (fig.III-13). However, in this experiment the pCTL's in the Helper cell population were not affected by the Ab plus complement treatment and produced almost as great a LU value as the Helper and Responder cell combination (183 vs 206 LU per culture well). In spite of this problem, the results of the experiment are still valid showing that some help is produced even after a dose of 12 gray. Because it has been shown that 6.0 gray reduces the LU production to less than 1% (fig. III-11), the doses at 6.0 gray or greater would have inactivated the pCTL's present and any increased response due to the addition of these cells was due to their helper activity and not due to any surviving pCTL's. Even after a dose of 12.0 gray, the radiation response curve should not fall below the level of lytic activity for 10^5

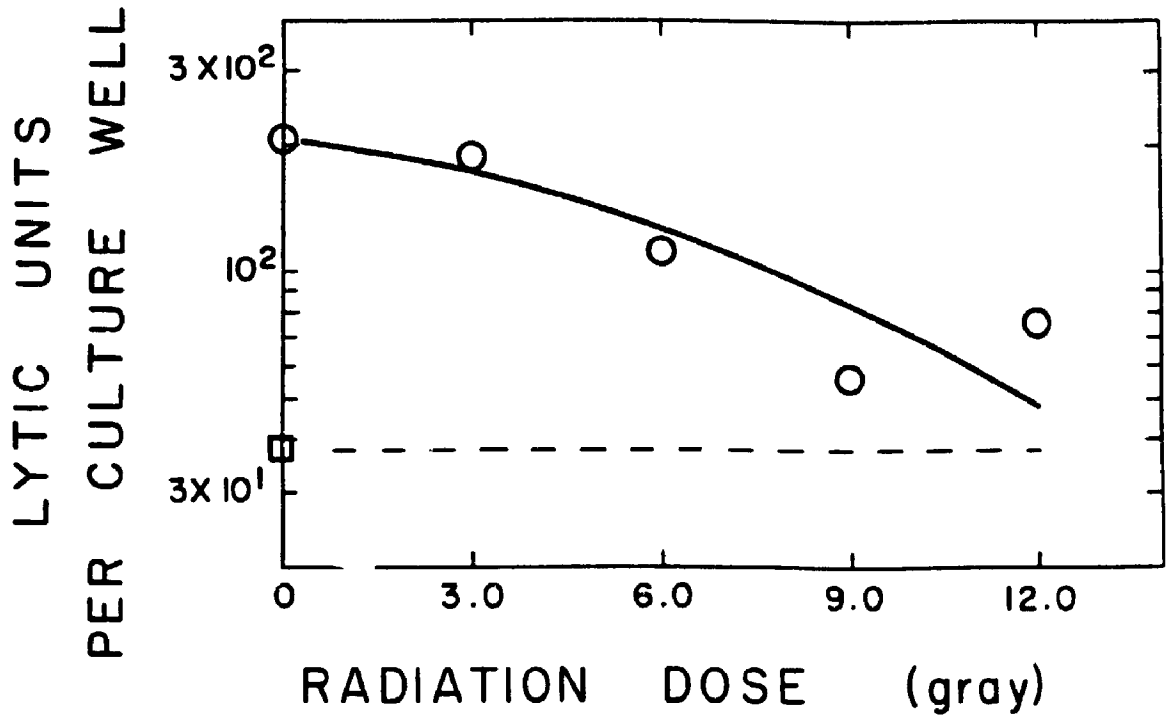


Figure III-13 Radiation sensitivity of Helper cells

○ - 10^5 unirradiated Responder cells and $2X10^5$ irradiated Helper cells

□ - 10^5 unirradiated Responder cells alone

note:

- Helper cells were from tumour - bearing animals
- anti-Ly-2 plus complement treatment did not remove pCTL's; however we know that at 6.0 gray, pCTL activity is reduced by at least 99% (figure III-11); therefore at 6 gray or greater, the contribution to the lytic activity from Helper cells is minimal
- the dotted line represents the lowest possible extent of the radiation response curve
- the solid line represents the least squares best fit to the expression

$S = e^{-(\alpha D + \beta D^2)}$

where : S = surviving fraction, D = radiation dose

Responder cells alone since those cells were not irradiated. Perhaps the helper cells are radioresistant relative to the pCTL's because cell division may not be a requirement for the expression of help.

It appears then that the pCTL's are relatively radiation sensitive with a dose of 6.0 gray reducing the lytic activity to less than 1% of that of unirradiated cells. On the other hand, the helper cells appear to be relatively radiation resistant with some helper activity being present even after 12.0 gray.

CHAPTER IV THE CORRELATION OF pCTL FREQUENCY WITH TUMOUR GROWTH

Chapter III presented the results of work done to optimize the culture and assay systems along with experiments to characterize the cells participating in the production of cell-mediated cytotoxicity against a syngeneic tumour.

The purpose of the work presented in this chapter is to provide answers to four questions:

1) Does the response (lytic unit production and pCTL frequency) change as a function of the time interval after tumour implantation?

2) a) Is there a correlation between tumour size and pCTL frequency in the spleens of tumour-bearing animals and b) Is there a correlation between tumour size and the presence of suppressor cells?

3) Is there a correlation between pCTL frequency in normal animals and tumour growth after tumours have been implanted in them?

4) Do pCTL's occur in other organs of the mouse and what is their frequency?

Each section in chapter four will deal with the experiments designed to answer one of these questions.

IV-I THE RESPONSE AS A FUNCTION OF THE TIME INTERVAL SINCE TUMOUR IMPLANTATION

Experiments were performed to determine whether there were changes in the pCTL frequency and in the lytic activity of the progeny of these

cells as a function of the time after tumour implantation. Spleen cells from both normal and tumour-bearing mice were studied using both the lytic unit assay and the LDA.

Preliminary studies - no Helper cells or SN2°MLC

Previous studies in this laboratory, using spleen cells as Responders in a Marbrook culture system, indicated that cells from normal animals produced no cytotoxicity while those from tumour-bearing animals generated lytic activity (241). In the research for this thesis, when no Helper cells or SN2°MLC was added to the culture microwells, the response with cells from normal animals was always very low or negligible compared to the response with cells from tumour-bearing animals (fig. IV-1).

Since spleen cells from tumour-bearing animals could be stimulated in vitro to develop cytotoxicity against the P815X tumour, it was determined whether spleen cells from normal animals, after an attempted immunization, would show an equal response. The injection of either $3X10^6$ or $3X10^7$ lethally irradiated P815X cells did not produce an appreciable cytotoxic response when the spleen cells were later used as Responder cells in culture (fig. IV-2).

Early experiments were done to assess the cytotoxicity produced (in lytic units) as a function of time after tumour implantation. No Helper cells or SN2°MLC were used in these studies and therefore the results can be compared with previous work in this laboratory (241). In each of five experiments, cells were cultured from pools of animals which had been carrying tumours for various times and lytic unit production was measured (fig. IV-3).

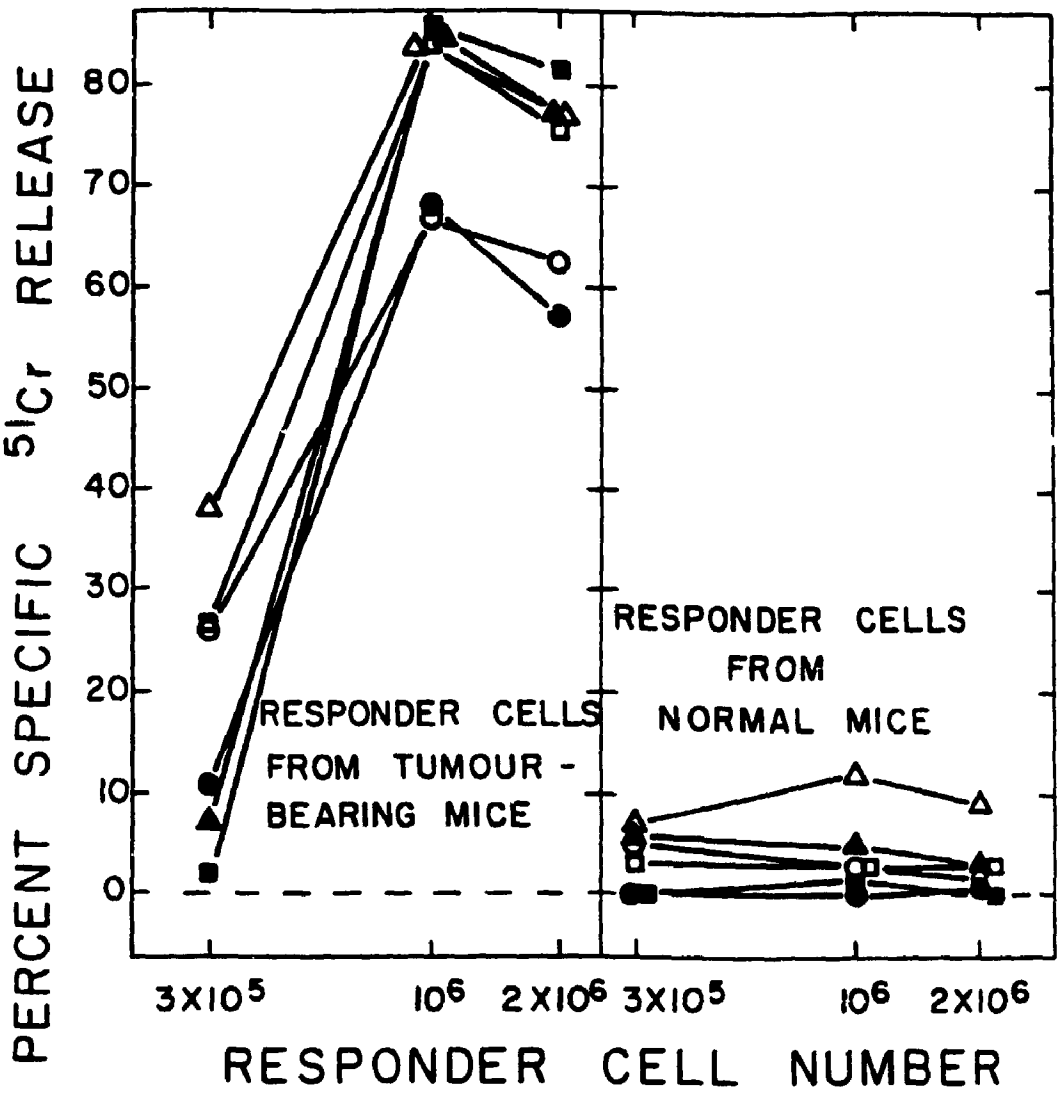


Figure IV-1 Response from normal spleen cells versus that from tumour-bearing animals - early studies

Responder cells from either normal (non-tumour-bearing) or tumour-bearing animals were cultured with either $3X10^3$ or 10^4 Stimulator cells. The resulting Effector cells were assayed with varying Target cell numbers.

	Stimulator Cell Number	Target Cell Number
● -	10^4	10^4
■ -	10^4	$3X10^3$
▲ -	10^4	10^3
○ -	$3X10^3$	10^4
□ -	$3X10^3$	$3X10^3$
△ -	$3X10^3$	10^3

culture conditions:
- $5X10^{-5}$ M 2-me present
- no SN2°MLC or Helper cells present

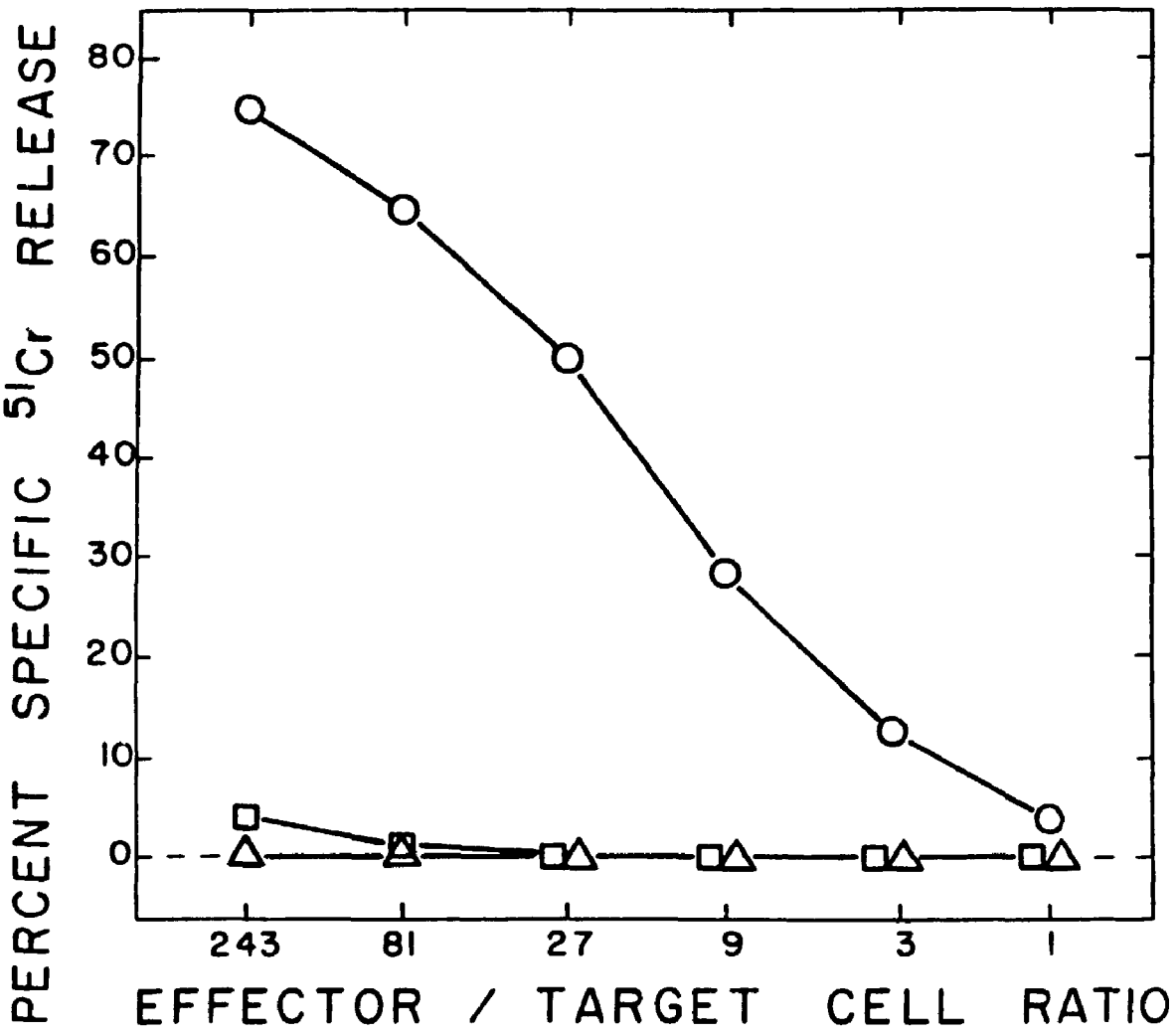


Figure IV-2 The response after an attempted immunization of normal animals

An attempt was made to immunize mice by a subcutaneous injection of either $3X10^7$ or $3X10^6$ lethally irradiated P815 tumour cells. After ten days, Responder cells from the spleens of these animals were tested, together with cells from tumour-bearing animals ($3X10^6$ live tumour cells injected ten days previously) in a ^{51}Cr release assay. Cells from tumour-bearing animals produced 23.0 LU per culture well.

Responder Cell Source

○ - tumour-bearing animals
□ - mice "immunized" with $3X10^7$ irradiated P815 cells
△ - mice "immunized" with $3X10^6$ irradiated P815 cells

culture conditions:
- 10^6 Responder cells, $3X10^3$ Stimulator cells, 10^3 Target cells per well
- no SN2°MLC or Helper cells present

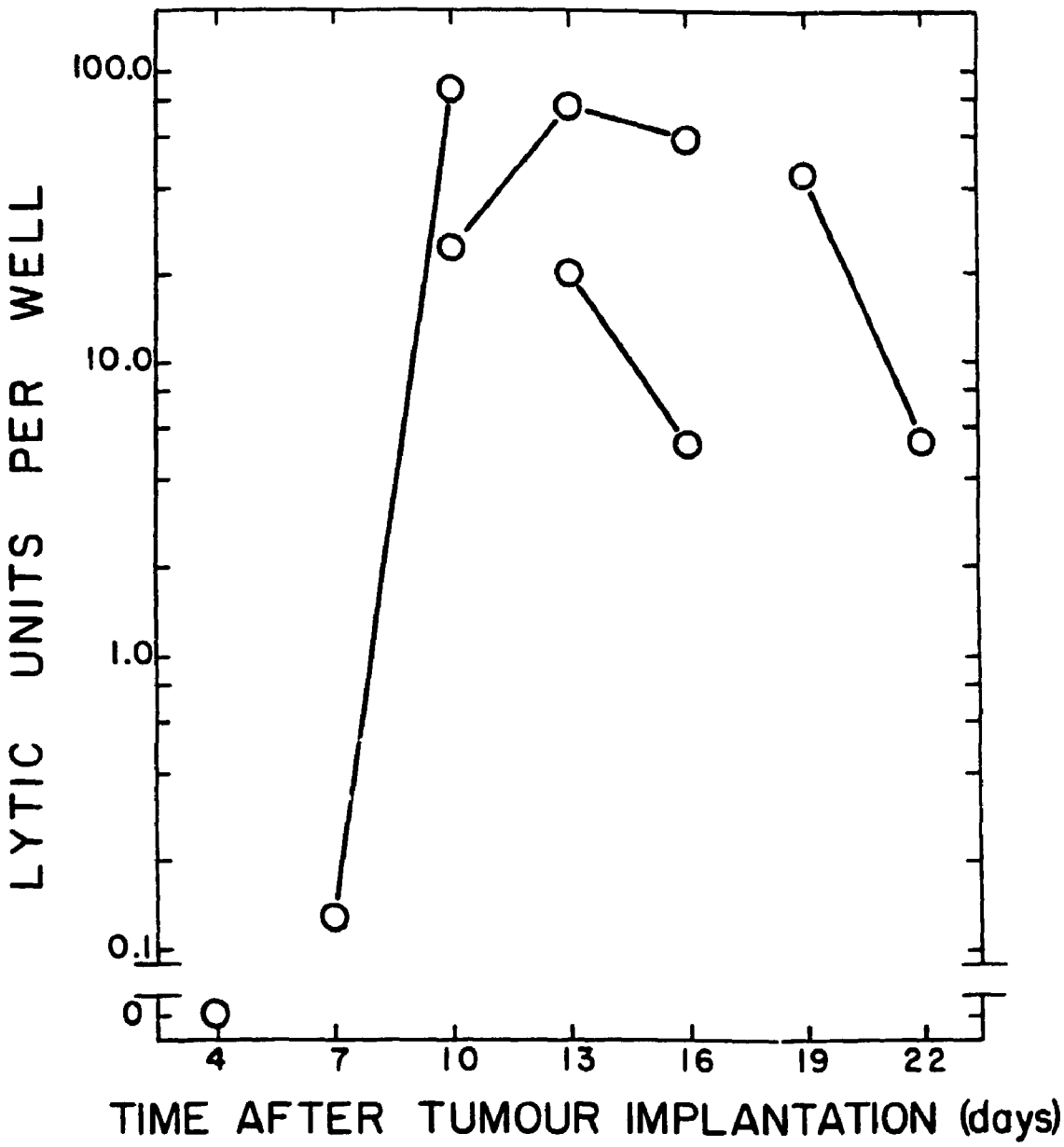


Figure IV-3 Effect of the length of tumour growth on lytic unit production - early studies

The results of five different experiments are presented. Solid lines join data points within the same experiment.
culture conditions:
- 10^6 Responder cells and $3X10^3$ Stimulator cells per culture well
- no SN2°MLC or Helper cells present
- points below the break in the Y axis have zero magnitude

The general shape of the response curve was similar to that generated in the original study (241). Cells from animals injected four days previously yielded no response; the peak activity occurred at about two weeks of tumour growth, after which the lytic unit production declined.

When spleen cells from tumour-bearing animals were used, there was a possibility that they were receiving a second stimulation in vitro: the first stimulation would be provided by the environment in the tumour-bearing host. Reculturing the Effector cells in vitro might stimulate cells from normal mice to produce CTL's to the same extent as those from tumour-bearing animals. Spleen cells from normal animals did not produce lytic activity upon a second culture period in vitro of five days, while cells from tumour bearers responded (table IV-1).

Studies with Helper cells and SN2°MLC

The results of 46 determinations of lytic activity, each measured in pooled cultures of spleen cells from groups of two or three animals, are presented in figure IV-4. The number of lytic units per culture well is plotted as a function of the time interval after tumour implantation, each point representing a single experiment. The results from normal animals are at time zero. Each culture well contained $3X10^4$ Responders (spleen cells) and $3X10^3$ Stimulators. Culture wells containing 50% SN2°MLC are indicated by open circles.

Whereas there was no response in any of the experiments without SN2°MLC, lytic activity was seen in 43 out of 46 experiments when SN2°MLC was present. There appeared to be an increase in the lytic activity of cells derived from pCTL's from mice two weeks after tumour injection

SOURCE OF RESPONDER CELLS	RESPONDER CELL NUMBER		LYTIC UNITS PRODUCED	
	First Culture	Second Culture	per 10^6 Effector cells	per well
Tumour-Bearing Animals	10^6	10^6	168.4	35.9
	10^6	$3X10^5$	307.7	31.4
	$3X10^5$	$3X10^5$	534.8	52.8
	10^5	$1.58X10^5$	0.0	0.0
	$3X10^4$	$3.6X10^4$	0.0	0.0
Non-Tumour-Bearing (normal) Animals	10^6	10^6	0.0	0.0
	10^6	$3X10^5$	0.0	0.0
	$3X10^5$	$3X10^5$	0.0	0.0

Table IV-1 The Effect Of Culturing Responder Cells On A Second Occasion

- there were two culture periods of five days each
- culture conditions:
  - no SN2°MLC or Helper cells
  - no 2-me used
  - 23 hour ^{51}Cr release assay
  - $3X10^3$ irradiated P815 Stimulator cells in each culture well

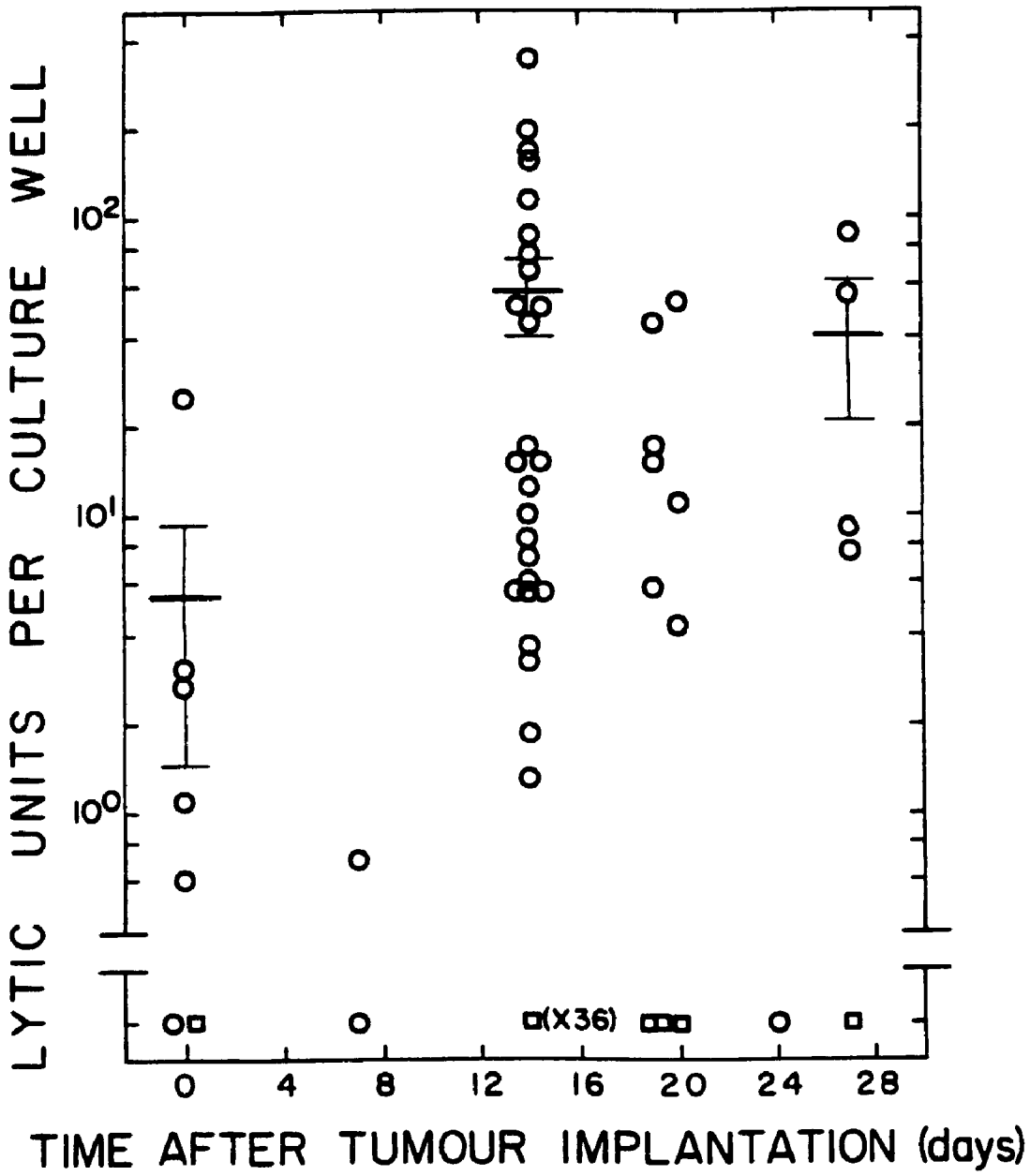


Figure IV-4 Lytic activity from cultured spleen cells versus the time interva since tumour implantation: $3X10^4$ Responder cells per culture well

O - with SN2°MLC in the culture medium
—- mean ± 1SEM for cultures with SN2°MLC at
day 0: 5.42 ±3.95
day 14: 56.7 ±16.0
day 27: 40.7 ±19.9
□ - without SN2°MLC in the culture medium
culture conditions:
- $3X10^3$ Stimulators per culture well
- with 2-me
- points below the break in the Y axis have zero magnitude

compared to normal animals. After two weeks, lytic activity leveled off or declined slightly.

When 10^5 Responder cells were cultured, the same increase in lytic activity from zero time to two weeks was observed (fig. IV-5). In this case there was positive activity in the 23 experiments which included SN2°MLC, but also in 19 out of the 33 experiments without it.

Sinclair et al. (241) have reported an increase in lytic unit production from zero for cells from normal animals to a maximum after two weeks of tumour growth. Although those experiments were done with the same syngeneic tumour system, Marbrook cultures were used without the addition of 2-me or SN2°MLC. With the addition of 2-me and SN2°MLC to the culture medium, we were able to obtain a response using cells from animals which had not been injected with tumour cells. As Sinclair et al. (241) found, no measurable response occurs in the absence of SN2°MLC. There was an increase in lytic activity from cultured cells during the first two weeks of tumour growth using either system.

The pCTL frequency in the spleens also increased after tumour implantation, thus corresponding to the increased lytic activity production in cells from tumour-bearing animals (fig. IV-6). The frequency appears to be low in normal animals at zero time and reaches a plateau at about two weeks of tumour growth. At zero time the mean was 57.4 (±17.6;SEM) pCTL per 10^6 cells and at two weeks, 167.4 (±63.9;SEM) pCTL per 10^6 cells.

To assess whether the increase in pCTL frequency over time was statistically significant has proved difficult. Normally the test used to assess differences of means among more than two groups is the analysis of variance (ANOVA). However the ANOVA has several important,

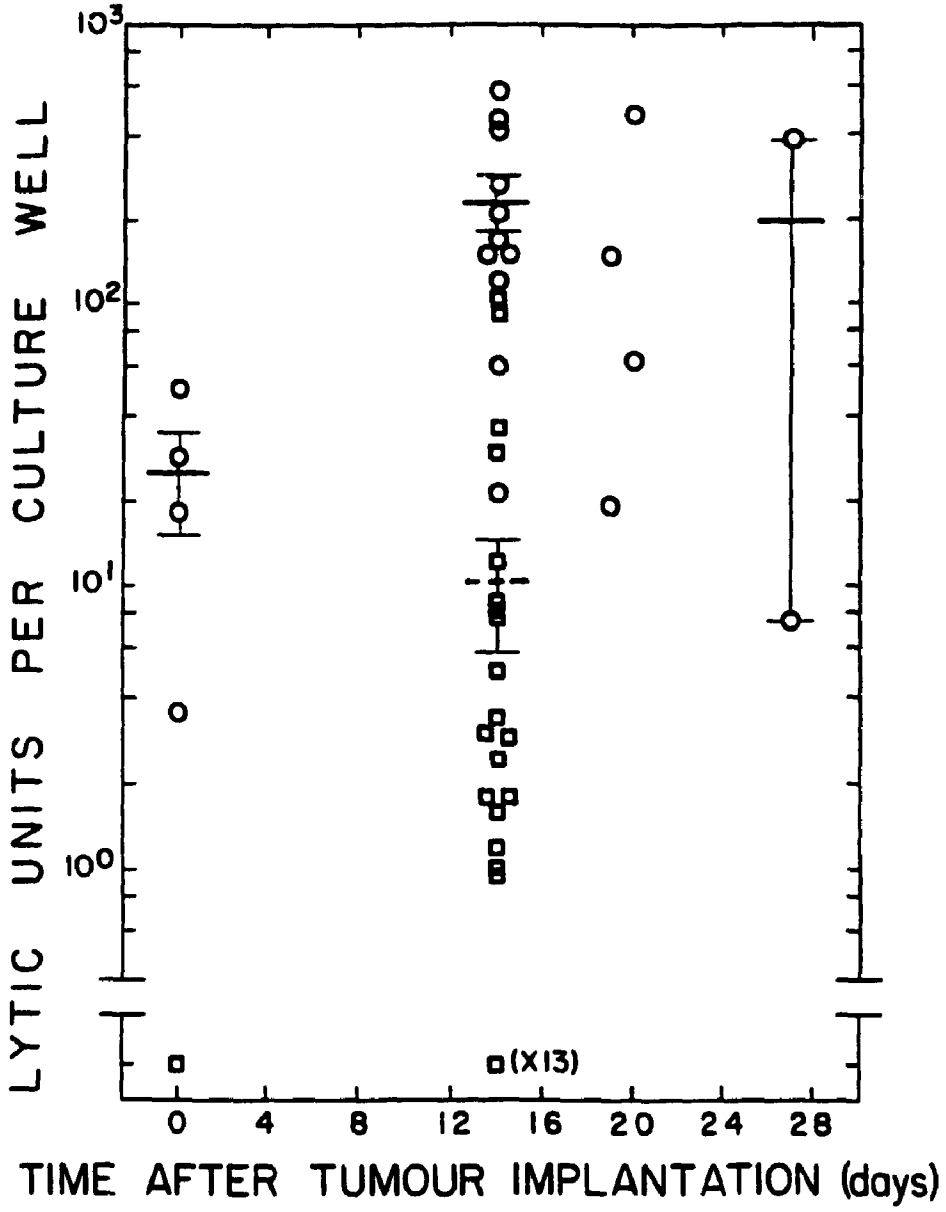


Figure IV-5 Lytic activity from cultured spleen cells versus the time interval since tumour implantation: 10^5 Responder cells per culture well

○ - with SN2°MLC in the culture medium
—— - mean ± 1SEM for cultures with SN2°MLC at
day 0: 25.4 ±10.0
day 14: 237.2 ±53.1
day 27: 198.3 ±190.9
□ - without SN2°MLC in the culture medium
– – - mean ± 1SEM for cultures without SN2°MLC at
day 14: 10.2 ±4.4

culture conditions:
- $3X10^3$ Stimulators per culture well
- with 2-me
- points below the break in the Y axis have zero magnitude

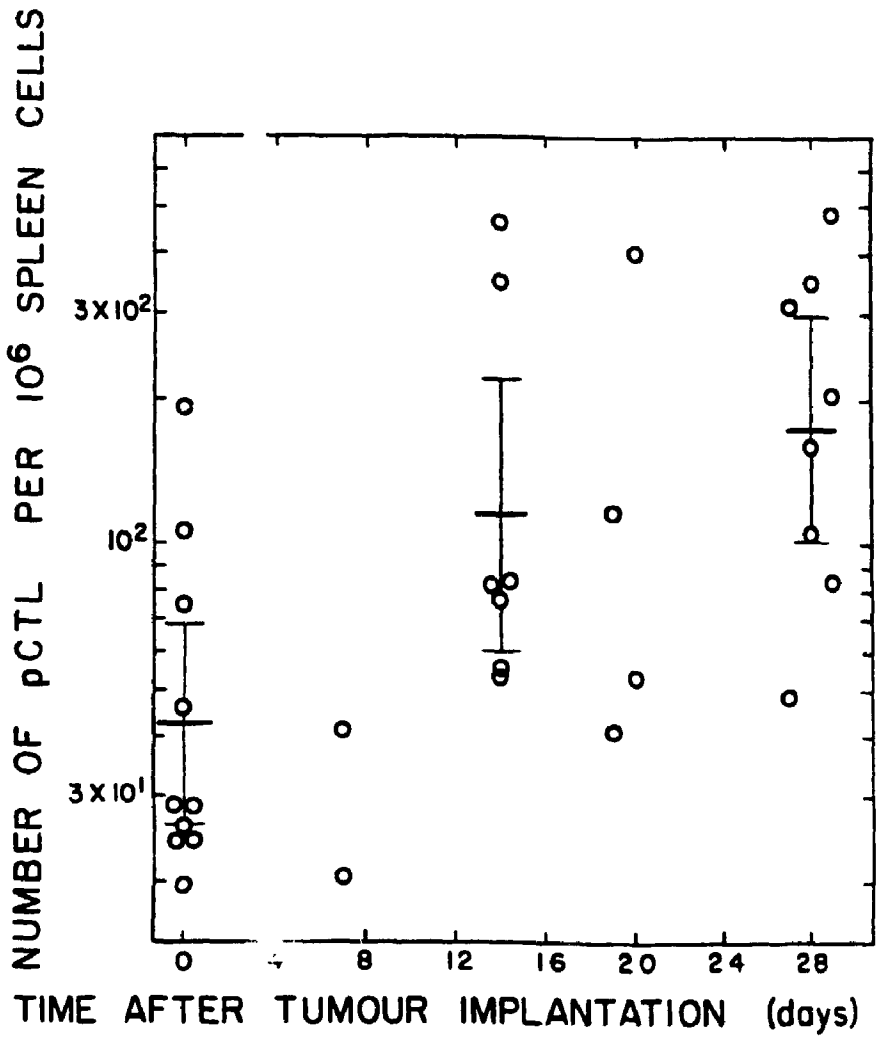


Figure IV-6 pCTL frequency in spleen cells as a function of time after tumour implantation

O - pCTL frequency measured by LDA
—— - mean and 95% confidence limits of log-transformed data at 0, 2, and 4 weeks

statistics:
untransformed data
- mean - time 0 = 57.4 (±17.6;SEM) pCTL per 10^6 cells
- time 2 weeks = 167.4 (±63.9;SEM) pCTL per 10^6 cells
- time 4 weeks = 220.6 (±53.6;SEM) pCTL per 10^6 cells

log - transformed data
- mean - time 0 = 42.3 (26.2-67.8; 95% confidence limits)
- time 2 weeks = 114.8 (59.7-220.8; 95% confidence limits)
- time 4 weeks = 174.2 (102.5-295.9; 95% confidence limits)

test for difference of means at 0, 14 and 28 days:
- ANOVA; F = 3.33 (0.05>P>0.025)
- ANOVA (log-transformed); F = 7.47 (0.005>P>0.0025)
- Kruskal-Wallis; H = 10.10 (0.01>P>0.005)

culture conditions:
- with SN2°MLC
- with 2-me

often over-looked, requirements (80,303). The first is that the variances of the samples be the same since a common population variance is estimated by the pooled variance. The second is that all the samples have come from normally distributed populations.

A test such as Bartlett's could be used to determine equality of variances but it is inefficient and adversely affected by non-normality (303). Moreover, there do not appear to be any good tests for normality when the sample size is less than twenty. Although we may use the ANOVA, we really cannot determine whether it is the appropriate statistical test. When faced with non-normal populations, Zar (303,p133) suggests transforming the data to their logarithms and performing an ANOVA. When sample variances are proportional to the mean values, a ln (natural logarithm) transformation would also have the effect of making the variances more equal.

There are a number of non parametric tests, such as the Kruskal-Wallis analysis of variance by ranks, and the Mann-Whitney-U (MWU) test, which can be used to test for differences between sample means. The Kruskal-Wallis test can be used instead of the ANOVA and it is not affected at all by inequality of variances nor by non-normal distributions. The Mann-Whitney-U test is used to compare two samples. Since transformations do not alter the ordering of values, none of the non-parametric tests is affected by manipulations such as ln transformations.

Because of the problems in deciding which statistical test was appropriate, we used three (i.e. ANOVA, ANOVA of ln transformed data and a non-parametric test) to determine differences between groups of data. If all three tests gave the same statistical result (i.e. all P

values < 0.05), then only one test may be reported. If the estimates of significance were different by the various methods, then all results are given and an attempt is made to determine which is the more correct, and what conclusions to draw.

Using an analysis of variance of the data presented in figure IV-6 and taking as sample populations the groups of data at times 0, 14 and 28 days, we found a significant difference between the means of the three groups ($F = 3.33$, $0.05>P>0.025$). An ANOVA of the ln-transformed data yielded an F value of 7.47 which also showed a significant difference between the three groups ($0.005>P>0.0025$).

The ANOVA can only tell us that there is a statistical difference among the sample groups being compared, not which groups are different. Even though the P values of these two tests are quite different, they both indicate significant differences among the sample means.

Because of the non linear and apparently bimodal distribution of the data at two weeks, a Kruskal-Wallis nonparametric analysis of variance by ranks was chosen to test for significant differences between the sample groups (i.e. pCTL frequencies at 0, 14, and 28 days of tumour growth). Since it yielded an H value of 10.10, and anything over 5.99 would have been significant, the conclusion was that the pCTL frequencies at 0, 14 and 28 days were statistically different ($0.01>P>0.005$).

Thus we have used three statistical procedures to test for significant differences between the three main sample groups of data presented in figure IV-6. They yielded P value ranges of ($0.05>P>0.025$) for the ANOVA, ($0.005>P>0.0025$) for the ANOVA of ln-transformed data and ($0.01>P>0.005$) for the Kruskal-Wallis test. Even though all of the

ranges are different and none overlap, we can conclude that, statistically they show a real difference in the data since all three tests yielded a P value of less than 0.05. This indicates that there is less than a 5% probability that all three sample groups came from the same population and we accept that criterion for statistical significance.

After the Kruskal-Wallis test has shown a significant difference between the groups, we can use a non-parametric, multiple comparisons test for the differences between pairs of sample groups (303;pp199-201). This test, which does not require equal numbers of values in each group, yields a Q statistic which is compared to Q values in a standard table. The test showed a significant difference between the pCTL frequencies at 28 days and zero time ($0.01>P>0.005$), but not between the frequencies at zero and 14 days ($0.20>P>0.10$) nor between the frequencies at 14 and 28 days ($P>0.50$).

While the preceding analyses seem to support the conclusion that there is a low background pCTL frequency in the spleens of normal mice and that this frequency increases with time after tumour implantation, there are some points that should be made concerning drawing this conclusion from the data. First, in only one experiment were the pCTL frequencies from normal and tumour-bearing mice compared at the same time. Second, there was some grouping of data by experiment; for instance, the six lowest values for normal mice at time zero all came from the same experiment. If this experiment was ignored, then the increase in pCTL frequency from zero time to two weeks became much less. Third, the data represents a large number of experiments done over an extended period of time. Fourth, the data at 20 and 28 days, as will be

shown in section IV-2, can be subdivided into two groups. One part comes from animals with small tumours and the other from animals with large tumours. At later times (20-28 days), spleen cells from animals with small tumours yield higher pCTL frequencies.

The results of the one experiment in which the pCTL frequencies from normal and tumour-bearing animals were obtained at the same time are presented in figure IV-7. These data actually represent the results of two experiments. In each experiment, LDA curves were obtained for pools of two or three spleens from normal and tumour-bearing animals (two weeks after tumour implantation) with the addition of SN2°MLC or Helper cells to the culture. The results from the two experiments were combined into complete LDA curves by treating all data as if they were derived from a single experiment (204,262).

With Helper cells, the measured pCTL frequency increased from 30 per 10^6 cells in normal spleens to 137 per 10^6 in tumour-bearing animals. With SN2°MLC present in the cultures, the measured pCTL frequency increased 2.4 fold from 193 per 10^6 cells in normal spleens to 468 per 10^6 cells in the spleens of tumour-bearers. This 2.4 fold increase was approximately the same as the overall increase presented in figure IV-6 (from 57 to 167 pCTL's per 10^6 cells) supporting the conclusion that there was an actual increase in pCTL frequency after tumour injection.

While the pCTL frequency increased by a factor of 2.4 from normal to tumour-bearing animals when SN2°MLC was used, the amount of cytotoxicity produced, measured by the number of lytic units per culture well, appears to have increased by a much higher factor (table IV-2). This was a limited experiment in which an attempt was made to do a LU and a pCTL frequency determination on cells from both normal and tumour-

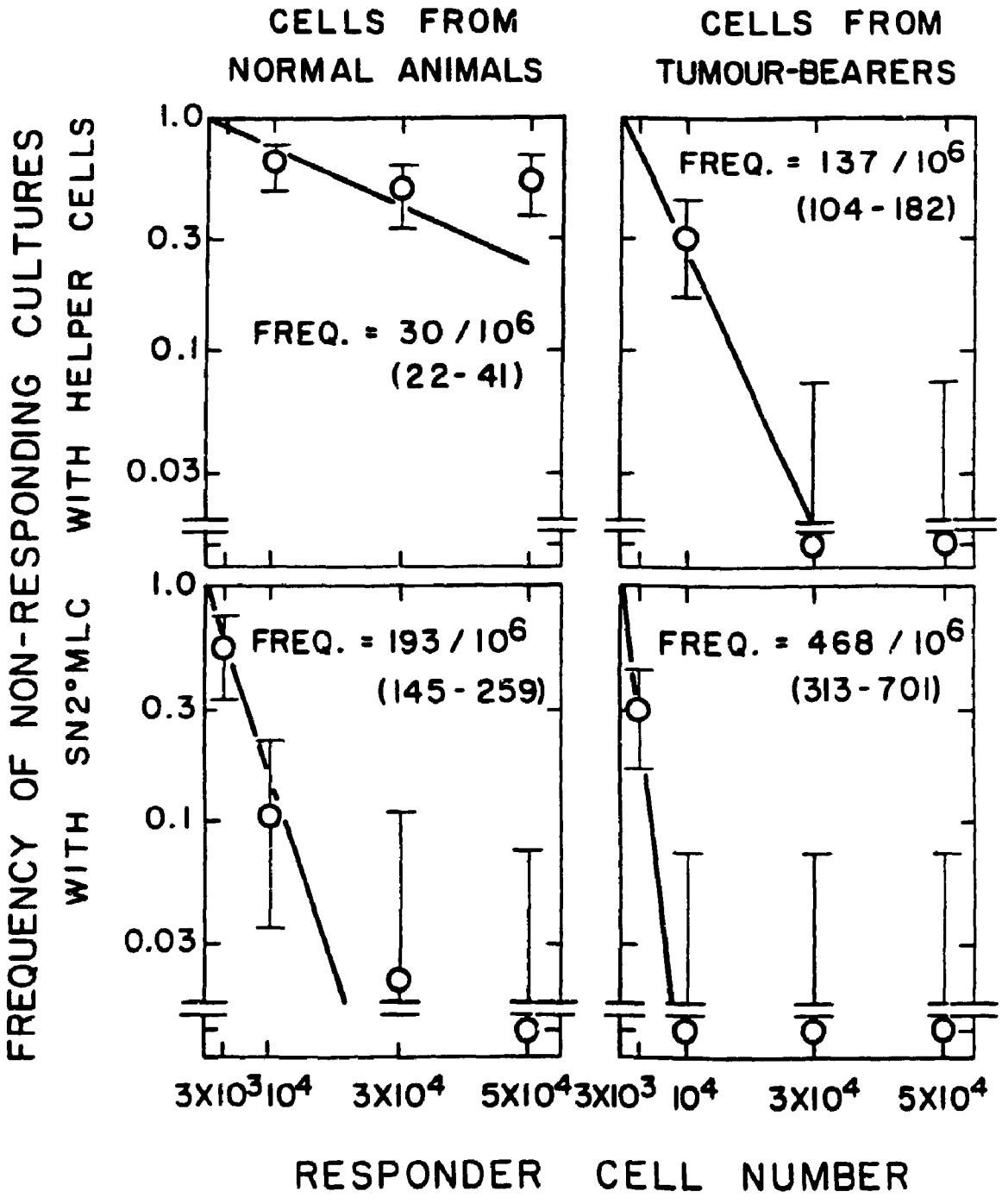


Figure IV-7 Combined results of two experiments using LDA's to compare pCTL frequencies in the spleens of normal and tumour-bearing mice

Frequencies of pCTL's per 10^6 spleen cells and 95% confidence limits (in brackets) are given. LDA results were obtained separately in two experiments and then combined into a single LDA for each condition. - points below the break in the Y axes have zero magnitude

	Cells From Normal Animals	Cells From Tumour-Bearing Animals
pCTL's Per 10^6 Cells	193 (145-259)	468 (313-701)
LU's Per Culture Well		
10^5 Responders	nd	294.5
$5X10^4$ Responders	5.9	nd
$3X10^4$ Responders	2.0	137.2

Table IV-2 A comparison of increases in pCTL frequency and lytic activity in cultured spleen cells from normal and tumour-bearing animals

- with SN2°MLC
- nd = not done
- in brackets - 95% confide e limits

bearing animals at the same time. These results, and those presented in figures IV-4, IV-5 and IV-6, might suggest that one pCTL from a tumour-bearing animal can be cultivated to produce greater lytic activity than can one pCTL from a normal animal. Because there is a non-linear correlation of LU production per culture with pCTL frequency (figures IV-8, III-2), it is difficult to compare, under optimal conditions, the lytic activity produced by given numbers of pCTL's. Such a comparison would be necessary to conclude that the total lytic activity derived from one pCTL from a tumour-bearing animal differs from that derived from a pCTL from a normal animal.

Because of the concerns raised about the interpretation of the data presented in figure IV-6, an attempt was made to ascertain whether there was, in fact, a significant change in the pCTL frequency in the spleens of mice as a result of tumour growth. Twenty mice of the same age (2 to 3 months) and weight were selected from a single stock cage and randomly distributed into two groups of ten One group received an injection of $2X10^6$ P815 cells in 0.1 ml of RPMI 1640 medium (without FCS) and the other group received a control injection of medium alone. The mice were numbered and 5 tumour-injected and 5 sham-injected mice were put into each of two cages. Over a two week period tumour growth was measured and fourteen days after injection, five tumour-bearing and five normal mice were killed, tumour masses were recorded and spleens were taken for a LDA. The next day the spleens of the other ten mice were processed for culture. Because of the size of the experiment, the cultures and assays were performed on two consecutive days.

The LDA results are presented in figure IV-9 where the pCTL frequency is plotted against the time after tumour implantation. The

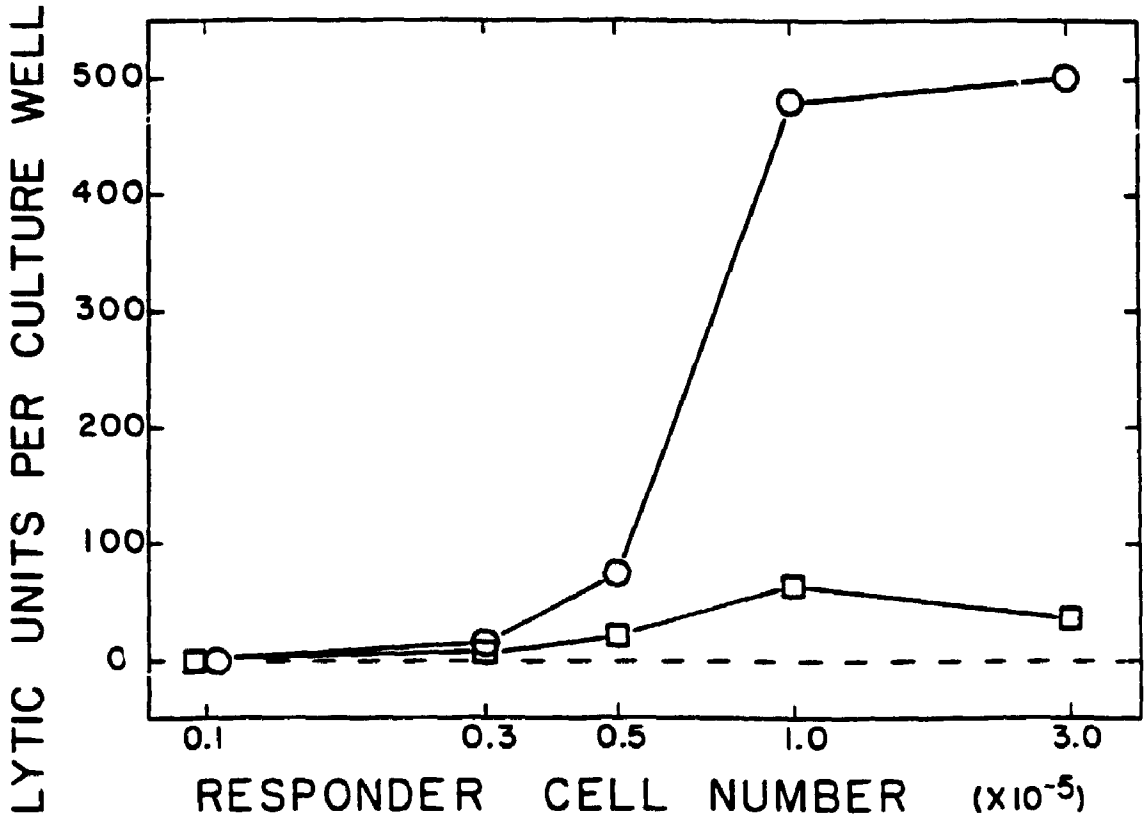


Figure IV-8 Lytic unit productior per culture well as a function of Responder cell number

Cells are from spleens of tumour-bearing animals 20 days after tumour injection.

○ - spleen cells from animals with small tumours
pCTL frequency = 400.9 (264.5-607.8; 95% confidence limits) per 10^6 spleen cells

□ - spleen cells from animals with large tumours
pCTL frequency = 53.0 (38.6-72.8; 95% confidence limits) per 10^6 spleen cells

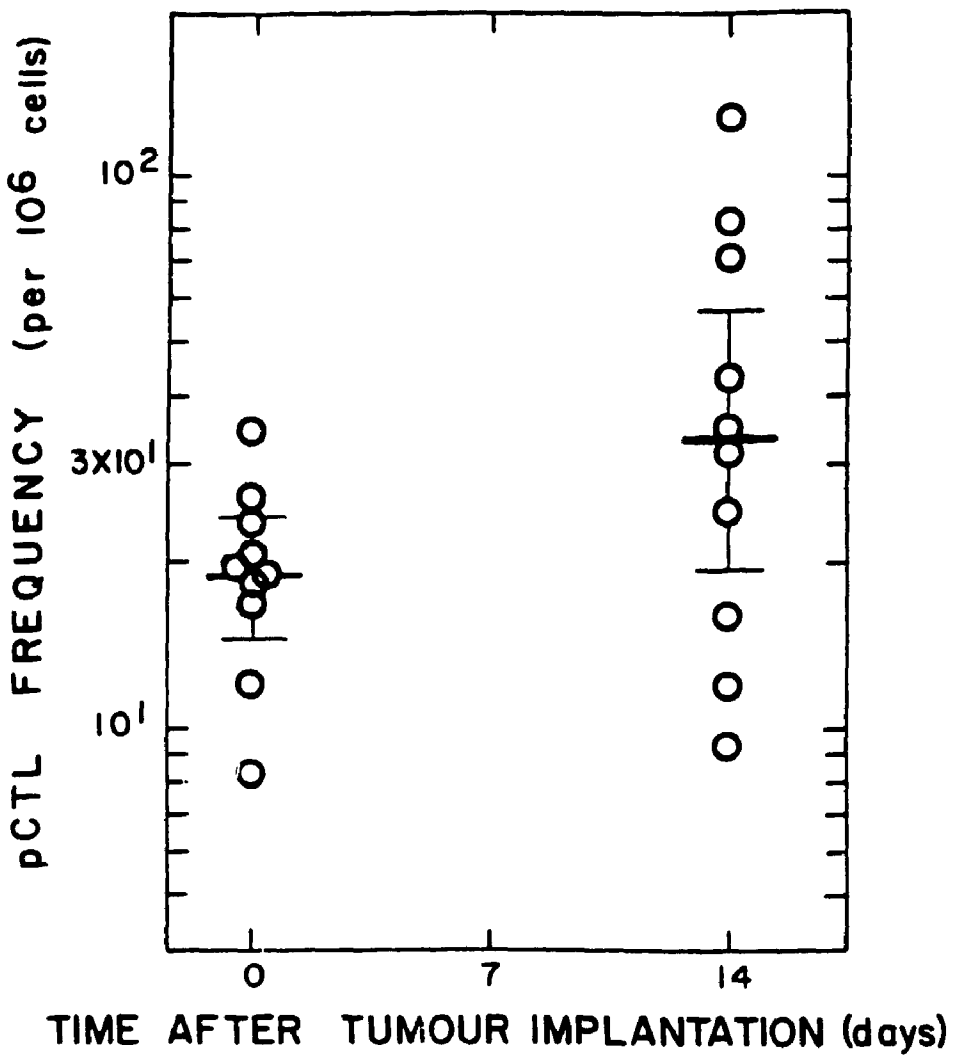


Figure IV-9 The number of pCTL's per 10^6 spleen cells in normal animals and in tumour-bearing animals injected two weeks previously

○ - pCTL frequencies for individual mice
—— - mean (±2SEM) of log-transformed data

statistics:
untransformed data
- normals mean = 19.8 (±2.3;SEM) pCTL's per 10^6 cells
- tumour-bearing mean = 45.3 (±11.9;SEM) pCTL's per 10^6 cells
  - ANOVA: F = 4.41 (P=0.05)
  - M-W-U test: (P=0.12)

ln-transformed data
- normals mean = 2.920 (±0.126;SEM) per 10^6 cells
- tumour-bearing mean = 3.500 (±0.270;SEM) per 10^6 cells
  - ANOVA: F = 3.78 (0.10>P>0.05)

culture conditions:
- with SN2°MLC
- with 2-me

mean pCTL frequency increased from 19.8 (±2.3;SEM) per 10^6 cells in normal spleens to 45.3 (±11.9;SEM) per 10^6 in the spleens from tumour-bearing animals. An ANOVA showed that the change was significant ($F = 4.41$, $P=0.05$). Because of the difference in variances between the two samples (52.9 for normals and 1415.9 for tumour-bearers), an ANOVA of ln-transformed data was performed. Ln-transformation gave a mean of 2.92 (±0.13;SEM) for normal animals and a mean of 3.50 (±0.27;SEM) for tumour bearers while the ANOVA showed a non-significant change ($F=3.78$; $0.10>P>0.05$). The non-parametric Mann-Whitney-U test, used to compare two samples, gave a P value of about 0.12.

Because the three different statistical tests on the same data gave results which may be interpreted as either significant or non-significant, there is difficulty in determining whether there was a change in pCTL frequency. With these statistics, we are using a two-tailed test to look for significant changes. Since we are interested in any change and, with a two-tailed test, the proper significance level to use is 0.05, we feel that the changes shown are not significant at the 5% level but are probably significant at the 10% level.

Perhaps we have failed to show a significant change because of the few data points. If the experiment were repeated a number of times to incorporate more data, the change would probably be significant. This conclusion is supported by the data presented in figure IV-6.

It is important to note the differences between this and the previous experiments. Here, individual spleens were assayed and all of the tumour-bearing mice were entered into the experiment. In previous experiments, 2 or 3 "average" tumour-bearing mice would be selected from 4 to 8 injected animals and those with very small or very large tumours

would be rejected. The selection was preferentially against relatively large tumours. Thus the analysis of the data in figure IV-9 includes results from animals with relatively large tumours and these would not have been included in prior experiments. Animals with large tumours tend to have low pCTL frequencies (as will be presented in section IV-2) and their inclusion here is, in fact, responsible for the relatively low pCTL frequencies in some of the mice after two weeks of tumour growth.

To this point, the analysis has been based on pCTL frequency per 10^6 spleen cells. We considered the possibility that the interpretation of the results might change if the pCTL determination per spleen were used instead. The number of pCTL's per 10^6 spleen cells might not correspond to the number of pCTL's per spleen. For instance, the number of pCTL's in a small spleen with a high frequency measured by pCTL's per 10^6 cells might be significantly smaller than the number in a large spleen with a lower frequency.

While determining whether calculating the data as pCTL's per spleen changed the results, we first checked for any differences in the number of nucleated cells recovered from the spleens of normal or tumour-bearing animals. The mean number of cells recovered from the ten normal spleens was 0.936 (±0.085;SEM) X 10^8 per spleen while from tumour-bearing animals it was 0.950 (±0.069;SEM) X 10^8 cells indicating no difference ($F=0.016$; $P>0.50$). As the experiment was actually split into two because of size, it was also determined that there was no difference in the cell yield in the two replications ($P>0.50$).

The number of pCTL's per spleen was then plotted against the time interval after tumour implantation (fig. IV-10). Again there was an

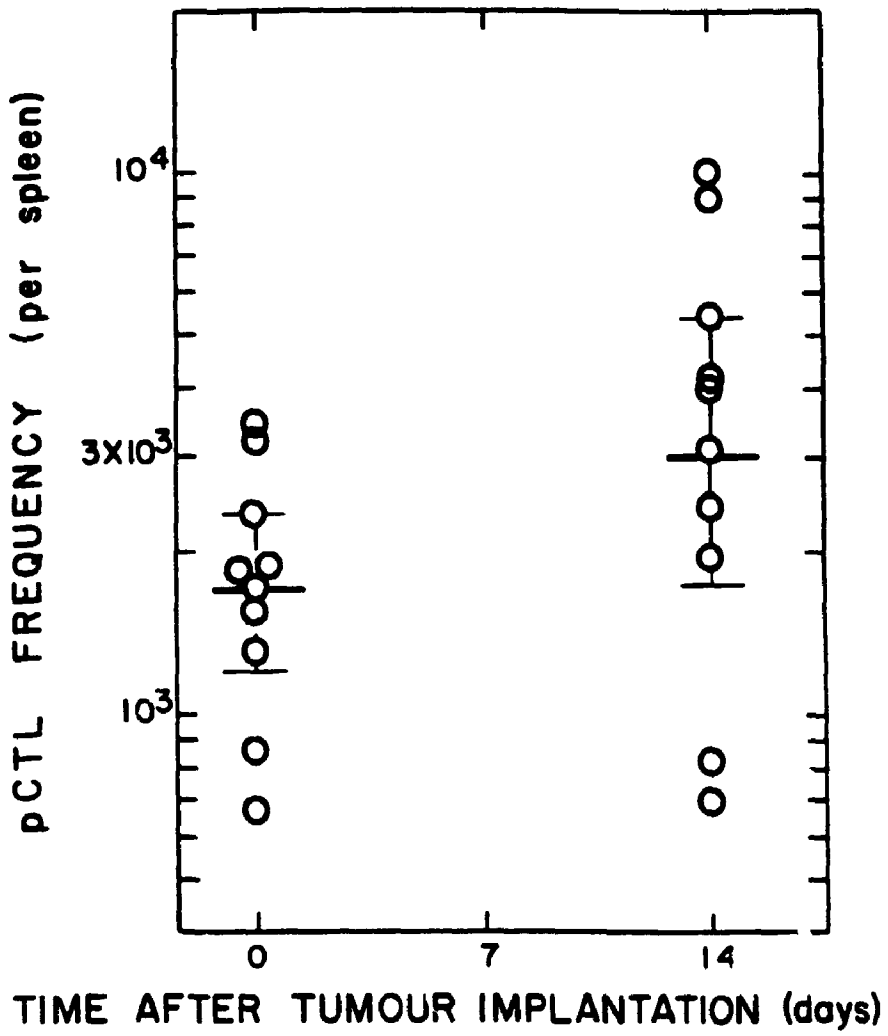


Figure IV-10 The number of pCTL's per spleen in normal animals and in tumour-bearing animals injected two weeks previously

○ - pCTL frequencies for individual mice
—— - mean (±2SEM) of log-transformed data

statistics:
untransformed data
- normals mean = 1.88 (±0.29;SEM) $X10^3$ pCTL's per spleen
- tumour bearing mice mean = 4.20 (±1.02;SEM) $X10^3$ pCTL's per spleen
  - ANOVA: F = 4.75 (0.05>P>0.025)
  - M-W-U test: (P=0.07)

ln-transformed data
- normals mean = 7.43 (±0.17;SEM) per spleen
- tumour-bearing mean = 8.03 (±0.28;SEM) per spleen
  - ANOVA: F = 3.37 (0.10>P>0.05)

culture conditions:
- with SN2°MLC
- with 2-me

increase in pCTL numbers from normal spleens (1.88 (±0.29;SEM) $X10^3$ per spleen) to spleens from tumour bearers (4.20 (±1.02;SEM) $X10^3$ per spleen). Although an ANOVA showed this difference to be significant ($F=4.75$; $0.05>P>0.025$), the ln-transformed data showed a change which was not significant (normals mean = 7.43 (±0.17;SEM); tumour bearers mean = 8.03 (±0.28;SEM); $F = 3.37$; $(0.10>P>0.05)$). The M-W-U test ($P=0.07$) supported the results of the ANOVA of the ln-transformed data. Again we are faced with the dilemma of one statistical test showing a significant change while two others show no statistical difference. Following the reasoning for the interpretation of the results of pCTL's per 10^5 cells, we would say that there is a change in the numbers of pCTL's per spleen which has not been proven statistically significant at the 5% level but which is significant at the 10% level.

In this section, we showed that there appears to be an increase in pCTL frequency in the spleens of mice after a tumour implantation. While the increase was not necessarily shown to be significant at the 5% level, (depending on statistical test one chooses) it was significant at the 10% level. The difference might very well be significant if more mice were to be tested.

IV-2 THE CORRELATION BETWEEN TUMOUR SIZE AND: A) pCTL FREQUENCY, and B) THE PRESENCE OF SUPPRESSOR CELLS, IN THE SPLEENS OF TUMOUR-BEARING ANIMALS

It was shown in the last section that pCTL frequency may be higher in the spleens of tumour-bearing compared with normal mice. There

appears to be a change as a result of tumour implantation and growth.

If pCTL's and CTL's participate in controlling the growth of a tumour, one might expect differences in pCTL frequency among animals to be accompanied by corresponding differences in tumour size. As well, there may be a correlation between tumour size and the presence (or absence) in the animal of cells which could suppress the immune response to the tumour. The purpose of the work presented in this section is to determine whether there is a correlation between tumour size and either pCTL frequency or the presence of suppressor cells in the animal.

The shape of a LDA curve can be used to determine the presence of suppressor cells in the Responder cell population (fig. IV-11). At low cell numbers the suppressor cells have been diluted out and we obtain a LDA curve with single hit kinetics and a frequency estimate based on the initial (low cell number) part of the curve. At higher cell numbers, there is a break in the line so that the expected decrease in the proportion of non-responding cultures in not seen. This is interpreted as indicating the presence of suppressor cells, which noticeably affect the response only at higher cell numbers.

The determination of pCTL frequencies was based of a maximum of three points on the LDA curve; this limit being imposed by the program used. The presence of suppressor cells was indicated if, at higher cell numbers, the LDA curve failed to follow single hit kinetics (i.e. produce a straight line). All possible combinations of data points, taken three at a time, were considered in determining whether or not the curve failed to follow single hit kinetics at higher cell numbers. Chi-squared values indicating the goodness of fit to a straight line, provided by the program, were used in this determination. When

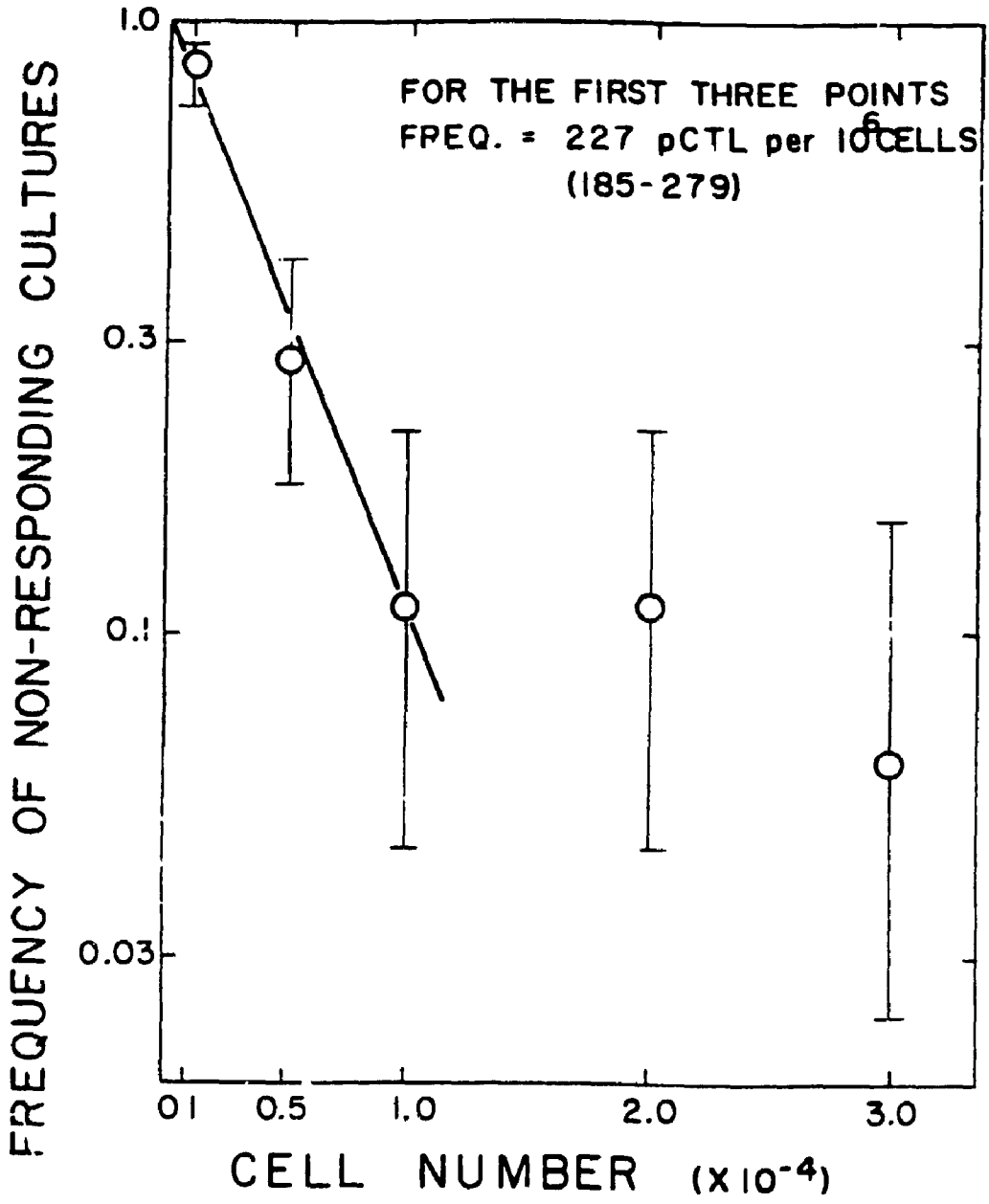


Figure IV-11 Typical LDA curve demonstrating suppression and calculation of pCTL frequency when suppression is present

The cells are lymph node cells from a mouse 14 days after tumour implantation. At low cell numbers, the suppressor cells are diluted out and a LDA curve with single hit kinetics is obtained. At higher cell numbers, suppression causes some of the normally responding cultures to not respond.

- for the first three points: frequency = 227 (185-279; 95% confidence limits) pCTL's per 10^6 cells

culture conditions:

- with SN2°MLC
- with 2-me

suppression was indicated by the shape of the LDA curve, pCTL frequencies were determined from at least two points, consistent with single hit kinetics, at the lowest cell numbers.

To demonstrate that suppressor cells were actually present in the spleens of mice with large tumours, we performed a mixing experiment (fig. IV-12). Spleen cells were obtained from pools of animals with either large or small tumours and ^{51}Cr release curves were generated. All cultures contained SN2°MLC. While cells from animals with small tumours produced high lytic activity, those from mice with large tumours produced no measurable lysis. When $2.5X10^5$ cells from animals with large tumours were mixed with $5X10^4$ cells from animals with small tumours, no lytic activity was produced. All of the cells were washed three times before culture so that the suppression came from the cells. This result demonstrates that cells from an animal with a large tumour are capable of suppressing the lytic activity normally produced by cells from animals with small tumours.

Figure IV-6 presented the pCTL frequency in spleen cells as a function of the time interval since tumour implantation. The data at 19 days or greater can be subdivided into animals with either large or small tumours (fig. IV-13). At days 19, 20 and 27 after tumour implantation, the spleen cells of two mice with either large or small tumours were pooled and LDA's were carried out. Selections were made from a single pool of 20 mice for days 19 and 27; animals with the largest and smallest tumours being chosen. For day 20, mice with the largest and smallest tumours were selected from a separate pool of 20 animals. The tumours were not measured. From the shape of the LDA curves, it was determined whether suppressor cells were present in the

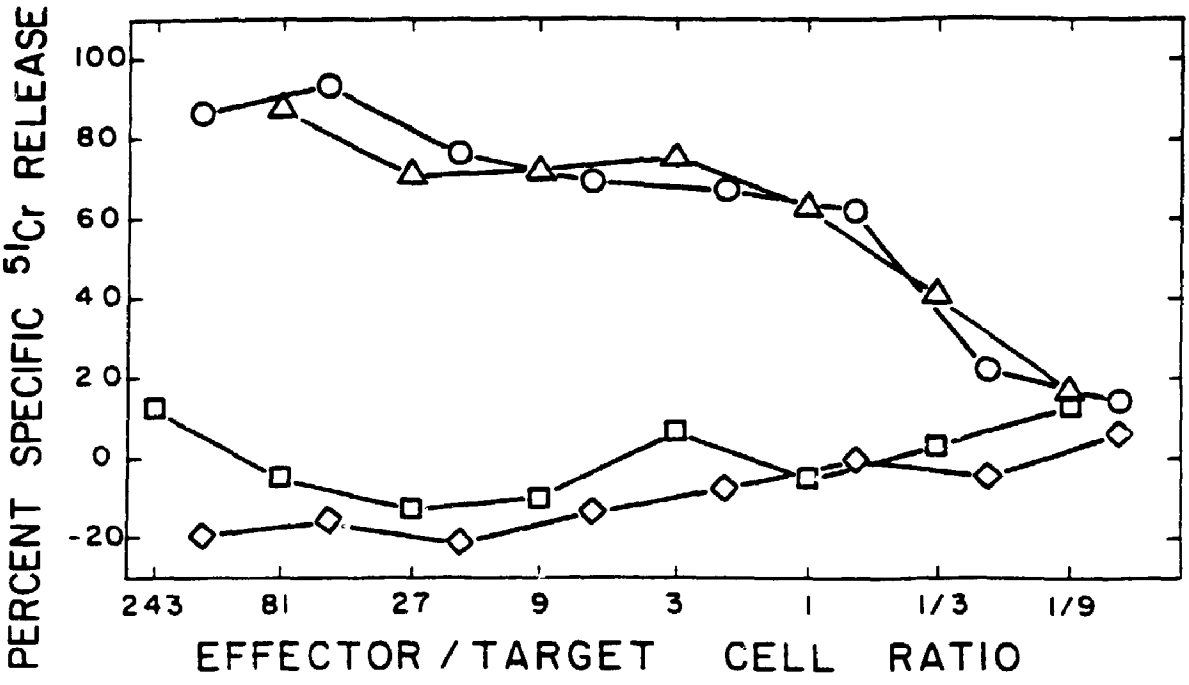


Figure IV-12 Evidence of Suppressor cells - mixing experiment

Chromium release curves (% specific ^{51}Cr release) versus Effector / Target cell ratio for:

- ○ - $2.5X10^5$ Responder cells (per culture) from spleens of animals with small tumours - 504.6 LU produced per well
- △ - $5X10^4$ Responder cells (per culture) from spleens of animals with small tumours - 352.0 LU produced per well
- ◇ - $2.5X10^5$ Responder cells (per culture) from spleens of animals with large tumours - 0 LU produced per well
- □ - $5X10^4$ Responder cells (per culture) from spleens of animals with small tumours, plus, $2.5X10^5$ Responder cells (per culture) from spleens of animals with large tumours - 0 LU produced per well

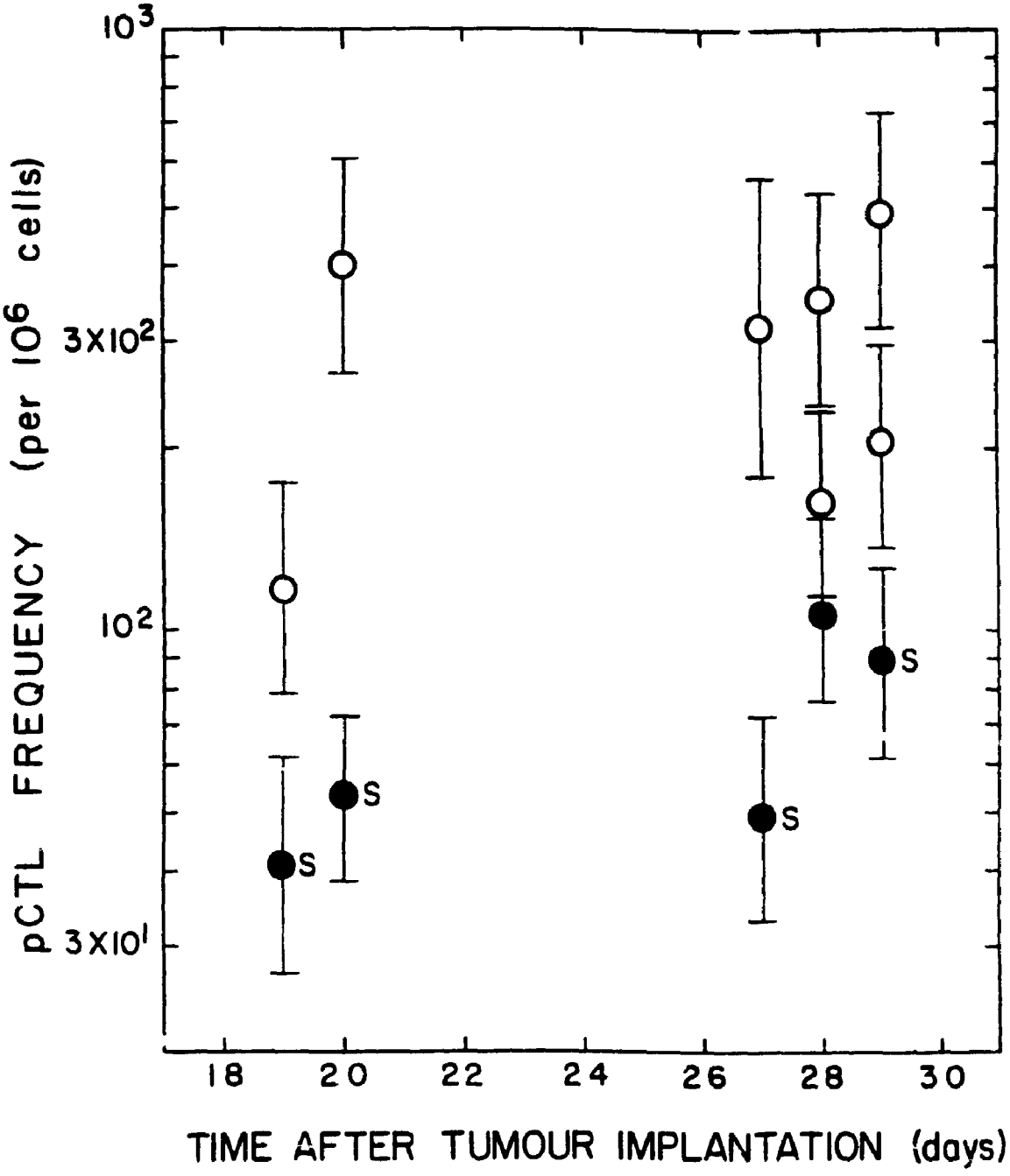


Figure IV-13 The pCTL frequency in the spleen versus time (more than two weeks) after tumour implantation. Animals with large or small tumours and evidence of suppression

○ - cells from pools of spleen cells from animals with small tumours
● - cells from pools of spleen cells from animals with large tumours
- S indicates evidence of suppression in the LDA curve
- error bars - 95% confidence limits

spleen cell suspension, and suppression, if present, is indicated in figure IV-13 by an "S" beside the data point. In another experiment, LDA,s were performed on spleen cells of individual mice at days 28 and 29 of tumour growth and pCTL frequencies as a function of the time after tumour implantation are included in figure IV-13 along with information concerning the tumour size.

In each instance, the pCTL frequency for animals with large tumours was considerably lower than the corresponding pCTL frequency for animals with small tumours. Suppression was evident in four of five of the LDA's of animals with large tumours and in zero out of seven of the LDA's of animals with small tumours.

The mean pCTL frequency for spleen cells from the seven samples of animals with small tumours was 291.0 (±53.1;SEM) per 10^6 and for the five samples of animals with large tumours it was 68.1 (±13.0;SEM) per 10^6. By ANOVA these were significantly different (F=12.74, P=0.005). Therefore for times greater than 14 days after tumour implantation, animals with small tumours had a greater frequency of pCTL's in their spleens than animals with large tumours. Evidence of suppression in the LDA was only present in the cells from animals with large tumours.

The complete LDA curves for the points at 19, 20 and 27 days from figure IV-13 are presented in figure IV-14. After the animals in those experiments had been injected with tumour cells at the same time, spleen cells were taken for culture at different times. These curves demonstrate the differences between the LDA's of cells from animals with large or small tumours. The typical levelling off due to suppression was evident in the curves with the cells from animals with large tumours; and the pCTL frequency was consistently higher in animals with

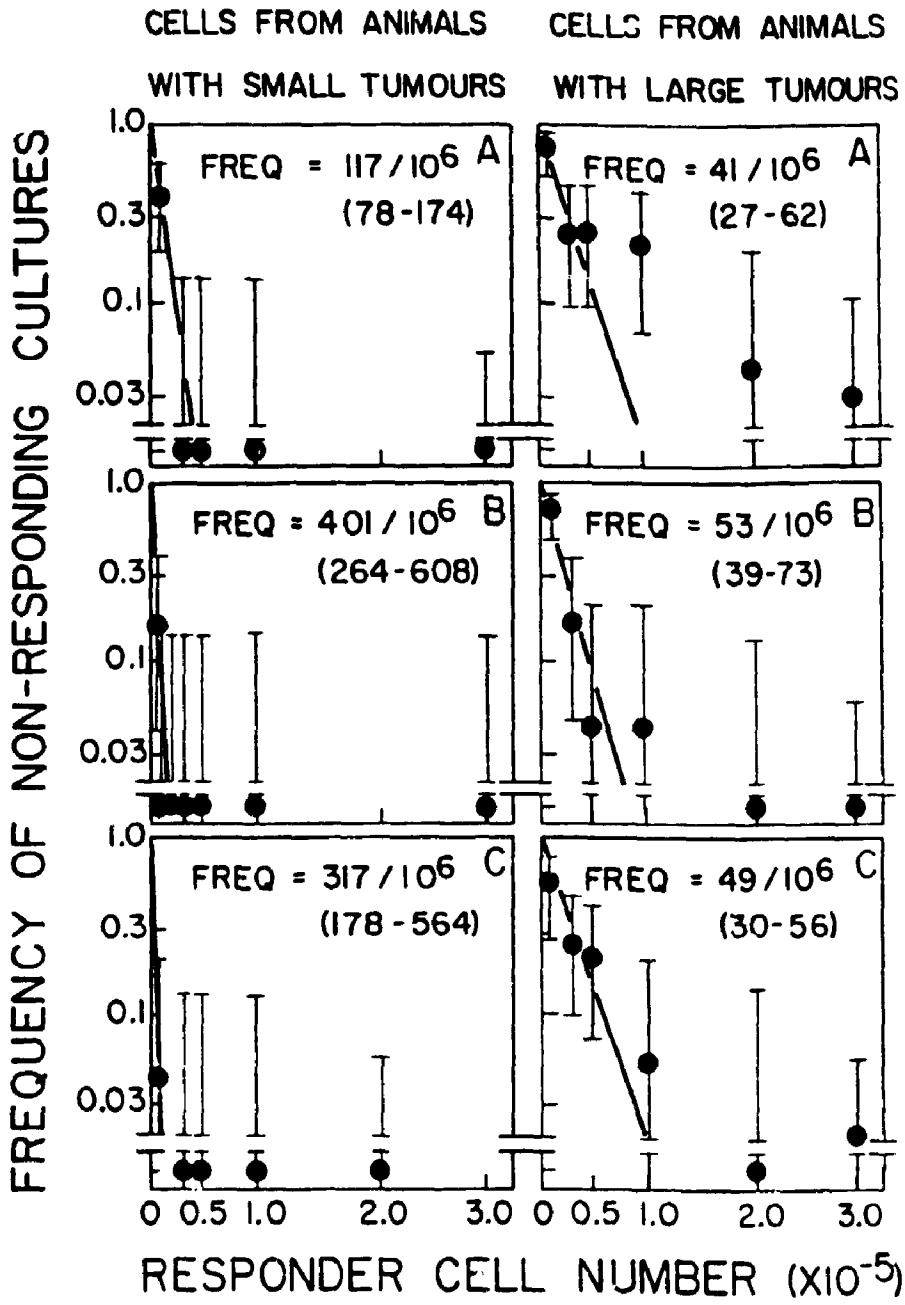


Figure IV-14 Limiting dilution assays for spleen cells, taken from mice with either small or large tumours at 19,20 or 27 days after tumour injection

Limiting dilution assays for spleen cells taken from tumour-bearing mice (A) 19, (B) 20 or (C) 27 days after tumour injection when mice bore either small (left panels) or large (right panels) tumours. Evidence for suppressor cells, as indicated by plateauing, is seen in animals bearing large tumours.
- (in brackets) 95% confidence limits of pCTL frequency
- points below the break in the Y axes have zero magnitude

small tumours.

Another way of demonstrating the differences in the cells from animals with large or small tumours is presented in figures IV-15a) and b). These are data from the experiments presented in figure IV-14. Frequencies of pCTL's and lytic units produced per culture well were calculated for a number of Responder cell concentrations. We could then calculate the average number of pCTL's and the LU's produced per culture well. The plots of log(LU per well) rsus log(pCTL's per well) are presented for cells from animals with large tumours (fig. IV-15a) and for cells from animals with small tumours (fig. IV-15b).

By comparing the two sets of curves, one can see that at a high pCTL concentration (>5 pCTL per well), there was suppression in the cultures from animals with large tumours and no evidence of suppression from animals with small tumours. At low frequencies (1-3 pCTL's per well), the lytic unit production is possibly higher in animals with large tumours. This might indicate a difference in the lytic unit production from individual pCTL's from animals with either large or small tumours when the effects of suppression are removed. However there is some overlap between the two groups of rather limited data and such a conclusion would have to be supported by further research.

Both sets of curves show a rough approximation of one LU per culture well when one pCTL is present. Data from other experiments also showed approximately the same relationship. This fact was useful in estimating the pCTL frequency and determining what cell numbers to use in a LDA when LU activity had already been determined.

An experiment was performed to determine whether there was a correlation between tumour mass and pCTL frequency in the spleens of

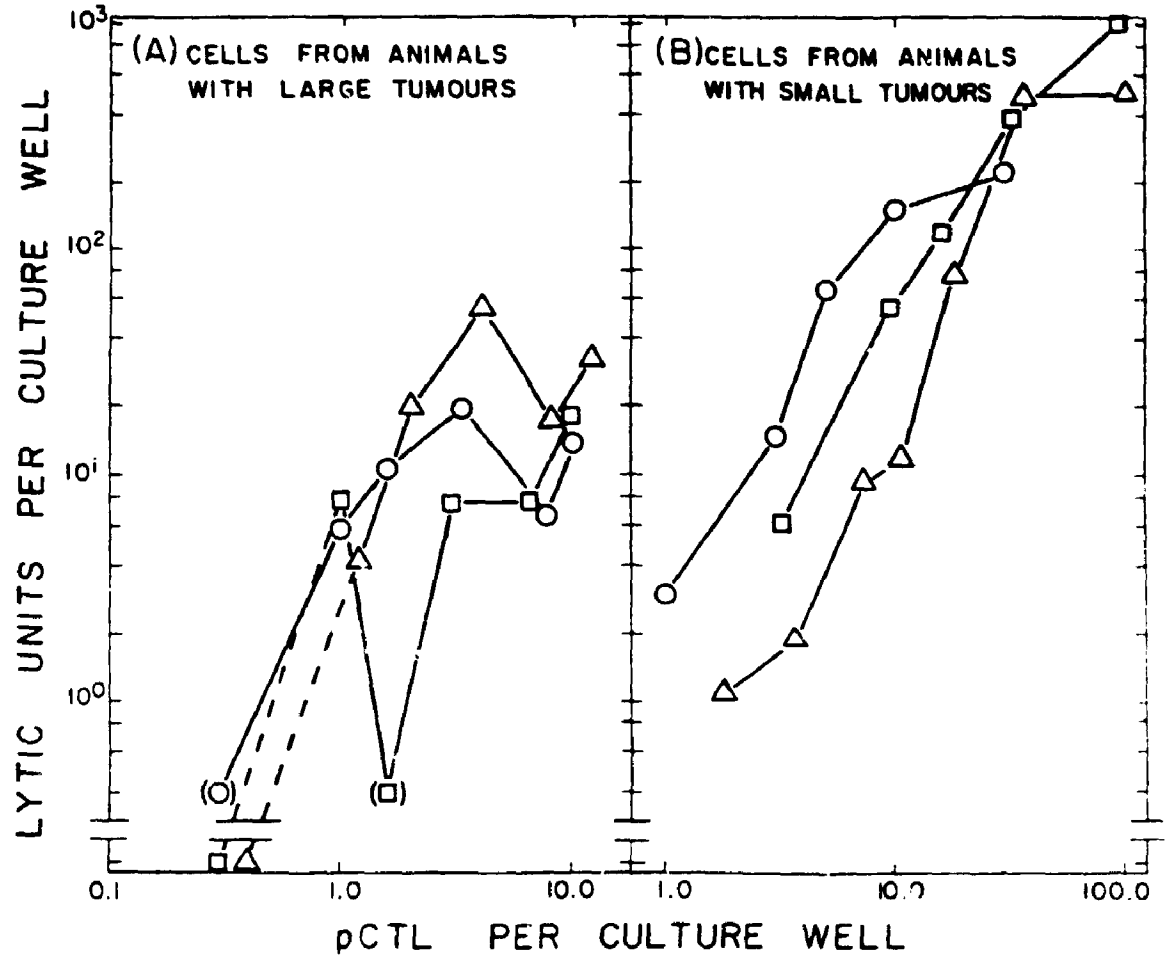


Figure IV-15a, b, Log-log plots of lytic units per culture versus pCTL's per culture for spleen cells from mice with either large (IV-15a) or small (IV-15b) tumours

The curves are for spleen cells from:
animals with large tumours
○ - 19 days after tumour implantation
△ - 20 days after tumour implantation
□ - 27 days after tumour implantation
animals with small tumours
○ - 19 days after tumour implantation
△ - 20 days after tumour implantation
□ - 27 days after tumour implantation
(brackets) indicate estimates
- points below the break in the Y axes have zero magnitude

animals injected 14 days previously with tumour cells. Figure IV-16 presents the results of a power ($y = a*x^b$) regression of tumour mass (y) against pCTL frequency (pCTL per 10^6 cells), (x) for 10 individual mice. The correlation coefficient for the power (or log-log) correlation was significant ($r = -0.848$, $0.002>P>0.001$).

The power regression shows a significant negative correlation between the logarithm of tumour mass and the logarithm of the pCTL frequency in the spleens of mice injected with tumour cells two weeks previously. The data indicates that there is a correlation between tumour mass and the pCTL frequency in the spleens and that this correlation may explain 72% (r^2) of the variation in tumour mass. However it is important to note that a correlation cannot prove a cause and effect relationship.

If there is a threshold effect, that is above a certain threshold frequency, the tumour mass would usually be small, then a regression curve with an increasingly negative slope as the pCTL frequency decreases would be expected to fit the data well. The power correlation was included as one example of this type of curvilinear correlation.

It is possible that the variable which best correlates with tumour mass is the total number of pCTL's per spleen and not the pCTL frequency (per 10^6 spleen cells). The power regression of tumour mass against the number of pCTL's per spleen is presented in figure IV-17. Again the log correlation coefficient was significant ($r = -0.752$, $0.02>P>0.01$). This shows a significant negative correlation between the logarithm of tumour mass and the logarithm of the number of pCTL's in the spleens of those animals.

It has been shown therefore, that by two weeks of tumour growth,

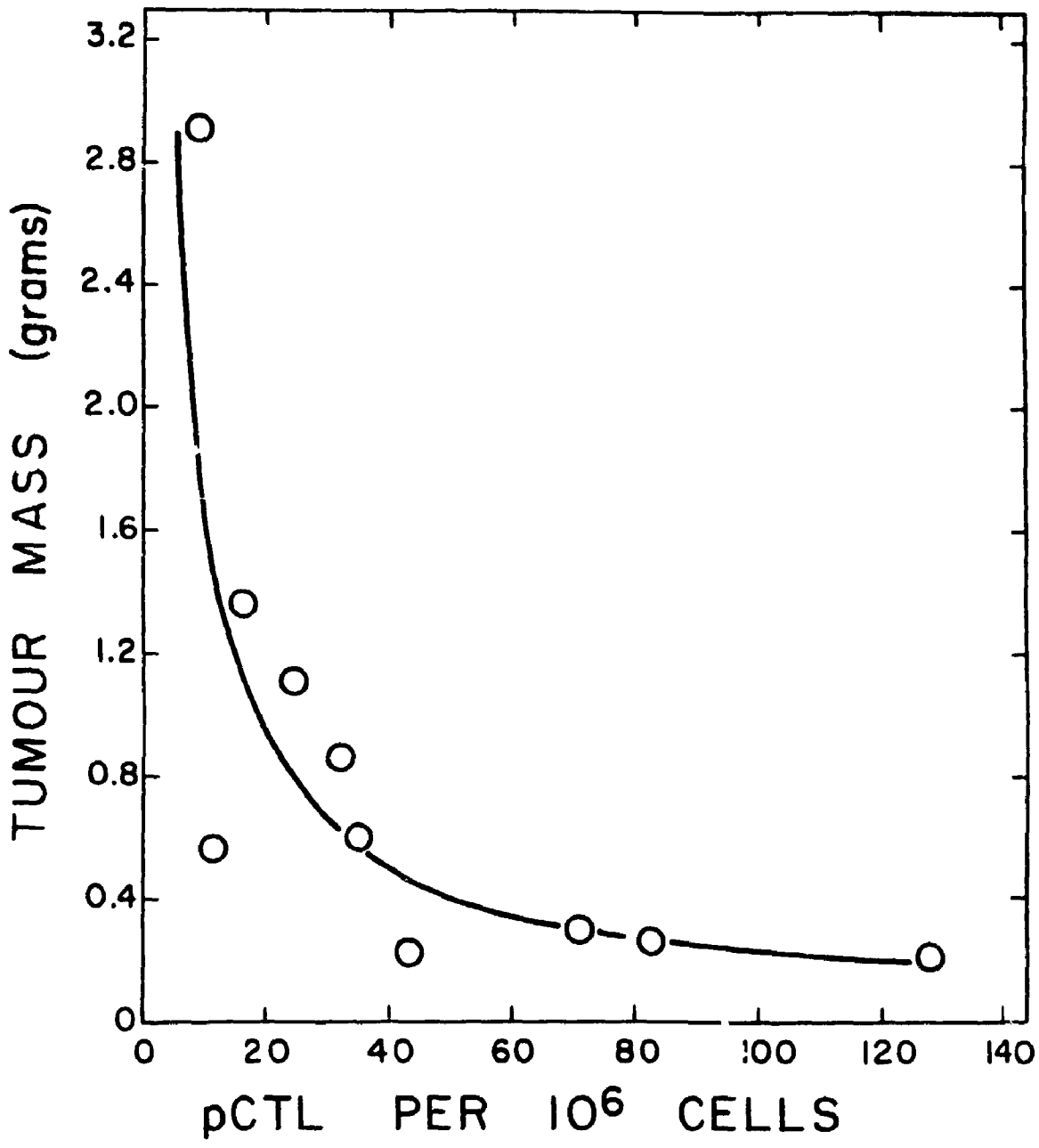


Figure IV-16 The correlation of tumour mass with pCTL frequency (pCTL's per 10^6 cells) in the spleens of mice two weeks after tumour implantation

○ - pCTL frequency and tumour mass data for individual mice
solid line - power regression $y_2 = 12.21x^{-0.866}$
$r^2 = 0.720$, $r = -0.848$
SEr = 0.187, t = 4.53, (0.002>P>0.001)

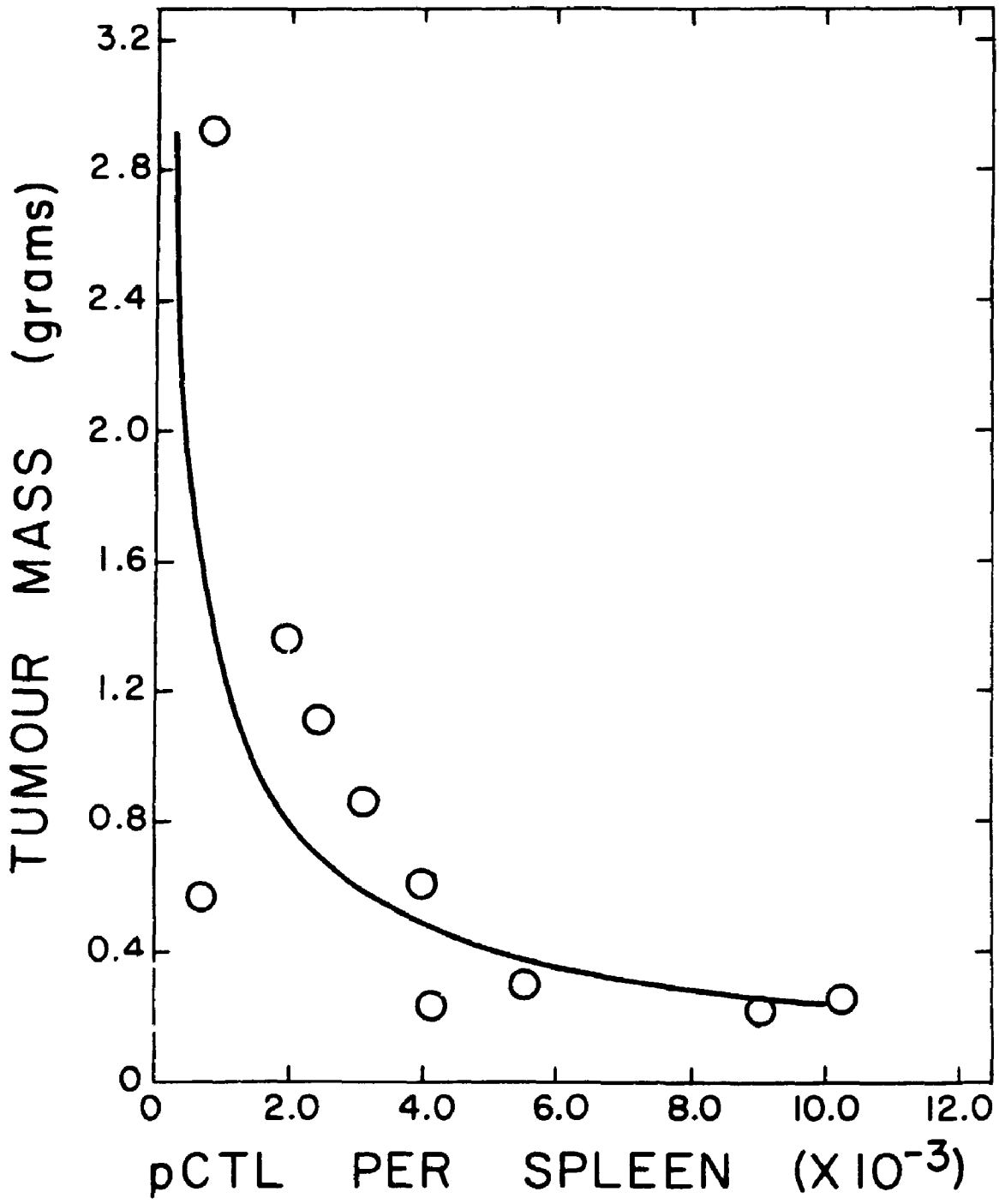


Figure IV-17 The correlation of tumour mass with the number of pCTL's in the spleens of mice two weeks after tumour implantation

○ - pCTL frequency and tumour mass data for individual mice
solid line - power regression $y = 206.2x^{-0.730}$
$r^2 = 0.566$, $r = -0.752$
SEr = 0.233, t = 3.23, (0.02>P>0.01)

mice with small tumours have significantly higher pCTL frequencies in their spleens than do mice with large tumours. Also, as might be expected, there is an inverse correlation between pCTL frequency and tumour mass at two weeks of tumour growth. With the LDA, we were able to detect the presence of suppressor cells in the spleens of only animals with large tumours.

IV-3 THE CORRELATION OF pCTL FREQUENCY IN NORMAL ANIMALS (PRIOR TO INJECTION) WITH TUMOUR GROWTH AFTER TUMOUR IMPLANTATION

In the first two sections of chapter IV, we have shown an increase in pCTL frequency in the spleens of mice with time after tumour implantation, and a negative relationship between pCTL frequency in the spleen and tumour size. It may be that some tumours were smaller because there were more pCTL's present, capable of being stimulated to produce an effective anti-tumour response. However, the cause and effect may be the reverse, i.e. a small tumour may bring about an increase in the number of pCTL's. Also, both of the variables may actually be correlated with a third, untested one.

We investigated whether there might be a correlation between pCTL frequency in animals prior to injection, and the size or mass of tumours after a tumour implantation. With our experimental tumour model, some of the animals develop progressive tumours and ultimately die. In other instances, the tumours grow, and then regress. If the regression is permanent, the mice survive. These different reactions to the presence of a tumour might be correlated not only with ongoing events which occur

during the exposure to the tumour, but also with some pre-existing immunological state or condition. We might be able to judge the potential for an effective immune response to an implanted tumour by measuring the pCTL frequency in animals prior to injection.

LDA's were done on PBL's of normal mice (female DBA's, 8-16 weeks old, weight 20-22 grams). From 0.2 to 0.4 ml of blood was taken by cardiac puncture and LDA's were performed to determine the pCTL frequency in the blood. After they had recovered for seven days from th blood-taking, the mice were injected subcutaneously with tumour cells. The growth of the tumours was followed by measuring tumour volume every 3 or 4 days.

In a series of experiments, 19 out of 31 tumours regressed while 12 progressed and the animals died. The animals with tumours that regressed could be further subdivided into two groups: permanent regression , and regression followed by redevelopment. Of the 19 regressing tumours, 10 shrank until there was no detectable tumour, and, after a further 60 days, the animals were still tumour free. Eight tumours regressed to an undetectable size for various lengths of time before redeveloping at the same site and growing progressively until the animals died. In the last case, the tumour regressed, from a volume of 1.08 cm^3 at day ten to a volume of 0.06 cm^3 at day forty-two, before regrowing and eventually killing the animal.

Therefore, there were three groups of animals: one group with progressing tumours, one with regressing tumours, and one with tumours which initially grew, regressed and then redeveloped at a later time. Tumour growth curves for these three groups are presented in figure IV-18.

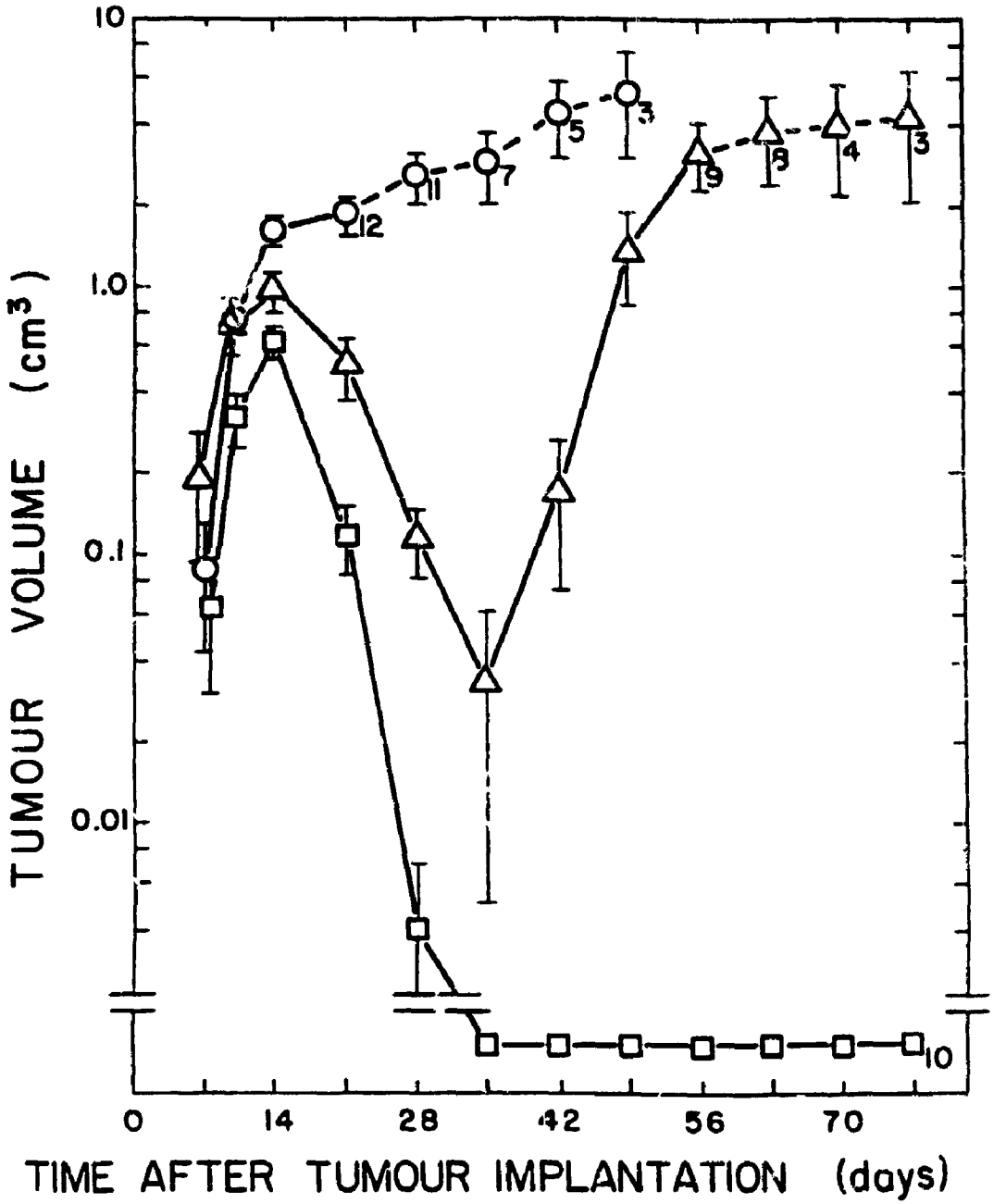


Figure IV-18 Growth curves of P815X tumours implanted in DBA/2 mice

- ○ - mean tumour volumes in mice with progressive tumours (n = 12)
- □ - mean tumour volumes in mice with regressive tumours (n = 10)
- △ - Mean tumour volumes in mice with tumours which initially progress, reach a maximum volume at about two weeks, and then regress (to zero volume in 8/9 cases) and then progress again (n = 9)

- numbers beside the symbols represent the number of surviving mice at that point
- error bars - ±1SEM
-points below the break in the Y axis have zero magnitude

to 0.1 g of the minced tumour in a fluted trypsinization flask containing a magnetic stirring bar. After the mixture had been stirred at low speed for 1/2 hour at 37°C, complete medium (RPMI-1640 containing FCS) was added to arrest the action of the enzymes. By shaking this mixture vigorously for sixty seconds, the viable cell yield was increased substantially. The suspension was then filtered through several layers of sterile cotton padding to remove undissociated cells. After three washings and counting, the cells were ready for use.

Solid tumours were produced in DBA/2 mice by injecting $2X10^6$ P815X cells in 0.1 ml of serum-free medium subcutaneously into the upper dorsal shoulder region of anaesthetized (ether) mice. After about seven days, solid tumours could be palpated at the site of injection.

Tumour growth was measured by two different methods. If the animal was killed, the tumour was excised and its mass measured. If the animal was to be kept alive, then the tumour volume was measured with calipers. Assuming the tumour to be approximated by a hemi-ellipsoid, the volume (V) was estimated as:

$$V = 0.5(4/3\pi)(L/2)(W/2)H$$

$$= 0.52L \cdot W \cdot H$$

where: L = length along the long axis

W = width orthogonal to the long axis

H = height above the normal curvature of the back

An attempt was made to allow for the skin thickness by subtracting an estimate of it from the measurements.

We wanted to combine the results of these experiments with others in which 12 mice were killed at day twenty-eight, after 9 of the tumours had regressed. We do not know whether the tumours in those 9 mice would have redeveloped. Because the tumours in the third group had temporarily regressed, probably due to the immune response of the mice, and because we wanted to combine the results of the various experiments, we decided, for further analysis, to combine the two classes of regressing tumours.

Part of the analysis, therefore, consisted of dividing the animals into two groups, those with progressing and those with regressing tumours, and then comparing them. Of the forty-three mice used, fifteen tumours progressed and twenty-eight regressed. Data concerning pCTL frequencies and tumour volumes at days seven and fourteen are given in table IV-3 for animals of both groups. These data were used for the following statistical analyses.

Figure IV-19 shows the linear relationship of tumour volume, fourteen days after tumour injection, to pCTL frequency in the animals before they were injected. The negative linear correlation between tumour volume and pCTL frequency ($r^2 = 0.173$, $r = -0.416$) was significant ($0.01>P>0.005$). The results of the regression of tumour volume on the logarithm of pCTL frequency (log regression; $r^2 = 0.266$, $r = -0.516$, $P<0.001$) is given in figure IV-20. Thus, both analyses show a significant negative correlation between pCTL frequency in the blood of normal animals before a tumour injection, and the tumour volume fourteen days after injection. The results suggest that, depending on the regression model chosen, between 17% and 27% of the variability of the volumes of tumours at fourteen days bears a mathematical relationship to

PROGRESSING TUMOURS			REGRESSING TUMOURS		
pCTL FREQUENCY (95% confidence limits)	TUMOUR VOL. (cc)		pCTL FREQUENCY (95% confidence limits)	TUMOUR VOL. (cc)	
	day 7	day 14		day 7	day 14
19.4 (8.2-45.9)	0.009	1.460	45.5 (25.6-81.0)	0.037	0.622
27.1 (13.1-56.2)	0.025	0.393	52.5 (29.7-92.8)	0.0	0.681
14.0 (3.7-53.5)	0.025	1.950	43.4 (23.5-80.0)	0.009	0.325
29.4 (18.8-46.0)	0.229	2.262	22.6 (14.7-34.7)	0.0	0.014
25.8 (16.0-41.4)	0.421	2.340	11.0 (5.5-22.0)	0.304	0.869
11.2 (6.3-19.7)	0.0	1.088	19.9 (12.7-31.3)	0.0	0.593
36.6 (24.1-55.6)	0.0	1.674	16.6 (10.2-26.9)	0.0	0.899
22.3 (11.6-42.7)	0.0	0.771	33.2 (21.5-51.4)	0.200	0.524
13.5 (7.3-25.0)	0.0	1.398	30.0 (17.0-53.0)	0.062	0.917
28.5 (14.4-56.2)	0.0	0.917	32.3 (16.3-64.0)	0.014	0.393
6.2 (3.0-12.9)	0.328	2.948	41.5 (22.5-76.5)	0.017	0.830
29.2 (14.8-57.9)	0.355	1.770	35.0 (20.6-59.7)	0.0	0.949
16.3 (8.7-30.6)	0.939	1.490	17.2 (7.2-41.3)	0.100	0.398
9.7 (5.1-18.5)	1.378	2.210	16.8 (9.5-29.8)	0.796	1.794
58.6 (31.5-108.9)	0.0	2.022	18.3 (9.3-35.8)	0.524	1.487
			36.5 (19.6-68.5)	0.014	0.949
			18.5 (11.7-29.4)	0.229	1.278
			53.3 (36.9-77.0)	0.060	1.100
			64.6 (45.3-92.2)	0.280	1.020
			45.6 (31.3-66.6)	0.250	0.420
			112.7 (79.3-160.2)	0.430	0.670
			71.3 (50.2-101.5)	0.050	0.230
			34.8 (23.3-52.1)	0.070	0.140
			65.6 (45.8-93.1)	0.014	0.040
			57.0 (39.1-83.2)	0.023	0.050
			50.2 (34.7-72.5)	0.003	0.190
			57.9 (32.8-102.3)	0.009	0.521
			29.0 (13.4-63.0)	0.017	0.711

Table IV-3 A comparison of animals with either progressing or regressing tumours. pCTL frequencies prior to injection and tumour volumes after implantation

- frequencies are expressed as pCTL per 10^6 PBL

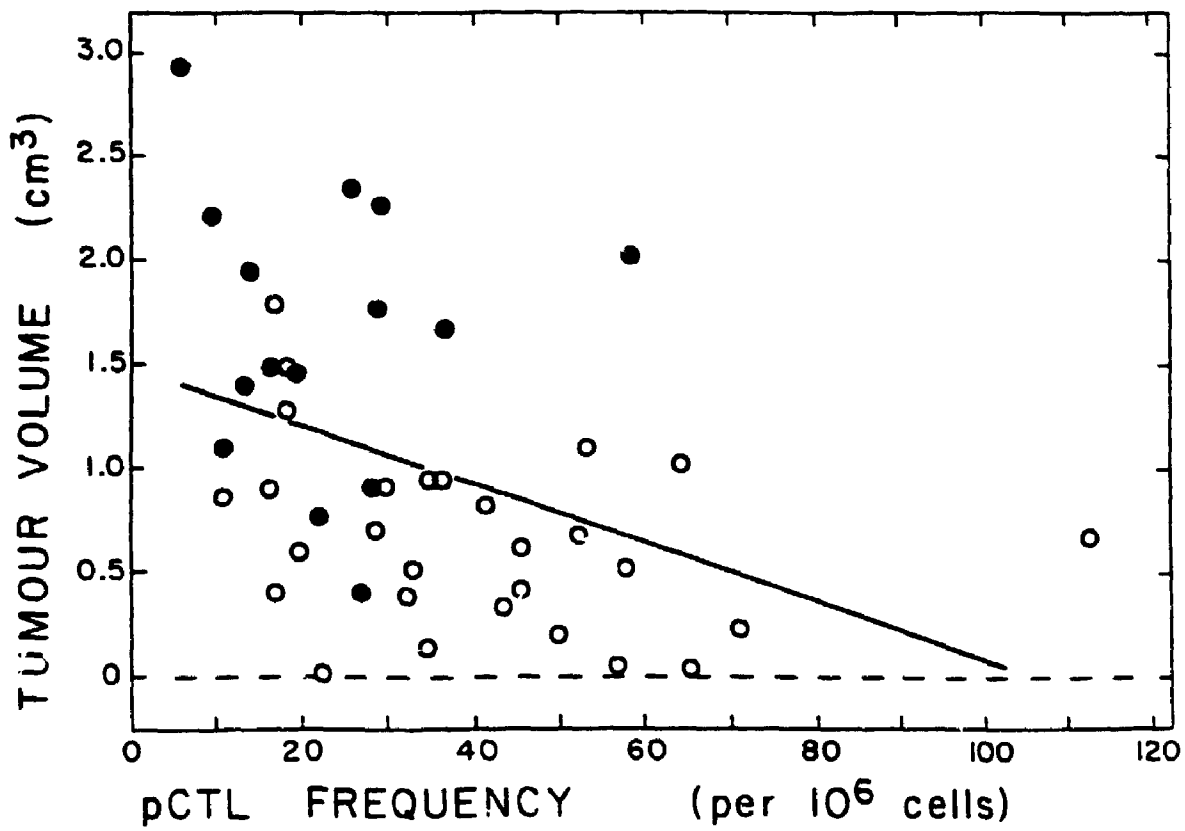


Figure IV-19 Regression of tumour volume, at 14 days, on pCTL frequency in the blood of mice before implantation of the tumour

Results are from experiments on individual animals
● - animals with progressing tumours
○ - animals with regressing tumours
- linear regression equation: $y = 1.491 - 0.0141x$
$r^2 = 0.173$, $r = -0.416$ $(0.01>P>0.005)$
(adjusted $r^2 = 0.153$)
- for statistics comparing animals with progressing or regressing tumours, see table IV-4

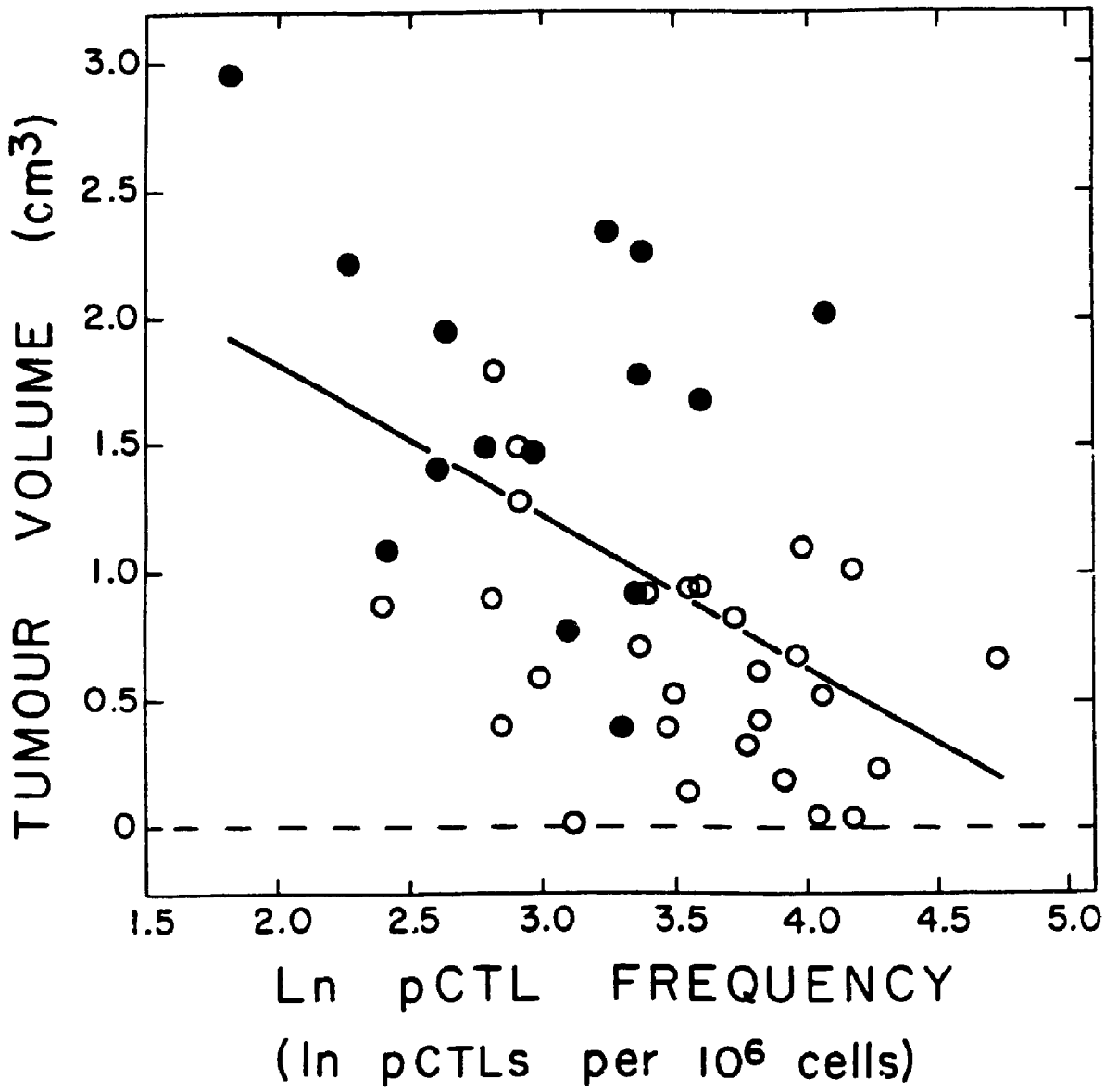


Figure IV-20 Regression of tumour volume, at 14 days, on the logarithm of pCTL frequency in the blood of mice before implantation of the tumour

Results are from experiments on individual animals
● - animals with progressing tumours
○ - animals with regressing tumours
- regression equation: $y = 3.001 - 0.593(\ln(x))$
$r^2 = 0.266$, $r = -0.516$ ($P<0.001$)
(adjusted $r^2 = 0.248$)
- for statistics comparing animals with progressing or regressing tumours, see table IV-4

the precursor frequency in normal animals before injection with tumour cells.

When comparing the progressing and regressing tumour groups, we found that animals with progressing tumours had, on average, fewer pCTL's in the blood prior to injection (23.2 (±3.4;SEM) per 10^6 PBL versus 40.5 (±4.2;SEM) per 10^6 PBL). An ANOVA showed this difference in pCTL frequency to be significant ($F = 7.69$, $0.005>P>0.002$; table IV-4). For the two groups, the tumour volumes were also significantly different at fourteen days; the time that the tumours that would regress reached a peak volume. The progressing tumours had a mean volume of 1.65 (±0.18;SEM) cm^3, while those regressing had a mean volume of 0.67 (±0.08;SEM) cm^3. At seven days after tumour implantation however, the tumour volumes of the two groups were not significantly different ($F = 1.78$, $0.25>P>0.10$; table IV-4).

In order to determine if there was a significant correlation as early as seven days, we performed a regression of tumour volume at seven days on the logarithm of the pCTL frequency in the blood before tumour injection (fig. IV-21). The negative correlation was significant ($r^2 = 0.123$, $r = -0.351$; $0.05>P>0.02$). But for the linear regression, the correlation coefficient was not significantly different from zero ($r = -0.212$; $0.20>P>0.10$).

We also considered data on the time of death of animals with progressively growing tumours; but there was no correlation with the pCTL frequency prior to tumour injection ($P>0.50$).

In a different analysis, we compared: a), the regression of the logarithm of the tumour volume fourteen days after tumour implantation, on the logarithm of the pCTL frequency in the blood of the mice before

	MICE WITH PROGRESSING TUMOURS (n=15)	MICE WITH REGRESSING TUMOURS (n=28)	ANOVA	M-W-U TEST
pCTL FREQUENCY (per 10^6 PBL)	23.19 ±3.39	40.46 ±4.17	F = 7.69 (0.01 > P > 0.005)	(0.005 > P > 0.002)
ln(pCTL FREQUENCY)	2.996 ±0.149	3.560 ±0.104	F = 9.33 (0.005 > P > 0.0025)	(0.005 > P > 0.002)
TUMOUR VOLUME (cc) DAY 7	0.247 ±0.105	0.125 ±0.036	F = 1.78 (0.25 > P > 0.10)	nd
TUMOUR VOLUME (cc) DAY 14	1.65 ±0.18	0.67 ±0.08	F = 33.06 (P < 0.0005)	nd

Table IV-4 A comparison of animals with either progressing or regressing tumours. Statistics of pCTL frequencies prior to implantation and tumour volumes subsequent to injection

- values are means ± 1SEM

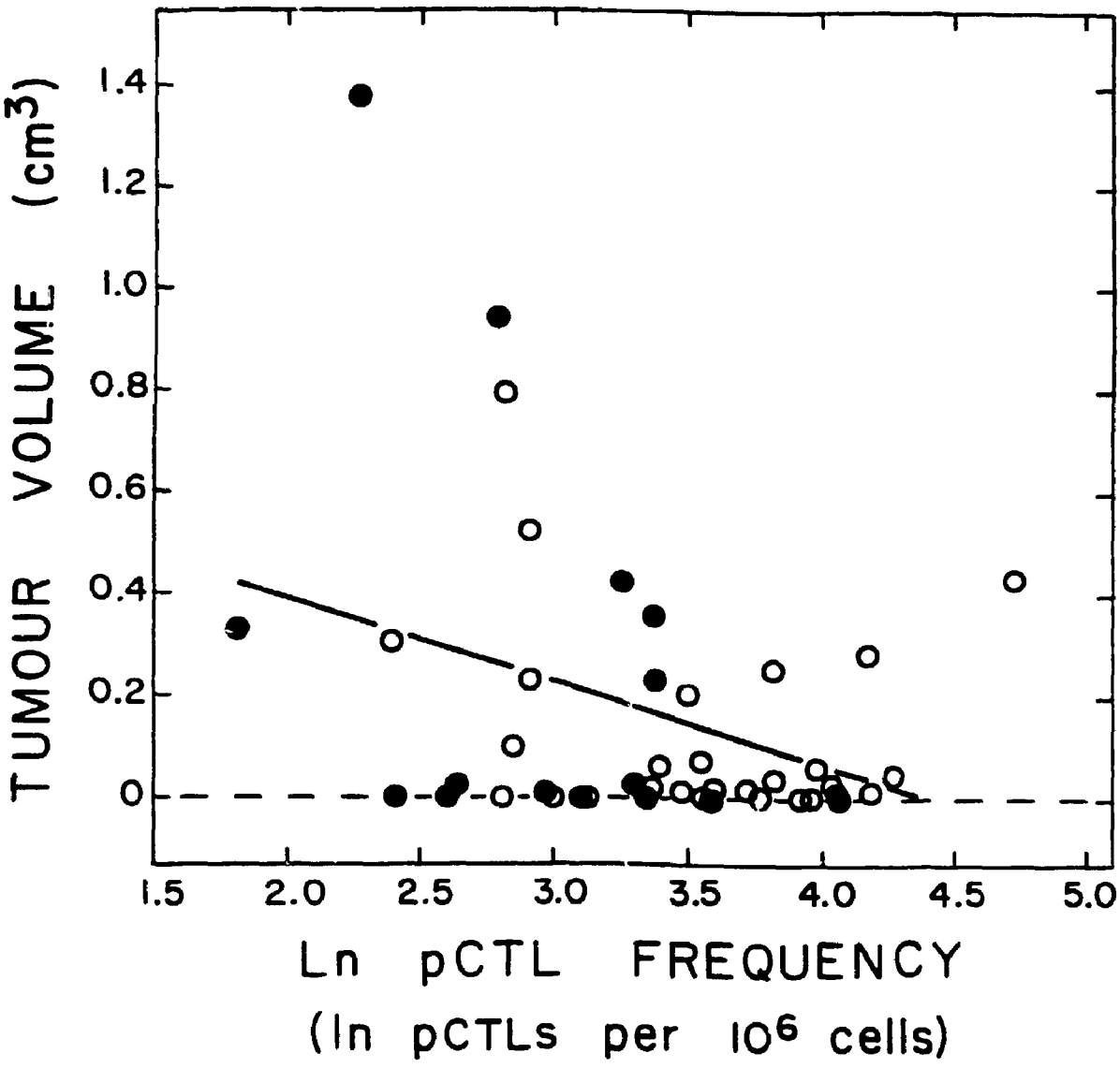


Figure IV-21 Regression of tumour volume, at 7 days, on the logarithm of pCTL frequency in the blood of mice before implantation of the tumour

Results are from experiments on individual animals
● - animals with progressing tumours
○ - animals with regressing tumours
- regression equation: $y = 0.719 - 0.164\ln(x)$
$r^2 = 0.123$, $r = -0.351$ $(0.05>P>0.02)$
- for statistics comparing animals with progressing or regressing tumours, see table IV-4

injection, with b), the regression of the logarithm of tumour mass versus the logarithm of pCTL frequency in the spleens of the same animals fourteen days after tumour implantation (fig. IV-22). The figure demonstrates that the spreads of the pCTL frequencies are similar. The apparently larger scatter for the data based on tumour volumes may be due to a greater random error in measuring tumour volume (with calipers in the live animal) than in measuring tumour mass.

For the power regression of tumour volume at day fourteen on the pCTL frequency in the blood of normal animals, the negative correlation coefficient was significant ($r^2 = 0.159$, $r = -0.399$, $0.01>P>0.005$) and the regression coefficient was -0.724. For the power regression of tumour mass on the pCTL frequency in the spleens, the correlation coefficient was again negative and significant ($r^2 = -0.720$, $r = -0.849$, $0.002>P>0.001$), while the regression coefficient had a value of -0.866.

In this section, we have shown a significant negative correlation between the pCTL frequency in normal animals and tumour growth after a tumour cell injection. When we divided the animals into two groups, those with progressing and those with regressing tumours, there were significantly more pCTL's, prior to implantation, in the blood of animals which would eventually reject(?) their tumours. Other variables also have an important influence on tumour growth since, for the regression of tumour volume on the pCTL frequency in the blood of animals prior to injection, the coefficient of determination (r^2) was only 0.16.

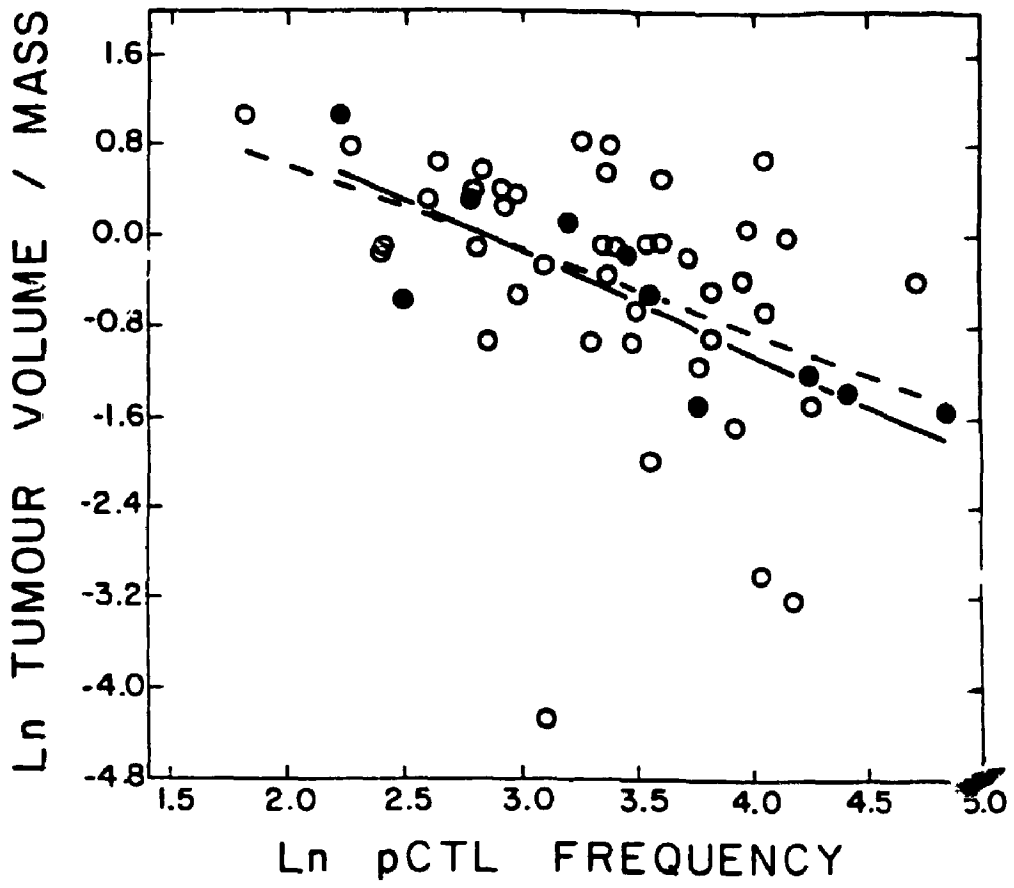


Figure IV-22 Regression of the logarithm of tumour size on the logarithm of pCTL frequency

Two regressions are shown:

● - and solid line: tumour mass 14 days after tumour implantation versus pCTL frequency in the spleens at the same time
- regression equation: $\ln(y) = 2.502 - 0.866\ln(x)$ (or $y = 12.21x^{-0.866}$)
$r^2 = 0.720$, $r = -0.849$ (-0.472 to -0.963; 95% confidence limits)

○ - and dashed line: tumour volume 14 days after tumour implantation versus pCTL frequency in the blood of the animals prior to injection
- regression equation: $\ln(y) = 2.059 - 0.724\ln(x)$ (or $y = 7.839x^{-0.724}$)
$r^2 = 0.159$, $r = -0.399$ (-0.113 to -0.625; 95% confidence limits)

- the correlation coefficients were significantly different ($0.05>P>0.02$)
- the regression coefficients (slopes of regression lines) were not significantly different ($P>0.50$)
- the regressions were done on results from different sets of mice

IV-4 ANATOMICAL LOCATION OF pCTL's

Most of the early work with LDA's involved taking spleens and working with those cells in suspension. Spleens were easy to obtain, and it was not required that the animal be kept alive. Because one of our goals was to follow the course of tumour growth after determining, by LDA, the number of pCTL's in the animal, prior to tumour injection, we were limited to determining the pCTL frequency in the blood. So the question arose whether the frequency in the blood was different from that in the spleen. Also, we wanted to know whether pCTL's were present in other lymphoid organs, such as the lymph nodes (LN), and the thymus.

The purpose of this section is to describe the distribution of the pCTL's in the organs of the mouse, other than in the spleen. We have found pCTL's in the blood, lymph nodes (axillary and brachial) and thymus.

As part of several experiments, the pCTL frequency in the blood of 55 individual normal mice was determined. The mean frequency was 33.3 (±2.8;SEM) pCTL's per 10^6 PBL.

The purpose of one experiment was to compare pCTL frequencies in the nucleated spleen cells and in the peripheral blood leukocytes of the same six normal animals (table IV-5). The mean pCTL frequency in the spleens was 25.3 (±1.4;SEM) per 10^6 cells and in the blood, 20.5 (±3.1;SEM) per 10^6 PBL. A paired t-test showed no significant difference in frequency between the two groups of data ($0.50>P>0.20$). Regression of pCTL's per 10^6 PBL versus pCTL's per 10^6 spleen cells were done and no significant correlations were found for linear and log regressions ($P>0.50$).

pCTL FREQUENCY IN THE SPLEEN (pCTL's/10^6 cells)	NUMBER OF pCTL's/SPLEEN	pCTL FREQUENCY IN THE BLOOD	ESTIMATED NUMBER OF pCTL's IN TOTAL BLOOD VOLUME
26.1 (16.8-40.4)	$5.01X10^3$	27.0 (15.4-47.2)	$5.05X10^2$
28.9 (19.0-44.1)	$4.65X10^3$	17.9 (10.8-29.7)	$3.35X10^2$
24.3 (15.5-38.2)	$4.50X10^3$	14.7 (7.4-29.0)	$2.75X10^2$
24.3 (15.5-38.2)	$2.89X10^3$	32.9 (20.9-51.7)	$6.13X10^2$
28.6 (18.7-43.8)	$4.95X10^3$	15.7 (9.2-26.8)	$2.94X10^2$
19.6 (12.0-31.9)	$1.76X10^3$	14.9 (8.7-25.6)	$2.79X10^2$
mean = 25.3 ±1.4(SEM)	mean = 3.96 ±0.54(SEM) $X10^3$	mean = 20.5 ±3.1(SEM)	mean = 3.84 ±0.58(SEM) $X10^2$

Table IV-5 pCTL frequencies in the spleens and blood of the same, normal mice

- in brackets - 95% confidence limits
- the estimated number of pCTL's in the total blood volume assumes a blood volume of 1.7 ml per mouse with a count of $11X10^6$ per ml
- a paired t-test showed no significant difference between pCTL frequencies in the spleens and in the blood ($0.50>P>0.20$)
- no significant correlation was found between the frequencies in the spleen and blood ($P>0.50$)

We were able to determine the pCTL number per spleen, and estimated the pCTL number in the blood of those animals by assuming a blood volume of 1.7 ml for a twenty-two gram mouse and a WBC count of $11X10^6$ per ml. This yields $18.7X10^6$ PBL per mouse. On this basis, and the results of the LDA, the total number of pCTL's in the blood of each mouse was calculated (table IV-5).

The mean number of pCTL's per spleen was found to be 3.96 (±0.54;SEM) $X10^3$ and the mean number in the total blood volume was estimated to be 3.84 (±0.58;SEM) $X10^2$. On this basis, we conclude that, in normal mice, there are about ten times as many pCTL's in the spleen as there are in the circulation. No correlation ($P>0.50$) was found between the total number of pCTL's per spleen and the concentration of pCTL's per 10^6 cells in the circulating blood of normal animals.

The presence of pCTL's in the thymus and lymph nodes of mice was determined as well. In figure IV-23 are presented the results of six experiments in which pCTL frequencies, in the thymuses and lymph nodes, were measured. The lymph nodes were from the axillary and brachial nodes. Essentially, these are near the "armpit" and "elbow" areas of the front legs. Because the tumours grew in the dorsal shoulder and neck region, the lymph nodes (especially the axillary nodes) may have been draining the tumour site.

In all five experiments where pCTL frequencies in LN's and thymuses were compared directly, the frequency in the lymph nodes was consistently higher (by an average factor of 12.5 (±2.5;SEM)). In the one experiment where the frequency of pCTL's was measured in the spleen, thymus, and lymph nodes of normal animals, the frequercy was highest in the lymph nodes and lowest in the thymuses. Those relative values were

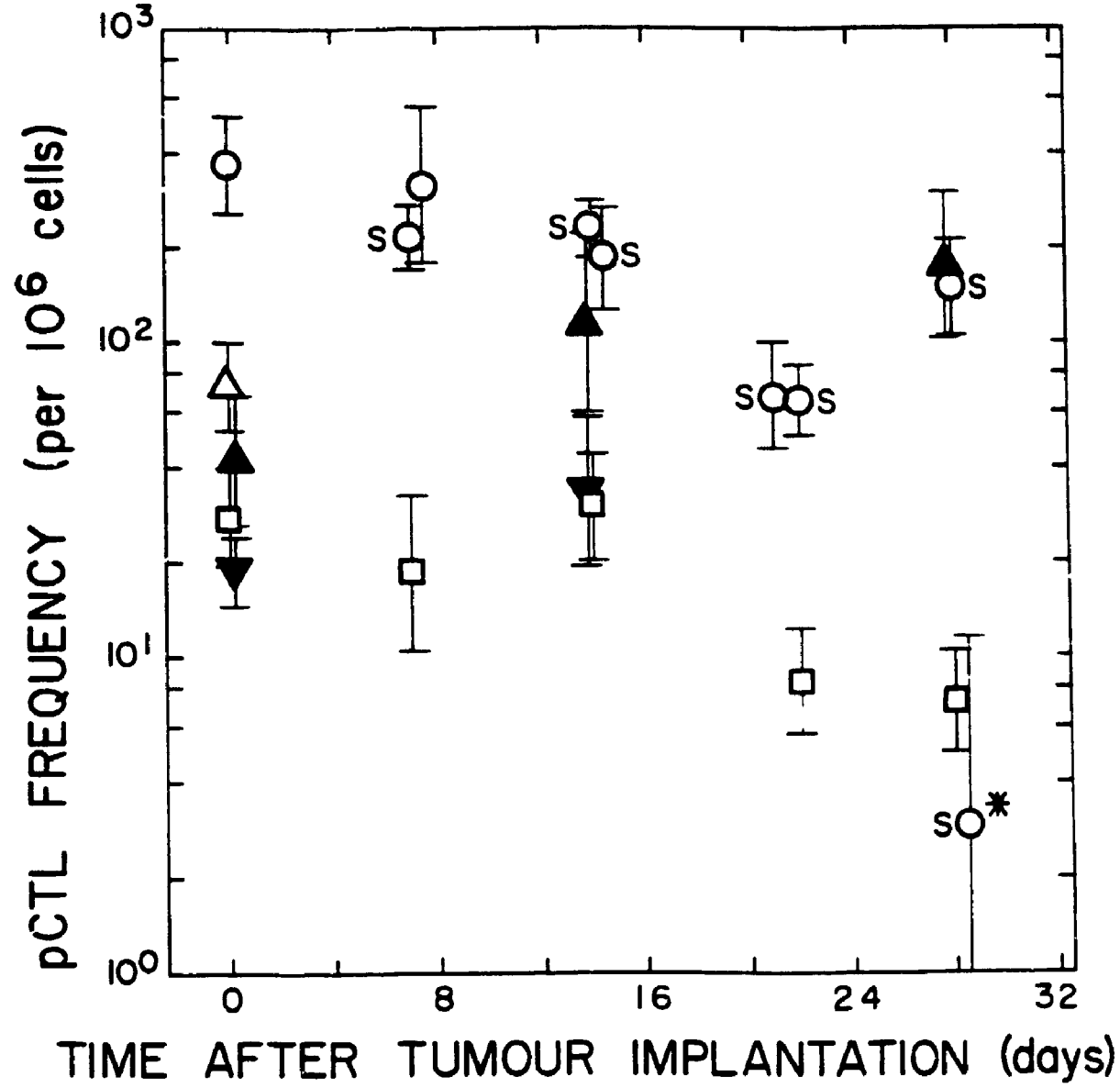


Figure IV-23 A plot of pCTL's per 10^6 cells from lymph nodes and thymuses as a function of the time after tumour implantation

- lymph node cells
- spleen cells
- thymus cells
- means (with 95% confidence limits) of spleen cells (from IV-6)
- mean pCTL frequency in spleens of 10 mice assayed individually (from figure IV-9)

- for each determination, cells were pooled from 2 or 3 mice
- "S" denotes evidence of suppression in LDA curves
- * - very large lymph nodes were present in one mouse and tumour cells appeared to be present in the cell suspension
- error bars - 95% confidence limits

also seen consistently in the LU measurements. That is, in tumour-bearing animals, the cells from the lymph nodes yielded the highest lytic unit activity, and from the thymus, the lowest activity. The LU activity of the spleen cells was intermediate.

Information about the presence of suppressor cells was obtained from the LDA curves. Figure IV-23 shows that suppression was found in all of the LN cell preparations after seven days of tumour growth and in one of the two tests at seven days. No suppression was observed in the LDA's of thymus cells.

In this section, we have determined the mean frequency of pCTL's in the blood of 55 normal mice to be 33.3 (±2.8;SEM) per 10^6 PBL's. In an experiment involving six animals, there was no difference in the pCTL frequency in the spleens and blood: however, there was also no correlation between the frequencies in the spleen and blood. We also concluded that, in normal mice, there are about ten times as many pCTL's in the spleen as in the circulating blood. In the lymph nodes tested, pCTL frequencies appear to be about 12 times higher than in the thymuses, and evidence of suppressor cells was found only in the lymph nodes.

CHAPTER V DISCUSSION

The conclusions which will be discussed are as follows:

1 We have developed a LDA which can be used to measure pCTL frequency in a syngeneic tumour model. As part of this development, optimization of the system was necessary and this has been achieved. As well, pCTL frequencies which were found are consistent with those reported in the literature.

2 We were able to use the LDA, and the quantitation of cytotoxicity produced (the LU), to measure the response to the presence of a tumour as a function of the time interval after tumour implantation.

3 The pCTL frequency and presence of suppressor cells, measured by LDA, are correlated with tumour development. We found the following. There is a correlation between pCTL frequency and tumour size; there is a correlation between the presence of suppressor cells and tumour size; there is a correlation between pCTL frequency in normal animals and development of the tumour after implantation.

4 The cells have been characterized as to their antigenic determinants and radiation sensitivity and the results are consistent with those reported in the literature.

5 We are able to measure pCTL frequencies in various lymphoid organs (spleen, thymus, lymph nodes, peripheral blood) of the mouse.

V-1 DEVELOPMENT OF A LDA TO MEASURE pCTL FREQUENCY IN A SYNGENEIC TUMOUR MODEL

V-1-1 Optimization of the system

The initial objective in this research was to develop and optimize procedures that would enable us to perform a LDA to measure pCTL frequencies in a weakly antigenic system. The importance of optimizing culture and assay conditions has been noted by other researchers (28,29,117, 118,119,280). This section will include a discussion of some of the results of optimization.

Various culture medium components were optimized. Because of the time taken to set up the ^{51}Cr release assays, and the resulting loss of CO_2 (a component of the buffering system), 10 mM HEPES buffer was added to the assay medium to maintain a proper pH. In other systems, 10 mM has been shown to be optimal, with 30 mM proving to be slightly toxic (117,118).

The addition of 2-mercaptoethanol to this and other culture systems has a dramatic effect on the cytolytic activity produced (28,29,61, 117,118,232). Our cultures contained $5X10^{-5}$ M 2-me; a concentration that is commonly used in similar <u>in vitro</u> systems to help generate a cell-mediated cytotoxic response (117,118).

The mechanism of action for 2-me is not known (29,61,119). It is a reducing agent and may act on sulfhydryl groups (29,61). It has been shown that 2-me can have a large effect even if added as late as 3 days after the initiation of the culture (61).

Foetal calf serum appears to be optimal in most culture systems across a range of perhaps 10 to 20% (117,118). We found no beneficial

effect of feeding with additional FCS during the first four days of culture.

The number of cells, or their ratios, are important in determining the strength of a response (28,29,117,118,119) and optima were determined for Responder, Stimulator, and Target cell numbers.

The addition of filler cells was an attempt to maintain a relatively consistent cellular environment in all of the culture wells. Others have found that adding filler cells (irradiated leukocytes) in mixed lymphocyte-tumour cell cultures (36,119), and in allogeneic mixed lymphocyte cultures (232), increases or stabilizes (makes it more consistent) the response.

The addition of SN2°MLC to the culture medium increased the response. IL-2 must be present for an activated pCTL to expand into a clone of CTL's (166). In a LDA designed for comparing precursor frequencies for proliferative and cytolytic responses in an allogeneic system, the most important parameter for a successful measurement of precursor frequency was thought to be the presence of SN2°MLC (215). It has been noted that the failure of certain cells to proliferate in response to antigen could be a consequence of the failure to produce sufficient IL-2, even with the expression of IL-2 receptors (1). In such a system, the addition of IL-2 (or SN2°MLC) would enhance proliferation.

In similar <u>in vitro</u> systems, where the response in the culture peaks at day 5, the decline at later times may be due to IL-2 depletion (199). If IL-2 is added at this time, the response can be boosted. A similar procedure was not attempted with our experiments for several reasons. Because the microculture wells were at capacity with 0.22 ml

of total fluid volume, the addition of extra SN2°MLC would have been difficult (recall that each well held 0.1 ml of SN2°MLC originally). Additions would have meant exposing the cultures to possible contamination. As well, the response of the system was sufficient so that limiting dilution assays were possible with SN2°MLC being added only at the initiation of the culture.

It seems likely that the spatial relationship between the various interacting cell types is important for the production of a maximum response and the shape of the culture well could affect that relationship since the cells settle to the bottom of the well. Others have found, for cultures containing approximately 5 ml of medium, that the response in tubes was greater than in flasks or dishes (117,118). They suggest that close contact between reacting cells is necessary for effective stimulation.

We found peak responses after a 5 or 6 day culture period and after a 24 hour chromium release assay period. Others have demonstrated that the cytotoxic T lymphocyte response *in vitro* peaks at 5 to 6 (28,29,117) or 5 to 8 (119) days with syngeneic murine mixed lymphocyte - tumour cell cultures (MLTC). It has been noted that an additional 18 to 21 hours of incubation in fresh culture medium at the end of a 5 day culture period could boost the response (29).

Since tumour cells continue to release ^{51}Cr for a period of time after they have been lethally damaged, a 4 hour incubation has been followed by a further one hour at 45°C to increase the rate of ^{51}Cr release from damaged cells (29). With a long incubation time (i.e. 24 hours), the amount of unreleased ^{51}Cr from damaged cells would be minimal.

Some parameters were not checked in this system. Stimulator cells were inactivated by irradiation. Burton et al. have shown that, for whatever reason, mitomycin C may be better, but this method had very little advantage (28). In other studies, they found that the method of inactivation was not important, with 50 gray of X rays being as effective as mitomycin C or glutaraldehyde treatment.

Hancock el al. found that the amount of help from thymus-derived Helper cells in a tumour-bearing animal depended on the number of injected tumour cells (94). If 10^4 rather than $2X10^3$ P815 cells were injected into DBA/2 mice, more animals contained T helper cells in the thymus and the magnitude of help was slightly greater. It is possible that in my experiments, there might have been different kinetics of appearance of helper and perhaps suppressor cells and pCTL's if different tumour cell numbers had been implanted.

No experiments were performed with stored cells but it has been demonstrated that cryopreserved normal splenocytes and leukemia cells were as efficient as fresh cells in generating allogeneic and syngeneic CTL's (117).

Culture volumes have been indicated as being an important parameter in the response (117,118). The total media volume (0.22 ml) in these experiments filled about 80% of the culture well. It was felt that altering the other factors such as cell numbers, ratios, concentrations of SN2°MLC etc. would have the same effect as altering the total fluid volume.

V-1-2 pCTL frequency estimates, comparisons; and the pCTL

pCTL frequencies obtained from these studies are consistent with

most of those reported in the literature. In experiments where pCTL frequencies for precursors responding to P815 tumour cells were determined, the pCTL frequencies in the spleens of tumour-bearing mice, two weeks after tumour implantation, were higher than in normal animals. In various series of experiments, the pCTL frequencies were found to be: 57.4 (±17.6,SEM;n=10) per 10^6 cells in normal versus 167.4 (±63.9,SEM;n=7) per 10^6 cells in tumour-bearing mice, 193 (145-259,95% limits,n=1) per 10^6 cells versus 468 (313-701,95% limits,n=1), and 19.8 (±2.3,SEM;n=10) per 10^6 cells versus 45.3 (±11.9,SEM;n=10) per 10^6 cells. For 55 determinations of pCTL frequency in the peripheral blood, the mean frequency was 33.3 (±2.8,SEM) per 10^6 peripheral blood leukocytes. There were frequency differences in the spleens at intervals greater than two weeks depending on whether the cells were from animals with small tumours (mean = 291.0 (±53.1,SEM;n=7) per 10^6 cells), or from animals with large tumours (mean = 68.1 (±13.0,SEM;n=5) per 10^6 cells).

Other researchers have also attempted to use the LDA to determine frequencies of pCTL's in DBA/2 mice. Lindahl and Wilson (146) measured the frequency of precursors capable of responding to trinitrophenol-conjugated (TNP-conjugated) syngeneic cells. After DBA/2 lymph node cells had been stimulated by DBA/2-TNP conjugated spleen cells, cytotoxicity was tested against P815-TNP target cells. The pCTL frequency was found to be 55 (±24,SEM;n=2) cells per 10^6 LN cells. Unlike the results of the present experiments, they were unable to stimulate <u>in vitro</u> DBA/2 LN cells by P815 cells to produce CTL's. Kilburn et al. (123), and Hancock and Kilburn (93) were able to obtain frequency estimates in <u>normal</u> DBA/2 mouse spleen cells responding to P815 tumour cells. Their experiments yielded estimates of 719 (462-

1118,95% confidence limits;n=1) per 10^6 cells (123) and 3350 (2060-5440,95% confidence limits;n=1) per 10^6 cells (93). For the higher estimate, they reported repeating the experiment five times with similar results.

The reasons why they obtained much higher estimates than in the present experiments are unclear. Their estimates are based on limited data (12 cultures per point were used for the LDA), and stimulators were treated with mitomycin-C and IL-2. However those differences should not lead to such large discrepancies. It is informative to consider that if the estimate of 3350 pCTL's per 10^6 spleen cells is correct, then what is the frequency of pCTL's in the total population of pCTL's that are capable of specifically responding to the syngeneic P815 tumour. It has been variously estimated that 11% (139) or 15% (38) of spleen cells are Ly-1^+,$2/3^+$ and therefore a potential source of pCTL's. Chen et al. (37) have estimated, by lectin-mediated cytotoxicity, that the frequency of all pCTL's in CBA mouse spleens is 9.2%. Assuming that as many as 15% of spleen cells are pCTL's, and that the estimate of 3350 pCTL's per 10^6 spleen cells is correct, then 3350 per $1.5X10^5$ pCTL's are specific for the syngeneic P815 tumour antigen(s). This would be a frequency of 1 per 45 cells and seems to me to be unreasonably high for an animal that has not previously been exposed to that particular tumour antigen.

In agreement with the present experiments, others have found much lower numbers in the spleens of DBA/2 mice. In a pool of 5 spleens from semi-syngeneic (DBA/2 X C57Bl/6)F1 mice, 9 days after tumour injection and at the peak of the CTL response, Johnson and North (111) estimated a frequency of 53.8 pCTL's capable of responding to P815 tumour cells per 10^6 spleen cells. This was equivalent to $5.9X10^3$ pCTL per spleen.

This estimate is similar to those reported in this thesis. When C parvum was injected together with the tumour cells to boost the immune response, estimates of 371.5 and 181 9 pCTL's per 10^6 spleen cells were reported (111).

pCTL frequencies in other syngeneic tumour systems have also been studied. In normal mice, Teh et al. (264) found the frequency in C57Bl/6 spleen cells responding to EL4 tumour cells to be 132 (±19,SEM;n=2) per 10^6 cells, and in C57Bl/6 spleen cells responding to C1498 tumours to be 231 (162-329,95% limits) per 10^6 cells.

In a series of experiments, Brunner et al. found the frequencies of pCTL's in the spleens of C57Bl/6 mice bearing regressing MBL-2 tumours to be 1730 (±270,SEM;n=3) per 10^6 cells (20) and 1240 (±190,SEM;n=6) per 10^6 cells (21) and, in the spleens of C57Bl/6 mice bearing regressing LSTRA tumours, to be 755 (±59,SEM;n=2) per 10^6 cells (20). Brunner et al. (24) found the pCTL frequency in spleens of C57Bl/6 mice bearing 15 day regressing MBL-2 tumours to be 2085 per 10^6 cells while in the spleens of normal mice, the frequency was much lower at 135 per 10^6 cells.

In sixteen patients with tumours, Vose has found the pCTL frequency of cells, reactive against their tumours, to be 167 per 10^6 peripheral blood leukocytes (276). The frequency in tumour infiltrating lymphocytes was higher at 526 per 10^6 cells. The frequency of proliferative cells was higher in both PBL ($265/10^6$) and TIL ($923/10^6$). Therefore the pCTL's are a part of the total number of cells which can be stimulated to divide in response to specific tumour antigens.

It is important to note that the LDA measures the <u>minimum</u> frequency of a given cell type. We can never be completely certain that

conditions are sufficient for the detection of a single pCTL in a culture well (except when only pCTL's are present and the pCTL frequency is found to be 100%). It is possible, under sub-optimal conditions, that some pCTL's (perhaps a particular subpopulation?) will not be stimulated to produce CTL's and therefore will not be detectable.

Even in the absence of suppressor cells, CTL production in vitro as a function of pCTL numbers is non linear. At high Responder cell numbers (> $3X10^5$ under optimal conditions with this system), there is a fall-off in the production of cytotoxic activity, probably due to either nutrient depletion or toxic waste buildup. The result is that LU production falls even as pCTL numbers increase. Therefore the measurement of precursor frequency by LDA may provide a better indication of at least the potential for an effective response than would the measurement of the amount of cytotoxicity produced by a given cell number.

There is a question as to whether the pCTL's, studied in this tumour system, are fully functional CTL's, capable of themselves lysing tumour target cells. In a study of alloantigen reactive T lymphocytes, the mean pCTL frequency in day 4-5 MLC's was 50 to 100 times higher than in normal spleen cells (164). Up to 25% of the cells in the MLC were found to be pCTL's. It appears that the cycle from pCTL to CTL to "memory" pCTL to CTL etc. can be repeated, perhaps indefinitely (153).

In the research for this thesis, there was no evidence that the pCTL's are directly cytotoxic to the tumour cells <u>in vitro</u>. All attempts to measure cytotoxicity directly in a ^{51}Cr release assay, without a prior <u>in vitro</u> incubation, have failed.

Because of the relative scarcity of these cells, they might <u>appear</u>

to be non-cytotoxic in an assay. They might be hindered from interacting with target cells by the presence of large numbers of non-cytotoxic cells. Recall that direct cell-cell contact is required for target cell lysis. It is only after the pCTL's have been stimulated to produce many cytotoxic cells that cytotoxicity can be measured.

For example, if we assume a pCTL (or CTL) frequency of 200 per 10^6 cells, an effector:target cell ratio of 100:1, and 1000 target cells (and therefore 100,000 effector cells), there would be 20 pCTL's and 99,980 non-cytotoxic cells and 1000 target cells. With such conditions, one would not expect much interaction between pCTL's and targets. With this tumour model, it is still an open question as to whether the pCTL's are functional CTL's. If the pCTL's could be isolated or concentrated, that question should be answerable.

V-2 THE RESPONSE AS A FUNCTION OF THE TIME INTERVAL AFTER TUMOUR IMPLANTATION

One of the objectives of this research was to determine whether the response, measured by lytic unit production and pCTL frequency, changes as a function of the time interval after tumour implantation.

The results demonstrate an apparent increase in potential lytic unit production for spleen cells derived from pools of animals two weeks after tumour implantation compared with spleen cells from normal, non-tumour-bearing mice. When pCTL frequencies were measured, a low background level was found in normal mice. The level increased at two weeks and became significantly different (from zero time) at four weeks.

When individual mice were tested within a single experiment, the results were similar. Tumour bearing mice had a higher frequency of pCTL's in their spleens after 2 weeks than did normal mice. The change in frequency was not significant at the 5% level (P is approximately = 0.10). Presumably, the pCTL frequency increases in response to the presence of the tumour. Assuming that the absolute number of pCTL's in the animal increases, these new pCTL's must be derived from pre-existing pCTL's.

Our conclusions are: there is a change in response correlated with the growth of a tumour; we can measure that change by the increased potential cytolytic response, or by the increased CTL precursor frequency; the increased response is consistent with results reported by others.

Cytolytic activity

Others have also reported, both with the P815 tumour model and with other systems, that more cytolytic activity can be generated from cells of tumour-bearing animals than from cells of normal ones (26,54,55,78,173, 174,185,186,191,199). The response usually reaches a peak and then declines, perhaps due to the advent of suppression. The LDA may provide a method of detecting cells capable of responding, even when suppressor cells or factors have diminished a potential cytotoxic cell response.

North found a peak in activity, at day 6 of tumour growth, in spleen cells capable, on adoptive transfer, of causing tumour regression in an immunodepressed animal (185,186). Other studies showed that cells responsible for concomitant immunity (active against the implantation of

a second tumour), reached a peak of activity 6 to 9 days after the tumour implantation (26,191). Two different cell types are responsible for the rise and subsequent decline in activity. Effector cells ($Ly\text{-}1^-2^+$ T lymphocytes) are acquired at about days 3 to 6, peak on day 9, and are lost by day 12. T suppressor cells ($Ly\text{-}1^+2^-$) arise after day 6 and increase in activity from days 9 through 15.

Cytolytic activity in DBA/2 spleen cells, directed against the P815 tumour, has been reported by Mills and Paetkau to peak at around day 20 of tumour growth (173,174,199). Although the same murine tumour model was used, their results differ from ours in some ways. When they cultured spleen cells with IL-2 alone, in the absence of tumour cells, there was a much greater response than with P815 stimulation in the absence of IL-2 (174,199). They also obtained a greater response from the spleen cells when neither P815 cells nor IL-2 were present than when tumour cells alone were added!

Our results show that the presence of P815 tumour cells is necessary for the stimulation of pCTL's and that there is no measurable response when either SN2°MLC alone is added or when neither SN2°MLC nor P815 cells are present.

The reasons for the differences between our results and those of Mills and Paetkau are unclear. Since their pCTL's can be stimulated by IL-2 alone, they resemble LAK cells rather than classic pCTL's. Their P815 cells were passaged <u>in vivo</u> in such a way as to select for a highly metastatic line. Our P815 cell source was from an ascites tumour, passaged <u>in vivo</u>. Perhaps accessory cells, able to present tumour Ag, were present (?). Their P815 cells were cultured <u>in vitro</u> and passaged once per month <u>in vivo</u> and therefore their source of stimulator cells

may have come from an *in vitro* passage, thus making the presence of accessory cells less likely. Although their CTL's did not lyse normal DBA cells, they did kill both the P815 and an unrelated syngeneic L1210 tumour *in vivo*.

Dye also has worked with the P815 tumour grown in (C57Bl/6 X DBA/2)F1 mice (54). He measured the cytotoxicity of lymph node and spleen cells directly in a ^{51}Cr release assay, at an effector:target ratio of 100:1. The percent specific cytotoxicity of spleen cells peaked at about 10% on day 10 of tumour growth while LN cells reached a peak activity on day 20. The response in syngeneic DBA/2 mice reached a peak of 7% on day 10. Although the difference between 7% for syngeneic DBA/2 mice and 10% for semisyngeneic B6D2F1 mice is slight, there is evidence that the response in semisyngeneic mice is stronger. The mean survival time of B6D2F1 mice injected i.p. with $2X10^6$ P815 cells was longer (by 6 days) than that of DBA/2 mice.

Dye has also shown that progressive growth of P815 tumours in an immunocompetent host results in an antitumour response, measurable by the concomitant immunity activity of Ly-1^+2^+ T cells. This activity is present by day 6, reaches a peak on day 9, and then declines.

pCTL frequency

There have been few studies using the LDA to measure a change in immune status. The LDA has been employed to monitor the change in immunological status after an *in vivo* immunization (216). Alloimmune spleen cells, obtained from C57Bl/6 mice immunized with DBA/2 tumour cells and tested against DBA/2 cells, show a pCTL frequency of 6500 per 10^6 cells compared with 2000 per 10^6 cells for spleens from unimmunized

mice.

Several studies have found, as ours did, increased pCTL frequencies associated with the growth of a tumour. Brunner et al. (20,21,24) have reported an increase in pCTL frequency in the spleens of tumour-bearing animals. Using the C57Bl/6-anti-MBL-2 tumour model, they found 2085 pCTL's per 10^6 spleen cells in mice 15 days after tumour implantation compared with 135 pCTL's per 10^6 cells in normal mice (24). These highly immunogenic tumours always regress after an initial growth period. In other model systems, they found precursor frequencies in animals bearing regressing tumours to be at least an order of magnitude higher than in normal mice (20).

Johnson and North performed a LDA to estimate the increased production of CTL's associated with C parvum-induced regression of P815 subcutaneous tumours in (C57Bl/6 X DBA/2) F1 semisyngeneic mice (111). Although Dye (54) reported a slightly increased response of these hybrids against the P815 tumour, Johnson and North reported there was no evidence of any difference in response. When the total number of pCTL's was measured in the draining LN, there was an increase of about 9 fold from day zero to day 9, at the peak of the response. When tumour cells were implanted together with C parvum, the increase was much higher, approximately 77 fold.

V-3 THE pCTL FREQUENCY AND PRESENCE OF SUPPRESSOR CELLS, MEASURED BY LDA, ARE CORRELATED WITH TUMOUR DEVELOPMENT

V-3-1 THERE IS A CORRELATION BETWEEN pCTL FREQUENCY AND TUMOUR SIZE

The results demonstrate a significant negative correlation between tumour mass and pCTL frequency in the spleens of mice, assayed individually, at day 14 of tumour growth (fig. IV-16). We believe this kind of relationship has not been reported previously; i.e. an immune response correlated with tumour size, at a specific time after implantation.

What has been shown by others is that the response can vary as a function of time after tumour implantation. For instance, when the lytic ability of tumour infiltrating lymphocytes from either progressing or regressing Molony sarcoma tumours is tested against ^{51}Cr-labelled tumour cells, there is no difference at day 11 (78). By day 13, T cells from regressing tumours are much more cytotoxic than those from progressing tumours.

It is difficult to determine whether there is a quantitative difference, depending on tumour size, in the cytotoxicity which can be generated. This is for the same reasons that it is difficult to determine the relative cytotoxicity produced by pCTL's from normal versus tumour-bearing animals. The amount of cytotoxicity depends on the number of precursors and on the presence of suppressor cells. It may also be true that the generation of maximum cytotoxicity requires different culture conditions for each precursor type (i.e. for pCTL's

from normal versus tumour-bearing mice). Determining a correlation between pCTL frequency and tumour size is much simpler to do.

Although there is a mathematical relationship between pCTL frequency and tumour size, we cannot assume there is a biological causal relationship (303). Even though we might believe that high pCTL frequencies are the cause of small tumours, it is possible that the cause and effect are reversed. That is, small tumours may lead to high pCTL frequencies. As well, both small tumours and high pCTL frequencies may both be dependent on a third, untested variable (for example; the presence or quantity of specific T suppressor cells).

Since there is a good correlation between pCTL frequency and tumour size, and assuming the size is indicative of the effectiveness of the immune response, then measuring the pCTL frequency in the peripheral blood may be useful in monitoring the strength of the immune response of a patient undergoing therapy, when viable tumour cells from a biopsy are available. We have shown this use of a LDA would also allow us to separate the effects of suppressor cells and pCTL frequencies.

V-3-2 THERE IS A CORRELATION BETWEEN THE PRESENCE OF SUPPRESSOR CELLS AND TUMOUR SIZE

Cells which suppress the anti-tumour immune response have been shown to arise late in the development of several animal tumour models including the P815X-DBA/2 one (56,57,59,70,144, 170,171,172,188, 201,257,258,259). It has been hypothesized that the P815 tumour grows progressively, in spite of its immunogenicity, because it evokes the

generation of suppressor T cells before enough effector cells are generated to reject it (193).

With the LDA, suppressor cells were detected only in the spleens of animals with large tumours and not in animals with small tumours. The amount of suppression was not measured in the LDA.

Plata et al. reported two suppressor cell populations (macrophages and T cells), in MSV-immune spleens, that can block the generation of CTL's (201). They noted that the Ts cells were particularly evident among day 9 to 10 spleen cells when mice had large, progressively growing tumours. Like the cells in the P815-DBA/2 model, these suppressor cells did not block the lytic activity of effector cells in a ^{51}Cr release assay.

Suppressor T cells have been shown to inhibit the production of cytotoxic T cells in adoptively immunized, T-cell deficient recipients, but not the antitumour function of cytolytic T cells already generated (171). Also, a tumour-specific Ts clone has been shown to act in vitro preventing the generation of specific syngeneic cytotoxic cells (96). These Ts cells would also suppress the response to tumour cells in vivo.

The results of mixing experiments indicate that suppressor cells act to prevent the production of CTL's from pCTL's. Since this occurs even when SN2°MLC is present, (known from the LDA experiments), it seems likely that the suppressor cells act on the pCTL's and not on the T helper cells. However we cannot rule out the possibility that the suppressor cells act directly on IL-2 or on IL-2 receptors on the pCTL's.

Other researchers have also postulated that suppressor cells act to inhibit the pCTL's and prevent production of CTL's (56,70,201). Some

have noted the effect of the suppressor cell (in the P815-DBA/2 system) might be on the helper cell (144).

The suppression can be specific. Cells from mice bearing a progressive P815 tumour were capable of suppressing adoptive T-cell-mediated suppression of P815 tumours, but not P388 tumours; and vice versa (57).

P815 tumour cells, in the spleens of tumour-bearing animals, have been shown to suppress a T cell-mediated response in vitro when the spleens were the source of Responder cells (272). The effect of tumour cells in the Responder cell population could be eliminated by the use of azaguanine-resistant P815 mutant cells that would die in culture medium supplemented with hypoxanthine, aminopterin and thymidine (HAT) (272).

V-3-3 THERE IS A CORRELATION BETWEEN pCTL FREQUENCY IN NORMAL ANIMALS AND DEVELOPMENT OF THE TUMOUR AFTER IMPLANTATION

The thesis results demonstrate a small but significant negative correlation between pCTL frequency in the blood of normal animals, seven days before a tumour injection, and the tumour volume fourteen days after injection. As well, animals with higher numbers of pCTL's active against the P815 tumour, prior to injection, are more likely to reject their tumour. The same comments made previously regarding correlation and cause and effect apply here. Simply because there is a statistically significant mathematical correlation between two parameters, does not mean that a cause and effect relationship exists.

One cannot adequately test whether preexisting conditions

potentially affect tumour growth in humans. Evidence in favor of such an effect comes from immunodeficient or immunodepressed patients who have a higher frequency of some types of tumours (98).

We might expect events occurring, or conditions existing, soon after tumour implantation to be even more important than preexisting conditions. It would be difficult to test, with this system, whether pCTL frequencies during the first few days of tumour growth are correlated with subsequent tumour volumes or eventual progression or regression of the tumour. Because about 0.3 ml of blood is required for the LDA test, the mouse may be somewhat traumatized, and this might affect subsequent tumour growth. If the experiment was performed on a larger animal, for instance a rat or rabbit, 0.3 ml of blood would still be adequate and this volume might be obtainable without unduly affecting the animal. With a larger animal, even serial determinations of pCTL frequencies might be feasible.

In the series of experiments discussed here, some tumours progressed and the mouse eventually died while other tumours grew and then regressed. Some tumours initially progressed, then regressed until no tumour mass was palpable, and eventually reappeared at the injection site, progressing until the animal died. Why did some tumours exhibit this growth pattern? Some possible explanations include 1) tumour antigen loss variants, 2) late development of suppression of the immune response, and 3) a virus, associated with the tumour, causing transformation and neoplasia.

If the immune system is responding to tumour-associated antigens, and if these specific antigens are lost for any reason from the tumour cell surface, or if there is a rare subclone of tumour cells not

expressing the appropriate antigens, then this growth pattern might occur.

The P815X tumour has been reported as belonging to a category of tumours in which the onset of an immune response can cause partial or temporary regression of the tumour (257,258,269). Experiments by Uyttenhove et al. (269), with ascitic P815X tumours, indicate that this pattern of tumour growth is due to antigen loss variants. The animals have been shown to be resistant to injection of fresh P815X tumour cells and therefore the immune system appears to have not been compromised.

Cytotoxic T lymphocyte receptors have a dual specificity for both tumour Ag and self-MHC Class I antigens (261). If Class I antigens are lost from the tumour cells (or altered), then the pCTL's or CTL's would not be able to recognize them, even if the tumour antigens per se remained.

The initial regression may be due to the onset of an effective immune response. If suppression were to develop later, when the tumour had almost been eliminated, then the tumour would be expected to regrow. This pattern is similar to the findings of Takei et al (257,258) and Dye (55). In one system, metastatic cells in the spleen and especially in the draining lymph nodes of tumour bearing animals follow this pattern - i.e. growth, reduction due to an immune response, and regrowth due to suppression of the response (55).

With either of these two possibilities, it is not clear why the tumour would be reduced to an undetectable state, for a period of days or weeks, before regrowing.

The major conclusions of this section are that, on average, animals with higher pCTL frequencies prior to tumour implantation have smaller

tumours at seven and fourteen days, and, their tumour is more likely to regress.

V-4 CHARACTERISTICS OF THE CELLS

The cells have been characterized as to their antigenic determinants and radiation sensitivity. It is important to do this kind of characterization in order to determine which cell types are involved in the response being measured. We want also to be able to compare the types and characteristics of cells in this system with those reported in the literature for other tumour models.

V-4-1 ANTIGENIC DETERMINANTS

From previous studies in this laboratory, it is known that the cytotoxic cells produced *in vitro* are T cells (Thy-1^+) and that they are Ly-2^+ and probably Ly-1^+ (241). The research for this thesis shows that the pCTL's are Thy-1^+, Ly-1^+, Ly-2^+ T cells. The predominant helper cell population has been shown to be Thy-1^+, Ly-1^+, Ly-2^- T cells.

These experiments were made difficult because of variable killing by Ab plus complement. In some experiments, anti-Ly-2 Ab plus complement treatment did not completely remove the pCTL population. Results were even more variable with anti-Ly-1 Ab plus C treatments. This variability was in some part due to an attempt to have sufficient killing so that all cells with that antigen would be lysed, and yet no

toxicity (from non-specific lysis, toxic complement etc.) would occur to other cell types.

There is considerable evidence that these results are in agreement with those obtained by other researchers with the same and similar systems. North, Dye and Mills have shown that the cytotoxic cells, harvested at the peak of response from draining lymph nodes of mice bearing syngeneic tumours (including P815X) are Ly-2$^+$ and partly Ly-1$^+$ (meaning that the activity was partly eliminated by anti-Ly-1 MoAb plus C) (57,58,170,171,187,193). These cells are cytotoxic in a ^{51}Cr release assay and are effective in a Winn type assay (170).

Maier et al. (160) showed that DBA/2 allogeneic killer cells were Ly-1$^+$2$^+$ and that cells cytotoxic to the syngeneic P815 tumour are predominately Ly-2$^+$. They also reported that a Ly-1$^+$ cell is essential in suppression. Suppressor cells, effective <u>in vitro</u>, lost about 80% of their ability to suppress the anti-P815 response after treatment with anti-Ly-1 Ab plus C but not after anti-Ly-2 Ab plus C. Others have demonstrated that Ly-1$^-$,2$^+$ T cells, suppressing a tumour-specific dth reaction, and Ly-1$^+$,2$^-$ T cells, suppressing the expression of adoptive immunity, are produced in the BALB/c-MethA fibrosarcoma syngeneic tumour system (49). We did not test for the Ly phenotype of suppressor cells.

Conflicting reports in the literature concerning cells responsible for an effective response against various murine syngeneic tumours have been noted (14,137). Those authors feel that the contradiction may be due to the tumour studied, or perhaps due to whether or not the assays were conducted <u>in vivo</u> or <u>in vitro</u>.

Shiku et al. showed that cells cytotoxic to syngeneic tumours are Thy-1$^+$, Ly-1$^+$ and Ly-2/3$^+$ (226,227). In studies with the Meth-A

fibrosarcoma growing in syngeneic BALB/c mice, it has been reported that excising the tumour at day 9, at the peak of host concomitant immunity, leaves the host in a long-lived immunocompetent state (27). At the time of excision, concomitant immunity is attributable to Ly-1^-,2^+ T cells while long lived immunity is due to Ly-1^+,2^+ memory T cells. Thus Ly-2^+ T cells are important in the response to syngeneic murine tumours other than the P815X mastocytoma.

Considering whether Ly-1^- T cells play an important role in the anti-tumour response, it is now recognized that all T cells have some Ly-1 molecules, even if some are at low density (31,139,158). Ly-1 has been shown to be present at relatively low levels on Tc and Ts cells (125,139). Brunner et al. have shown that various CTL clones are Thy-1^+ and Ly-2^+ (25). All were also Ly-1^+ although there was a greater heterogeneity in the density of Ly-1 on the cells and this marker appears to be less abundant than Ly-2.

It is now recognized that mouse Th cells bear the L3T4 (CD4) antigen and thus helper cells can be distinguished from cytotoxic T cells on the basis of this and the CD8 (Ly-2) marker. Th cells are known to be $CD4^+CD8^-$ and CTL's are known to be $CD4^-CD8^+$ (31,115,200). The L3T4 antigen marker was not used in these studies because, when these early experiments were done, it was felt that the combined use of Ly-1 and Ly-2 markers was sufficient for distinguishing subpopulations of T cells.

It is important to note that if the system allows time for differentiation or recruitment of the effector cells, then it is difficult to know which cell is ultimately responsible for the destruction of a tumour (228). For instance, if it is shown that Ly-

1^+2^- T cells, when injected into a tumour-bearing animal, are effective in mounting an anti-tumour response, it may be Ly-2^+ cells, recruited from the host, which are actually ultimately responsible for the tumour destruction.

The possibility that Ly-1^+2^- T cells can be CTL's (responding to Class II MHC antigens) may explain why some researchers have said the important cell is a helper cell (because of belief that a Ly-1^+2^- cell had to be a helper cell) when in fact they were CTL's. This is very speculative and I am not aware of any demonstrations of a Ly-1^+2^- CTL specific for syngeneic tumours.

V-4-2 RADIATION SENSITIVITY

The results indicate that, with the P815-DBA/2 tumour model, the effector cells are radiation resistant; 6 gray has no effect. T helper cells, obtained from tumour-bearing animals, are of intermediate radio-resistance and the pCTL's are most sensitive, with 4 gray reducing the lytic activity produced to about 5% of control.

The radiation response curves presented are not true survival curves since survival curves describe the relationship between the absorbed radiation dose and the survival of the cells, measured by the ability to divide (91). These response curves are based on cellular function, or the ability to produce lytic activity. For the response of pCTL's to radiation, there is a survival component because radiation will inhibit the division of the cells and the subsequent formation of CTL's. If curves had been generated based on pCTL frequency, they would

then have truly represented survival.

It has been reported that the state of lymphocyte activation can influence radiation response (148). Activated T cells are not killed as quickly as unactivated ones. After 10 gray, the viability of activated cells was reduced by 50% while that of unactivated cells was reduced by over 99% (148). Activation might affect the radiation response of the cells in the P815-DBA/2 model.

Six gray of radiation had little or no impact on the function of the Effector cells. This was expected because we are measuring the radiation effect on the cell's ability to express cytotoxicity and not to divide. Cell division is generally much more sensitive to radiation than is cell function (91). Also, the CTL's are activated and this may protect the cells somewhat. Others have also shown the CTL's in the P815 tumour model to be very resistant; 25 gray did not diminish the function of the cytotoxic cells (173).

The pCTL's were more sensitive than were the CTL's, probably because of the effect of radiation on the cell's ability to divide.

In some murine tumour systems, the pCTL has been reported to be less sensitive than the T helper cells (113,254). Although 5 gray whole body irradiation had no effect on the pCTL frequency increase measured in a secondary response, T helper cells were impaired by that dose (113). Here, there was some helper cell function even after a dose of 10 or 12 gray. Because of the method of producing the response curve, it is difficult to estimate what percentage of help remains after a given dose and then to make a direct comparison of the radiosensitivity of helper cells and pCTL's. Since there appeared to be some help, even following a dose of 12 gray, this may indicate that multiple divisions

are not required for the provision of effective help. Others have also found T helper cells in the P815 system that appear to be relatively radioresistant (94).

The radiation response of suppressor cells was not determined in these experiments. The suppressor function in some tumour models can be removed by 4 to 6 gray of whole-body irradiation (48,173,187). Successful immunotherapy via radiotherapy has been demonstrated in 3 syngeneic tumour models (5,189). Five gray of whole-body irradiation induced tumour regression by preferentially eliminating or preventing the production of T suppressor cells, thus allowing the host to generate an effective immune response. It was shown that the important radiation effect was on the immune system and not on the tumour cells themselves since infused suppressor cells from tumour-bearing mice inhibited the tumour regression. Variability in the model was demonstrated when 1 out of 5 P815 tumours regressed completely and 4 out of 5 were delayed and then continued to grow after radiation treatment (5).

V-5 ANATOMICAL LOCATION OF pCTL'S

We are able to measure pCTL frequencies in various lymphoid organs (spleen, thymus, lymph nodes, peripheral blood) of the mouse.

When six normal animals were tested, there was not a significant difference between pCTL frequencies in the spleens and in the blood. At the same time, there was no significant correlation between the pCTL frequency in the peripheral blood and in the spleen of the same animal. Because the ranges of frequencies in the spleen (20-29 per 10^6 cells) and

in the blood (15-33 per 10^6 PBL) were quite narrow and similar in the experiment where direct comparisons were made, they might not have been expected to lead to a significant correlation.

Because the frequency of pCTL's in the spleen increases after tumour implantation (figures IV-6, IV-9), the question arises whether the pCTL frequency in the blood also rises, and whether data including both normal and tumour bearing animals might indicate a correlation between pCTL frequencies in the spleen and in the blood. Data for frequencies in the blood of tumour-bearing animals was not collected and this question remains unanswered.

In studies where pCTL frequencies have been measured in the blood of animals at the onset of tumour regression, the frequency in the peripheral blood (2320 (±420,SEM) per 10^6 cells) was found to be higher than in the spleen (1240 (±190,SEM) per 10^6 cells), but not nearly as high as in TIL preparations (12,100 (±1700,SEM) per 10^6 cells) (21). Others have also reported, in experiments with human tumours, that the pCTL frequency in TIL's (526 per 10^6 cells) is higher than in the peripheral blood (167 per 10^6 cells) (276). The pCTL frequency in the spleen and peripheral blood may be decreased by the accumulation of pCTL's at the tumour site. This might explain the lower pCTL frequencies in the spleens of animals with large tumours compared with animals with small tumours. It should be possible to use centrifugal elutriation (235) to separate tumour cells from host cells in order to quantitate pCTL's and determine the lytic activity of CTL's at the tumour site.

The results from normal mice indicate that the pCTL frequency in spleen cells is intermediate between LN cells the thymus cells with the

frequency in LN cells being the highest. When LN and thymus cells in tumour-bearing mice were compared over time, the frequency in the LN's was about 12 fold higher. While results show that the pCTL frequency in spleens increases after tumour implantation, the frequency in thymuses and lymph nodes appears to remain the same or decrease.

In experiments where the generation of lytic activity, from cells from various organs, was tested against syngeneic tumours in vitro, it was found that spleen and pooled lymph node cells of normal mice gave comparable results while thymus cells produced a negligible cytotoxic response (117,118). With the DBA/2-P815X system, it has been reported that thymus cells from tumour-bearing mice don't produce increased cytolytic activity compared with normal mice, while spleen cells demonstrate an increase in lytic activity which can be generated (174,199). This is similar to our results.

Frequencies of pCTL's have been reported to be about three times higher in lymph nodes than in spleens (166). As well, precursor frequencies of anti-alloantigen responding cells in the lymph nodes of C57Bl mice appear to be much higher (3283 per 10^6,95% limits 2110-5105) than in the thymus (178 per 10^6 cells, 95% limits 100-299) (69).

Evidence of suppressor cells in the LDA was noted only in lymph node cells (and as early as 7 days after tumour implantation - earlier than in the spleen) and not in the thymus cells. This is in contrast to reports of suppressor T cells, specific for P815 tumours, being found in the thymus 4 to 8 days after tumour injection, earlier than in the spleen (Takei, Levy and Kilburn; 259). Differences between results obtained with our system and those of Kilburn, Talbot and Levy (123), and Hancock and Kilburn (93) have already been discussed.

It is noted that the frequency of pCTL's and lytic activity produced per 10^6 Responder cells has been consistently higher in lymph nodes than in spleens of tumour-bearing animals. And yet suppression, measured by the LDA, is found in the lymph nodes as early as day 7 after tumour implantation. The pCTL frequency can certainly be high, even in the presence of suppressor cells; but it is not clear why lytic activity should remain high in the presence of suppressor cells. We did not determine how effectively the suppressor cells from LN's, their presence being indicated by a LDA, could diminish the production of lytic activity in the culture system. Perhaps the amount of suppression was not sufficient to overcome the increased lytic activity produced by an increased number of pCTL's.

V-6 CLINICAL APPLICATIONS AND FUTURE STUDIES

Clinical Applications

If viable tumour cells are available from a biopsy, then the pCTL frequency measured in the peripheral blood may be a useful determination of the immune response of a patient.

Enough blood for a LDA (0.5 ml) is easily obtained when a sample is taken for other tests. The tumour cells could be used immediately as either stimulator or target cells, or stored at the temperature of liquid nitrogen. Both murine and human lymphoid cells have been successfully cryopreserved for at least several months without compromising their ability to respond in allogeneic MLC's (119). As well, frozen and thawed murine and human leukemia and solid human tumour

cells can stimulate the generation of CTL's in syngeneic mixed lymphocyte-tumour cell cultures (119).

It has been pointed out that therapists should consider the possible effects of the therapy on the immune response (206). The LDA could be used to determine pCTL frequency when a patient is undergoing conventional (radiation, chemotherapy etc.) therapy. If a different procedure (eg. a change in timing etc.) were as effective as the conventional one, and yet would enhance the immune response, then adopting it could be advantageous to the patient. The LDA could be especially useful in monitoring attempts to boost the immune response of a patient undergoing immunotherapy.

Since suppression of T cells may be an obstacle to immunotherapy (119,190,206), the LDA may be important in separating the effects of suppressor cells and pCTL frequencies. The information might signal when a suppressor cell population is being stimulated, as opposed to a decrease in precursor frequency occurring.

Future Studies

It is important to understand the interrelationship between pCTL's, CTL's, helper cells, suppressor cells, and the growth of tumours. Some suggestions for future research are as follows:

1) Determine the relative cytotoxicity produced by pCTL's from normal animals versus those with small or large tumours. Although the pCTL frequency may be an important factor in influencing tumour growth, the number of CTL's produced by each pCTL, as well as their cytolytic activity on a per cell basis, will directly affect any possible anti-tumour activity.

2) Determine whether pCTL's are functional CTL's. The question arises whether the pCTL's and helper cells in tumour-bearing animals are already antigen activated. Does the environment in the animal allow exposure of the pCTL's to tumour antigens resulting in activated cells? Experiments with SN2°MLC and helper cells have shown the importance of IL-2 and helper cells. Are helper cells from normal and tumour-bearing animals different and how important is the frequency of helper cells and the relative help per cell?

3) Tumour infiltrating lymphocytes (TIL's), stimulated by culturing with tumour cells and expanded with IL-2, may be an important source of anti-tumour effector cells for immunotherapy. Since the tumour site may be the most important site of response, a determination of the pCTL frequency there should be of value in gauging the overall response.

4) Since there is a correlation between pCTL frequency in normal animals, and growth of a tumour after implantation, animals can be selected with low pCTL frequencies and therefore with poor prognoses. It should be possible to test various immunotherapeutic techniques designed to increase the potential T cell response in these animals and thus determine if the response to the tumour can be improved

V-7 SUMMARY

By the development and use of a LDA, we have found a correlation between pCTL frequency in normal mice and what happens after a syngeneic tumour is implanted. When the pCTL frequency is high, the tumour will tend to be smaller at seven and fourteen days and will be more likely to

be rejected. As the tumour grows, there is an increase in pCTL frequency. In an animal with an established tumour, a higher pCTL frequency is correlated with a smaller tumour size; and the presence of suppressor cells is correlated with a larger tumour.

These observations suggest two things. The frequency of precursors of cytotoxic T lymphocytes, specific for a syngeneic tumour, is important for the growth regulation of that tumour. The generation of suppressor cells, capable of down-regulating the immune response to the tumour, also appears to be important for tumour growth regulation.

APPENDIX: SOME IMPORTANT CELL TYPES WHICH MAY BE INVOLVED IN THE IMMUNE RESPONSE TO A TUMOUR

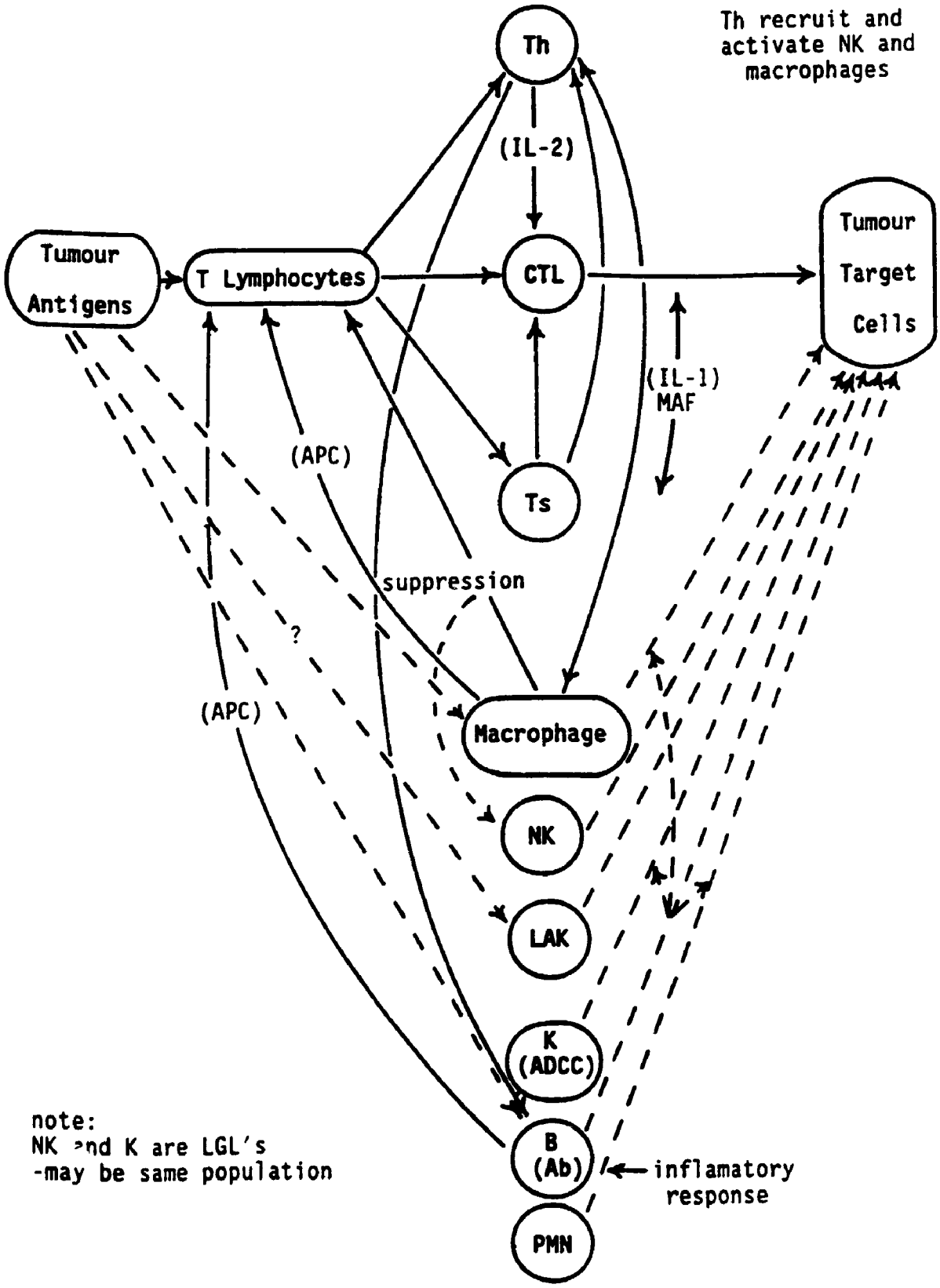
The response of various cell types to a tumour has been described in several recent general reviews (9,209,234,278,294). Cells thought to be involved are: T lymphocytes including the cytotoxic T lymphocyte (CTL), T suppressor cells (Ts) and T helper cells (Th); so-called 'null' cells including natural killer (NK) cells, Killer (K) cells responsible for antibody-dependent cell-mediated cytotoxicity (ADCC), and large granular lymphocytes (LGL) - a morphologic distinction which may include NK and K cells; lymphokine-activated killer (LAK) cells; macrophages; polymorphonuclear leukocytes (PMN's), and B lymphocytes - responsible for antibody production (figure A-1). As well, various factors produced by many of these cell types also participate in the immune response to tumours. An overall picture of the cells involved and their interactions is presented below. Each cell type is committed to at least one functional role and is modulated by factors produced by other cell types (234).

Section I-3-3 contains a description of T cells and how they interact in an anti-tumour response. In the rest of this section I will describe the role of cells, other than T cells, in the immune response to a tumour.

Natural Killer (NK) Cells

The term 'natural killers' has been applied to a population of large granular leukocytes (LGL's, 2-5% of peripheral blood leukocytes; (105,209,213) which can kill certain tumour target cells by direct

Fig A-1 Cells Which May Be Involved In The Immune Response To A Tumour



contact, without prior sensitization. A similar population of LGL's, the K cell, is responsible for antibody-dependent cell-mediated cytotoxicity (ADCC) of certain tumour cells. With ADCC, recognition and killing is dependent on the presence of antibody (Ab) bound to the target cells as well as Fc receptors for this Ab on the K effector cells. Other ADCC effector cells include monocytes, activated macrophages and PMN's (9).

Both NK and K cells have LGL morphology and it has been suggested that they are the same population (127,234). However, Ab and Fc receptors are not involved in NK cell mediated killing. The terms natural cell-mediated cytotoxicity (NCMC) or natural cytotoxicity (NC) have also been applied to NK cells (234,252). These cell types were originally called 'null' cells because they were thought to be lymphoid cells but apparently lacked detectable T and B cell markers and were functionally and phenotypically different from these cells (122,127).

The term 'natural killer' is an operational one. These cells have the power to kill tumour cells even though the donor has not been exposed (and therefore sensitized) to the target cells (9,128). Typically NK activity is assayed for by the lysis of certain tumour cell lines <u>in vitro</u> (19,234).

NK cells have an undefined lineage. They are nonadherent and nonphagocytic (175,234), have azuraphilic granules in the cytoplasm (175,209), are low density cells (129), and have a high cytoplasmic to nuclear ratio (209,301). In the mouse, NK activity decreases with age.

NK cells have characteristics common to both mononuclear phagocytes and to T lymphocytes. Like macrophages, they express myelomonocytic antigen, produce IL-1 and are cytotoxic against a range of tumour

targets (175). They express some T cell antigens and like T cells, respond to IL-2 (175). In the mouse, there appears to be a close association with the T cell lineage. NK-like activity can be mediated by CTL's stimulated by IL-2 or IFN (19). A single clone of cells has been shown to express activities of both NK cells and CTL's. The specificity, or lack thereof, of cultured human NK cells may be due to variations in the level of differentiation or activation (301). Therefore, there is not an absolutely clear division between NK and T cells.

There is heterogeneity in the antigenic markers found on NK cells. They have Fc receptors which are not involved in the lytic process (209,234). The HNK-1 antigen is expressed on human NK cells (19,126) and asialo GM1 is found on mouse NK cells (9,101,213). These cells have receptors for interferon (IFN), IL-2 and sheep red blood cells (213). They also express a variety of T cell-associated markers (9,126,175,213,234) including Thy-1 on at least a portion of the population (101). Since the Ly-2 marker appears to be selectively expressed on the mouse CTL but not on the NK cell (19,101), we have a means to differentiate between the activity of CTL's and NK cells in vitro.

The normal role of NK cells <u>in vivo</u> is uncertain. They display a broad specificity in being able to lyse a wide range of target cells including tumour cells (9,175,209), virally infected cells (127,175) and immature cells of normal tissue including embryonic cells (127,175,209), bone marrow stem cells (104) and a thymocyte subpopulation (175,209). The recognition and lysis of a target is not MHC restricted (234), and mouse NK cells can kill human target cell

lines (209).

The normal role of NK cells may be to attack virally-infected cells (104,175,213), or to regulate cellular differentiation or proliferation in the thymus and bone marrow (127,175,213). NK cells may also help regulate B cell differentiation and Ab production (126,175) and may be immunoregulatory through the production of lymphokines (104).

NK activity is greatest in the blood and spleen and relatively poor or non - existent in lymph nodes, thymus, thoracic duct and bone marrow (175), If NK cells are important in the anti-tumour response, then the most probable site of action is where they occur the most, in the blood - perhaps against circulating tumour cells (278). Depressed NK cell function can lead to tumour growth and metastatic disease (209). In patients with head and neck cancer, the levels of NK cells and NK function are reduced (294). However, not all patients or tumour types show this correlation (126,209).

Although it has been postulated that NK cells play a role in surveillance against primary tumours (126,127,213,252), there is no clear evidence that they can protect against such tumours (128), and the majority of primary tumour cells are relatively resistant to natural cytotoxicity (126). It has been noted that <u>in vitro</u> activity against sensitive targets, the way NK cells are assayed, may not be relevant to the response <u>in vivo</u> against autologous tumours (234).

The evidence that NK cells help control metastases is more persuasive (103,104,127,213,278). Animals with experimentally depleted NK function have a diminished ability to destroy tumour cells injected into the circulation. These animals develop increased numbers of experimental lung 'metastases' (7,292). When NK activity is restored by

adoptive transfer or by chemical stimulation, the number of lung 'metastases' decreases.

One might wonder whether NK cells are active at the tumour site. Although some virally and chemically induced animal tumours show the presence of NK activity within the tumour (278), NK cells are barely detectable among human tumour infiltrating lymphocytes (TIL's) (209,278). However when TIL's are stimulated with IL-2, NK-like killing of K562 cells occurs (278). The nature of the cytotoxic cells was not determined. Among TIL's in humans, the NK precursor frequency may be low and the activity suppressed.

Because IL-2 can activate NK cells (9,19,127,209), and can lead to enhanced killing (127,175,218) or expansion of NK-like cell populations (19,127,218,277), experimental conditions which lead to the production of IL-2 may cause expansion of NK-like cells and thus assays for T cells, under such conditions, may instead detect NK-like cells (277). The activation of NK cells by IL-2 may place them somewhat under T cell control with Th cells producing IL-2. Conversely, it has been demonstrated that clones of NK cells can themselves produce IL-2 (104). IL-2 may act by stimulating the production of IFNgamma - a potent stimulator of NK cell activity (19,209).

Another cell type which may control NK cell activity is the macrophage. Macrophages can suppress NK cell activity <u>in vitro</u>, possibly through the production of prostaglandins (126,127,209).

Lymphokine-Activated Killer Cells (LAK)

A population of cells can be isolated from the peripheral blood of cancer patients as well as normal individuals which, upon incubation

with the lymphokine interleukin-2, can kill a variety of freshly isolated and cultured tumour cells (87,88,147,207). These cytolytic cells have been termed lymphokine-activated killer (LAK) cells since the activation apparently depends only on the incubation with IL-2 (TCGF) and not exposure to the tumour cells. Since interferon is not effective in activation of LAK cells, it is not the primary stimulus in the preparations containing TCGF (87).

Since PBL's from normal, tumour-free individuals can be activated in this manner, the response does not depend on a prior exposure to tumour. When tested, allogeneic LAK cells from normal individuals are capable of lysing fresh tumour cells from all specimens tested (87). These cells have been isolated from over 90% of cancer patients tested (88).

LAK cells constitute a system, distinct from the classical CTL and NK cells, which is potentially active against tumours. As well as being found in the blood, LAK precursors exist also in the thoracic duct, the thymus and in the bone marrow (88). Because of their antitumour reactivity and wide distribution in the body, it has been suggested that they may be important in immune surveillance against tumours (87,88). Since they differ functionally from NK cells and can lyse NK resistant tumour cell lines, they may be important in surveillance against NK-resistant solid tumours.

By serologic and morphologic selection procedures, precursors of LAK cells have been shown to be different from NK cells, T lymphocytes, memory cytotoxic T cells and their precursors, monocytes and B lymphocytes (9,87,88). LAK cells are nonadherent and adherent cells are not required in their activation.

Although LAK cells are distinct from CTL's, they do express many T cell markers (87,88). The stimulus that is required and the time course for activation distinguish the two cell types. Also, the specificity of lysis of the two cell types is quite different. Single clones of LAK cells are able to lyse multiple fresh human tumour targets, suggesting that LAK cells recognize determinants shared by many different tumour cells (207).

Macrophages

Macrophages are large, motile, phagocytic monocytes. They are located throughout all tissues of the body (248) and have been isolated from various tumours (278).

They may act in an anti-tumour response in several ways. Through Fc receptors on their cell surface, they play a role in ADCC (antibody dependent cell-mediated cytotoxicity), much like large granular lymphocytes (131,213,248,278). Activated macrophages can kill Ab-coated tumour cells through a two step process involving close contact or binding with the tumour cell and then a separate lysis stage (131,132,248). Phagocytosis of tumour cells or debris is a late effect and occurs only after the tumour cells have been killed. Macrophages have been shown to be non-specifically cytotoxic for tumour cells in vitro (MTC or macrophage-mediated tumour cytotoxicity) (9,248). Even though this antibody-independent killing of tumour cells appears to be non-specific, it is selective in that normal cells are spared (131).

Macrophages are also important as accessory or antigen presenting cells (APC) in the T cell response to tumours (9,234,248). After processing tumour antigen, they present it to T cells in an immunogenic

form. In some model systems, T cells require processing of antigen by APC's for the effective production of an anti-tumour proliferative response *in vitro* and for an effective tumouricidal response *in vivo* (52).

Macrophages play a regulatory role in the immune response and produce factors that can stimulate (i.e. IL-1 which induces production of IL-2 by T lymphocytes) or suppress the proliferative responses of lymphocytes to tumour antigens (9,133,213,278). Monocytes themselves can be activated by IL-1 and this positive feedback may be part of an autoregulatory system (132). Through interaction with tumour cells, they can produce prostaglandins which initiate suppressor elements. Cultures of mouse spleen cells have yielded suppressor macrophages which strongly inhibit the response of T and B lymphocytes to stimuli such as mitogens and specific antigens (145).

Macrophages may also contribute to tumour progression by stimulating vascularization of the tumour by the host (131). As well, tumour cell variants may be selected for, following the interaction of macrophages with tumour cells, and metastatic growth can actually be accelerated (46).

Macrophages are themselves regulated by other immune system cells. Recruitment and activation of macrophages at the tumour site can entail the production, by T cells, of factors which attract (CFM - chemotactic factor for macrophages), inhibit movement of (MIF - migration inhibition factor), and activate macrophages (MAF - macrophage activation factor - probably gamma interferon). Activated macrophages have been shown to be directly cytotoxic or cytostatic to tumour cells.

Even though macrophage infiltration of human tumours is frequently

seen, there doesn't appear to be a significant association of this with the rate of metastatic spread (294). They do however appear to participate in the immune-mediated destruction of tumours once that process has been initiated by modulation of the immune response (248).

Macrophages go through several stages before they become fully active, capable of killing tumour cells (248). Following the initial nonresponsive stage when they are relatively quiescent, they enter the responsive stage where they have elevated Fc and C3 receptor numbers and are capable of receptor-mediated phagocytosis. An initial signal (lymphokines such as IFNgamma, from Ag-stimulated T cells), triggers macrophages into the primed stage where a second signal (e.g. LPS) can push them to a completely activated state where they are capable of killing tumour cells (9,181,234). Activated macrophages show many alterations in metabolism and structure and this state can be down-regulated by a number of agents (i.e. PGE, protease complexes etc.) (131).

More than forty distinct surface receptors have been identified on macrophages. These include receptors for: Fc portions of various IgG isotypes, complement components, hormones, and other molecules (131,248). One surface component important for the triggering of T lymphocytes is the Ia determinant (Class II histocompatibility complex antigen). When activated, macrophages express more Ia antigen.

Polymorphonuclear Leukocytes (PMN's)

Another cell type included in natural immunity is the polymorphonuclear granulocyte (PMN), classed into neutrophils, basophils, and eosinophils on the basis of histological staining. These

comprise 60-70% of normal blood leucocytes. They possess Fc receptors for IgG and also complement receptors, as do activated macrophages. These cells are non-specific and their predominant role is one of phagocytosis. Therefore they are probably not involved in the immune response to viable tumour cells.

B Lymphocytes

B lymphocytes comprise approximately 5-15% of the circulating lymphoid pool. Until activated, they are morphologically identical with T cells. When activated they may produce antibodies which, combined with complement, may be lytic for tumour cells, or, which may aid in ADCC via K cells or macrophages, through Fc receptors (278). Each B cell will produce Ab directed against a specific Ag and it has been estimated that as many as 10^6 different antigens may be recognizable by B cells (9). Since approximately 15% of tumour infiltrating lymphocytes express surface IgG's (indicative of B cells), B cells may be active in the immune response at the tumour site (278). B cells are now recognized as an antigen presenting cell and have been shown to have helper activity ($Ly\text{-}1^+$ B cell).

Through various mechanisms, such as formation of immune complexes, Ab may act to block the immune response to tumour antigens (278).

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We wanted to combine the results of these experiments with others in which 12 mice were killed at day twenty-eight, after 9 of the tumours had regressed. We do not know whether the tumours in those 9 mice would have redeveloped. Because the tumours in the third group had temporarily regressed, probably due to the immune response of the mice, and because we wanted to combine the results of the various experiments, we decided, for further analysis, to combine the two classes of regressing tumours.

Part of the analysis, therefore, consisted of dividing the animals into two groups, those with progressing and those with regressing tumours, and then comparing them. Of the forty-three mice used, fifteen tumours progressed and twenty-eight regressed. Data concerning pCTL frequencies and tumour volumes at days seven and fourteen are given in table IV-3 for animals of both groups. These data were used for the following statistical analyses.

Figure IV-19 shows the linear relationship of tumour volume, fourteen days after tumour injection, to pCTL frequency in the animals before they were injected. The negative linear correlation between tumour volume and pCTL frequency ($r^2 = 0.173$, $r = -0.416$) was significant ($0.01>P>0.005$). The results of the regression of tumour volume on the logarithm of pCTL frequency (log regression; $r^2 = 0.266$, $r = -0.516$, $P<0.001$) is given in figure IV-20. Thus, both analyses show a significant negative correlation between pCTL frequency in the blood of normal animals before a tumour injection, and the tumour volume fourteen days after injection. The results suggest that, depending on the regression model chosen, between 17% and 27% of the variability of the volumes of tumours at fourteen days bears a mathematical relationship to