# ENHANCEMENT OF AN ANTI-TUMOR IMMUNE RESPONSE USING BACTERIOPHAGE T4 AS A LINKED HELPER DETERMINANT ON P815 TUMOR CELLS

by

MARGOT M. BRIDGETT

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Department of Microbiology

The University of British Columbia
1956 Main Mall
Vancouver, Canada
V6T 1Y3

Date Oct. 6 / 1986

# ABSTRACT

Heterogenization is defined as the enhancement of weak anti-tumor responses by attachment of strong antigenic helper determinants to the surface of tumor cells. Anti-tumor immune responses may be further amplified by addition of T helper cells specific for the helper determinant. I investigated the effectiveness of bacteriophage T4 (ØT4) as a helper determinant in the heterogenization of P815 tumor cells. Experiments were performed to quantitate and optimize chemical coupling of ØT4 to irradiated P815 tumor cells (P815r). Proliferation assays showed that T cells primed in vivo to ØT4 could recognize ØT4 that was chemically linked to P815r (P815r-Ø). T cells primed in vivo with P815r-Ø responded in the proliferation assay to free ØT4. An in vivo adoptive transfer system was used to examine the helper ability of ØT4-primed spleen cells. ØT4-primed, irradiated spleen cells and SRBC-primed B cells were transferred into irradiated host mice. The anti-SRBC response of the transferred SRBC was augmented only when the mice were challenged with SRBC linked to ØT4. Challenge with SRBC and free ØT4 did not increase the response.

A ØT4- specific T helper cell line was established and maintained in vitro. The cell line was cloned by limiting dilution and the clones were characterized as to their proliferative response to ØT4 and their production of interleukin 2. When added to cultures of spleen cells that were stimulated with either P815r-Ø or P815r and free ØT4, the cell line dramatically enhanced the killing of P815 tumor cell targets by cytotoxic T cells. Elimination of in vitro primed T helper cells using antibody plus complement was essential to detect this result.

The T helper cell line and clones were tested in in vivo immunotherapy trials. Mice were treated with cyclophosphamide to reduce suppression in the animals, injected with T helper cells and immunizing doses of P815r-Ø, and challenged with viable P815 tumor cells. The cells line and clones demonstrated in vivo helper activity. Optimal conditions for performance of the immunotherapy experiments are discussed.

# TABLE OF CONTENTS

Page

Page

Page

Page

LIST OF TABLES

Page

Table

LIST OF FIGURES Page

Figure

Page

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# ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| Ab | antibody |
| BCG | *Bacillus Calmette-Guerin* |
| BSS | balanced salt solution |
| C | complement |
| Con A | concanavalin A |
| cpm | counts per minute |
| $^{51}Cr$ | chromium-51 |
| $CrCl_3$ | chromic chloride |
| CTL | cytotoxic T cell |
| D2 | DBA/2 mouse strain |
| DNase | deoxyribonuclease |
| *E. Coli* | *Escherichia coli* |
| EDAC | 1-ethyl-3(3-dimethylaminopropyl) carbodiimide |
| FCS | fetal calf serum |
| $^{3}H$-TudR | methyl-$^{3}H$-thymidine |
| Ig | immunoglobulin |
| IL2 | interleukin 2 |
| i.p. | intraperitoneal |
| i.v. | intravenous |
| KLH | keyhole lympet hemocyannin |
| LB | luria broth |
| LN2 | a ØT4-reactive T helper cell line |
| LPS | lipopolysaccharide |
| mAb | monoclonal antibody |
| NaCl | sodium chloride |
| NMS | normal mouse serum |

| | |
|---|---|
| $^{32}P$ | Phosphorus-32 |
| P815 | a methylcholanthrene-induced mastocytoma of D2 mice |
| P815r | irradiated P815 tumor cells |
| P815r-Ø | irradiated P815 tumor cells chemically linked to bacteriophage T4 |
| PBS | phosphate buffered saline |
| PFU | plaque forming unit |
| PMA | phorbal myristic acetate |
| PPD | purified protein derivative |
| PPO-BIS | 2,5-(diphenyloxazole/1,4-bis-(2 methyl-stryryl)-benzene |
| O.D. | optical density |
| rpm | revolutions per minute |
| SRBC | sheep red blood cells |
| SRBC-Ø | sheep red blood cells chemically linked to bacteriophage T4 |
| ØT4 | Escherichia coli bacteriophage T4D |
| TL | thymic leukemia |
| TSA | tumor specific antigen |

# INTRODUCTION

## 1. Background

Tumors consist of cells that for various reasons do not obey the growth control signals to which normal cells respond. Thus, tumor cells can grow "out of control" in the host animal. Animals do have anti-tumor immune defense mechanisms such as cytotoxic T cells (CTLs), helper T cells, macrophages , natural killer cells and antibodies (Bach et al.,1980; Carlson and Terres,1976; Djeu et al.,1980; Evans and Alexander,1970; Herberman and Holden,1978; North et al.,1978; Ruco and Meltzer,1977). How then do tumor cells manage to escape these immune defenses?

There are several " escape mechanisms" shown to be used by tumors to avoid destruction. The major categories of tumor escape mechanisms are: (i) masking of tumor cell-surface antigens; (ii) shedding of antigen; (iii) formation of antibody-antigen complexes; (iv) antigenic modulation and loss of surface antigen, and (v) tumor interaction with the host immunoregulatory apparatus (Henney, 1977; North et al.,1978; Ozer,1982).

The research described in this thesis mainly concerns the fifth mechanism, which is the interaction of the tumor with the host's immune system, specifically with anti-tumor cytotoxic T cells, helper T cells, and suppressor T cells.

In the past decade, there has been extensive research on the relationship between growth of tumors and the concomitant suppression of anti-tumor cellular immune responses. This research is the subject of reviews by Broder and Waldmann, (1978); Naor, (1979); and Hakim, (1979). In our laboratory, the cellular response to the syngeneic mouse mastocytoma P815 has been studied. It was observed that when a mouse is injected with $1x10^4$ P815 tumor cells there is a gradual increase in the activity of anti-tumor cytotoxic T cells in the animal until approximately the sixteenth day after injection of the tumor, followed by a rapid decline in CTL activity.The increase in CTLs is matched by greater activity of tumor-specific helper T cells, whereas the

decline in CTL activity correlates to an increase in tumor-specific suppression (Takei et al., 1977, Hancock et al., 1981).

The anti-tumor immune response might be more effective if one could increase the immunogenicity of the tumor and at the same time overcome the suppression.This might be accomplished by attaching a strong antigenic helper determinant to the tumor cell surface, as was originally suggested by Mitchison (1970). Helper cells reactive to the strong helper antigen would send helper signals to activate CTLs reactive to tumor cell antigens. The helper signals would stimulate CTL proliferation and increase the anti-tumor cytotoxic response. A further increase in the anti-tumor response might be realized by priming animals for T cells reactive to the strong antigenic determinant or adding antigen-reactive helper T cells or factors.

In my project, I explored the possibilities of using the Escherichia coli bacteriophage T4 (ØT4) that was chemically linked to P815 tumor cells as a helper determinant. ØT4 was chosen as the antigenic determinant because it elicits a strong immune response and could potentially be used in a plaque inhibition assay for antigen-binding factors. Experiments designed to increase the immune response against tumors were performed, in vitro and in vivo.

## II. Tumor escape mechanisms

Tumor cells can grow in a syngeneic host animal because the host does not mount an immune response strong enough to reject the tumor. There are two basic reasons why tumors are exempt from immune attack: (i) weak tumor immunogenicity ( i.e. the tumor cells do not trigger the host immune response) (Weiss, 1977), and (ii) suppression of the anti-tumor response (Berendt and North,1980; Broder and Waldmann,1978; Cantor and Gershon,1979; Fujimoto et al.,1976; Kamo and Freidman,1977; Kobayashi,1982).

Different kinds of tumors vary in their degrees of immunogenicity. The multiple levels of immunogenicity among tumors depend on factors including host/tumor interactions, and whether the tumor is chemically induced, virally induced, or spontaneously occurring. For example, many virally induced tumors are highly immunogenic and are efficiently rejected by the host

(Kobayashi et al., 1973; Moloney, 1966). Chemically induced tumors are variably immunogenic. Tumors induced with the chemical methylcholanthrene range from being of high to low immunogenicity with most being moderately immunogenic (Takeda et al.,1968). Some tumors provoke no demonstrable immune response. Examples of non-immunogenic tumors are diethylnitrosamine induced mouse lung cancer (Baldwin, 1977), cellophane film induced tumors (Klein et al. , 1963), and some spontaneously occurring tumors (Hewitt et al., 1976).

In order to design effective anti-tumor immunotherapy it is important to understand why some tumors are so weakly immunogenic. Researchers have described many tumor "escape mechanisms" that enable tumors to survive in an animal. These have been reviewed in detail (Ozer, 1982). Below is a brief overview of the reasons for the low immunogenicity of tumors.

One explanation for weak tumor immunogenicity is that they simply have weak antigens. This was the basic assumption behind the so-called "sneaking through" theory (Old et al., 1961). This theory postulated that tumors initially go unnoticed by the host immune system because they have weak antigens. The tumor cells proliferate and by the time the the immune system has recognized the presence of the foreign tumor cells, the tumor load is too large for an immune response to be effective. The sneaking through theory is a simple explanation for tumor cell survival in a host animal. However, new evidence shows that tumors are able to evade host immune responses by using more sophisticated, if not more simple mechanisms.

One of these ways is known as "masking" (Sanford,1967; Watkins et al.,1971). Tumors produce chemicals such as sialic acid, hyaluronic acid, and chondroitin sulphate which bind to tumor antigens, hiding them from immune defence mechanisms. Treatment of tumor cells with the enzyme neuraminidase, extracted from Vibrio cholera , has been shown to increase tumor immunogenicity (Currie and Bagshawe, 1969). The enhanced immunogenicity is directly related to the enzymatic release of sialic acid from the tumor cell surface, which exposes hidden tumor antigens.

Tumors also shed their surface antigens (Alexander,1974; Wolf et al.,1976). Tumor antigens shed from large, solid tumors may induce high-zone tolerance in effector T and B cells,

by attachment directly to effector cell surfaces (Baldwin et al., 1973). Low-zone tolerance may be caused by weakly immunogenic antigens that block responses to highly immunogenic determinants by saturation of receptors on the effector cells (Currie and Alexander, 1974). Small amounts of weakly immunogenic tumor antigens, such as those shed from metastatic tumor growths, may cause clonal deletion of effector cell precursors (Nossal, 1974). Shed tumor antigens may also activate suppressor cells that counteract anti-tumor responses.

One mechanism of tumor escape that has been extensively studied is the formation of antigen-antibody complexes and "blocking factors" (Hellström and Hellström, 1974; Hawrylko, 1978). The blocking factors, found in the sera of tumor-bearing animals and humans, are thought to be non-cytolytic anti-tumor antibodies. By binding to tumor antigens on tumor cell surfaces, these antibodies may block the antigenic targets for the cytotoxic T cell and helper cell receptors. The blocking antibodies may also function by preventing the induction of pre-CTLs, presumably by binding and sequestering stimulatory antigen . A third blocking mechanism may occur when antigen-antibody complexes bind directly to effector cells, inhibiting differentiation and proliferation.

Some tumors may escape host detection by antigenic modulation (Stackpole and Jacobson, 1978). The modulation of the thymic leukemia (TL) antigen from TL+ to TL- upon transplantation into immune mice is the classic example of this form of escape (Boyse et al., 1965). The immune response is effective against the cells that bear the TL+ form of the antigen, but cannot recognize the TL- leukemia cell.

Clearly, tumor cells have several mechanisms enabling them to avoid attack and destruction by host cell defenses. Other escape mechanisms under intensive study involve the complex interaction of tumors with the regulation of the host immune system. The immune system is a finely balanced system regulated by feedback mechanisms and a network of helper and suppressor cells that take part in anti-tumor defense (Gershon, 1974; Cantor and Gershon, 1979; Diener and Feldman, 1972; Green, 1982; Uhr and Möller, 1968; Waksman and Namba, 1976; Ozer, 1982). Possibly one of the most important tumor escape mechanisms, at least from our perspective, is

the ability of tumors to interact with the balance of helper and suppressor cells to induce immune unresponsiveness. Tumor growth may stimulate suppressor T cells that down-regulate the host's anti-tumor immunity. Fujimoto et al. (1976a, 1976b) showed that growth of methylcholanthrene-induced sarcomas in mice was promoted by suppressor T cells. As mentioned earlier, a model that demonstrates the dynamics of help and suppression in the cellular immune response to the mastocytoma P815 has been studied by Takei et al., (1976, 1977). They showed that when P815, a methylcholanthrene induced tumor, is injected into syngeneic mice, a cytotoxic T cell response is mounted against the tumor. In addition to cytotoxic T cells, helper T cells and suppressor T cells are stimulated by the presence of tumor cells in the animal. The suppressor cells inhibit the ability of the helper/ cytotoxic arm of the anti-tumor response to function. Thus the tumor can grow, metastasize, and kill the host. Introduction of additional helper cells to this system might counter-balance the suppression and lead to enhancement of anti-tumor immunity.

## III. Types of tumor antigens

Over the past three decades, researchers have been trying to identify antigens that are unique to tumor cells. These tumor specific antigens (TSAs) may be related in some way to the neoplastic characteristics of tumor cells. If there were antigens that were found only on tumor cells, then anti-tumor immunotherapy could be aimed at those specific antigens. Immunotherapy could be made specific for any and all tumors, directing effector cells at cells bearing the TSAs and avoiding damage to cells not bearing those antigens.

Unfortunately, the putative tumor specific antigens have yet to be found. Few if any true TSAs have been demonstrated however many tumor associated antigens ( i.e. antigens found on some tumor and also on some normal cells), have been described and may be classified into two categories: (i) differentiation antigens (Sell,1978; Uriel,1979), and (ii) induced antigens (Livingston et al.,1982).

Differentiation antigens occur on normal cells only at certain specific stages of differentiation. They are also found on some tumor cells. An example is retrograde differentiation

antigens, that are found on normal fetal cells and persist on cells that undergo "retrograde" differentiation to become tumor cells. Another type of differentiation antigen is the thymus leukemia antigen that is found on normal mouse thymus cells but also occurs on leukemic cells (Old et al., 1961).

The second category of antigens, induced antigens, are found on virally induced or chemically induced tumor cells. Viral associated antigens are usually the same on tumors induced by the same virus, wheras tumor associated antigens, found on chemically induced tumors, usually differ on different tumor types induced by the same chemical carcinogen.

The search for tumor specific antigens is continuing, but since these antigens have not yet been clearly demonstrated, researchers in anti-tumor immunotherapy have investigated other strategies that do not depend on the existence of TSAs.

## IV. Models of immunotherapy

If tumors are sufficiently immunogenic, injection of the tumor cells into animals can induce immunologically activated lymphocytes and antibodies that recognize tumor antigens. The tumor-reactive immune cells or sera can be removed from the original animal and transferred to host animals, which are then challenged with injections of viable tumor cells . Such experiments, termed adoptive transfer experiments, have been more successful in reducing tumor size and increasing survival of mice when the host animals were challenged with live tumor cells after having received a transfer of tumor reactive immune cells. There are very few examples of successful adoptive transfer therapies in which the host animals already have established tumors before they receive the anti-tumor cells (Rosenberg and Terry, 1977). As mentioned earlier, since suppression of anti-tumor responses is an escape mechanism for tumors, the failure of immunotherapy to control established tumors may be due to suppression of the immune system by tumor growth. Recent models of tumor therapy have addressed the problem of suppression by employing anti-suppressive drugs and irradiation . For example, anti-tumor effector cells have

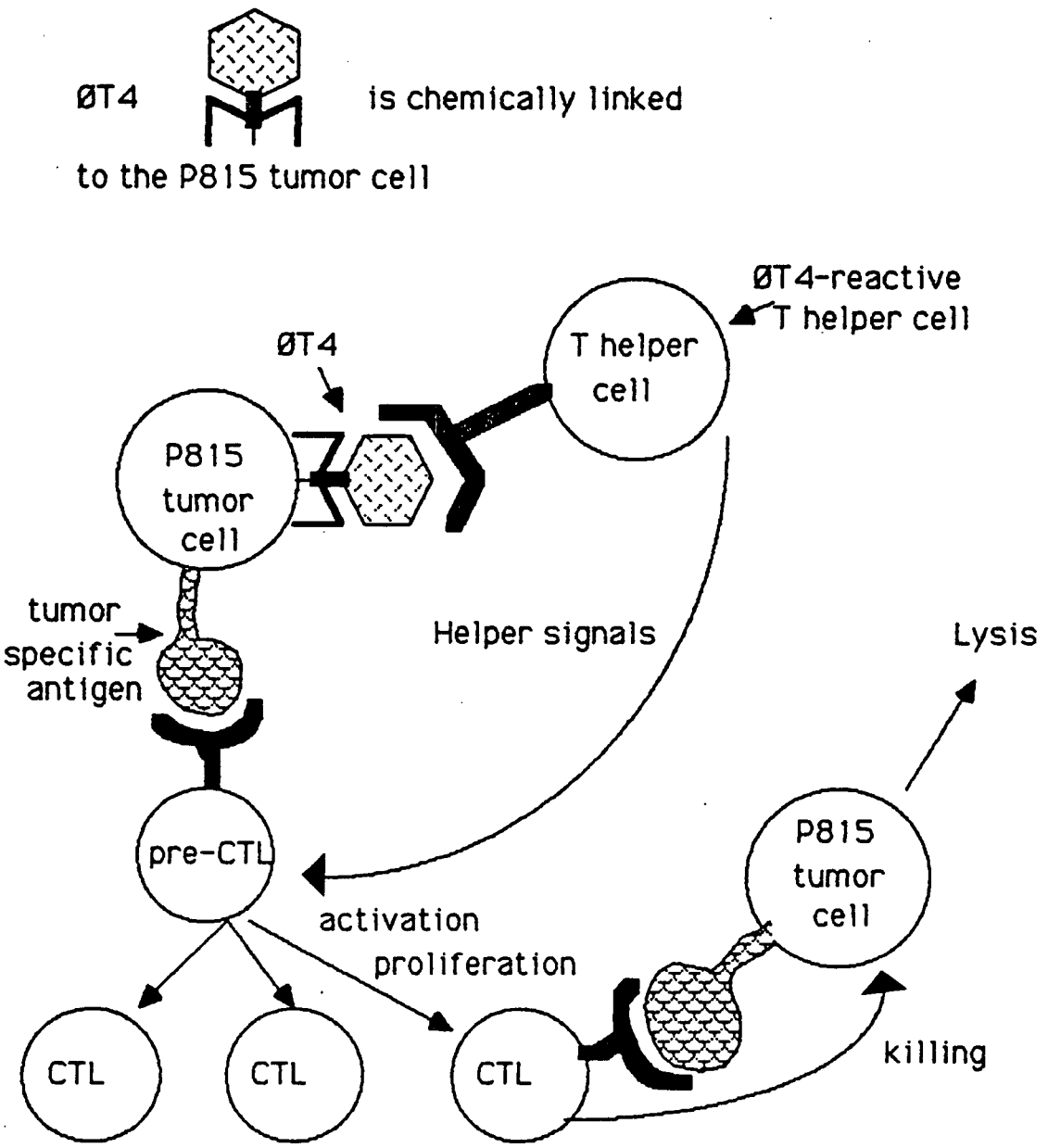
been grown in long-term culture using Interleukin 2 (IL2) to increase the number of cells available for transfer, and injection of IL2 into the host after cell transfer has also been used to improve the viability of the transferred cells. The lymphocyte transfer experiments of Cheever et al. (1986) employed tumor-reactive T cells lines along with cyclophosphamide treatments and injections of IL2. This combination therapy, called chemoimmunotherapy, was successful in eradicating disseminated FBL-3 leukemia in mice. Cheever's work is an example of tumor immunotherapy models that use transfer of T cells in combination with other methods. (reviewed in Fefer and Goldstein,1982; Mihich, 1982).

A different strategy for immunotherapy, designed to overcome weak immunogenicity of tumors and suppression of anti-tumor responses, has been to attach strong antigenic determinants to the surface of tumor cells. When Hamburg and Svet-Moldavsky (1964) first artificially infected tumor cells with virus, they described the acquisition of new viral antigens on the tumor cell surface as "heterogenization". Since then, several other workers have described increased tumor antigenicity due to viral antigens on tumor cell surfaces. Stück et al. (1964), and Sjögren and Hellström (1965), called this phenomenon "antigenic conversion". Kobayashi et al. (1969) were the first to report regression of rat tumors artificially infected with murine leukemia virus, a process they dubbed "viral xenogenization". Other reports of enhanced immune responses due to heterogenization of tumor cells with viruses followed (Lindemann and Klein, 1967; Kobayashi et al.,1973; Kuzumaki et al., 1978; Iglehart et al., 1981; Yamaguchi et al., 1982).

Viruses were not the only antigens that were attached to tumor cell surfaces. Martin et al. (1971) reported enhanced immunogenicity of mouse tumor cells by coating the cells with conconavalin A or dinitrophenyl caproate. Heightened immunogenicity using chemical modification of tumor cells was achieved by Galili et al. (1976) and Hashimoto and Yamanoha (1976). A third method used to alter tumor antigens and increase immunogenicity was mutagenisis of the tumor cells (Uyttenhove et al., 1980; Calvelli et al., 1982).

Mitchison, (1970) was the first to propose a unified theory of enhanced immunogenicity due to artificially induced antigenic determinants on tumor cell surfaces. He suggested that a

Figure 1: Schematic representation of enhancement of an anti-tumor response via associative recognition



strong new antigen on a tumor cell surface would act to focus help from antigen-specific T helper cells reactive to the new antigen onto cytotoxic cells that recognize tumor antigens. (see Fig. 1). Because the new antigen, or helper determinant, is linked to tumor antigens on the tumor cell surface, the mechanism could be similar to the "antigen-bridge" model of T cell help for B cell responses in a hapten-carrier system (Mitchison, 1971b). The process in which a cellular response to a principle antigenic determinant is helped by a concomitant immune response by a different cell reacting to a second helper determinant is called "associative recognition" (Lake and Mitchison, 1976), because the principle determinant and the helper determinant are thought to form an "associative unit".

The demonstration by Lanzavecchia (1985) that B cells process carrier determinants on a hapten- carrier molecule and present them to T helper cells helped to clarify the process by which carrier-specific T cells help hapten-specific B cells. The mechanism by which helper T cells help anti-tumor cytotoxic T cells in response to specific antigen linked to tumor cells remains obscure. Questions regarding the mechanisms of processing and presentation of the linked antigen remain to be answered.

Lake and Mitchison (1976) felt that lack of T cell help was the primary reason for weak immune responses against tumors, and suggested that heterogenization of tumor cells with T helper determinant antigens could be a successful form of immunotherapy, as both problems of weak tumor antigenicity and suppression of anti-tumor immune responses would be dealt with.

Since Mitchison put forward the idea of cellular cooperation in tumor immunity, several groups have investigated systems of linked helper determinants on tumor cells. Essentially, the experimental design of these experiments is similar. The experimental animals, usually mice, are immunized with a helper antigen to amplify the number of antigen-specific helper T cells. Then, to prime for anti-tumor CTLs, the mice are given injections of irradiation-inactivated tumor cells with the helper antigen attached to the tumor cell surface. Finally, after antigen-reactive helper T cells and tumor-reactive CTLs are primed, the mice are challenged with injections of viable tumor cells that do not have the helper antigen linked to their cell surfaces.

Boone et al. (1974) showed evidence for the helper-determinant mechanism of linked recognition in experiments with virally-heterogenized tumor cells. Takatsu et al. (1980) used purified protein derivative (PPD) linked to the chemically-induced syngeneic plasmocytoma X5536 to achieve prolonged survival of mice and tumor regression. The enhanced survival occurred only in mice that were primed with Bacillus Calmette-Guerin (BCG) which elevates the number of PPD-reactive helper T cells in the animal. Lachmann and Sikora (1978) and Vyakarnum et al. (1981) showed that anti-tumor immunity could be enhanced in BCG-sensitized mice that were immunized with PPD-coupled, irradiated tumor cells. They have also reported (Lachmann et al., 1985) some success with clinical trials in humans immunized with BCG and subsequently with PPD-linked human tumor cells. Fujiwara et al. (1984) was successful using in situ heterogenization of tumor cells with trinitrophenyl (TNP) in mice with amplified TNP helper cell activity. They found that only when suppression in the mice was inactivated with cyclophosphamide treatments could they achieve prolonged survival and tumor regression. Zoller (1985) haptenized a non-lymphoid rat tumor with TNP. She discovered that adoptive transfer of effector CTLs and helper T cells was necessary to avoid suppression in in vivo immunotherapy. Complete protection against haptenized tumor cells and partial protection against native tumor cells was achieved following the transfer of hapten-specific helper T cells and in vitro stimulated, tumor reactive CTLs into irradiated hosts.

The preceeding experiments used animals immunized with the helper antigen as a source of helper T cells, and immunization with heterogenized tumor cells to stimulate anti-tumor CTLs. Rather than activating helper T cells in vivo, cloned T cell lines may also be transferred into host animals. Sia et al. (1984) found that clones of T cells specific for PPD could potentiate anti-tumor immunity in BCG-negative mice immunized with PPD-coupled, irradiated sarcoma cells. Kilburn et al. (1979, 1981) reported a PPD-specific helper factor that enhanced in vitro cytotoxicity of tumor cells. This helper factor could replace PPD-primed T helper cells in in vitro heterogenization experiments (Kilburn, unpublished results). Substitution of antigen-specific helper T cell lines, clones, or factors is a promising alternative to in vivo priming of the

host with helper antigen for therapy of tumors. It offers a more precise method to study the mechanism of immune enhancement by heterogenization, and to define the specific populations of cells involved in anti-tumor responses.

V. Objectives of this investigation

The aim of this study was to investigate the use of bacteriophage T4 as a strong antigenic determinant for the heterogenization of the the syngeneic mouse mastocytoma P815. I used ØT4 for the heterogenization of P815 tumor cells in in vitro and in vivo experiments. This involved chemically linking ØT4 to P815r and testing the linked antigen-tumor cells (P815r-Ø) for the ability to stimulate helper T cells. Adoptive transfer experiments were performed to see if ØT4-primed T cells would help in a classic hapten-carrier system. A T helper cell line reactive to ØT4 and clones of the T helper line were established and maintained. The cell lines and clones were characterized and tested for their response to various antigens and mitogens. The cell lines were also checked for the ability to help an in vitro CTL response against P815 tumor cells. Finally, heterogenization of P815 with ØT4 along with injection of T cell lines and clones was tested in vivo for immunotheraputic potential.

# MATERIALS AND METHODS

## I. Mice

DBA/2J female mice (D2), 6-10 weeks of age, were obtained from the Jackson Laboratory, (Bar Harbour, ME.), or from the Animal Unit, Department of Microbiology, U.B.C..

## II. Media

### A. Tissue culture medium.

10.38 grams per litre of RPMI 1640 powder (Flow Labs, Mississauga, Ont.), containing glutamine but not bicarbonate, was dissolved with 10 mM Hepes (Sigma H-3375, St. Louis, MO.) and 2.38 mM sodium bicarbonate (Fisher S233,Fair Lawn, N.J.) in distilled water. The mixture was sterilized by filtration through a 0.2 micron filter (Millipore 14250, Bedford, Mass.) and stored at 4°C in 450 ml batches. Before use, each 450 ml bottle of RPMI 1640 +Hepes + bicarbonate was supplemented with 50 ml of fetal calf serum (FCS)(Grand Island Biological Company (GIBCO), Chagrin Falls, Ohio),5 ml of a100 x antibiotic- antimycotic solution (GIBCO), and 0.5 ml of 0.05 M 2-mercaptoethanol (Sigma). FCS was heat inactivated at 56°C for 30 minutes before use. The supplemented medium was called complete RPMI. Medium containing all the supplements except FCS was called serum-free medium (SFM). In some experiments, 0.5% normal mouse serum (NMS), heat-treated at 56°C for 30 min, was used instead of 10% FCS.

### B. Luria broth and Bottom layer agar

Luria broth (LB) was prepared as follows: 10g tryptone (DIFCO Labs 0123-01,Detroit, Mi.), 5g yeast extract (DIFCO 0127-05), 5g sodium chloride ( NaCl ), and 1g dextrose, were dissolved in 1 litre distilled water. 5 ml/l of 10x M9 salt (see below) were added. The pH was adjusted to 7.2-7.4 with sodium hydroxide and the luria broth was heat sterilized.

Bottom layer agar was prepared using the same recipe as luria broth, except that M9 salts were omitted, and 11 g/l agar (DIFCO) was added before autoclaving.

10x M9 salt

58g sodium hydrophosphate, 30g potassium di-hydrophosphate, 5g sodium chloride, 10g ammonium chloride, and 1 ml of 0.1M ferric chloride were dissolved in 1 litre of distilled water and heat sterilized.

C. Phosphate buffered saline

Phosphate buffered saline (PBS), pH 7.2-7.4, was made up in distilled water and heat steralized. It contained 8g/l sodium chloride , 0.2 g/l potassium chloride, and 0.2 g/l sodium phosphate dibasic hepta-hydrate.

D. Balanced salt solution

Balanced salt solution (BSS) was prepared as described in Mishell and Shiigi, (1980).

E. Buffered salt solutions

Solutions consisting of 100 mM NaCl in distilled water were used for storage of bacteriophage T4 . The salt solutions were maintained at various pH levels for use in coupling reactions and for ØT4 storage. The following buffers were used to maintain the different pH levels:

100 mM TRIS (Baker) ----- pH 7.2

100 mM MOPS (Sigma)----- pH 7.2

100 mM MES (Sigma)------ pH 5.5

These solutions were heat sterilized.

III. Tumor cells

P815, a subline of a methylcholanthrene-induced mastocytoma of D2 mice, (Dunn and Potter, 1957) was obtained from Dr. B. Smith, Institute for Cancer Research, Philadelphia, Pa.. P815 was maintained in ascitic form in D2 mice. Every 5-7 days, tumor cells were withdrawn from the peritoneal cavity of a mouse, washed, suspended in PBS, and $10^7$ cells re-injected intraperitoneally (i.p.) into a naive D2 mouse. Tumor cells were stored at $10^7$ cells/vial (Nunc, GIBCO Can. Inc. 3-68632, Burlington, Ont.) in 1 ml of 10% dimethylsulphoxide (Sigma) + 90% FCS. The vials were stored frozen at -80°C in liquid nitrogen. Every 3-4 months, new vials of P815 were thawed for use.

IV. Monoclonal antibodies

Jlj, a rat hybridoma producing anti-mouse monoclonal antibody (mAb) anti-Thy1.2, was a gift from Frank Symington, Seattle, Wa..

GK1.5, a rat anti-mouse hybridoma producing a monoclonal IgG2b antibody that recognizes the L3T4a determinant on helper T cells, (Dialynas et al., 1983) was obtained from the American Type Culture Collection.

V. Cell Preparations

A. Single cell suspensions

Spleen cells or lymph node cells for use in tissue culture were prepared as follows. Spleens or lymph nodes were dissected from freshly killed mice and put in sterile glass petri dishes containing 10 ml of complete RPMI. Single cell suspensions of spleens or lymph nodes were prepared by pressing the tissues through sterile stainless steel screens (60-80 mesh) using the flat end of a 1cc disposable syringe plunger.The cell suspension was drawn into the syringe (no

needle attached) and squirted onto the bottom of the petri dish several times to break up clumps of cells. The cell suspensions were transferred to a test tube ( 17x100mm, Evergreen, Los Angeles, Ca. ), spun for 7 min at 1000g, and resuspended in medium. The viability of the cells was determined by the trypan blue exclusion method (Direct blue 14, 0.07% in saline, Matheson, Coleman and Bell, TX1580, Norwood, Ohio) in an eosinophil counter chamber (Speers-Levy, 2/10 mm deep x 1/16 $mm^2$, C.A. Mausser and Son, Philadelphia, PA.).

B. Irradiation of cells and mice

Spleen cells, tumor cells, and mice were irradiated in the chamber of the $^{60}$ Co-gamma radiation source (Gammacell 220, Atomic Energy of Canada, Ltd.) in the Department of Chemistry, U.B.C.. Spleen cells were suspended in complete RPMI + 10% FCS, and tumor cells were suspended in cold PBS, before irradiation. The dosages of irradiation used were as follows: spleen cells- 2,000-3,300 rad; tumor cells- 10,000 rad; and mice- 600 rad for the host mice in adoptive transfers, and 150 rad to reduce suppression in mice.

C. Treatment of cells with anti-Thy 1.2 antibody + complement

For anti-Thy-1.2 antibody treatment, cells were washed once in complete RPMI and resuspended at a concentration of $10^7$ cells/ml with 5 ml/tube, in anti-Thy 1.2 antibody (Ab) ( 1:20 dilution in BSS). Cells were then incubated at 23°C for 40 min with gentle shaking, washed once in BSS, and resuspended at a concentration of $10^7$/ml in rabbit complement (C) (Cedarlane Lo-tox M, CL3051, Guelph, Ont.) ( 1:10 dilution in BSS). Cells were incubated for 50 min in a 37°C water bath. Immediately after this incubation, cells were plunged into ice and aliquots of cells were counted using trypan blue exclusion. Cells were then washed twice in BSS, resuspended in PBS, and used for injection.

D. Treatment of cells with mAb GK1.5 + complement

Spleen cells to be treated with mAb GK1.5 + C were washed once in complete RPMI , counted, and resuspended at a concentration of $5x10^6$ cells/ml in GK1.5 cell free supernatant

( 1:1 dilution in complete RPMI) . A control group of cells, to be treated with C only, was suspended in complete RPMI only. Cells were incubated for 60 min at 23°C with frequent gentle mixing, washed once in SFM, and then resuspended at a concentration of $10^7$ cells/ml in rabbit C ( 1:10 dilution in SFM ). The diluted C was filtered ( Nalgene 0.45 micron filter, F3200-2, Rochester, N.Y. ) before use. Cells were incubated for 50 min at 37°C in a 5% $CO_2$ incubator. After incubation, cells were put on ice and an aliquot of cells from test and control groups were counted and scored for live and dead cells using trypan blue exclusion. Cells were then washed twice in complete RPMI, resuspended in medium, and set up in assays.

E. Passage of spleen cells over nylon wool

Spleen cells were passed over nylon wool columns to enrich the spleen cell population for T cells. The procedure used was essentially as described by Henry ( 1980), with the following changes. Nylon wool ( Travenol, Type 200,Fenwal Laboratories, Deerfield, Ill. ) was boiled and washed in distilled water. The wool was dried and packed in disposable syringes. To pack the syringe- columns, the nylon wool was soaked with distilled water, and packing was done with the columns submerged in a large beaker of disilled water. After packing, the columns were thoroughly washed with PBS and checked with a pasteur pipette to ensure that no bubbles had formed in the nylon wool. The packed columns were soaked in PBS, sealed with aluminum foil and autoclaved with the nylon wool wet. Cell separation was performed using complete RPMI plus 25mM Hepes as the medium. The column was incubated in a 37°C incubator for the incubation steps. Recovery of viable, T-enriched spleen cells using this method was between 15-20% of input cells.

VI. Establishment and maintenance of T helper cell lines and clones

A. Establishment of T helper cell lines

T cell lines were established according to Kimoto and Fathman ( 1982), with changes as noted. Briefly, mice were immunized subcutaneously at the base of the tail with 10 µg ØT4 in

incomplete Freund's adjuvant. Seven days later, cells draining from the paraartoric and inguinal lymph nodes were removed, prepared as in Materials, V-A, and cultured in 24-well plates (Costar 3424, Cambridge, MA.) at a density of $5x10^6$ cells/well. The cells were cultured in 1.5 ml complete RPMI plus 5µg/ml ØT4. After 4 days, the cells were harvested and washed twice in complete RPMI, counted, and set up in resting culture. $2x10^5$ blast cells were cultured with $5x10^6$ irradiated (3,300 rad) syngeneic spleen cells as filler cells in 2 ml complete RPMI. The cells were rested for 10-14 days, and were then re-stimulated with ØT4. $2x10^5$ resting lymph node cells were cultured with $5x10^6$ irradiated syngeneic spleen cells in 2 ml complete RPMI plus 5 µg/ml ØT4 for 4 days. This cycle of 4 day stimulation and 10-14 day rest was the maintenance routine for the in vitro cell line.

B. Cloning the ØT4-reactive helper cell line

Limiting dilution cloning of T helper cell lines reactive to soluble antigen has been described previously by Kimoto and Fathman (1982). I cloned ØT4-reactive T helper cell line cells after they had been through four cycles of stimulation and rest. 96-well, flat-bottom plates (Linbro, Flow Labs, 76-032-05) were prepared by adding $1x10^6$ irradiated (3,300 rad) syngeneic filler cells per well, in 100 µl of complete RPMI containing 5µg/ml ØT4 + 1% EL4-IL2. To each well was added 100µl of the cell line, containing either 1.0 cell/ml, 10 cells/ml, or 30 cells/ml. Plates were incubated at 37°C in a 5% $CO_2$ atmosphere, carefully observed for growth, and fed with complete RPMI + 1% EL4-IL2. Approximately 3 weeks after initial culture, wells with growing cells were recultured in 24-well plates (Costar), in complete RPMI + 2% EL4-IL2. These clones were expanded and maintained on a cycle of 1 week rest/ 4 day stimulation with ØT4, as described for the T cell line (Materials, VI-A), except that the medium used was complete RPMI + 2% EL4-IL2.

C. Freezing and reviving the cell lines and clones

The method of freezing was obtained from Dr. H. Mostowski of the National Institutes

of Health, Bethesda, Maryland. Cells were washed in complete medium and $5x10^6$ cells were resuspended in 0.5 ml of a Dextran (Sigma)+ 90% FCS solution. Cells were incubated at 23°C for 30-60 min. Then 0.5 ml of a cold mixture of 10% Dextran, 15% dimethylsulphoxide, and 75% FCS was added to the cells, drop by drop, while vortexing the cells at a very low speed. The cell mixture was transferred to a freezing vial (Nunc). Vials were frozen by suspension in the vapor phase of liquid nitrogen for 18 hr, then submerged in the liquid phase of nitrogen for storage. To reculture frozen cells, vials were thawed in a 37°C water bath, shaking gently, and as soon as the last ice in the vial had melted, cells were transferred into 5 ml of warm complete RPMI + 1% EL4-IL2. Cells were washed 1x in complete RPMI, counted, and cultured in 24-well plates at a cell density of $2x10^5$ cells/ml in a total volume of 2 ml/well. Each well also contained $5x10^6$ irradiated (3,300 rad) syngeneic spleen cells and 5 µg/ml ØT4. Complete RPMI + 2% EL4-IL2 was the medium used to culture clones. After revival, clones were maintained in culture as described in Materials, V-B.

## VII. Preparation of bacteriophage T4D

### A. Standard preparation of phage

1 litre of Escherichia coli, strain B23 (E.coli) was grown to a density of $3x10^8$ cells/ml. A stock preparation of bacteriophage T4D was used to infect the E.coli host (multiplicity of infection =5).The culture was incubated at 37°C for 2-3 hr in a shaking air incubator. The lysate was saturated with chloroform, and incubated at 4°C for 18 hr. The lysate was poured into a flask containing 3 mg of bovine pancreatic deoxyribonuclease(DNase) (Calbiochem 260912, La Jolla, Ca.), leaving the chloroform in the original flask; then incubated in the air shaker at 37°C for 15 min. The preparation was filtered (sterile Whatman #1 filter) and centrifuged at 8000 rpm for 20 min, using a JA-10 rotor (Beckman). The ØT4-containing supernatant was spun at 17,000 rpm for 20 min, in a JA-20 rotor (Beckman). The pellets were left for 1 hr on ice to let them loosen up; then pooled and resuspended in cold TRIS-NaCl. A second high-speed spin (17,000 rpm, 20 min ) was done on the pooled ØT4. The pellet was resuspended in 4 ml TRIS-NaCl, left for

12 hr at 4°C to facilitate resuspension, and purified before use over a sepharose column (Materials, VII-C ).

B. Preparation of $^{32}P$-labelled phage

Twenty-five ml of E.coli B23 was grown to a density of $3x10^8$ cells/ml. Twelve ml of the cells were washed once in 10 ml of 1/5 Phosphate TCG, and resuspended in 50 ml of 1/5 Phosphate TCG + Phosphorus-32 ($^{32}P$)(New England Nuclear NEX053, Lachine, PQ.) (specific activity = 5µCi/µg P). The cells were grown to a density of $3x10^8$ cells/ml. Ø T4, from a stock preparation, was added (multiplicity of infection=5). The culture was incubated at 37°C for 90 min, in a gently shaking water bath. A few drops of chloroform were added and the lysate was chilled on ice for 10 min to complete lysis. The supernatant was poured into a flask containing pancreatic DNase and incubated for 10 min at 30°C. The lysate was centrifuged for 5 min at 6000 rpm. The pellet was resuspended in 10 ml TRIS-NaCl and spun for 20 min at 17,000 rpm. This high speed pellet was resuspended in 1 ml buffer and centrifuged for 5 min at 6000 rpm to remove cellular debris. The ØT4-containing supernatant was removed, purified over sepharose CL-4B, counted in a scintillation counter, and titred.

C. Phage titration

Phage was titred on bottom layer agar using the soft agar overlay technique (Adams, 1959). Spectophotometric estimates of ØT4 titre were made by measuring the optical density at 260 nm ($OD_{260}$). Using spectrophotometric measurements, the ØT4 titre was calculated as follows:

at $OD_{260}$ : Absorbance=1 is equivalent to 50 µg ØT4D protein

for ØT4D: 1µg protein = $5x10^9$ plaque-forming units (PFUs)

D. Purification of phage

ØT4 was purified from E.Coli host contaminants such as DNA and cellular debris by

passage over sepharose CL-4B (Pharmacia, 17-0151-01). For purification of $^{32}$P-ØT4, a sepharose column was made using a 10-cc polystyrene disposable pipette as the column support. When a 1 litre preparation of non-radioactive ØT4 was purified, a 1.5 x 50 cm column( Bio-Rad, Mississauga, Ont.) was used. TRIS-NaCl or MOPS-NaCl were the buffers used to pour the columns and for elution of the phage. Columns were poured to within 4 cm of the top of the large column, and to the 10 ml mark of the small column, and were washed with 3 column volumes of buffer before use. Phage was loaded on the column in a volume of buffer that was not more than 5% of the total column volume. 1ml fractions were eluted at the rate of 0.6ml/4 min. The phage titre of each fraction was estimated by spectophotometric measurements at $OD_{260}$. Fractions having an $OD_{260}$ greater than1 were pooled and titred on BLA plates. After purification, the phage was stored in the appropriate buffer, in a glass tube, over chloroform, at a density of between $5x10^{11}$and $4x10^{12}$ ØT4/ml.

VIII. Production of Interleukin 2

Interleukin 2 (IL2) was produced from EL4 thymoma cells, obtained from Dr. V. Petkau of the University of Alberta. The original line was from the laboratory of Farrar. The production of IL2 by stimulation of EL4 thymoma cells with phorbal myristic acetate (PMA) has been described previously (Farrar et al., 1980). Briefly, EL4 cells were washed and cultured in complete RPMI + 5% horse serum at a concentration of $1x10^6$ cells /ml. 10ng/ml PMA (Sigma) was added. After 18-24 hr the cell-free supernatant was filter sterilized over a 0.45 micron filter (Nalgene 450-0045) and stored at -20°C. The supernatant was tested before use for its' ability to maintain growth of the IL2 dependent cell line, CTLL.

IX. Coupling ØT4 to P815 tumor cells

ØT4 was coupled to P815 cells using a modification of the method of Takatsu et al. (1978). 1-ethyl-3(3-dimethylaminopropyl) carbodiimide, (EDAC),(Bio-Rad,153-0990) was used to cross-link ØT4 and P815 membrane proteins. Irradiated (10,000 rad) P815 tumor cells

(P815r) were washed 3x in saline, pH7.2, and resuspended to a concentration of $10^7$ cells/ml in MES-NaCl, pH 5.5, containing 0.5mg/ml EDAC and 50-80 µg/ml ØT4. The EDAC was dissolved in saline just before addition to the coupling mix. ØT4 in MOPS- NaCl, pH 7.2, was diluted in saline 1 hr before use. Coupling was done in polystyrene tubes ( 17x100 ml, Evergreen) in a final volume of 1.0 ml. The mixture was incubated for 1 hr at 23°C with frequent gentle shaking. After coupling, the cells were washed 3x in PBS or complete RPMI and used in experiments the same day.

X. Coupling ØT4 to sheep red blood cells

A. Sheep red blood cells

Heparinized sheep red blood cells (SRBC) were obtained from the Animal Care Unit, U.B.C.. Cells were diluted 1:1 in sterile Alsever's solution, stored at 4°C for at least 1 week, and washed 3x in saline, pH 7.2, before use. SRBC were used for 6-8 weeks after receipt, after which time lysis of the cells made them useless.

B. Chromic chloride coupling

ØT4 was coupled to SRBC using the chromic chloride ($CrCl_3$) method, essentially as described by Poston ( 1974). The only change to be noted was the use of new glass tubes as the reaction tubes, to eliminate phosphate contamination that could inhibit the reaction.

C. Hemagglutination assay

The hemagglutination procedure, used for testing anti-SRBC serum titres, and to check coupling of ØT4 to SRBC, was done essentially as described in Hudson and Hay ( 1976). The buffer used for hemagglutination was PBS + 10% FCS. Briefly, 8 doubling dilutions of the antisera to be tested for anti-SRBC Ab titre were made in V-bottom, 96-well plates (Linbro,76-023-05 ). Dilutions of sheep cell hemolysin were made in control wells. 25µl/well of SRBC, at a concentration of 0.5% ( $1x10^8$ SRBC/ml) were added for a final concentration of 0.25% SRBC per

well. Cells were mixed by gently tapping the plates from every side, and left for at least 1 hr at 23°C until hemagglutination in control wells could be observed. To check $CrCl_3$ coupling of ØT4 to SRBC, dilutions of anti-ØT4 antiserum were made in the wells, and the coupled ØT4-SRBC was added for a final concentration of 0.25%. Controls were SRBC treated with $CrCl_3$ only.

XI. Phage plaque inhibition assay

D2 mice were injected in the hind footpad with 5µg ØT4. Seven days later, mice were bled and the anti-ØT4 antiserum was used in this assay. ØT4 was incubated at 4°C with an equal volume of a $10^{-2}$ dilution of anti-ØT4 antiserum, normal mouse serum, or TRIS-NaCl alone. At times 0, 24, and 48 hr after the start of incubation, the phage was titred. Duplicate samples of 0.1 ml of the ØT4: serum mixture were added to 0.1 ml of E. coli, and titred using the soft agar overlay method. The number of ØT4 PFUs were counted for each plate.

XII. Proliferation assay

The proliferative responses of spleen cells, cell-line cells, or clones (responder cells) were measured by methyl-$^3$H-Thymidine ($^3$H-TudR) incorporation by the responder cells. Culture medium was complete RPMI + 0.5% NMS when spleen cells were the responders, or complete RPMI + 10% FCS when cell-line cells or clones were tested. $1x10^4$ - $1x10^5$ responder cells were cultured in triplicate in flat-bottom wells of 96 well plates (Linbro 76-032-05). When nylon-wool enriched spleen cells, cell-lines or clones were tested, $5x10^5$ irradiated (3,300 rad) syngeneic spleen cells were added to each well as filler cells. Various concentrations of stimulating antigens or mitogens were added at the initiation of culture. Total volume of cultures was 0.2 ml/well. Cultures were incubated at 37°C, in a 5% $CO_2$ incubator, for from 48-120 hr. Eight hr before harvesting, cultures were pulsed with 1µCi/well $^3$H-TudR (2.0 Ci/mmol in aqueous solution, Amersham, U.K.) in 50µl medium, using a Hamilton pipetter. Cultures were harvested with a 12-channel harvester onto glass-fibre filters (Gelman, 61638). Filters were put into vials,(Packard 6000192, Downer's grove, Ill.), dried, and 3 ml of 2,5-

Figure 2 : Cytotoxic assay for helper cells in primed spleen cell populations.

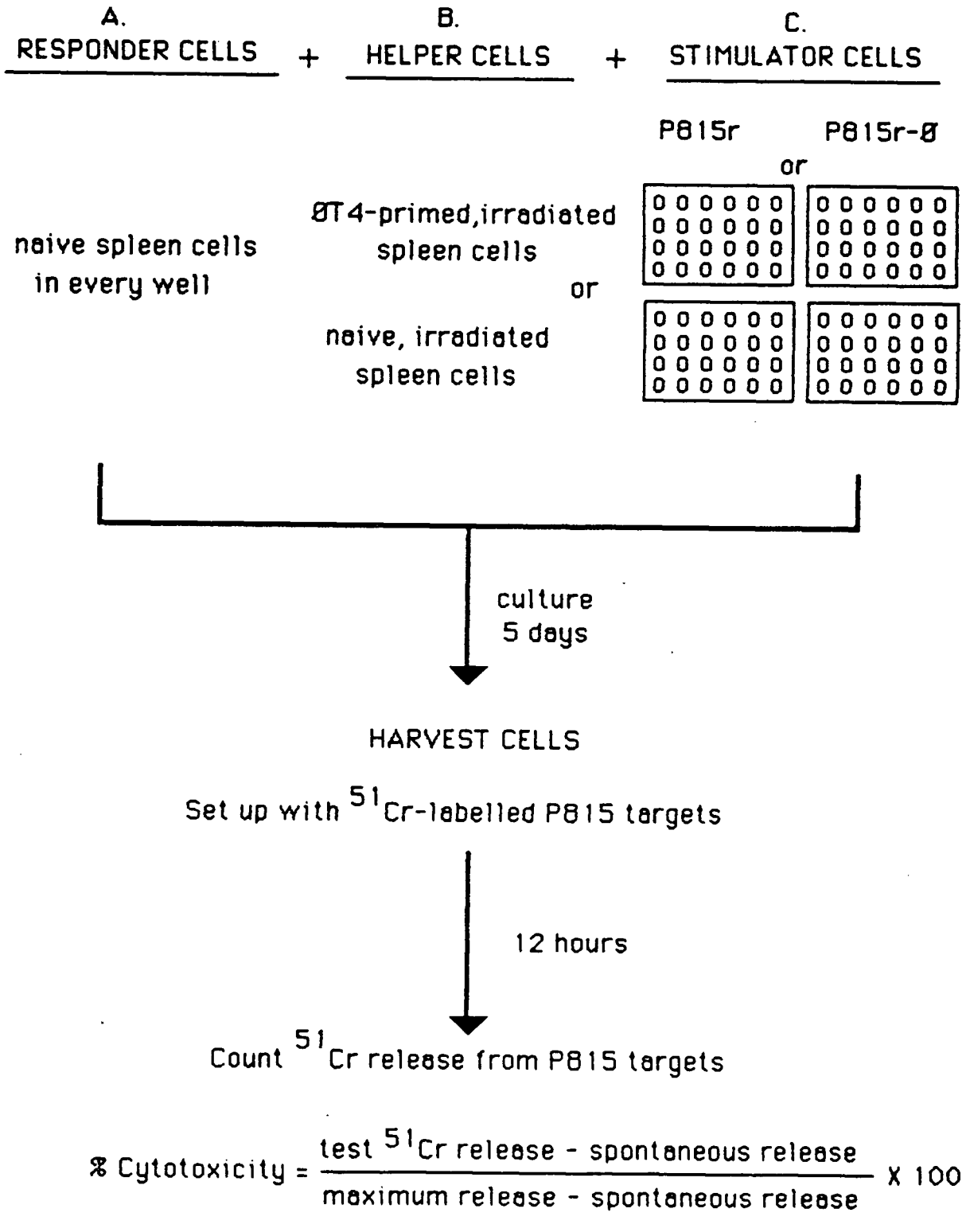
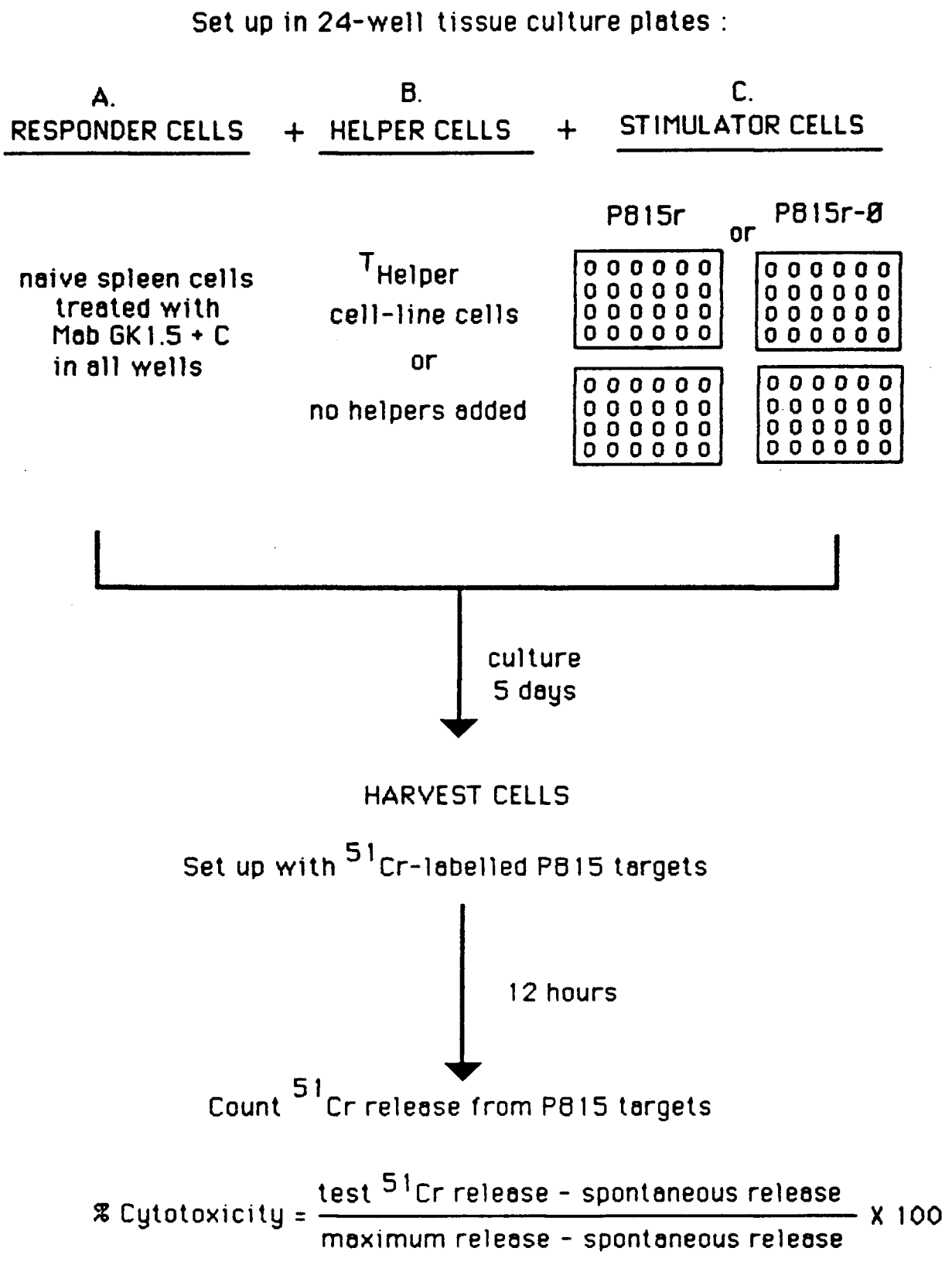


Figure 3 : Revised cytotoxic assay for helper cells.



(diphenyloxazole/1,4-bis-(2 methyl-stryryl)-benzene (PPO-Bis), dissolved 4g/l in toluene, was added to each vial. Vials were wiped with an ionic detergent (C-Tab) and counted in a scintillation counter. Results were expressed as mean counts per minute (cpm) of triplicate cultures.

XIII. Cytotoxic assays

A. Assays for helper activity

The assays for helper activity are illustrated in Figs 2 and 3. Normal D2 spleen cells (responder cells) were removed from mice, processed, and cultured at a concentration of $2.5 \times 10^6$ cells/ml, in quadruplicate 2 ml cultures in 24-well plates (Costar). In some experiments, the responder cells were treated with mAb GK1.5 + C before addition to culture (Materials, V-D). $5 \times 10^5$ $P815_r$ or $P815_r$-Ø were added to each well as stimulators cells. Cells to be tested for the ability to help the cytotoxic response (helper cells) were added in numbers ranging from $1 \times 10^3$ - $1 \times 10^6$ helper cells/well. The cells were cultured at 37°C in a 5% $CO_2$ incubator for 5 days, then harvested and set up in a chromium-51 ($^{51}Cr$)-release assay as described in Materials, XIII-B.

B. $^{51}Cr$-release assay

After 5 days in culture, groups of quadruplicate wells (Materials, XIII-A) were pooled, the cells were counted and used as effector cells in the $^{51}Cr$-release assay (Brunner et al., 1968). Cells were suspended at a concentration of $5 \times 10^6$/ml, and 0.2 ml of this suspension was added to triplicate wells of the top row of 96-well round-bottom plates (Linbro,76-042-05). Six to eight doubling dilutions of cells were made down to the bottom row of the plate. $5 \times 10^3$ $^{51}Cr$-labelled target cells (Materials, XIII-C) were added to every well. Cells were gently sedimented by centrifugation for 5 min at 150 x g, and incubated for 12 hr at 37°C, in a 5% $CO_2$ incubator. After incubation, 0.1 ml of cell-free supernatant was harvested and counted in a gamma counter (Isoflex automatic, Micromedic systems Inc.). Maximum $^{51}Cr$-release controls were $5 \times 10^3$ labelled targets lysed with detergent ( 5% Triton-X 100, Sigma 76878). Spontaneous

release was measured from target cells cultured in medium alone. Results were expressed as % cytotoxicity.

$$\% \text{ cytoticity} = \frac{\text{test } ^{51}\text{Cr-release} - \text{spontaneous release} \times 100}{\text{maximum release} - \text{spontaneous release}}$$

C. Labelling P815 tumor cells with $^{51}$Cr

P815 cells were the targets in all cytotoxicity assays. P815 cells were washed once in PBS, counted, and 4 -5x$10^6$ cells were suspended in 0.1 ml of $^{51}$Cr (sodium chromate in aqueous solution containing 0.9% NaCl, Amersham) diluted to a concentration of 5µCi/ml in PBS. Cells were incubated for 60 min at 37°C, in a 5% $CO_2$ atmosphere with occasional gentle mixing. Cells were washed twice in complete RPMI and further incubated for 2 hr at 37°C, in 10 ml complete RPMI. The second incubation was necessary to reduce spontaneous release of $^{51}$Cr from the targets in the final assay setup. After the second incubation, cells were washed once in complete RPMI and suspended at a concentration of 5x$10^4$ cells/ml in complete RPMI. 0.1 ml of this suspension was added to each well of the assay.

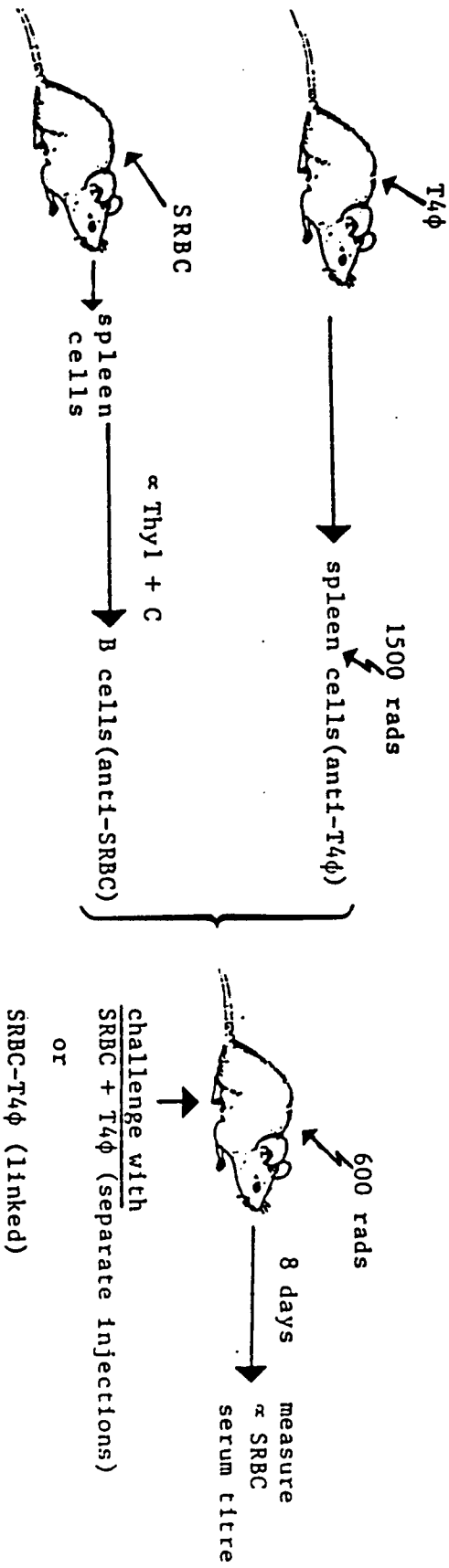
XIV. Adoptive transfer assay

Conditions for performing adoptive transfer experiments have been described previously (Mitchison, 1971a). Fig.4 is a flow chart illustrating the basic procedure. Two different kinds of cells were transferred to host D2 mice that had been irradiated (600 rad) 1 day before cell transfer. Each mouse received:

i). 2x$10^7$ spleen cells from normal or ØT4-primed D2 mice. Mice were irradiated with 150 rad 1 day before intraperitoneal (i.p.) injection of mice with 10µg ØT4 in incomplete Freund's adjuvant. Primed spleen cells were used 3 months after priming of the mice.

ii). 1x$10^7$ spleen cells from mice injected i.p., 1- 3 months before cell transfer, with 2x$10^8$ SRBC. These cells were treated with anti-Thy 1.2 Ab + C to remove T cells. (Materials, V-C).

Figure 4 : In vivo adoptive transfer assay for T cell help.



Transferred cells were suspended in PBS and injected intravenously (i.v.). To avoid mouse deaths, 10 units of heparin were injected i.p., 10 min before the i.v. injection. One day after cell transfer, mice were challenged with i.p. injections of $2x10^8$ SRBC , SRBC linked to ØT4 (SRBC-Ø), or SRBC and a separate injection of free ØT4. To estimate the amount of free ØT4 for injection, 5% of the input ØT4 was assumed to be coupled to the SRBC in the coupling reaction, and an equivalent amount of free ØT4 was injected. Coupling of SRBC-Ø was checked by hemagglutination using anti-ØT4 antiserum, before the coupled cells were injected.(Materials, X-C). Nine days after challenge, mice were tail-bled and the sera from individual mice were titred for anti-SRBC Ab using the hemagglutination assay.

XV. Immunotherapy experiments

Several different protocols for the in vivo immunotherapy experiment were tested. The basic procedure was as follows (see Fig. 17). Mice, at least 10 weeks of age, were injected i.p. with 20mg/kg/mouse of cyclophosphamide (Cytoxan, Bristol Labs 9407-01, Belleville, Ont.). Two days later, $10^6$-$10^7$ ØT4-reactive T cells were mixed with $1x10^5$ $P815_r$-Ø, or $P815_r$ alone. The cell mixture was resuspended in PBS and injected i.p., in a total volume of 0.4 ml/mouse. One week later, each mouse was challenged subcutaneously on the left flank with $5x10^3$ viable P815 tumor cells resuspended in 0.1 ml PBS. In some experiments, mice were given a second injection of ØT4-reactive cells mixed with coupled or uncoupled $P815_r$ and the tumor challenge was given 2 days after the second injection. Tumor growth on each mouse was measured with calipers and survival of the mice was recorded.

# RESULTS AND DISCUSSION

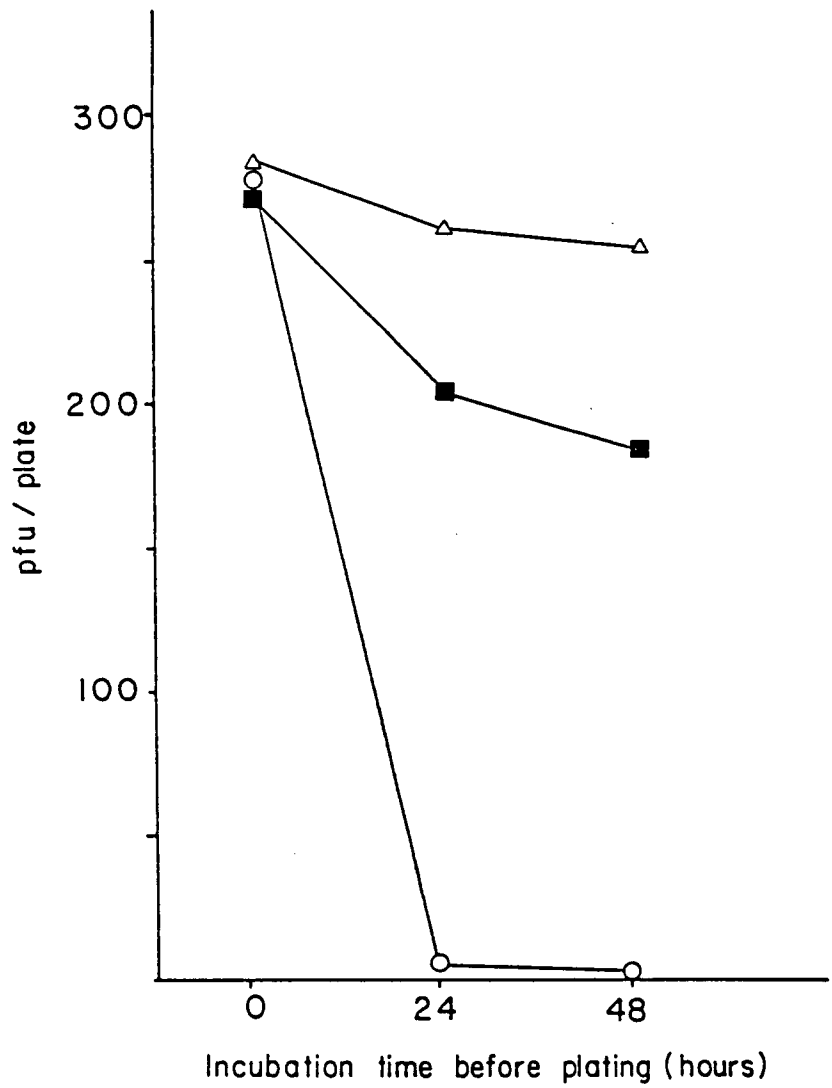
## CHAPTER I. USE OF BACTERIOPHAGE T4 AS A LINKED HELPER DETERMINANT ON TUMOR CELLS

This laboratory chose bacteriophage T4 as the helper determinant to be chemically linked to the surface of P815 tumor cells in our experiments. This novel choice of antigen was made because phage has previously been used for detection of very small quantities of antibodies in phage inhibition assays (Mäkelä et al. , 1980). Our lab hoped to use an assay similar to the phage inhibition assay to detect putative antigen-specific ( ie. ØT4 specific) helper factors. The helper factors might bind to ØT4, inactivating it and resulting in a decrease in the number of plaques formed in a lawn of host bacteria.

### A. Phage inhibition assay

Fig. 5 shows the results of a ØT4 inhibition assay using anti-ØT4 antiserum from a mouse, previously injected with 5µg ØT4 (Methods, XI). A 1:100 dilution of the anti-ØT4 serum completely inhibited the formation of phage plaques after incubation of phage and serum for 24 hr. In contrast, incubation in normal mouse serum seemed to be protective, since ØT4 incubated in a 1:100 dilution of the normal mouse serum maintained a higher titre over time than ØT4 incubated in the standard phage storage medium, TRIS-NaCl. The reason that the control solution of ØT4 in TRIS-NaCl dropped in titre over the 48 hr incubation period is because the concentration of phage (3000 ØT4/ml) in the solution was much lower than the optimum phage storage concentration, which is $1 \times 10^{12}$ ØT4/ml. This experiment demonstrated that the plaque-inhibition assay could be useful for the detection of antibody that binds and inactivates ØT4. Before using ØT4 in my project, further preliminary work was necessary to determine whether it would function as a helper determinant on the surface of tumor cells.

Figure 5: The inhibition of ØT4 by anti-ØT4 antiserum. ØT4 was incubated with $10^{-2}$ dilutions of anti-ØT4 antiserum ( ○ ), or normal mouse serum ( △ ). At 0, 24, and 48 hr after the start of the incubation, aliquots of the ØT4/serum mixtures were titred by the soft-agar overlay method (Materials XI). The control was ØT4 incubated with Ø maintenance medium, TRIS-NaCl ( ■ ).



B. Optimization of the conditions for attachment of ØT4 to irradiated P815 tumor cells

There are many reports of using bacteriophage coupled to simple chemicals, proteins, nucleic acids, or polysaccharides in sensitive assays for antibodies. In addition, tumor cells have been coupled to a number of viruses and chemical antigens. To my knowledge, this is the first example of the use of ØT4, chemically linked to tumor cells, as a helper determinant. My next set of experiments were designed to quantitate and optimize the coupling of ØT4 to irradiated P815 cells (P815r).

1. pH and buffer

Previous work has been done in this laboratory using EDAC, a water-soluable coupling reagent, for the linkage of PPD to P815r cells. The EDAC reaction mechanism involves 2 steps. The first step, activation of carboxyl groups on the ligands, which in our experiments are ØT4 or tumor cells, requires a low pH of 4.7-5.0 (Bio-Rad catalogue, 1984). The second step involves reaction of amino groups with the activated carboxyl groups.

To achieve the optimal degree of coupling, I altered the previous coupling protocol (Takatsu et al., 1978) to improve conditions for the reaction.

Kipp and Miller (1980) used saline rather than phosphate buffer in EDAC reactions because phosphate reacts with EDAC and may interfere with the reaction. It was also important to eliminate TRIS in the coupling reaction since amino groups in TRIS could compete with amino groups on the ØT4 or on the tumor cells. For these reasons, MOPS and MES were chosen as phosphate and TRIS-free buffers. MOPS is an effective buffer in the pH range 6.5-7.9, so was the choice for storage of ØT4 to be used in coupling reactions. MES has a buffering capacity in the pH range 5.5-6.7 and was used in experiments to test the optimal pH for coupling, and as the coupling reaction buffer. ØT4 viability was tested in both buffers and found to be equal to that in standard TRIS-NaCl storage buffer.

Experiments were done to test the effect of different pH levels in the EDAC reaction.

Table I: Optimal pH for coupling ØT4 to P815 tumor cells using EDAC coupling reagent.

| pH | INPUT $^{32}$P-ØT4 (cpm) | % BOUND $^{32}$P-ØT4 [a] |
|---|---|---|
| 4.9 | 284,875 | 1.9 |
| 5.5 | 295,120 | 2.5 |
| 6.0 | 296,750 | 0.3 |
| 6.5 | 315,220 | 0.4 |
| 7.0 | 308,980 | 0.1 |

[a] $$\%\ \text{bound}\ ^{32}\text{P-ØT4} = \frac{(\ \text{cpm [+EDAC]}\ ) - (\ \text{cpm [-EDAC]}\ )}{\text{input cpm}} \times 100$$

Representative results from these experiments are shown in Table I. The best level of coupling resulted when MES, pH 5.5, was the reaction buffer. In subsequent coupling reactions, MES buffer pH 5.5 was used.

2. Concentration of phage

Reports of the use of EDAC to couple antigens to cells have varied according to the concentration of antigen used in the coupling reaction (Golub et al., 1968; Johnston et al., 1966; Henney, 1970; Takatsu et al., 1978; Takatsu et al., 1980). Using $^{32}P$-labelled ØT4 (Materials, VII-B), I did several experiments testing different concentrations of phage in the EDAC reaction.

Table II shows the results of a representative experiment testing various concentrations of ØT4. The percent input phage that bound to P815r cells increased as the number of input phage increased. Thus, the number of phage bound per P815r cell also increased with increasing concentration of input phage. A limiting factor in doing these experiments was the number of $^{32}P$-ØT4 that could be used as input phage. To limit the $^{32}P$ to a safe working level, low concentrations of input ØT4 were used, i.e., approximately 1-4µg/ml. The results indicate that approximately 2.7% of input phage was coupled to tumor cells in this reaction. This is slightly higher than the level of 2.5% coupling of PPD to tumor cells, reported by Takatsu et al., (1980). In all further coupling experiments, a final ØT4 concentration of 80-100 µg/ml was used. In these experiments, I assumed that 3% of input phage was coupled to the tumor cells. Since an increase in input phage concentration resulted in an increased percentage of phage bound per P815r cell, 3% may be a conservative estimate of phage binding when a higher concentration of input phage is used in the coupling reaction.

3. Other coupling methods.

Other methods besides the EDAC method have been used to attach antigens to cell surfaces. Lachmann et al. (1981) coupled PPD to the lectin, concanavalin A (CON A). The lectin-CON A was then incubated with tumor cells, binding to the tumor cells and creating a PPD - CON A - tumor

Table II: Coupling $^{32}P$-labelled ØT4 to P815r cells using EDAC: measurement of coupling efficiency.

| INPUT $^{32}P$-ØT4 (cpm) | INPUT $^{32}P$-ØT4 (µg/ml) | % BOUND $^{32}P$-ØT4 [a] (cpm) | NUMBER OF BOUND ØT4 PER P815r |
|---|---|---|---|
| 294,456 | 1.0 | 1.8 | 9 |
| 587,347 | 2.0 | 2.7 | 27 |
| 704,817 | 2.4 | 2.6 | 31 |
| 881,804 | 3.0 | 3.5 | 52 |
| 1,174,695 | 4.0 | 3.1 | 62 |

a $$\%\ \text{bound}\ ^{32}P\text{-ØT4} = \frac{(\text{cpm}\ [+\text{EDAC}]) - (\text{cpm}\ [-\text{EDAC}])}{\text{input cpm}} \times 100$$

Table III: Comparison of EDAC and $CrCl_3$ as coupling reagents.

| ADDITION TO CULTURE | ØT4 CONCENTRATION IN COUPLING REACTION (ØT4/ml) | $^3$H-TudR INCORPORATION BY ØT4-PRIMED SPLEEN CELLS (cpm) |
|---|---|---|
| ----- | ---- | 618 (26) |
| ØT4 | | |
| 1.0 µg/ml | ---- | 5,339 (488) |
| 0.1 µg/ml | ---- | 1,876 (239) |
| 0.01 µg/ml | ---- | 890 (255) |
| P815r-Ø | | |
| EDAC coupling | $10^{12}$ | 12,393 (777) |
| | $10^{11}$ | 8,271 (1,074) |
| | $10^{10}$ | 3,005 (208) |
| | ---- (EDAC only) | 2,917 (350) |
| P815r-Ø | | |
| $CrCl_3$ coupling | $10^{12}$ | 6,147 (1,087) |
| | $10^{11}$ | 2,975 (228) |
| | $10^{10}$ | 1,940 (315) |
| | ----($CrCl_3$ only) | 2,691 (135) |

EDAC and $CrCl_3$ were used to couple P815r to ØT4, using different concentrations of ØT4 in the coupling reactions. $1x10^5$ P815r-Ø, or various concentrations of ØT4 alone, were added to cultures of $1x10^5$ ØT4-primed, nylon-wool enriched spleen cells plus $5x10^5$ irradiated syngeneic spleen cells. On day 5, cultures were pulsed for 8 hours with $^3$H-TudR and assayed for proliferation. Values are the mean of triplicate cultures. Numbers in brackets represent the standard deviation from the mean.

cell "sandwich". I tested the CON A method of coupling ØT4 to P815r, and found it unsatisfactory for this study since the CON A used to link ØT4 to P815r induced more active proliferation of spleen cells than the proliferation induced by the ØT4.

Chromic chloride ($CrCl_3$) is effective in coupling antigens to red blood cells (Poston, 1974). Several experiments using $^{32}P$-ØT4 to measure the level of coupling of ØT4 to P815r with $CrCl_3$ were inconclusive. To compare the effectiveness of EDAC and $CrCl_3$ as coupling reagents, a proliferation assay was set up (Methods, XII) with P815r-Ø coupled using either EDAC or $CrCl_3$, as stimulators for ØT4-primed, nylon-wool enriched spleen cells. As shown in Table III, P815r-Ø coupled using EDAC was more effective than P815r-Ø coupled with $CrCl_3$ for stimulation of the ØT4-primed spleen cells. P815r cells that were incubated in the coupling reagents EDAC or $CrCl_3$ only, without addition of phage to the reaction, were nearly equal in their ability to stimulate the spleen cells. These results indicated that EDAC effected a higher level of coupling than did $CrCl_3$. They also demonstrated that sufficient phage was attached to the P815r surface to stimulate proliferation of ØT4-primed spleed cells. Thus, EDAC was used as the reagent for coupling ØT4 to P815r in all further experiments.

C. Summary

ØT4 was chosen to be the helper determinant antigen in this project. A preliminary study showed that the phage-inhibition assay could detect small amounts of anti-ØT4 antibody. Conditions for optimization of the EDAC coupling reaction were examined. The use of MES buffered saline, pH 5.5, as the reaction buffer, with a final ØT4 concentration of 80-100 µg/ml, produced effective coupling of ØT4 to P815r. Other coupling methods were tested and compared to the EDAC method. EDAC was found to be the best reagent for my purposes.

## CHAPTER II. T CELL RESPONSES TO PHAGE LINKED TO THE SURFACE OF P815 TUMOR CELLS

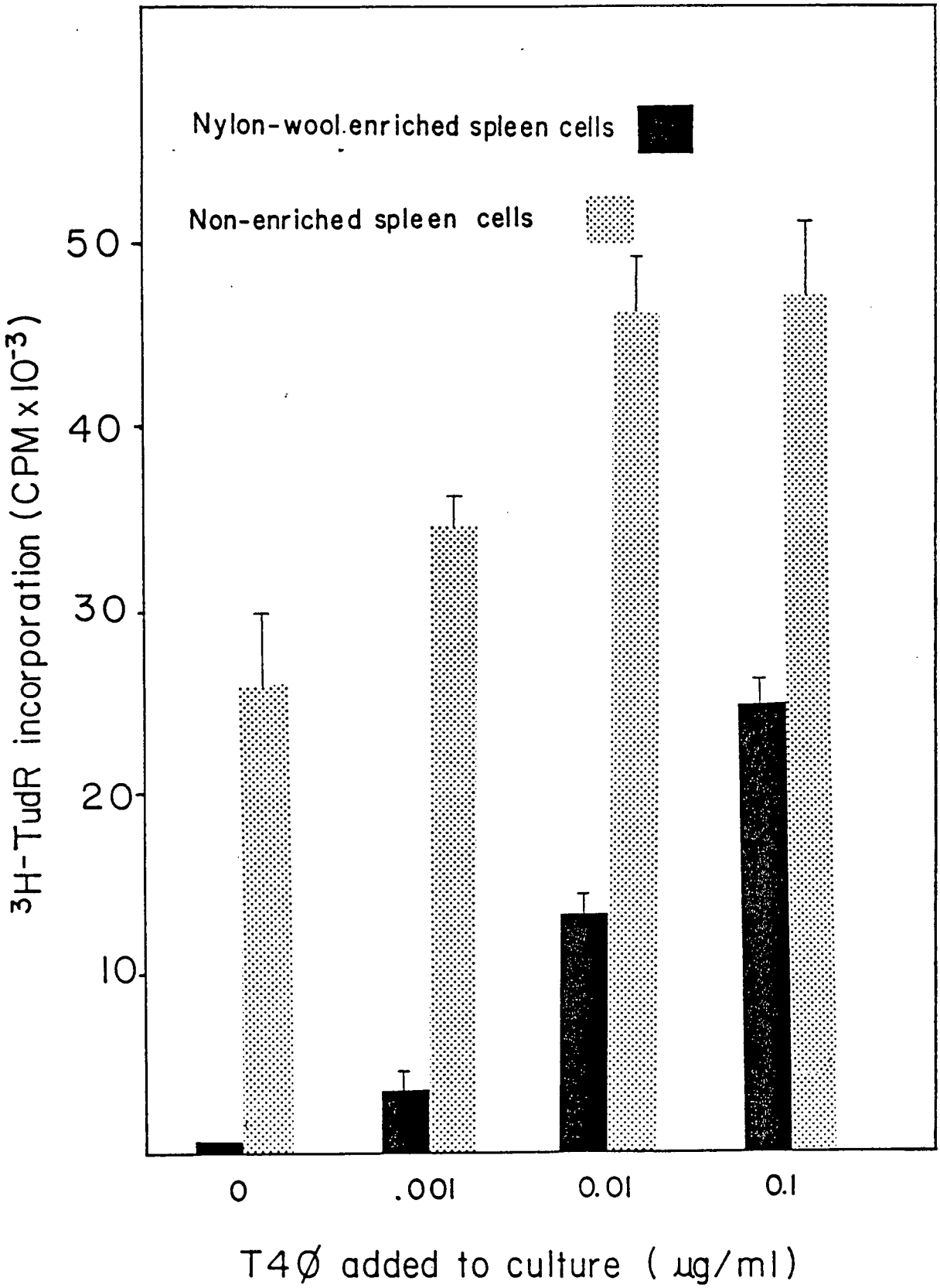
Once I had ascertained that ØT4 could be chemically linked to P815r, the next step was to test the linked determinant for its ability to stimulate T helper cells, *in vitro* and *in vivo*. A series of proliferation assays were performed for this purpose, as described below.

### A. Nylon wool treatment of spleen cells

Several experiments were designed to compare spleen cells from D2 mice primed with ØT4, and spleen cells from naive D2 mice, for their proliferative responses to ØT4. The results showed a very high background response from both primed and naive spleen cells, with and without antigenic stimulation. To reduce the background, the experiments were repeated using complete RPMI plus 0.5% normal mouse serum (NMS), instead of complete RPMI plus 10% FCS as the medium. This serum substitution reduced the over-all level of proliferation, because the mouse spleen cells had been stimulated to proliferate by the FCS, but did not react to normal mouse serum.

A second change that was made to lower the background in proliferation experiments was the use of nylon-wool enriched spleen cells as responder cells. As shown in Fig.6, ØT4-primed spleen cells had a basal incorporation of 26,000 cpm after an 8 hr pulse with $^{3}H$-TudR in a standard proliferation assay with $10^5$ spleen cells cultured /well (Materials, XII). When cells from the same spleen were enriched for T cells by passage over nylon wool, (Materials, V-E), and set up at the same concentration of $10^5$ cells/well in the proliferation assay, the background proliferation dropped to less than 1,000 cpm. The decrease in proliferation may be attributed to the removal of B cells, plasma cells, and accessory cells from the spleen cell population. The proliferation of these nylon-wool adherent cells obscured the response of T cells alone. In all further proliferation experiments using *in vivo* primed spleen cells, complete RPMI +0.5% NMS was the medium used,

Figure 6 : The effect of enrichment of Ø-primed spleen cells over nylon wool on the response of the spleen cells to ØT4 in a proliferation assay.



and responder cells were enriched for T cells by passage over nylon wool.

B. Kinetics of phage-primed T cell proliferation

Experiments were initiated to define the kinetics of the secondary proliferation of ØT4-primed, nylon-wool enriched T cells, in response to stimulation with ØT4. Fig. 7 shows the dose-response curves derived from one such experiment. Cultures of ØT4-primed, nylon-wool enriched spleen cells were stimulated with two different concentrations of ØT4. On each of days 3-8 after culture, incorporation of $^{3}$H-TudR was measured for an 8-hr pulse period. When cells were stimulated with 0.1 µg/ml ØT4, the proliferative response peaked on day 5 of culture. Stimulation with 0.01 µg/ml ØT4 resulted in a much lower proliferative response that peaked on day 4. In other experiments not shown, naive spleen cells were set up and stimulated in the same way as the cells in Fig 7. The naive spleen cells responded to both concentrations of ØT4 stimulation with low levels of proliferation, less than 1000 cpm, when pulsed on days 2-5 after culture, indicating that ØT4 is not a non-specific T cell mitogen.

C. In vitro and in vivo responses of spleen cells to P815r-Ø

It was important to determine whether murine T cells could "see" and respond to ØT4 when it was chemically linked to a tumor cell surface. Mice were primed to ØT4, and the primed, nylon-wool enriched spleen cells were tested for their ability to respond in vitro to ØT4 linked to P815r, in a proliferation assay (Table IV). Controls in this experiment were naive, nylon-wool enriched spleen cells. Test and control cells were stimulated with either ØT4 alone, P815r-Ø, or P815r alone. The primed cells responded in a dose-related manner to stimulation with three different concentrations of ØT4. Incubation of cells with P815r alone stimulated approximately a 2-fold greater proliferation over background levels. The same number of P815r with ØT4 linked to the surface stimulated twice the proliferation than when the cells were incubated with P815r alone. Naive T cells did not respond to stimulation with any of the antigens. These results demonstrated that primed T cells can recognize, in vitro, ØT4 that is chemically linked to P815r.

Figure 7: Kinetics of ØT4-primed T cell proliferation in response to stimulation in vitro with various concentrations of ØT4. $10^5$ nylon-wool enriched, ØT4-primed spleen cells were cultured with $5x10^5$ irradiated (2,000 rads) syngeneic spleen cells in 0.2 ml complete RPMI + 0.5% normal mouse serum. ØT4 was added at the initiation of cultures in the following concentrations: 0.1 µg/ml ( ▲ ), 0.01 µg/ml ( ■ ), no antigen added ( ◢ ). Eight hours before harvest, cultures were pulsed with 1µCi/ml $^3$H-TudR and assayed daily for proliferation. Each point represents the mean of triplicate cultures.
Priming protocol of mice for Ø-primed spleen cells: 150 rads, 24 hr before injection i.p. of 10 µg ØT4 in IFA, 8 weeks before experimental set-up.

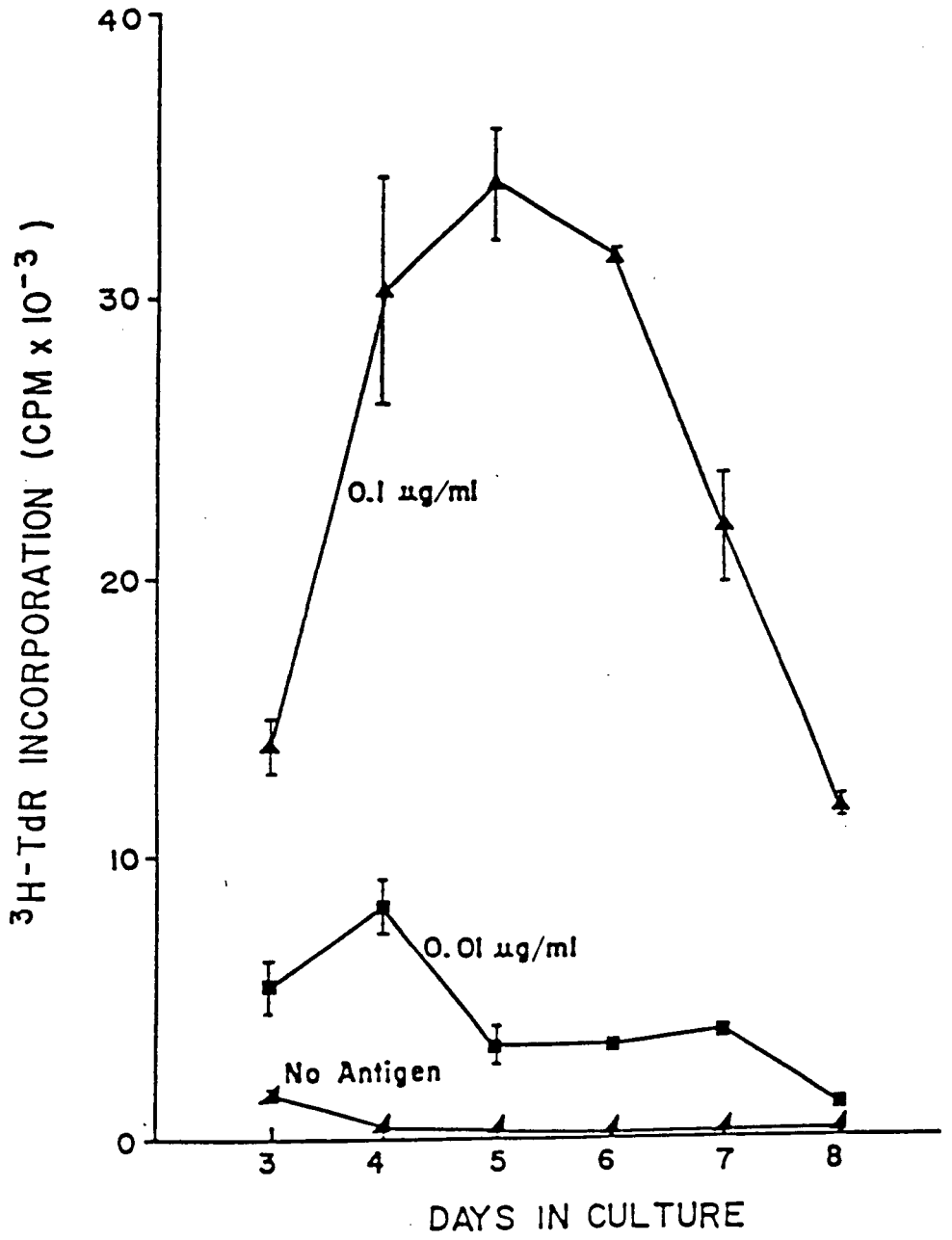


Table IV: T cells from mice primed to ØT4 respond to ØT4 linked to P815r

| ANTIGEN | DOSE | PROLIFERATIVE RESPONSE (cpm) | |
|---|---|---|---|
| | | ØT4-PRIMED T CELLS | NORMAL T CELLS |
| ----- | ----- | 4,597 (450) | 93 (27) |
| P815r | $10^5$ cells/well | 10,817 (737) | 268 (24) |
| ØT4 | 1.0µg/ml | 67,362 (778) | 101 (19) |
| ØT4 | 0.1µg/ml | 29,725 (3,976) | 267 (24) |
| ØT4 | 0.01µg/ml | 8,315 (291) | 98 (26) |
| P815r-Ø | $10^5$ cells/well | 21,623 (727) | 342 (38) |

Spleen cells from ØT4-primed or normal mice were passed over nylon wool to enrich for T cells, and set up in a proliferation assay. $1x10^5$ nylon-wool enriched spleen cells were cultured with $5x10^5$ irradiated (2,000 rad) syngeneic spleen cells in 0.2 ml complete RPMI + 0.5% NMS. P815r, P815r-Ø, or ØT4 were added at the initiation of culture. On day 4, cultures were pulsed for 8 hr with $^3$H-TudR and assayed for proliferation. Results represent the average of triplicate samples. Numbers in brackets represent the standard deviation from the mean.

Table V: Proliferation of P815r-Ø primed T cells in response to ØT4 and P815r.

| ADDITION TO CULTURE | PRIMED SPLEEN CELLS TAKEN FROM MICE INJECTED WITH: $10^7$ P815r-Ø | $10^6$ P815r-Ø | $10^5$ P815r-Ø | ----- |
|---|---|---|---|---|
| ---- | 675 (128) | 2,722 (396) | 2,970 (53) | 1,102 (55) |
| ØT4 | | | | |
| 1.0 µg/ml | 5,204 (1,754) | 5,866 (837) | 12,815 (178) | 4,265 (373) |
| 0.1 µg/ml | 4,824 (967) | 10,447 (810) | 14,505 (3715) | 4,337 (1191) |
| 0.01 µg/ml | 2,044 (1,397) | 3,110 (362) | 4,316 (825) | 1,505 (256) |
| P815r | 735 (92) | 1,354 (237) | 1,462 (180) | 1,357 (55) |

Values are cpm $^3$H-TudR incorporated. Each represents the mean of triplicate cultures. Numbers in brackets represent the standard deviation from the mean.
4 mice/ group were injected with $10^7$, $10^6$, or $10^5$ P815r-Ø. One week later, spleen cells from mice in each group were pooled, enriched for T cells over a nylon wool column, and set up in the standard proliferation assay. $1 \times 10^5$ nylon-wool enriched spleen cells were cultured with $5 \times 10^5$ irradiated (2,000 rad) syngeneic spleen cells in 0.2 ml complete RPMI + 0.5% NMS. P815r and ØT4 were added in various concentrations at the initiation of culture. On day 4, cultures were given an 8 hr pulse of $^3$H-TudR, harvested, and counted.

The next questions were, could mice be primed in vivo with P815r-Ø; and could spleen cells recognize, in vivo, ØT4 that was chemically linked to a tumor cell?

Mice were injected intraperitoneally with three different doses of P815r-Ø. One week after injection, spleen cells from the primed mice were set up in a proliferation assay with ØT4 alone or P815r alone as stimulators. When the in vivo primed, nylon wool enriched spleen cells were presented with P815r alone, the proliferative response in all groups was equal to or less than the response when no antigen was present in the culture (Table V). Thus, the effect of injection of mice with P815r-Ø was suppression of in vitro proliferation of the T cells when presented with P815r. However, in spite of the suppressive effect of injections of P815r-Ø on the T cell response to P815r, the T cells did proliferate in response to ØT4 alone added to the cultures.

These results showed that injections of mice with P815r-Ø primed a population of nylon-wool non-adherent spleen cells. The primed spleen cells proliferated specifically to ØT4 in the proliferation assay. There are several reasons that might explain why the proliferative response of the nylon-wool enriched spleen cells to P815r was negative. The injection of P815r-Ø may have activated P815-reactive suppressor cells which suppressed proliferation of P815r-reactive CTLs and helper T cells. Alternatively, coupling of ØT4 to the tumor cell surface might have masked the tumor antigens so that they couldn't be recognized. A control for this possibility would be to use spleen cells from mice injected with P815r alone as responder cells in the assay.

D. Summary

Nylon-wool treated spleen cells, enriched for T cells, were tested in proliferation assays for their responses to antigenic stimulation with ØT4, P815r cells, or P815r-Ø. T cells from mice primed with ØT4 responded strongly to both phage alone and also to ØT4 that was chemically linked to P815r cells. Injection of P815r-Ø into mice stimulated phage-reactive T cells, but at the same time caused suppression of the T cell proliferative response to P815r. The results demonstrated that T cells could recognize and respond to ØT4 bound to P815 tumor cells.

## CHAPTER III. ADOPTIVE TRANSFER OF PHAGE-REACTIVE HELPER CELLS

### A. Adoptive transfer experiments

The next question to be answered was whether ØT4-primed spleen cells could provide in vivo help for a cellular response to another antigen that was chemically linked to ØT4. We decided to investigate the ability of phage-primed T cells to help an in vivo B cell response to sheep red blood cells. We chose to look at help for a B cell response to SRBC instead of help for an anti-tumor CTL response because the T helper-B cell system has been studied (Mitchison, 1971b), and may have a mechanism similar to the mechanism of T cell help against heterogenized tumors. In addition, the in vivo anti- SRBC responses by B cells that make anti-SRBC antibody can be measured in a simple hemagglutination assay, whereas the assay for in vivo anti-tumor responses is much more complex and not as clear-cut to interpret.

In initial experiments, mice were primed by injection of ØT4; the primed mice were then injected i.p. with SRBC alone or with SRBC-Ø; and finally the mice were given i.p challenge injections of SRBC alone. The mice were bled and the individual mouse sera were titred in a hemagglutination assay for anti-SRBC antibody (Materials, X-C). The results from these experiments were variable. In some trials, the mice receiving injections of ØT4 and SRBC-Ø had lower anti-SRBC titres than mice that were challenged with SRBC only. In no experiments did the primed mice have greater anti-SRBC responses than the naive mice. This suggested that injection of SRBC and ØT4 was causing suppression in the animals. In an attempt to overcome the suppression, we decided to use the adoptive transfer protocol (Materials, VIII) (Fig. 4 ).

Fig.8 illustrates the results from the first adoptive transfer experiment. In this trial, all mice received intravenous transfers of SRBC- primed B cells, plus either naive, phage-primed, or phage-primed, irradiated ( 1500 rad) spleen cells. All mice were challenged with SRBC-Ø. Mice receiving phage-primed, irradiated spleen cells developed anti-SRBC serum titres 4-8 fold

Figure 8 : Adoptive transfer experiment #1 to test for the in vivo helper activity of ØT4-primed spleen cells. See Figure 4 for a flow chart of this assay.

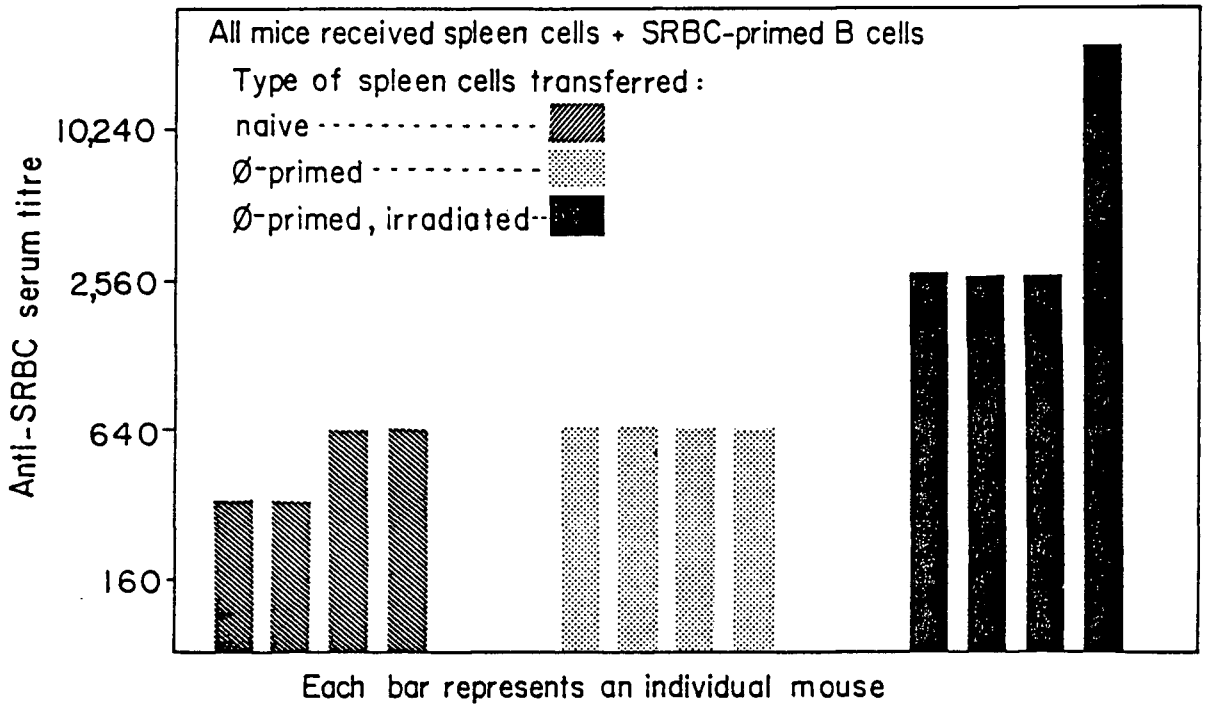
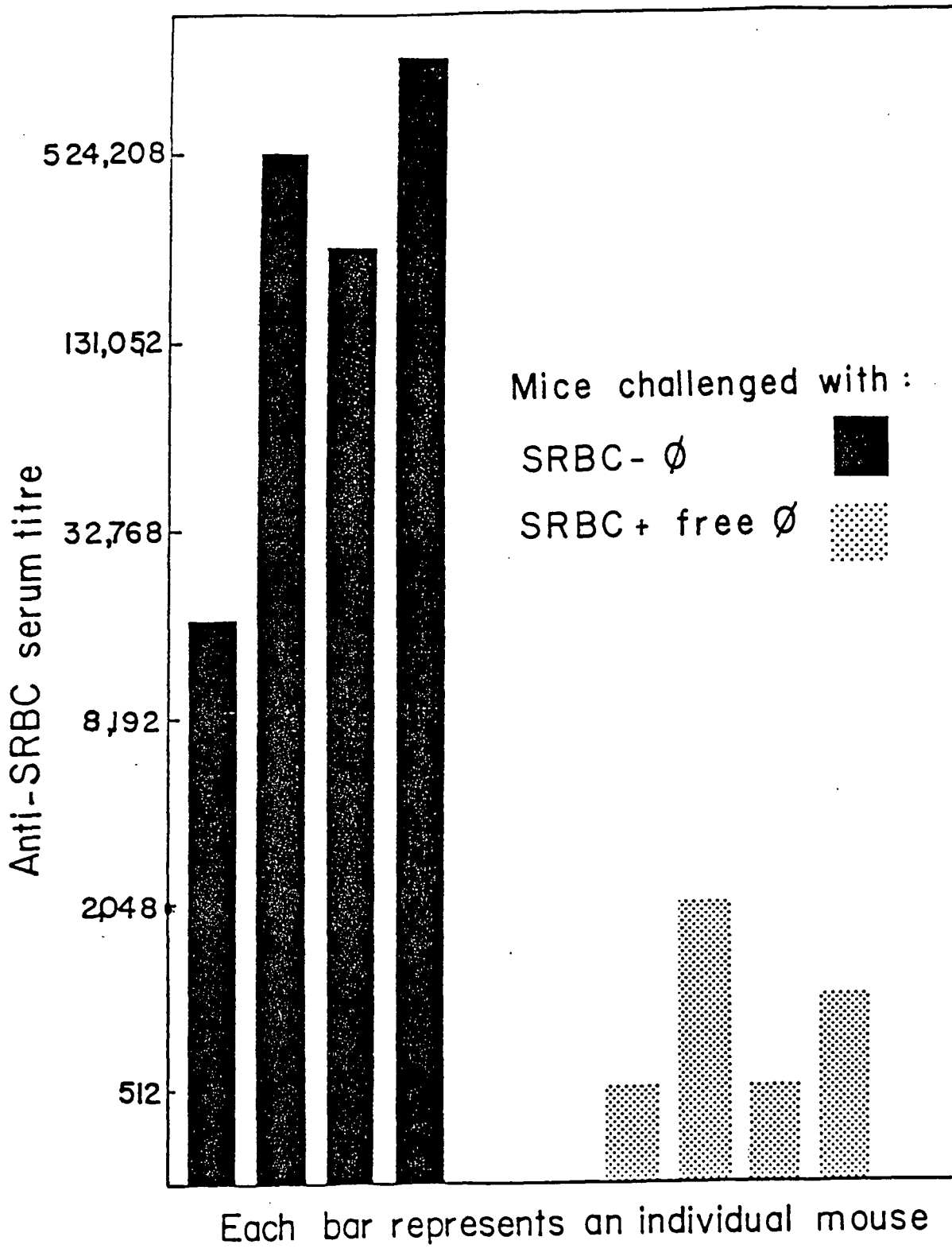


Figure 9 : Adoptive transfer experiment #2 to test for the in vivo helper activity of ØT4-primed spleen cells. See Figure 4 for a flow chart of this assay.



greater than mice injected with naive spleen cells or phage-primed, non-irradiated spleen cells. This experiment suggests that there were radiation sensitive suppressor cells in the primed spleen cell population. When treated with a dose of 1500 rads, which preferentially inactivates suppressor cells, the phage-primed spleen cells plus the SRBC-primed B cells reconstructed the strongest anti-SRBC response.

A second adoptive transfer experiment was performed (Fig.9 ). Two groups of 4 mice/group were given injections of SRBC-primed B cells plus phage-primed, irradiated spleen cells. This time, one group was challenged with SRBC-Ø, and the second group was challenged with SRBC and a separate injection of free ØT4. Mice challenged with SRBC-Ø had anti-SRBC serum titres up to 500 fold greater than the highest serum titre of the mice challenged with SRBC and free ØT4. This experiment clearly showed that attachment of ØT4 to the SRBC surface greatly increased the amount of help delivered by phage-primed spleen cells to B cells in the secondary response against SRBC.

B. Summary

Adoptive transfer of ØT4-primed, irradiated spleen cells and SRBC-primed B cells into irradiated host mice was done to test the in vivo helper ability of phage-primed T cells. ØT4-primed spleen cells had to be irradiated to eliminate suppression before transfer into the host animal. The results agreed with previous reports (Lachmann and Sikora, 1978; Mitchison, 1971) indicating that the helper determinant must be physically linked to the cell surface for enhancement of the anti-cellular response to occur. These experiments showed that ØT4 functions as a helper determinant in an in vivo anti-SRBC antibody response. A similar system of associative recognition may occur in an anti-tumor cellular response.

# CHAPTER IV. T HELPER CELL LINES AND CLONES

Instead of using primed spleen cells as a source of ØT4-reactive helper T cells, a ØT4-reactive cell line was established for use in immunotherapy experiments. The use of a T helper cell line could by-pass the problem of suppression that had been encountered with in vivo priming. A cell line, and clones derived from this line, would provide a consistant source of T helper cells that could be better characterized than primed spleen cells. Also, by first priming with ØT4 in vivo, and repeatedly stimulating primed cells in vitro with the same antigen, the in vitro cell line could be enriched for cells that react specifically to ØT4.

## A. Characteristics of the ØT4 reactive helper cell line,LN2

The T cell line used was established from ØT4-primed murine lymph node cells. (Materials, VI-A). The cell line was called LN2. I tested the cell line to check its antigen specificity and the effect of mAb GK1.5 + C on the LN2 cells.

### 1. Antigen specificity and mitogen responsiveness

The proliferative response of LN2 to various antigens and mitogens was measured using the proliferation assay (Materials, XII). I tested LN2 several times after the line was established. Table VI shows the results of an assay done one month after initiation of the line. LN2 proliferated in response to ØT4 and CON A but not to lipopolysaccharide (LPS) or keyhole lympet hemocyannin (KLH). The results indicated that LN2 was specific for the ØT4 antigen. The cell line proliferated 40- fold more in response to CON A, a T cell mitogen, than to LPS, a B cell mitogen, indicating that LN2 was predominantly T cells.

### 2. Effect of treatment of LN2 with mAb GK1.5 plus complement

Treatment of cells with complement-fixing antibody plus complement will kill only those cells that bind the specific antibody. I measured the percentage of LN2 cells that were killed after

Table VI : Antigen specificity and mitogen responsiveness of the T cell line, LN2.

| ANTIGEN or MITOGEN | µg/ml | $^{3}$H-TudR UPTAKE (cpm) | |
|---|---|---|---|
| ----- | ----- | 2,958 | (2,557) |
| ØT4 | 5 | 26,837 | (2,266) |
| KLH | 5 | 850 | (154) |
| CON A | 4 | 40,695 | (1,965) |
| LPS | 20 | 1,162 | (98) |

Cells were tested after 10 days in resting culture. $5x10^{4}$ viable cells were assayed for reactivity on a panel of antigens or mitogens in the presence of $5x10^{5}$ syngeneic irradiated (3,300 r) filler cells. Proliferative response was assayed on day 4. Values are the mean of triplicate cultures. Numbers in brackets represent the standard deviation from the mean.

Table VII: Treatment of LN2 cell line cells and mouse thymocytes with mAb GK1.5 plus complement.

| TREATMENT OF CELLS | % SPECIFIC LYSIS [a] | |
|---|---|---|
| | LN2 CELL LINE CELLS | D/2 THYMOCYTES |
| complement only | 0 | 14 |
| mAb GK1.5 + complement | | |
| dilution of GK1.5 supernatant: | | |
| undilute | 81 | 75 |
| 1/5 | 75 | 81 |
| 1/10 | 84 | 76 |
| 1/100 | 75 | 29 |
| 1/1000 | 7 | 13 |

[a] $\% \text{ SPECIFIC LYSIS} = \dfrac{\% \text{ dead cells (experimental)} - \% \text{ dead cells (control)}}{100\% - \% \text{ dead cells (control)}} \times 100$

treatment with mAb GK1.5 + C (Materials, V-D). The cells were tested 3 months after the line was established. D2 mouse thymocytes were tested as controls. GK1.5 is a monoclonal antibody specific for the L3T4a antigen found on T helper cells.

As can be seen in Table VII, 84% of the LN2 cells were lysed when the mAb GK1.5 supernatant was used at a concentration of 1:10. At a mAb supernatant concentration of 1:1000, the specific lysis dropped to 7%. This indicated that at least 84% of the LN2 cell line cells bear the L3T4a antigen, and are therefore helper T cells. The killing of D2 thymocytes decreased more sharply than the killing of LN2 with decreasing concentrations of mAb supernatant. This may indicate that normal mouse thymocytes have fewer copies of the L3T4a antigen on their cell surfaces than the LN2 cell line cells have, or that a lower percentage of thymocytes carry the L3T4a marker.

B. Characteristics of helper clones

Clones established from the helper T cell line by limiting dilution (Materials, VI-B), were tested for their proliferative responses to ØT4, and for their production of IL2. Table VIII shows the results from the proliferation assay measuring the proliferation of each of the clones 1-7 in response to three concentrations of ØT4. Clones 3, 4, 6, and 7 had well defined responses to ØT4, clone 4 having the largest incorporation of $^{3}$H-TudR over the 8 hr pulse period. Clone 1 had a consistantly high level of proliferation that did not decrease as the phage concentration decreased. Clone 2 did not respond to ØT4 and died shortly after this experiment.Proliferation of clone 5 appeared to be slightly inhibited by addition of ØT4 to the medium. It was interesting to note the physical appearance of the clones before and after stimulation with ØT4. Before stimulation, and after the clones had been resting for seven days, the cells were dull and ragged-looking, with approximately 30% of dead cells in the culture. After the four-day stimulation period, the cells were highly refractory with sharply defined cell walls. There were very few dead cells after stimulation, and pseudopodia were visible on many of the cells.

Each clone was also assayed for its production of IL2 after ØT4 stimulation (Table IX). The clones were set up in culture with 5µg/ml ØT4 in complete RPMI without addition of IL2.

Table VIII : Proliferation of T cell clones in response to ØT4: incorporation of $^{3}H$-TudR (cpm).

| | ØT4 ADDED TO CULTURES (µg/ml) | | | |
|---|---|---|---|---|
| CLONE | ---- | 10 | 2 | 0.4 |
| 1 | 15,810 | 9,084 | 24,741 | 18,665 |
| 2 | 129 | 605 | 181 | 104 |
| 3 | 228 | 4,997 | 362 | 86 |
| 4 | 204 | 27,508 | 6,022 | 490 |
| 5 | 2,477 | 1,304 | 1,117 | 1,237 |
| 6 | 112 | 3,105 | 322 | 90 |
| 7 | 129 | 3,470 | 5,041 | 238 |

$1x10^{4}$ cloned T cells were cultured with $5x10^{5}$ syngeneic irradiated spleen cells in 0.2 ml complete RPMI + 1% EL4-IL2, plus various concentrations of ØT4. At 96 hr after initiation of culture, wells were pulsed for 8 hr with 1 µCi/well $^{3}H$-TudR, harvested, and counted on a scintillation counter. Values are the mean of triplicate cultures. All values for standard deviations are less than 10%.

Table IX : Interleukin 2 production by T cell clones

| | 48 HOUR CULTURE SUPERNATANT | | | | 72 HOUR CULTURE SUPERNATANT | | | |
|---|---|---|---|---|---|---|---|---|
| | DILUTION OF CULTURE SUPERNATANT | | | | | | | |
| CLONE | 50% | 25% | 12.5% | 6% | 50% | 25% | 12.5% | 6% |
| 1 | 7,302 | 3,280 | 914 | 21 | 248 | 69 | 76 | 28 |
| 2 | 496 | 196 | 143 | 78 | 169 | 74 | 61 | 37 |
| 3 | 1,368 | 320 | 131 | 45 | 139 | 84 | 80 | 39 |
| 4 | 31,123 | 19,134 | 7,208 | 3,417 | 5,843 | 1,182 | 331 | 81 |
| 5 | 4,329 | 1,285 | 380 | 113 | 286 | 94 | 118 | 87 |
| 6 | 5,903 | 1,497 | 511 | 178 | 1,493 | 309 | 92 | 36 |
| 7 | 4,134 | 1,438 | 505 | 109 | 1,123 | 225 | 54 | 35 |

Values are $^{3}$H-TudR incorporated by the IL-2 dependent cell line, CTLL. Supernatants from the T cell clones were sampled at 48 hr and 72 hr after culture. 0.1 ml of each supernatant was added to 0.1 ml of CTLL cells for a cell concentration of $5x10^{4}$/well. Medium used was complete RPMI + glutamine + pyruvate. $5x10^{6}$ irradiated (3,300 rad) syngeneic filler cells were added per well. Control wells were set up with 2% E14-IL2, or no IL2 added to the CTLL cultures. After 24 hr, 1µCi/well $^{3}$H-TudR was added. Cells were harvested 18 hr later, and counted in a scintillation counter. Results are the mean of triplicate cultures. Standard deviations of the mean are less than 10% in all cases.

Control values (cpm): 2% EL4-IL2 added to the medium------50,480
no IL2 added to the medium---------- 61

Supernatant samples from the cultures were taken at 48, 72, and 96 hours after initiation of the cultures, and stored at 4°C. The supernatants from the clones were then assayed for IL2 content by checking their ability to sustain growth of the IL2-dependent cell line, CTLL, as described in Table IX. Wells with supernatants added were compared to control wells with 2% EL4-IL2 or no IL2 added to the medium.

All of the clones produced some IL2, although clone 2 produced a very small amount. Clone 4 produced considerably more IL2 than any other clone. It is interesting to note that clone 4 had the greatest response to ØT4 and produced the most IL2. In comparison, clone 3 had a much lower proliferative response to ØT4 and produced little IL2. The amount of IL2 present in the clone supernatants decreased significantly at each subsequent 24-hr sample timepoint. By 96 hr, all values for cpm incorporation were less than 500 cpm. The decrease in IL2 in the proliferating cultures was because the clones used the IL2 that they produced, for their own proliferation. This production and use of IL2 by helper T cells is an interesting example of an autocrine system.

C. Summary

Characterization of the cell line, LN2, proved it to be specifically reactive to the antigen, ØT4, and to the mitogen, CON A. At least 84% of the LN2 cells were specifically killed with mAb GK1.5 + C. These results indicate that LN2 is a ØT4-specific, T helper cell line.

The seven clones derived from LN2 were compared for their proliferative responses to ØT4 and their production of IL2 upon stimulation with ØT4. The responses of the individual clones differed markedly indicating the heterogenous nature of the parent cell line LN2.

## CHAPTER V. IN VITRO T CELL HELP

The next set of experiments were designed to investigate whether ØT4-reactive helper cells could help in the generation of cytotoxic T cells to P815 tumor cells in vitro. The assay system is described in Materials, XIII, and in Figs. 2 and 3.

### A. In vitro priming of a T helper cell population

Initially, spleen cells taken from ØT4-primed mice were used as the helper cells in this assay. Figs. 10 and 11 depict the results of two such experiments. In both experiments, the effector cells that were stimulated with P815r-Ø had higher cytotoxicity against P815 targets than did effector cells stimulated with P815r alone. In Fig. 10, the addition of ØT4-primed, irradiated spleen cells enhanced cytotoxicity more than addition of naive, irradiated spleen cells. In the repeat of this experiment, Fig. 11, no significant difference in cytotoxicity resulted when ØT4-primed, irradiated spleen cells were added compared to addition of naive, irradiated spleen cells. As in Fig. 10, the main difference was between groups of cells stimulated with P815r and P815r-Ø, stimulation with P815r-Ø causing a much higher degree of cytotoxicity.

Taken together, the results from the two experiments suggest that in vitro priming of an endogenous population of helper T cells was occurring in response to stimulation with ØT4 linked to the P815r cell surface. This would explain why, no matter whether primed or naive helper cells were added to the cultures, cytotoxicity was enhanced by stimulation of cultures with P815r-Ø, wheras no enhancement resulted upon stimulation with P815r alone.

### B. Use of mAb GK1.5 to eliminate in vitro priming

To check for in vitro priming of helper T cells, the cytotoxicity assay was set up essentially as before, except that the spleen cells comprising the responder population were treated with mAb GK1.5 + C before they were set up in the assay (Materials, V-D). This treatment was intended to

Figure 10. Cytotoxicity against P815 target cells generated in cultures of normal spleen cells stimulated with P815r (●) or P815r-Ø(■). $10^5$ or $10^6$ ØT4-primed (——) or naive (- - -) irradiated spleen cells were also added to cultures. After 5 days in culture, cells were harvested and set up with P815 targets in a 12-hour $^{51}Cr$-release assay with P815 targets.

panel a. $10^5$ irradiated spleen cells added to cultures
panel b. $10^6$ irradiated spleen cells added to cultures

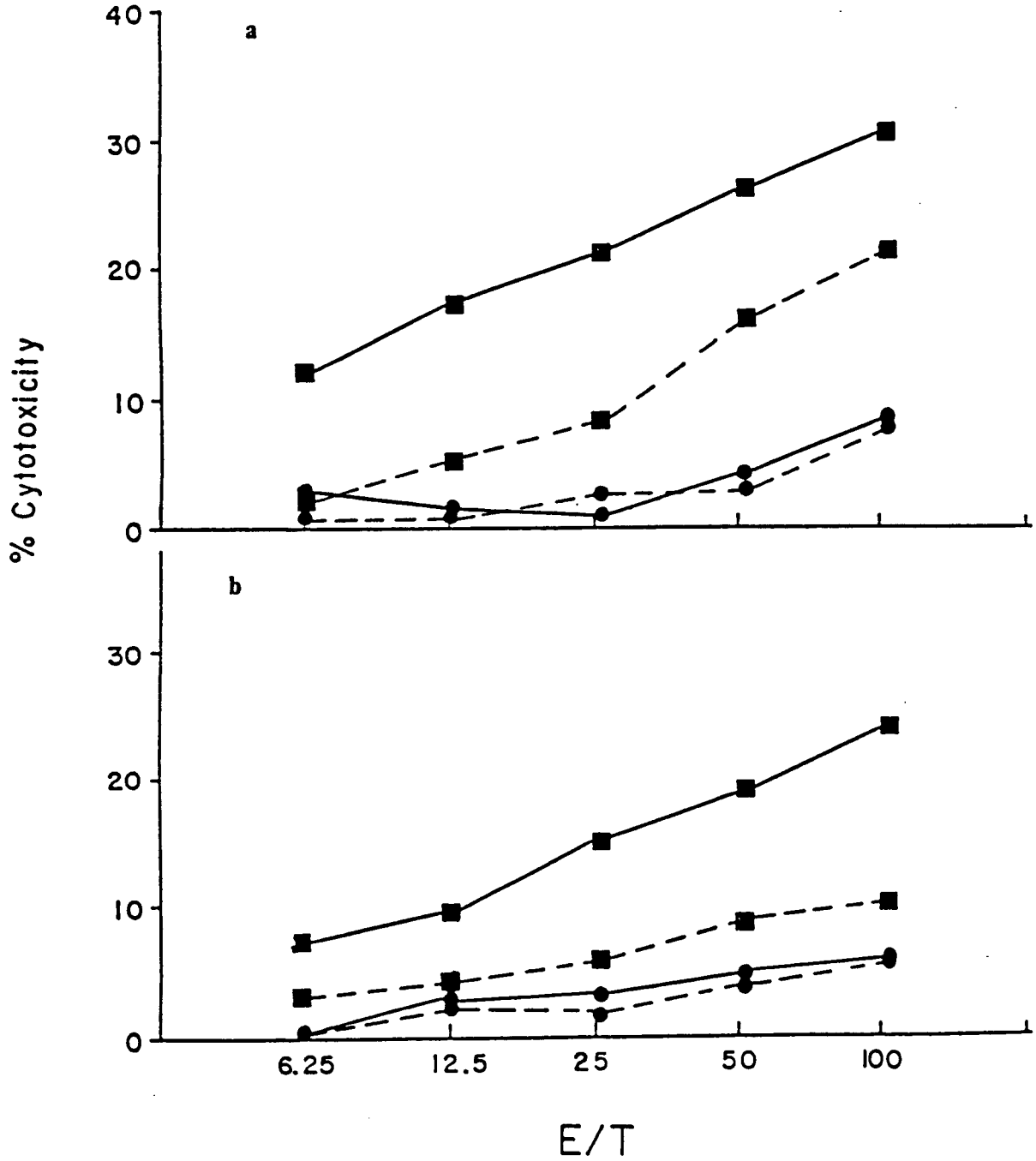
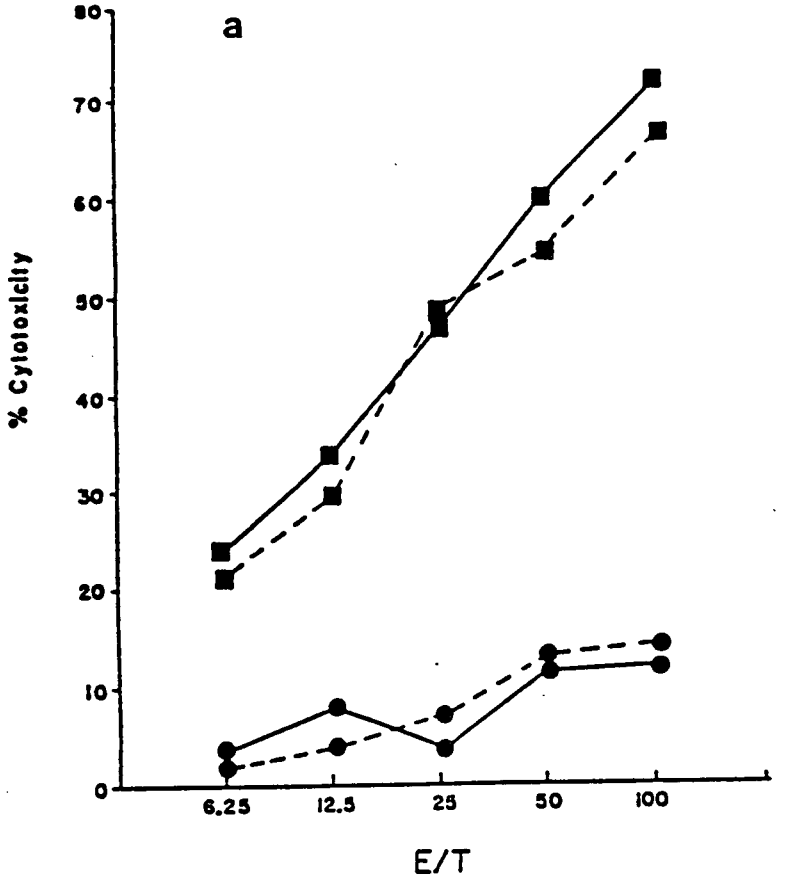


Figure 11. Cytotoxicity against P815 targets generated in cultures of normal spleen cells stimulated with irradiated P815 (●), or P815r linked to ØT4 (P815r-Ø) (■). ØT4 primed (——) or naive (- - -) irradiated spleen cells were also added to the cultures. After 5 days in culture, cells were harvested and set up in a 12-hour $^{51}Cr$-release assay with P815 targets.

panel a. $2.5 \times 10^5$ irradiated spleen cells added to cultures
panel b. $2.5 \times 10^4$ irradiated spleen cells added to cultures


a
% Cytotoxicity
80
70
60
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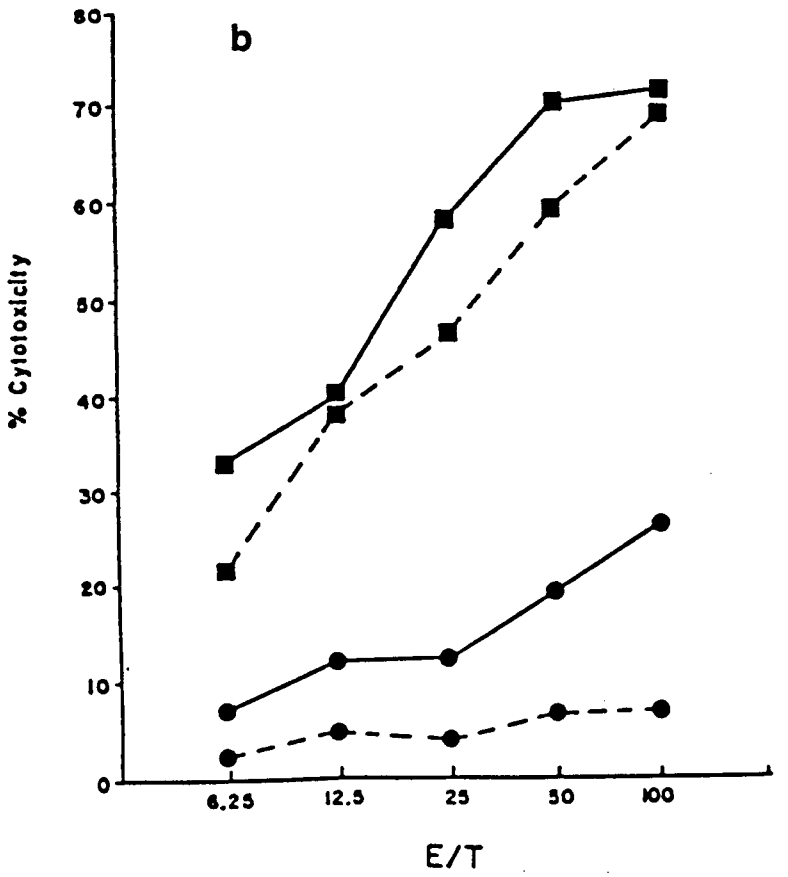
30
20
10
0
6.25
12.5
25
50
100
E/T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% Cytotoxicity
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6.25
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100
E/T

eliminate endogenous helper T cells in the responder cell population. The T helper cell line, LN2, instead of ØT4-primed, irradiated spleen cells was used as the helper population.

The results of this experiment are shown in Fig. 12. When $1x10^4$ LN2 cells were added to cultures stimulated with P815r-Ø, a dramatic increase in cytotoxicity occurred, compared to cultures with added LN2 cells but stimulated with P815r. Stimulation with P815r-Ø without addition of LN2 cells had no effect. The dashed lines indicate groups in which the responders were treated only with complement without mAb GK1.5. As seen in the previous experiments, these groups had enhanced cytotoxicity both when stimulated with P815r-Ø or with P815r. This experiment showed that the ØT4-reactive helper T cell line, LN2, was effective in helping the cytotoxic response only when the responder cells were stimulated with P815r-Ø. Elimination of endogenous helper cells by treatment of the responder population with mAb GK1.5 + C was essential to detect this helper activity.

Fig. 13 depicts a repeat of the previous experiment, with the addition of one extra test group. In this experiment, mAb GK1.5 + C treated responder cells were cultured with either P815r, P815r-Ø, or P815r plus free ØT4, with $1x10^4$ LN2 helper cells added per well. The results indicate that stimulation with P815r-Ø or P815r plus free ØT4, conjunct to LN2 cell help, enhanced <u>in vitro</u> cytotoxicity against P815. This result seemed to contradict the doctrine that helper determinants must be physically linked to the cell in order for associative recognition of helper and cell antigens to occur. However, since this was an <u>in vitro</u> experiment, the enhancement that occurred with linked or unlinked P815r and ØT4 might be explained as follows:

One reason that linked helper determinants boost anti-tumor CTL responses might be that the linked antigenic determinant on the tumor cell surface focuses helper T cells, via the specific antigen receptors on the T cells, to the area near the tumor cells where anti-tumor pre-CTLs are activated by tumor antigens. The actual requirement for linkage of helper determinant and tumor may exist so that antigen-presenting cells can process both helper antigens and tumor antigens as one unit, present them as one unit on the presenting cell surface, and thereby localize the cells involved in the cooperative anti-tumor response. Since in <u>in vitro</u> experiments, the helper cells

Figure 12. Effect of addition of ØT4-specific T helper cell line cells to cultures of spleen cells, on the generation of cytotoxicity to P815 target cells. Responder spleen cells were treated with Mab GK1.5 + C (——) or C only (- - - -) before being cultured, and were stimulated with P815r (●) or P815r-Ø (■) for the duration of the 5 day culture period. $10^4$ T helper cell line cells (LN2) were added to cultures where indicted (↙). The LN2 cell line was rested in culture for 9 days without addition of ØT4 before use in this assay. The set-up for this experiment is illustrated in Figure 3.

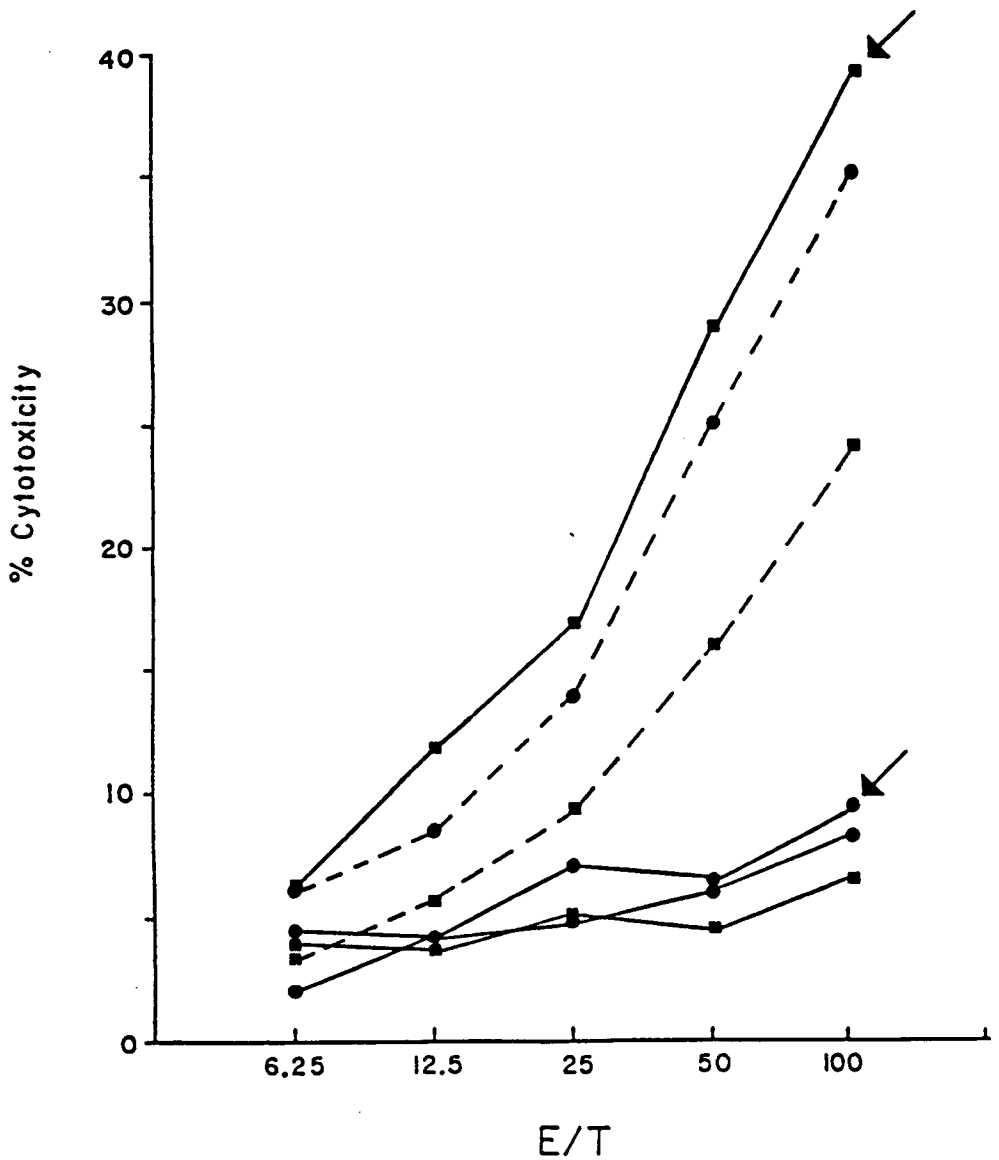
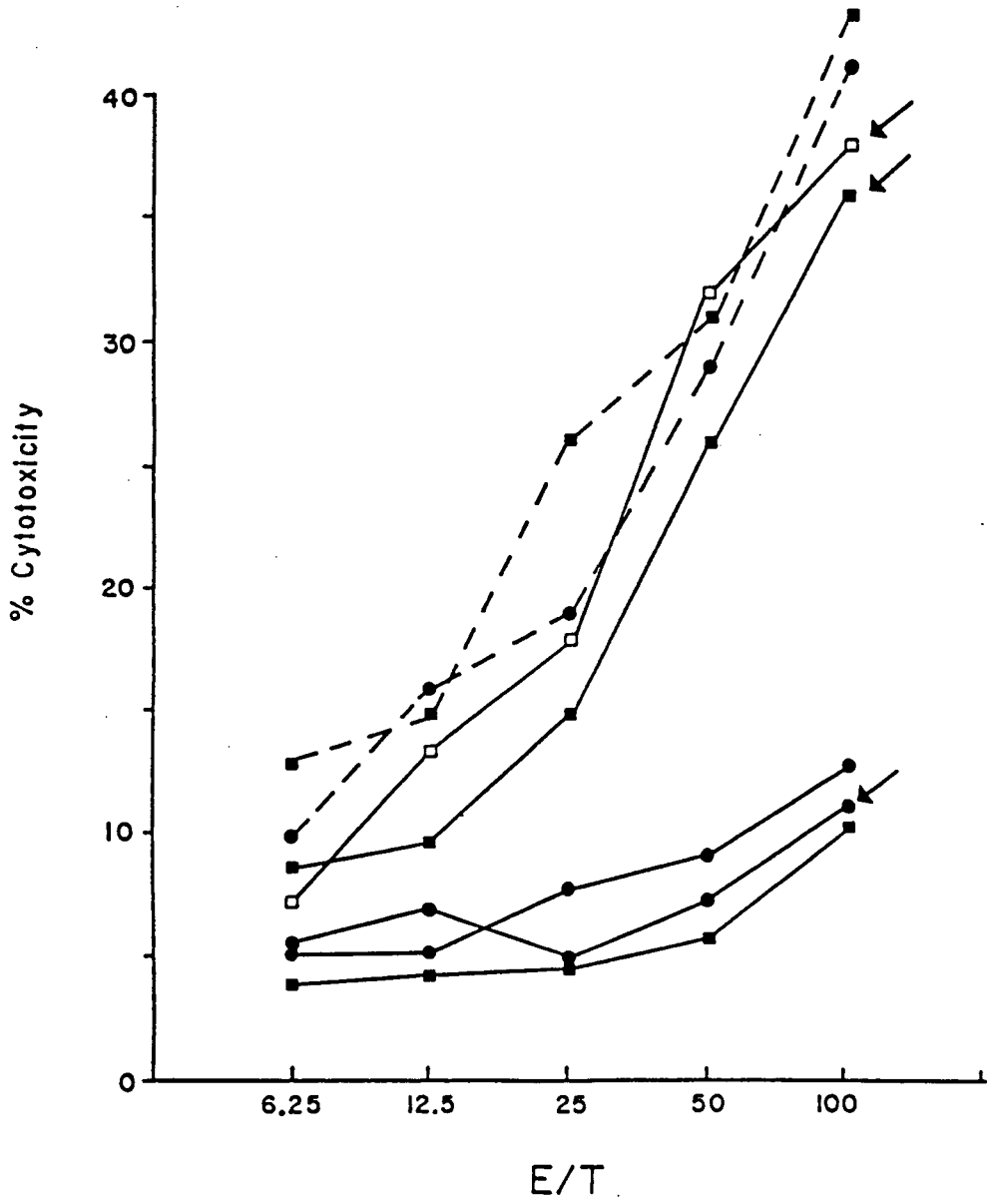


Figure 13. Effect of addition of ØT4-specific T helper cell line cells to cultures of spleen cells, on the generation of cytotoxicity to P815 target cells. Responder spleen cells were treated with Mab GK1.5 + C (——) or C only (- - - -) before being cultured, and were stimulated with P815r ( ● ), P815r-Ø ( ■ ), or P815r plus free Ø ( □ ) for the duration of the 5 day culture period. $10^4$ T helper cell line cells (LN2) were added to cultures where indicated ( ↙ ). The LN2 cell line was rested in culture for 9 days without addition of ØT4 before use in this assay.



and responder cells are already in close proximity, the linkage of helper antigen to the tumor cell surface may not be necessary. This may be one explanation for the in vitro increase in cytotoxicity observed when responders are stimulated with either linked or unlinked P815r and ØT4.

There is at least one other possible explanation for this result. When LN2 is stimulated by ØT4 that is either linked or unlinked to P815r, the cell line produces IL2. In experiments not included here, I have shown that stimulation of responder cells with P815r and addition of 1% EL4-IL2 supernatant to cultures of responder cells will cause equivalent enhancement of cytotoxicity to that seen when responder cells are cultured with P815r-Ø and LN2. Therefore, even if antigen specific helper factors are produced by LN2, their effects may be hidden by non-specific stimulation of cytotoxicity by IL2, produced by the helper cell line in reponse to ØT4 . If the helper factor could be isolated , a specific response due to the helper factor might be studied independent of the effects of IL2. We are presently testing two of the helper cell clones in the helper assay; clone 3, that produces little IL2, and clone 4, that produces substantial amounts of IL2. These experiments may be helpful in determining the role of antigen specific and non specific help in this assay.

C. Summary

A dramatic increase in cytotoxicity against P815 tumor cells was achieved by addition of the helper T cell line, LN2, to cultures of mAb GK1.5 + C treated spleen cells stimulated with P815r-Ø. Addition of LN2 concomitant to stimulation with P815r and inclusion of free ØT4 in the culture medium also enhanced cytotoxicity, whereas stimulation with P815r alone and addition of LN2 was not effective in augmenting the CTL response. To detect exogenous helper T cell activity, it was necessary to eliminate endogenous helper cells in the responder cell population by treatment of responder cells with mAb GK1.5 + C, which specifically kills helper T cells.

## CHAPTER VI. IN VIVO IMMUNOTHERAPY OF P815 TUMORS

The final stage in this project involved testing the T helper cell line and clones in tumor immunotherapy models *in vivo*. The protocol for *in vivo* immunotherapy experiments is described in Materials, XV and in Fig. 17.

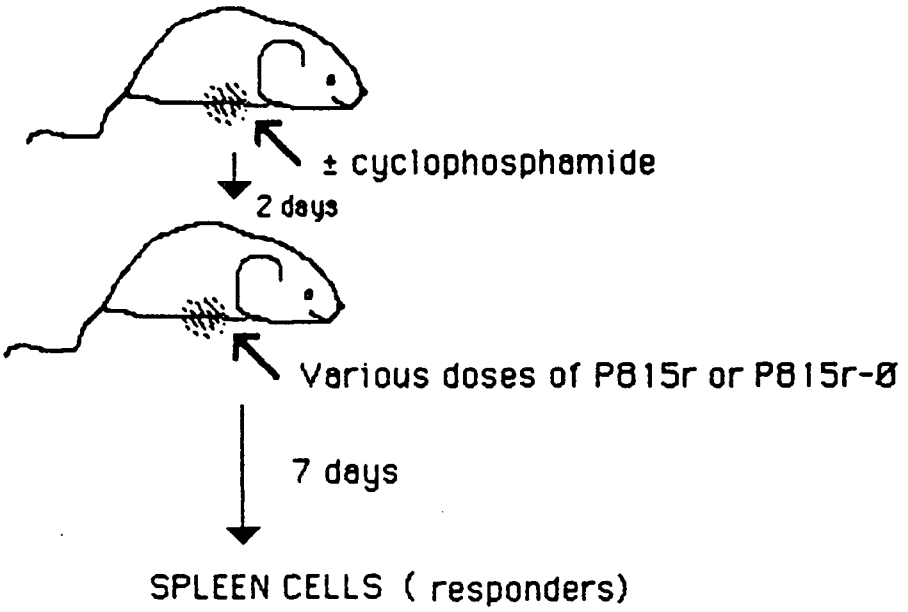
### A. Suppression of anti-tumor responses by injection of P815r into mice

Initially, *in vivo* experiments were performed without pre-treatment of the test mice with cyclophosphamide. In those experiments, mice that were not given injections of either helper cells or irradiated tumor cells survived significantly longer than the test mice that received immunizing shots of helper cells and irradiated tumor cells. In light of previous reports of cell-mediated suppression which occurs in mice bearing P815 tumors (Takei *et al.*, 1976; Takei *et al.*, 1977; North *et al.*, 1982) we suspected that suppression was occurring in our experiments due to the "immunizing" injections of irradiated tumor cells. Figs. 15 and 16 depict the results of experiments designed to investigate what effect injections of P815r had on the generation of cytotoxicity in mice. Fig. 14 is a flow chart of the design of these experiments.

In the first experiment (Fig. 15) mice were given i.p. injections of P815r or P815r-Ø. The spleen cells from these mice were removed one week later, stimulated *in vitro* with P815r or P815r-Ø, and then assayed in a cytotoxicity assay to test the ability of the spleen cells to mount a cytotoxic response against P815. Four different combinations of P815r and P815r-Ø were used for injection and in the *in vitro* stimulation, as described in Fig. 15.

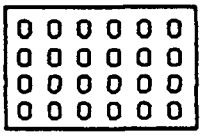
The most striking result in this experiment was that spleen cells from control mice that were not injected with either P815r or P815r-Ø had consistantly higher cytotoxic responses against P815 targets than mice receiving the injections. This was clear evidence that injection of P815r caused suppression of anti-P815 cytotoxicity in the animal. It was interesting to note that when

Figure 14 : Assay for the effect of injection of P815r or P815r-Ø into mice on the cytotoxic response of spleen cells against P815 targets.




SPLEEN CELLS ( responders)

+ P815r or P815r-Ø (stimulators)



5 days

HARVEST CELLS

Set up with $^{51}$Cr-labelled P815 targets

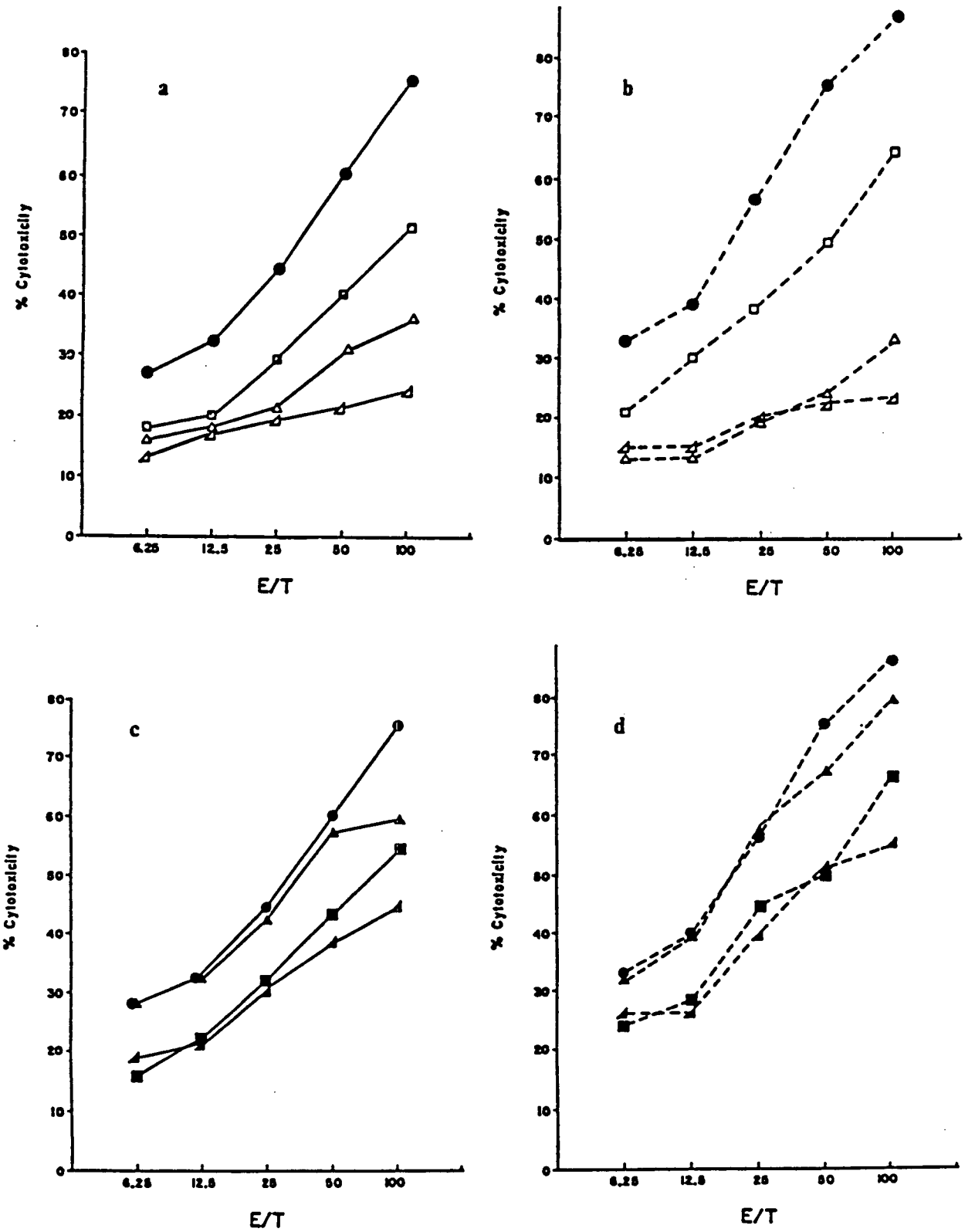
12 hours

Count $^{51}$Cr release from P815 targets

$$\% \text{ Cytotoxicity} = \frac{\text{test } ^{51}\text{Cr release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

Figure 15. Effect of injection of mice with $10^6$ (◿), $10^5$ (□), or $10^4$ (△) P815r or $10^6$ (◢), $10^5$ (■), or $10^4$ (▲) P815r-Ø on the generation of cytotoxicity to P815 tumor cell targets. Mice were injected with various doses of P815r or P815r-Ø. One week later, $5x10^6$ spleen cells from immunized mice were cultured with $5x10^5$ P815r (——) or P815r-Ø (– – – –) for 5 days, harvested, and set up in a 12-hour $^{51}Cr$-release assay with P815 targets. Non-immunized control cultures are indicated (●). See Figure 14 for a flow chart of this experiment.

| Panel | Injection in vivo | Stimulation in vitro |
|---|---|---|
| a | P815r | P815r |
| b | P815r | P815r-Ø |
| c | P815r-Ø | P815r |
| d | P815r-Ø | P815r-Ø |


a
b
c
d
% Cytotoxicity
E/T
6.25
12.5
25
50
100
0
10
20
30
40
50
60
70
80

mice were injected with P815r, the least suppressive dose was $1x10^5$ cells/mouse. Injections of lower ( $10^4$) and higher ( $10^6$) numbers of P815r cells resulted in reduced killing of the tumor cell targets. However, the suppression due to injections with P815r-Ø was the least at the smallest dose of cells ( $10^4$ cells/mouse) and increased with injections of increasing numbers of P815r-Ø. The reason for the difference in optimal dosage for injection of P815r and P815r-Ø is not clear. It may be that when P815r was injected, high or low zone tolerance occurred, and when P815r-Ø was injected, the ØT4 somehow enhanced suppression of the CTL response.

B. Effects of treatment of mice with cyclophosphamide

In the next experiment, we tested the effect of pretreating mice with cyclophosphamide on the cytotoxic response of spleen cells taken from the mice after injection with P815r or P815r-Ø (Fig.16).

The dose of cyclophosphamide used in this experiment ( 20 mg/kg/mouse) was based on previous work done in this laboratory ( Hancock, 1983.) She found that up to 50 mg/kg/mouse of cyclophosphamide enhanced the cytotoxic response to P815 tumor antigens, probably due to removal of cyclophosphamide-sensitive suppressor cells. Higher doses of cyclophosphamide ( 75-200 mg/kg) reduced or eliminated the anti-tumor response. I had also tested spleen cells from mice injected with 75 mg/kg/mouse cyclophosphamide in a proliferation assay for response to P815r, and found that this dose almost completely obliterated the proliferative response. Therefore, a low dose of 20 mg/kg/mouse was chosen for my experiments.

Mice were injected i.p. with cyclophosphamide two days before receiving injections of P815r or P815r-Ø. The rest of the experiment was as described for Fig. 15. In contrast to the results seen in Fig. 15, injection of cyclophosphamide not only removed a significant amount of the suppression in the animals, but also dramatically decreased the basal response of CTLs from mice that received injections of cyclophosphamide only and no in vivo shots of P815r or P815r-Ø. Naive mice in Fig. 15 that were not treated with cyclophosphamide had a healthy anti-P815 response when stimulated in vitro with P815r.

However, it is apparent that treatment with cyclophosphamide at the dose of 20 mg/kg/mouse did not completely obliterate T cells in mice, since in this experiment injection with P815r or P815r-Ø after cyclophosphamide treatment significantly enhanced the in vitro killing of P815 targets by CTLs.

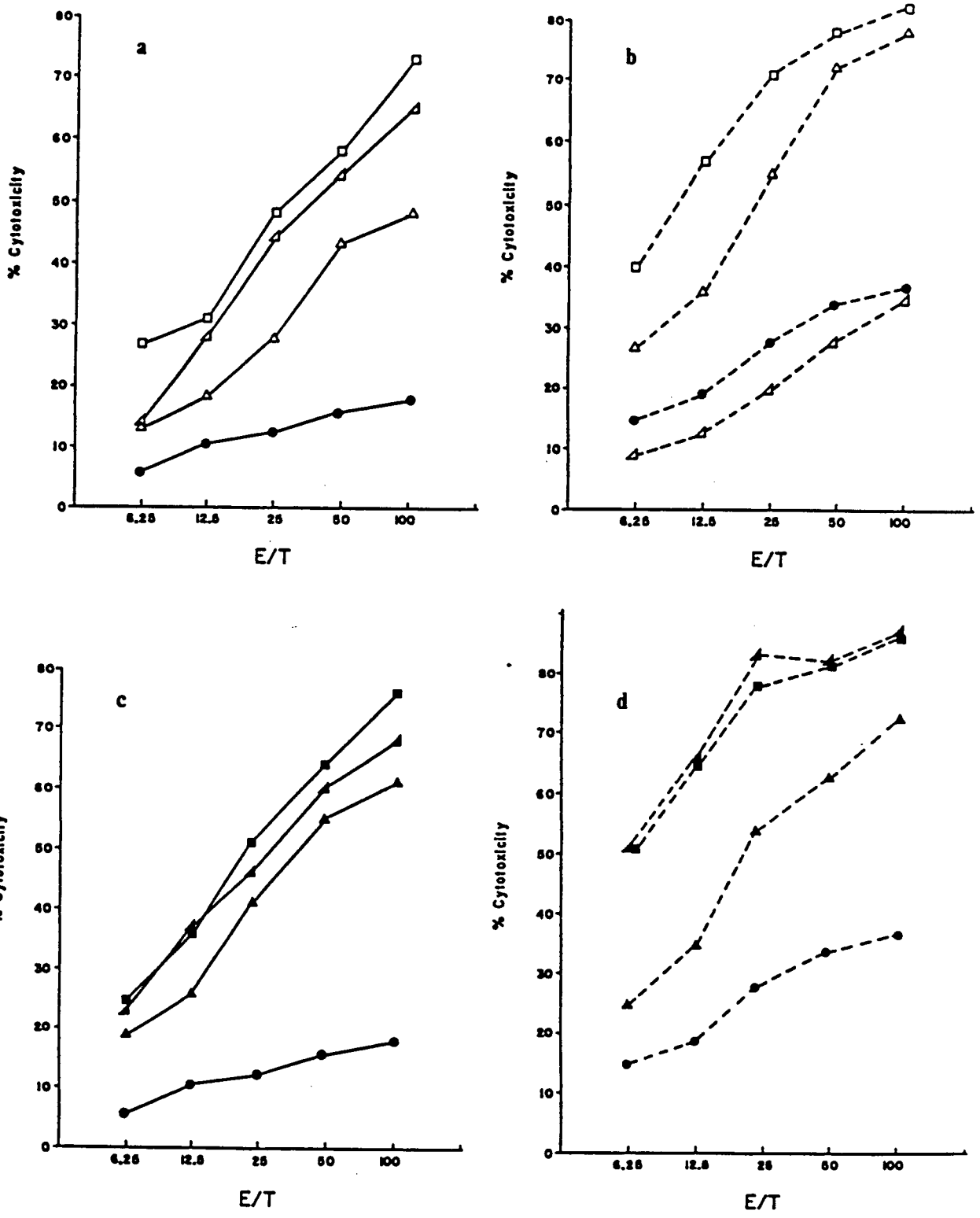
The results of the four experiments in panels (a) to (d) in Fig. 16 were similar in that injection of $1x10^5$ P815r or P815r-Ø was the best dose for priming anti-tumor CTLs. In addition, in all cases injection of either P815r or P815r-Ø increased the cytotoxicity above that level seen when only cyclophosphamide was injected, with an interesting exception in panel (b). In panel (b), priming with $10^6$ P815r, and then stimulation in vitro with P815r-Ø, caused dramatic suppression of the CTL response. Comparison of this exception with the results of similar experimental groups in panels (a) and (d) may provide clues as to why the suppression in panel (b) occurred.

In experiments (a) and (b), all mice were injected in vivo with P815r, but the in vitro stimulation of cells in the two groups was different. In panel (a), mice were stimulated in vitro with P815r, whereas in panel (b) mice were stimulated in vitro with P815r-Ø. One explanation for the low response that resulted when $10^6$ P815r were injected and then stimulation was with P815r-Ø may be that the high dose of P815r primed for suppressor cells in vivo, and that subsequent stimulation in vitro with P815r-Ø activated suppressor-inducer cells that further increased suppression of the CTL response.

If we next compare the experiments shown in panels (b) and (d), it can be seen that in both experiments cells were stimulated in vitro with P815r-Ø, but the difference was in the in vivo injections. In panel (d), mice were injected with different numbers of P815r-Ø, whereas in panel (b) P815r was injected. The results show that in vivo injection with $10^6$ P815r-Ø did not appear to induce equivalent suppression to that caused by injection of $10^6$ P815r. Maybe the ØT4 on the P815 cell surface primes T helper cells in vivo that counter-balance the suppressor cells reactive to the tumor cell antigens. Alternatively, linkage of ØT4 to the P815 cell may mask the tumor antigens that cause suppression at high cell doses.

Figure 16: Effect of cyclophosphamide treatment and injection of mice with P815r or P815r-Ø on the generation of cytotoxicity to P815 tumor cell targets. Mice were injected on day -2 with 20 mg/kg/mouse cyclophosphamide. On day 0, mice were injected with $10^6$ (◿), $10^5$ (□), or $10^4$ (△) P815r, or $10^6$ (◢), $10^5$ (■), or $10^4$ (▲) P815r-Ø. One week later, $5x10^6$ spleen cells from immunized mice were cultured with $5x10^5$ P815r (——) or P815r-Ø (- - - -) for 5 days, harvested, and set up in a $^{51}Cr$-release assay with P815 targets. Non-immunized control cultures are indicated (●). See Figure 14 for a flow chart of this experiment.

| Panel | Injection in vivo | Stimulation in vitro |
|---|---|---|
| a | P815r | P815r |
| b | P815r | P815r-Ø |
| c | P815r-Ø | P815r |
| d | P815r-Ø | P815r-Ø |


a
b
c
d
% Cytotoxicity
E/T
6.25
12.5
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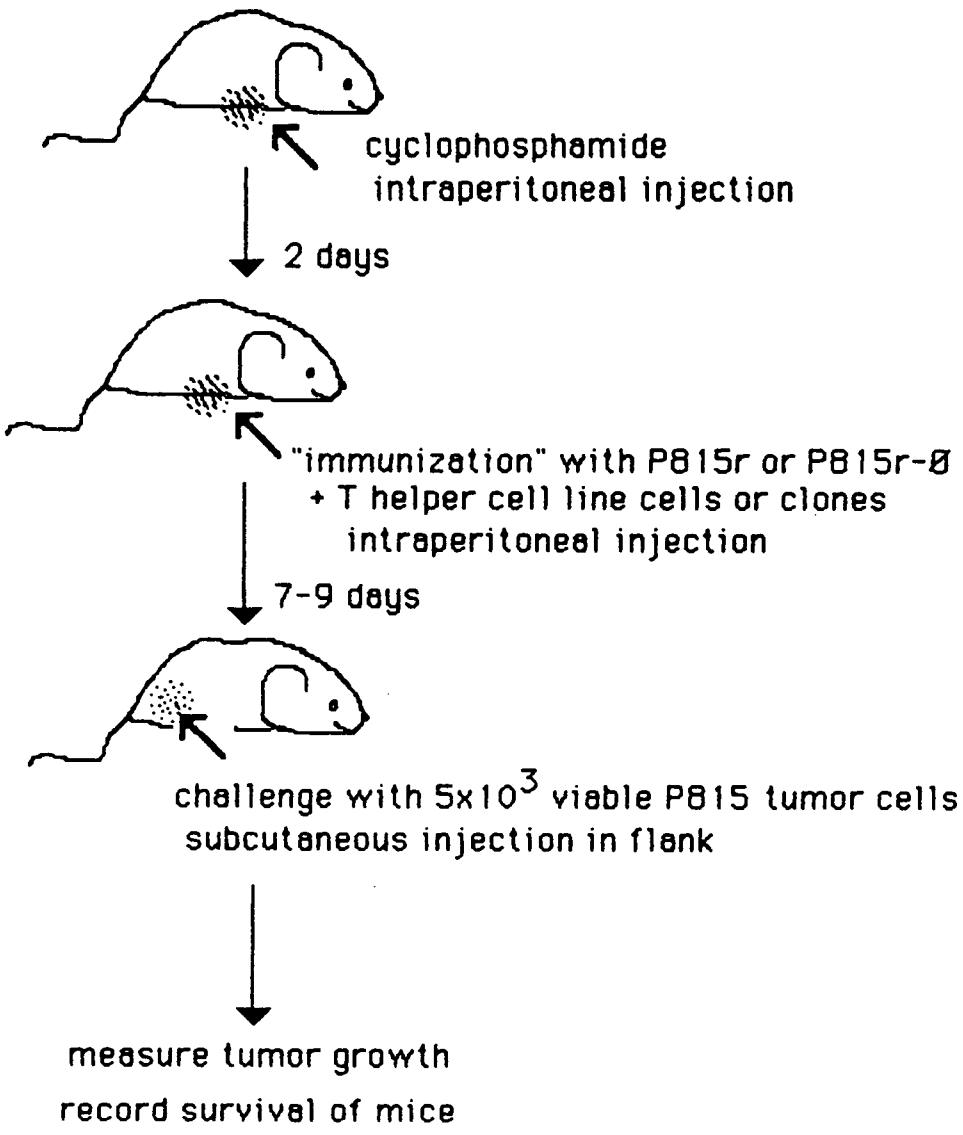
80

Figure 17 : Protocol for in vivo immunotherapy for P815 tumors.



Over all, with the exception of the experiment in panel (b), the results from these experiments demonstrated that a dose of $10^5$ P815r or P815r-Ø could prime CTLs directed to tumor cell antigens. Pretreatment of mice with cyclophosphamide was necessary to reduce suppression caused by injection of the irradiated tumor cells. The cytotoxic responses were higher when the primed cells were stimulated in vitro with P815r-Ø, than when stimulated with P815r. This same effect was seen earlier in the in vitro experiments in Chapter V, where in vitro stimulation with P815r-Ø primed endogenous ØT4-reactive helper cells that enhanced the CTL response against tumors.

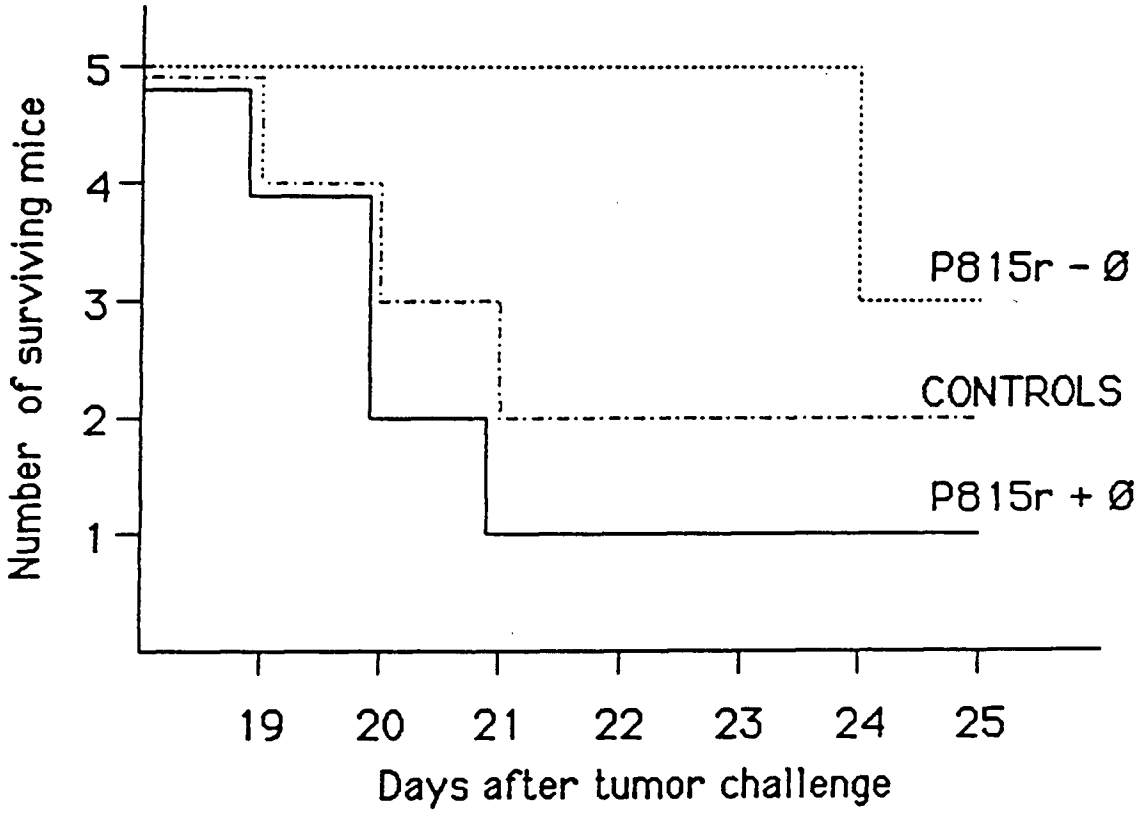
The experiment in panel (b) that did not follow the pattern seen in the other experiments indicates that the dose of cells used for immunization against tumors is critical, and that relatively small changes in dose can cause dramatic changes in the cellular response by upsetting the balance between help and suppression.

C. Immunotherapy experiments

The final, and ongoing stage of this work was to test the the use of ØT4-reactive helper cells in conjunction with injections of ØT4-linked P815r tumor cells and cyclophosphamide treatment for in vivo tumor immunotherapy.

The immunotherapy protocol was patterned after the model of Sia et al. (1984). The core of the procedure for the immunotherapy experiments is described in Materials, XV, and Fig. 17.
The first glimpse of success, after several unsuccessful immunotherapy trials without pretreatment of the mice with cyclophosphamide, was seen in the experimental results shown in Fig. 18. In this experiment, 5 mice/group were given injections of 20 mg/kg/mouse cyclophosphamide, followed 2 days later by $10^7$ LN2 cell line cells plus either P815r-Ø , P815r and a separate injection of free ØT4, or no injection (control group). One week after the injection of the cells, the mice were challenged with $3x10^3$ viable P815 tumor cells injected subcutaneously on a hind flank. After the tumor challenge, the mice were checked daily for survival and growth of the tumor.

Figure 18 : Survival of mice after receiving immunotherapy against P815 tumor. All mice were injected with 20 mg/kg body weight of cyclophosphsmide, and 2 days later were injected with LN2 cell line cells plus either P815r- Ø ( ····· ), P815r + free Ø (———— ), or PBS only (controls) ( -.-.-.-.- ). One week later, mice were challenged with viable P815 tumor cells, and their survival was recorded.



As shown in Fig. 18, the first mice to die in the control group, and in the group receiving unlinked ØT4, died on day 19 after the tumor challenge. The first mouse to die in the group receiving shots of P815r-Ø did not die until 5 days later, that is, 24 days after tumor challenge. After the first death in each group, however, mice in all groups began to die daily and all were dead by day 30.

The tumor size did not vary significantly among the various groups. Tumors were generally palpable by day 9 after challenge, and grew quickly therafter, reaching a size of approximately 15-20 $mm^2$ by the time the mouse was dead. Dissection of the dead mice revealed extensive metastases of tumor cells to the liver.

A repeat of this experiment did not reveal any enhancement of survival in any of the three groups of mice. Because the cell line consisted of a mixture of different cell types, which may have included helper cells for suppressor cells (suppressor-inducers) as well as helpers for CTLs, we decided to concentrate our research efforts on the clones that had been established from the cell line.

Three of the clones, clones 1, 3, and 4, were tested next in the immunotherapy model. This time, the therapy routine consisted of injection of 20mg/kg/mouse of cyclophosphamide on day -2, followed by injections of each of the clones plus either P815r, P815r-Ø, or PBS (controls) on days 0 and 9. Numbers of the clones injected varied: $3x10^6$ cells/mouse (clone 4), $6x10^6$ cells/mouse (clone 3), and $1x10^7$ cells/mouse (clone 1). On day 11, the mice were challenged with $5x10^3$ viable P815 tumor cells. The results from this experiment are shown in Fig. 19.

Sixty percent of the control mice that received only PBS injections after the cyclophosphamide treatment were dead by day 31 after tumor challenge. The group of mice receiving clone 3 plus P815r alone followed much the same survival pattern as the control group. In contrast, 100% of mice receiving clone 3 plus P815r-Ø were dead on day 31. This may indicate that clone 3 has ØT4-specific suppressor-inducer activity that turns on suppression of the anti-tumor response, instead of helping cytotoxic cells.

Both groups of mice that received clone 4 fared the best in this experiment. On day 31 after

Figure 19 : Survival of mice after immunotherapy against P815 tumor. All mice were injected with 20 mg/kg body weight of cyclophosphamide, and 2 days later were injected with P815r or P815r-Ø plus one of the T helper clones 3 or 4, as follows :

Clone 3 + P815r

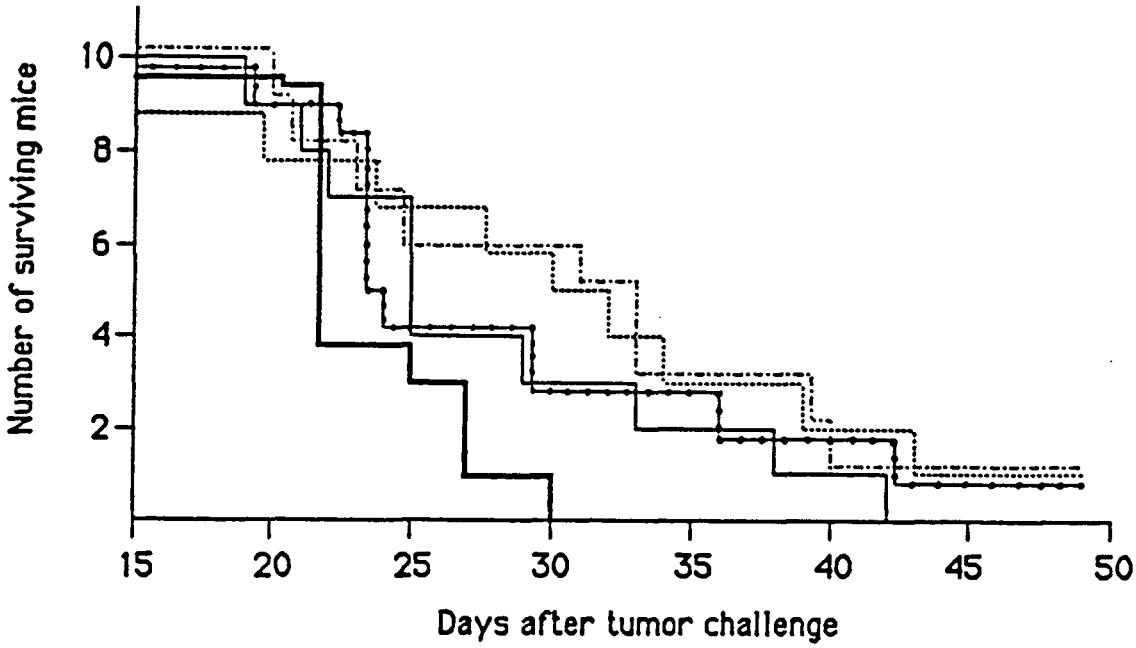
Clone 4 + P815r

Clone 3 + P815r-Ø

Clone 4 + P815r-Ø

PBS only

Repeat injections of the Ø- linked or unlinked P815r plus clones were given on day 9, and on day 11 mice were challenged with subcutaneous injections of viable P815 tumor cells.



tumor challenge, 60% of animals receiving injections of clone 4 plus P815r-Ø were alive, and 50% of the group that got clone 4 plus P815r only were alive, compared to 30% of the control mice. Injection of clone 4 extended the survival of mice until day 31, after which the survival curves for all the remaining groups merged. By day 50, all the mice were dead.

Injection of Clone 1 with either P815r or P815r-Ø did not have a significant effect on survival of the mice or tumor growth (results not shown).

The tumor growth in this experiment did not follow any identifiable trends, with the exception of the group that received clone 3 plus P815r-Ø. The tumors in these animals were the last to become palpable and grew more slowly than the other tumors for a few days, but rapidly caught up in size by the time of the early demise of these animals.

Although this treatment did not cure the mice, or cause regression of the tumors, injection of clones 3 and 4 had interesting effects. Clone 4 appeared to enhance survival of the animals, although under the conditions of this experiment the enhancement did not appear to be specific for ØT4. Clone 4 may be producing a non-specific factor, such as IL2, in large enough amounts to have a transient effect on survival of the animals. Clone 3 effected a decrease in survival only when injected concomitant with P815r-Ø, not with P815r alone. As mentioned earlier, clone 3 may be a helper clone, but one that helps suppressor cells to down-regulate the anti-tumor response in this experiment. Clone 3 was not a large producer of IL2 (Chapter IV-B); therefore it is possible that this clone may be producing an antigen-specific factor.

The <u>in vivo</u> experiments described above were complicated by several variables that may have affected the final outcome of the experiments. Some of the complications that I encountered are described below.

Cyclophosphamide is a toxic drug, belonging to the family of nitrogen mustards. Treatment of the mice even at the low dose used in my experiments had adverse physical side effects on the mice. Approximately 2 weeks after the cyclophosphamide injections, many of the mice developed alkali burns on the skin around the anal area, probably due to elimination of the drug in the urine. This irritation, which in some cases led to infection, weakened the animals and may have led to

premature deaths. The use of healthy mice of at least 8 weeks of age, and preferably of 10-12 weeks of age, is therefore advisable, and was our practise in the later experiments. Irradiation of test mice at a dose that preferentially inhibits suppressor cells is an alternative to drug therapy that has been used effectively by some workers. Notwithstanding the irritation that was observed in my experiments, I believe that testing larger doses of cyclophosphamide up to 75mg/kg/mouse may be a worthwhile experiment, to try to eliminate suppressor cells.

A technical problem arose with the injection of the tumor cell challenge. In my hands, cells injected subcutaneously in 0.1 ml of PBS seemed to be more prone to leakage back out of the animal than when injected i.p. or i.v.. Even though I did become quite proficient at these injections, in order to control for inconsistancy in the numbers of cells actually injected into the animal I believe that the minimum number of animals in each test group should be 10 mice. When $3x10^3$ viable P815 tumor cells were injected subcutaneously, in groups of 10 mice usually 1 mouse would not develop any tumor. For future experiments, I would suggest injection of $1x10^4$ P815 cells for the tumor challenge. These measures would improve the consistancy of the tumor challenge.

A factor of major importance in cell transfer therapy is the viability of the helper cells after transfer into the host animals. To ensure that enough cells are actually functioning in these experiments, either a large number of cells must be transferred, or some way of maintaining viability of the transferred cells must be practised. Cheever et al. (1986) used "antigen-driven" clones reactive to tumor cells antigens in transfer immunotherapy. He prolonged the survival of the cells in vivo by injection of the host mice with $2.4 x10^3$ units of IL2, which increased the total number of donor T cells eight fold. In Sia et al.'s experiments (1984), two injections of helper cells were given before tumor challenge. He found that injection of $10^6$-$10^7$ cloned T helper cells was more effective at retarding tumor growth than was immunization of the host mouse to prime for antigen-specific helper cells. However, in his experiments, $1 x10^5$ transferred cells were too few to be effective.

In my experiments, there seemed to be definite effects due to injection of cloned T cells, but

these effects were transient. This short-lived effectiveness might be prolonged if the transferred cells were longer-lived in vivo. Repeated infusions of T helper cells, and injections of IL2 after tumor challenge might improve the efficacy of the therapy. Over all, the timing and dose of injection of the T cells and irradiated tumor cells are critical factors that must be determined empirically to achieve prolonged survival in immunotherapy with transferred cells. Thus, further work is required to define the in vivo conditions for experiments in which the effect of the helper clones can be fully realized.

D. Summary

Injection of $10^5$- $10^7$ irradiated P815 cells into mice resulted in suppression of the cellular cytotoxic response against P815 tumor cells. Treatment of the mice with low doses of cyclophosphamide before injection of irradiated tumor cells decreased suppression so that priming of anti-tumor CTLs by injection of P815r or P815r-Ø could be observed. Differences in CTL activity were also seen between cells stimulated in vitro with P815r and cells stimulated with P815r-Ø.

The T helper clones appeared to have specific and non-specific activities in the immunotherapy experiments. However, the large number of variables in the in vivo experiments and the difficulty in controlling the conditions in vivo make it necessary to verify the results in repeated experiments. After the experiments are repeated using the suggested conditions, it will be possible to judge whether the use of .-specific helper cells significantly enhances anti-tumor responses in vivo.

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