MACROPHAGE REGULATION OF THE T CELL ALLOGENEIC RESPONSE DURING TUMORIGENESIS

by

Kevin Michael Connolly

Thesis submitted to the Graduate Faculty of the

Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

Microbiology

APPROVED:

K.D. Elgert, Chairman

R. C. Bates

N. R. Krieg

February, 1977

Blacksburg, Virginia

ACKNOWLEDGEMENTS

I wish to thank my major professor and mentor , not only for the contribution of his considerable store of scientific acumen but for the opportunity to work in, what I consider a "challenging scientific environment". I also wish to acknowledge the debt I owe to my committee members, and for contributing a seemingly inexhaustable supply of that intangible yet very real substance known as "moral support." Finally, I deliver a heartfelt "Thank-you" to , whose meticulous care of animals and cleaning of glassware aided immeasureably in the successful completion of these experiments.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

I. INTRODUCTION

Cancer, a disease which is pre-eminent in annual mortality statistics, has been avidly examined by tumor immunologists of the past two decades. Though it arises from the host's own tissues, a neoplastic cell possesses its own foreign "tumor-specific" or "tumor-associated" antigens. Theoretically, cancerous tissue should be recognized and attacked by the body's immune system. However, the immuno-surveillance system is usually ineffective in removing the cancer; allowing it to proliferate and metastasize. Since Winn's experiments in the early 60's (199) tumor recognition has been most often attributed to the cell-mediated immune (CMI) system, made up of macrophages and various thymus-derived lymphocyte (T cell) populations. The ability of the tumor to evade destruction by cell-mediated immunity has created certain areas of inquiry, which will be examined in this thesis.

a) Does it actively debilitate cells of the immune system, rendering them incapable of responding to any foreign antigen? Is this destruction directly caused by the tumor cells or an ancillary killer cell?

b) Does it merely create a reversible inactivation of the CMI response? Is it contact dependent or caused by release of a soluble suppressor factor? What kind(s) of cells are affected?

c) By what mechanism does tumorigenesis affect the ability of the tumor to depress the CMI response.

In vivo human studies, while most directly applicable to the questions at hand, are often impractical due to the experimental nature of the research. Consequently, it is hoped that current in vivo animal

studies are sophisticated enough to answer at least some of the questions concerning the CMI response during human tumorigenesis.

The manifestation of tumor-associated antigens is closely related to the gene governing expression of histocompatibility antigens (105). This indicates that there may be a correlation between tumor and allograft rejection. A close *in vitro* correlate to the CMI response to alloantigens is the mixed lymphocyte reaction (MLR), involving measurement of a blastogenic response to foreign lymphocytes. Therefore, the purpose of my research is to perform parallel MLR studies using normal and tumor-bearing animals to allow examination of the effects of tumor growth on *in vitro* T cell blastogenesis. By determining the factors involved in maximizing the cell-mediated MLR response, it may be possible to draw conclusions as to what enhances or retards the T cell response to *in vivo* tumor proliferation.

II. LITERATURE REVIEW

A. General

Until 1940, the immune system was thought to consist solely of lymphocytes which released humoral antibodies cytotoxic only in the presence of complement. Experiments on delayed hypersensitivity in the 1940's revealed that certain types of immune reactions could be induced in the absence of humoral antibodies or complement. The *in vivo* experiments of Brent (19), Winn (199), Astrom and Waksman (6), and Rubin (162) pointed to a cellular (as opposed to antibody) involvement in transplantation rejection, tumor immunity, autoimmune disorders and viral immunity.

In vitro techniques examining the CMI response were developed only slowly because of the difficulties in maintaining cell viability in tissue culture. Bain *et al.*, (11) working with human peripheral blood, discovered that one *in vitro* indicator of lymphocyte stimulation was blast transformation upon exposure to foreign lymphocytes. Blast transformation, a morphologically detectable change from small to large proliferating lymphocytes, was most easily quantified by measuring increased ^{3}H-thymidine uptake due to enhanced DNA synthesis (17, 69). Thus, Dutton (35) was able to measure degrees of lymphocyte immunocompetence by assaying ^{3}H-thymidine uptake in the presence of foreign lymphocytes (the "mixed lymphocyte reaction" or MLR).

Cunningham (31), however, did not view lymphocyte MLR reactivity as an *in vitro* correlate of *in vivo* allogeneic recognition. He felt that "lymphocyte interaction involved some different recognition system which was not immunological--an abnormal form of induction." Studies by

Gordon and MacLean, and Schellekens and Eysvoogel (68, 169) demonstrated that non-viable allogeneic lymphocytes or cell-free allo-antigens did not stimulate lymphocyte blastogenesis. Therefore, Cunningham (31) theorized that viable allogeneic lymphocytes released a unique T lymphocyte signal which, when received by the responder lymphocyte, triggered MLR blastogenesis. Howe et al. (85) working with immature T cells from neonatal mice proposed a similar mechanism after noting their inability to act as stimulator cells in the MLR. Satte and Pachman (85) in the same article, contested the validity of the "unique T lymphocyte signal," since lymphopenic patients suffering from DiGeorges syndrome and Nezelof's syndrome possessed lymphocytes that could act as stimulator cells in the MLR. Other in vitro assays, done by Berke et al. (14), Main et al. (129), Hersh (79) and Kountz et al. (109), showed that lymphocytes could be stimulated by allogeneic mouse embryo fibroblasts, epidermal cells and fibrosarcoma cells. As Ling and Kay (124), and Ling and Hardy (120) pointed out, the MLR differs from normal in vivo antigen specific activation in that there is no prior sensitization to stimulator antigens. But, they added, the degree of MLR activity generally paralleled in vivo activity of the graft vs. host reaction (20, 127, 143). In defense of Dutton's use of the MLR as a measure of in vivo immunocompetence, Ling and Kay (124) concluded that the MLR is neither a special type of reaction occurring only between two lymphoid cells nor a reaction between a lymphocyte and just any nucleated foreign cell.

The prevalent theory, at the present time summarized by Ling and Kay (124) and Howe et al. (85) is that the MLR is a special but valid example of lymphocytic response to allo-antigens. Certain types of cells

(i.e. lymphocytes and epithelial cells) possess a concentrated number of allo-antigens and are far more immunogenic than other cells containing serologically non-detectable amounts of allo-antigens.

B. Mechanics of the Mixed Lymphocyte Reaction

1. Lymphocyte Source

In addition to an understanding of the theory behind the MLR, a knowledge of the mechanics of this reaction is important if confidence is to be placed in the MLR as an *in vitro* reflection of *in vivo* activities. The MLR assay has been developed in a variety of animal models (123). Early histocompatibility work by Chapman and Dutton (27) was accomplished using rabbit lymphocytes. Ling and Kay (124) have catalogued MLR experiments using dog, chicken, monkey and pig lymphocytes. In his analysis of T cell-macrophage interactions, Rosenthal *et al.* (159) used a guinea pig model, while Folch and Waksman (53) obtained rat lymphocytes in order to work on T cell suppressor activity. The investigations of Bach *et al.* (7) into human T cell-monocyte synergism is most directly applicable to man since extrapolation from animal to human model is unnecessary. However, syngeneic spleen, lymph node, thymus, and thoracic duct lymphocytes have been most frequently obtained from inbred strains of mice.

The H-2 histocompatability locus of the mouse has often been compared to the human HL-A histocompatibility locus. Working with mouse and human models, Bach *et al.* (10) as well as Polet (153), Ling and Kay (124), and others (8, 18, 84, 181, 191, 198) showed that the locus governing the allogeneic response in the MLR was closely linked, but not identical, to the histocompatibility locus governing *in vivo* allograft rejection. In general, high MLR reactivity denoted H-2 or HL-A incompatibility as

shown by the studies of Festenstein (50), Tridente et al. (185), and Tanaka et al. (178). Exceptions to this rule (9, 38, 106) have led Ling and Kay (124) to conclude that "there is no reason for regarding the MLR as the identical in vitro counterpart of a typical homograft reaction." Though the genetic locus governing expression of histocompatibility antigens is not identical to the locus controlling the degree of MLR activity, the genes are so closely linked that, in the vast majority of cases a difference in the MLR locus indicates a difference in the histocompatibility locus. For this reason the MLR continues to be used as an in vitro approximation of the allograft rejection mechanism.

2. Media Preparation and Maintenance of Viability

Obtaining viable lymphocytes is of no great difficulty compared to the problem inherent in maximizing cell viability while minimizing lab artifact. In the MLR, a certain degree of non-specific blastogenesis is attributable to protein serum factors found in the media. Proper concentration of serum is of primary importance, since, as Katz-Heber et al. (98) mentioned in 1973, "MLR culture systems are generally plagued by several inherent complications arising from the requirement for serum, plasma or some constituent thereof. For example: 1) variation in results occurring with different sources and lots of serum, 2) use of homologous serum possessing natural antibody interferes with antigen recognition; 3) heterologous serum results in extraneous stimulation; 4) other serum factors result in non-specific stimulation or inhibition." Fowler et al. (55) reported that in a single experiment conducted with non-pooled spleen cells from eight syngeneic mice, the amount of fetal calf serum needed to induce maximum MLR activity ranged in concentrations from

1.25% to 20%. To minimize background blastogenesis, Heber-Katz and Click (75) as well as Lemke and Opitz (112) substituted a 5×10^{-5} M concentration of 2-mercaptoethanol for serum, its action as a reducing agent substituting for unknown cell potentiating factors in the serum. Though Vischer (194) felt no need to add serum or 2-mercaptoethanol to his media, the general consensus among immunologists was that serum, whether it be mule (130), burro (131), human (1), or calf (151), should be added to media already supplemented with 2-mercaptoethanol. Striving to duplicate the *in vivo* lymphocyte environment, addition of autologous mouse serum theoretically should create optimal conditions for maintenance of lymphocyte viability. Nelson and Schneider (141) found that mouse serum inhibited blastogenesis when present in concentrations of more than 1.5%, though Katz-Heber *et al.* and Peck and Click (98, 150) showed it to be mildly stimulatory at .5% concentration, when used in conjunction with 2-mercaptoethanol.

3. Cell Separation Techniques

In vivo and *in vitro* work with the CMI response revealed that the immune system was composed of a heterogeneous population of lymphocytes. Cooper *et al.* (29) showed that, in the chicken, all antibody producing cells had originally passed through the bursa of Fabricus (hence the term B cells). Later, Miller and Mitchell (135) discovered that all non-antibody-producing lymphocytes initially were "processed" by the thymus (thymus-derived lymphocyte or T cell). Because this lymphocyte is responsible for all observed CMI reactions great efforts have been made to isolate it *in vitro*. One of the most widely employed *in vitro* cell separation techniques makes use of the adherence properties of various

lymphocyte subpopulations. Julius et al. (91) and Trizio and Cudkowicz (186), perfected an "adherence assay" in the early 1970's. They showed that pure T cell populations, due to their non-adherent properties, were able to pass through a column of nylon wool, leaving behind adhering B cells and macrophages. The B cells and macrophages could subsequently be removed by agitating the nylon wool and/or eluting with EDTA. Making use of the non-adherent properties of T cells, others have obtained "purified" non-adherent (NA) populations by eluting them from plastic tissue culture plates (110) or columns of glass beads (71). Folch and Waksman (53) "graded" leucocytes according to their adherent properties, dividing the immune system into: a) non-adherent T cells, b) nylon wool adherent B cells, T suppressor cells, and null cells (categorized as mildly adherent), and c) tissue culture plate-adherent macrophages (strongly adherent).

Cell separation by adherence properties, favored because of its rapidity, simplicity and reproducibility, is but one means of classifying leucocyte populations. Bovine serum albumin gradients used by Hartzman et al. (71) and Gery et al. (62) enabled them to separate buoyant macrophages from T cells and dense B cells. Differences in types of T and B cell surface antigens have allowed recognition of T cells by their theta antigens and B cells by their immunoglobulins. Howe (83), making even a finer distinction, divided T cells into subpopulations containing varying quantities of theta antigen. Homogeneous T or B cell populations can be selected for by adding complement and antibody directed against the appropriate surface antigens (56, 204), thus destroying the unwanted types.

Many cell separation techniques have been designed to selectively remove macrophages as well as B cells. Subpopulations of macrophage-free lymphocytes may be obtained either by making use of the macrophage's adhesive properties or its phagocytic abilities. Erb and Feldman (42), looking at the role of macrophages in the generation of T helper cells, allowed macrophages to engulf iron filings and then pulled them out of suspension with a magnet. Levy and Wheelock (117) noted that the compound carageenan, once phagocytized by macrophages, caused lysozomes to rupture. Subsequent macrophage death left pure lymphocyte populations.

In addition to macrophage and B cell elimination, NA cell preparations must be rid of autologous erythrocytes. Ling and Kay (123), citing the work of Tarnvik (180), Yachnin et al. (200), and Johnson et al. (89) concluded that red blood cells augmented the T cell response to phytohemagglutinin (PHA). In the MLR, however, separate studies by Mardiney and Wren (131), Peck and Click (150), and Fowler et al. (55) demonstrated that red blood cell (RBC) contamination inhibited lymphocyte blastogenesis by as much as 40%. When working with human peripheral blood, Polet (153) utilized cellular sedimentation coefficients to separate dense RBC's from light white blood cells on a dextran gradient. However, Mangi and Mardiney (130) have shown that dextran was toxic to murine lymphocytes and substituted 50% mule or burro serum to precipitate out contaminating RBC's (131). Many investigators have made use of the osmotic properties of the relatively sensitive RBC membrane and selectively lysed erythrocytes with distilled water (98) or ammonium chloride (70, 176).

4. Verification of Cell Purity

Mitogenic profiles of lymphocyte subpopulations have been used to evaluate efficiency of various purification procedures. Daguillard (32) and Betel et al. (15) working with whole mouse spleen cells and NA cell preparations showed that the NA cell response to the T cell mitogens concanavalin A (Con A) and phytohemagglutinin (PHA) was heightened. Since Con A and PHA have generally been accepted as specific stimulators of T cell blastogenesis (32, 153), pure NA populations of T cells, when exposed to PHA, should have a much higher level of ^{3}H-thymidine incorporation than adherent or whole spleen cell preparations. However, if, in eluting NA cells, the columns are agitated too severely, the NA PHA response will be low due to the dislodged, mildly adherent T suppressor cell observed by Folch et al. (52, 53, 54). PHA stimulation of NA cells in also contingent upon the type of donor animal the lymphocytes are taken from. Contrary to the data of Vischer (195) and Levis and Robbins (114), purified NA mouse lymphocytes will respond to PHA but only at the optimal mitogen concentration. Even when macrophages are present, an extremely high or low dose of mitogen will not stimulate T lymphocytes (136). Addition of macrophages enhances T cell response to sub-optimal concentrations of PHA and Con A (62, 202), but at the optimal concentration of mitogen, macrophage-free NA cells will undergo extensive blastogenesis (147). Lipopolysaccharide, a B cell mitogen and dextran sulfate do not stimulate NA cells (137, 163). Therefore they can be used to assess levels of B cell contamination in a purified NA population of lymphocytes.

Other techniques have also been used to determine the purity of the

T cell preparation. Using the Jerne plaque assay, Julius et al. (91) showed a 50% recovery of viable T cells from nylon wool columns with less than 5% B cell contamination. Fluorescent antibody studies by Handwerger and Schwartz (70) and others (91, 176) showed only a 2%-5% contamination by B cells, correlating quite well with Julius' data.

To assay the degree of macrophage contamination, Handwerger and Schwartz (70) counted cells containing phagocytized latex beads, while Erb and Feldman (42) looked at the number of cells capable of engulfing fluorescent-conjugated corynebacteria. In both cases, purified NA populations contained less than .5% macrophages. Detection of macrophages could also be made by specifically staining for esterases found only in phagocytic cells (94, 118, 169).

5. Admixture of "R" and "Sm" Populations

Having obtained a purified NA population for use as responder cells in the one-way MLR, it becomes necessary to keep histoincompatible stimulator cells from undergoing blastogenesis. Chapman and Dutton (27), Bain et al. (11), Dutton (35), and Bach et al. (10) have found that Mitomycin C treatment or X-ray irradiation halted DNA synthesis without lowering viability or altering antigenicity of the stimulatory cell. Elves (39) believed that Mitomycin C treatment did not totally eliminate DNA synthesis and that it inhibited in vitro blastogenesis of responder cells exposed to stimulator cells treated with Mitomycin C. Lebrun (111) confirmed the observations of Bach that Mitomycin C was a reliable inhibitor of DNA synthesis. Currently, to inactivate DNA production, the methods of choice are, exposure to Mitomycin C or X-ray irradiation.

Mixing of NA responder populations with Mitomycin C-treated stimu-

lator populations required calculations of: (a) cell numbers, (b) cell ratios, (c) total incubation time, and (d) length of ^{3}H-thymidine pulse. The MLR assays of Adler et al. (1) were done in tubes with relatively large ($1 \times 10^{6} - 1.5 \times 10^{7}$) numbers of cells and a short incubation period (three days). Dutton's (35) "macro" assays were incubated for one to two days while the assay by Phillips et al. (151) was incubated for 72 hours. Adler et al. (1) varied responder to stimulator cell ratio from 1:1 to 1:2, while Katz-Heber et al. (98) kept the ratio at 1:2. With the introduction of the Multiple Automated Sample Harvester (72, 132, 182), a microplate technique could be employed that reduced cell numbers and media volume at least five-fold. Incubation time was lengthened to five days, but harvesting was reduced to minutes since 24 cultures were filtered simultaneously. The optimal cell ratio was established at 1:1 or 1:2 while ^{3}H-thymidine uptake did not substantially change when the pulse occurred anywhere during the last 18 hours of the 120 hour incubation period. Phillips et al. (151), noting a conflict in length of the macro and micro method incubation period, ascribed it to differences in cell density present in the two types of MLR assays. More area and more cells in the macro assay allowed for a greater degree of contact with foreign histocompatibility antigens and, therefore, more rapid blastogenesis.

C. Cellular Interactions and the Allogeneic Response

1. T_1-T_2 Cell Interactions

Mastery of the techniques involving the MLR allowed a detailed examination of the many types of cellular interactions which occur in the in vitro CMI response to foreign histocompatibility antigens.

The discovery that T cell subpopulations can both enhance and inhibit the CMI reaction was one of the first indications that the CMI system was far more complex than initial investigations indicated. Independent studies by Rich and Rich (155) and Anderson and Hayry (5) showed that addition of murine lymph node cells to a splenic T cell population enhanced its response to foreign antigens. "Amplifier" spleen cells have been found by Cantos and Asofsky (23, 24) and "enhancing peripheral blood lymphocytes" by Cohen and Howe (28).

Often, the suppressor function is ascribed to the thymocyte. Gershon et al. (61) described a thymocyte suppressor cell which appeared to control the CMI reaction through a "feedback inhibition mechanism." When spleen cell blastogenesis occurred rapidly, addition of thymocytes suppressed the reaction; in times of slow blastogenesis thymocyte addition enhanced the reaction. Other studies by Gershon et al. (60) characterized the suppressor thymocyte as a cortisone resistant cell, a characterization which is in agreement with Kontiainen and Feldman (108), and Feldman and Schrader (49). Hirano and Nordin's (81) cytotoxicity studies showed that cytolytic lymphocytes were inactivated by cortisone-sensitive suppressor cells. Fitch et al. (51) have also discovered a splenic suppressor T cell generated in MLR cultures which closely resembles the suppressor cell described by Hirano and Nordin (81).

Splenic lymphocytes have drawn much attention as suppressor cells (36, 46, 97). Kappler and Marrack's work (96) in 1974 showed it to be a sessile, short-lived, anti-theta serum sensitive lymphocyte found primarily in the spleen or thymus. Eardley and Gershon (36) isolated a splenic suppressor T cell that differed from the cell described by

Kappler and Marrach (96) in that it was resistant to lysis by complement and anti-theta serum. Folch and Waksman (52) and Rich and Rich (156) noted that splenic suppressor cells exhibited their inhibitory activity when exposed to high doses of PHA, Con A or macrophages. Such cells were also triggered by *in vitro* cell concentrations greater than 10^6 cells per milliliter. These nylon wool-adherent suppressor cells were more abundant in spleens of young rats than in those of aged animals.

The "activated" splenic suppressor cell described by Rich and Rich (156) secreted a soluble factor which non-specifically depressed blastogenesis in responder cells. Tardieu *et al.* (179) described a thoracic duct suppressor cell that also exhibited non-contact dependent suppression. There has been considerable debate over the exact nature of the soluble suppressor cell factor. Tadakuma *et al.* (177) and later, Kuhner and David (110) isolated a soluble suppressor factor biochemically identical to MIF. Release of this suppressor factor could be the mechanism by which MIF releasing blast cells in tumor-bearers suppress T cells, thereby enhancing tumor proliferation. Namba and Waksman (138), as well as Jeffes and Granger (88), argued for two types of soluble suppressor: (a) a DNA synthesis inhibitor, and (b) a proliferation inhibitory factor (PIF). In concentrated doses, PIF was found to be cytotoxic to target cells and possessed all the biochemical characteristics of T cell lymphotoxin. Kasahard and Shiori-Nakano (97), as well as Opitz *et al.* (145), attributed no cytotoxic abilities to their suppressor factor, which was electrophoretically characterized as thymidine and thymidine monophosphate. They felt that "suppression" was an *in vitro* artifact caused by lymphocyte release of "cold" thymidine which

favorably competed with ^{3}H-thymidine for DNA incorporation. What Kasahard and Shiori-Nakana failed to explain was why the supernatant also inhibited ^{3}H-uridine and ^{3}H-leucine incorporation. Such results do not indicate in vitro thymidine competition but actual DNA, RNA, and protein synthesis suppression.

2. T Cell-Macrophage Interaction

When macrophages were added to the various T cell subpopulations, the complexity of the cellular interactions was compounded. There is an abundance of evidence indicating macrophage-T cell involvement in mitogen stimulation (12, 15, 54, 63, 76, 167, 168, 179, 197, 202) and in B cell-T cell helper activity (45, 47, 49, 80, 87, 139). Macrophages can also influence blastogenesis in the in vitro T cell response to foreign histocompatibility antigens. However, such blastogenesis, depending upon experimental conditions, can be enhanced or depressed by macrophage presence. In addition, the macrophage influence may or may not be dependent on cell contact.

One of the earliest theories of blastogenesis, proposed by Jones (90), acknowledged the necessity for macrophage-T cell contact, but required polymorphonucleocytes to stimulate monocyte transformation into macrophages. Gordon (67), working with human cells, also showed a need for macrophage contact to promote T cell stimulation. However, macrophage enhancement was not impaired by use of allogeneic responder cells. This bears out the later human lymphocyte work done by Hersh and Harris (80), Rode and Gordon's (157) conclusions, like those of Rosenthal et al. (158), varied from those of Hersh and Harris (80) on one major point--they could demonstrate no T cell blastogenic inhibition at ex-

cessive concentrations of macrophages. However, Nguyen and Stadtsbaeder (142) showed that lymphocytes in vitro spontaneously attached to macrophages when macrophage concentrations were raised above 1%. Such contact was cytotoxic to the lymphocytes.

Macrophage cytotoxicity is not necessarily contact dependent (74, 188). Soluble macrophage factors have been isolated which exhibited low dose enhancement and high dose inhibition of T cell stimulation (3, 148).

3. T_1-T_2 Cell Interactions During Tumorigenesis

Heretofore, examination of cellular immunity has been confined to "normal" animals. When tumor-bearing animals are experimentally used, complexities of the cellular interactions again increase. In recent years, several comprehensive treatises have attempted, with varying degrees of success, to present an overview of tumor immunology (13, 77, 78, 104).

Since the early work of Kaliss and Malomut (93) in 1952, it has been known that in vivo injection of alloantiserum can enhance tumor growth. One of the most recent and startling findings is that in vivo injection of purified T cells also enhances tumorigenesis. Gillette and Fox (65) and Umiel and Trainin (187) working with thymectomized, X-irradiated bone marrow reconstituted (TXB) mice found them to possess more slowly developing tumors than normal tumor-bearing animals. Nude mice also manifested a slower rate of tumorigenesis than normal tumor-bearers. Injection of spleen cells from normal and tumor-bearing animals, enhanced the growth rate of tumors. When spleen cells were exposed to anti-theta serum plus complement, tumor growth enhancement was eliminated. Injection of spleen cells from thymectomized adult

mice showed very limited enhancement of tumor growth (65, 187). Such findings force a re-examination of the T cell immune surveillance system. Apparently, there is a short-lived population of suppressor T cells in normal and tumor-bearing animals, which can abrogate in vivo tumor destruction. Using T cells from tumor-bearers Fujimoto et al. (56) substantiated these conclusions, though he was unable to induce tumor enhancement with normal T cells. He did succeed in isolating an immunosuppressive soluble factor from tissue-cultured thymus and spleen cells of tumor-bearers. In vivo and in vitro T cell cytotoxicity studies by Treves et al. (183) showed in vivo T cell promotion of tumor growth, but in vitro T cell cytotoxicity towards tumor cells. Subsequent studies by Gorczynski and Norbury (66) have shown an in vivo inhibition of tumor growth due to CMI.

Not only can T cells stimulate or depress tumorigenesis, but tumor cells can stimulate or depress blastogenesis. Recent kinetic studies with methylcholanthrene-induced fibrosarcomas show initial T cell stimulation and subsequent suppression. Following up on in vivo work done by Gershon et al. (59), Vaage (190), and Decker et al. (34), Paranjpe and Boone (149), using an in vivo delayed hypersensitivity reaction, were able to demonstrate early tumor cell stimulation, and later depression, of the CMI response. This work correlated with the in vitro studies done by Bhatnagar et al. (16), and Jurin and Suit (92) who showed maximum T cell stimulation 6-12 days post tumor transplantation. Depression of the T cell response sets in rapidly with animals possessing tumors over 100 cubic millimeters. "Depression," as Kondo pointed out, is a relative term. In their work, even though MLR reactivity on a per

cell basis was severely diminished, over all numbers of cells per spleen tripled. Therefore they argue, from the viewpoint of the mouse, tumorigenesis, in all its phases, stimulates the CMI response (107, 174, 175).

4. T Cell-Macrophage Interactions During Tumorigenesis

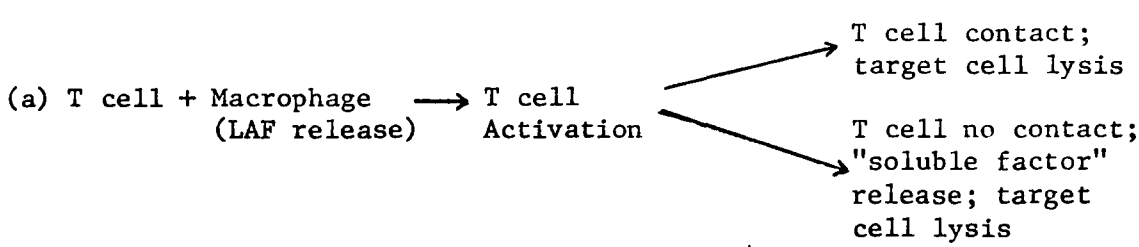
The term "cell-mediated immune response," when used in reference to tumor immunology, is perhaps a misnomer. It implies that the role of cytotoxic "effector" lymphocyte belongs solely to the T lymphocyte. Recent studies have shown that macrophages and T lymphocytes can take the part of accessory as well as effector cells (141), though Haskill et al. (73) pointed out that the works of Cerottini et al. (26), Miller et al. (134), Wagner et al. (196), and Rouse et al. (161), did not acknowledge the role of macrophages as possible effector cells. Kearney et al. (99) showed T cell-macrophage synergy in tumor cell rejection. Contrary to previously cited papers (65, 187), his results showed that TXB mice exhibited no resistance to tumor allografts. In his experiments, in vitro lysis of tumor cells required both macrophages and T cells. Further work by Simes et al. (172) showed that T cells in the peritoneal exudate of tumor-bearing animals exhibited a measurable level of cytotoxicity even after glass adherent macrophages had been removed from the cell population. It could be postulated that this T cell cytotoxicity was due to lymphocyte activating factor (LAF) released by macrophages in the peritoneal cavity. The model of Shevach (170) and Treves et al. (184) depicted lymphocyte activation as a contact dependent process requiring macrophages to "present" the antigen to the responder lymphocytes. Piper and McIvor (152) described the effector cell as a peritoneal macrophage from tumor-bearers that released a specific cytotoxic factor

upon re-exposure to the tumor antigen. Release of macrophage protein toxin was independent of T cell presence. The Schaeffer et al. (166) model for a tumor-bearing effector cell was also a T cell-independent macrophage. Through peritoneal lavage, it could be obtained after a single injection of the bacterial antigen, Corynebacterium parvum, into the peritoneal cavity of tumor-bearing TXB mice. Unlike the effector cell described by Christie and Bomford (30), macrophage cytotoxicity was triggered only after second exposure to the tumor antigens. Christie and Bomford (30) demonstrated that T cell independent effector macrophages produced non-specific contact cytotoxicity by exposing them to two doses of C. parvum and bacterial antigen-immune T cells. Meltzer et al. (133) duplicated Christie's work, substituting Bacillus Calmette-Guerin for C. parvum. Finally, Evans (44) and David (33) emphasized macrophage dependence on immune T cell release of "specific macrophage activating factor" (SMAF) to sustain macrophage contact cytotoxicity.

The interactions between macrophages and T cells in tumor-bearers was outlined eloquently in two recent review articles by Alexander, et al. (2) and Levy and Wheelock (117). The following schematics summarize their presentation.

I. T CELL AS EFFECTOR AND MACROPHAGE AS ACCESSORY CELL

Kearney et al. Model (99)



Shevach and Treves et al. Model (170, 184)

(b) T cell + Macrophage antigen "presentation" → T cell Activation → T cell contact: target cell lysis; T cell no contact: "soluble factor" release; target cell lysis

II. MACROPHAGE AS T INDEPENDENT EFFECTOR CELL

Piper and McIvor Model (152)

(a) Tumor antigen immune macrophage + "Shocking" dose of tumor antigen → Activated macrophage → Specific toxic soluble factor; cytolysis; Non-specific toxic soluble factor; cytolysis

Shaeffer et al. Model (166)

(b) Tumor immune macrophage + *In vivo* bacterial antigen → Tumor immune macrophage + Tumor antigen → Non-specific contact dependent lysis

Christie and Bomford Model (30)

(c) Macrophage + *In vivo* bacterial antigen → Bacterial antigen immune macrophage + "Shocking" dose of bacteria + Tumor antigen → Non-specific contact dependent lysis

III. MACROPHAGE AS EFFECTOR AND T CELL AS ACCESSORY CELL

Christie and Bomford Model (30)

(a) Macrophage + Bacterial immune T cell (SMAF release) + Bacterial antigen ⟶ Armed macrophage

+ Tumor target ⟶ "Activated" macrophage ⟶ Non-specific contact cytotoxicity

Evans et al. Model (44)

(b) Macrophage + Tumor antigen immune T cells (SMAF release) + Tumor target cells ⟶ Tumor target cells +

Armed macrophage ⟶ "Activated" macrophage ⟶ Non-specific contact cytotoxicity

As with any young science, cellular immunology and more particularly, tumor immunology, abounds in hypotheses, speculations, and unanswered questions. It is hoped that, using such tools as the MLR, many of the perplexities involving cellular interactions during tumorigenesis will be satisfactorally resolved.

III. MATERIAL AND METHODS

A. General Protocol

1. Media Preparation

For preparing media, procedures described by Ling and Kay (124) were generally followed. Powdered RPMI 1640 medium (10.38 grams/liter) already containing 2 mM L-glutamine (Flow Labs, Rockville, Md.) was dissolved into doubly glass distilled water. To the medium was added: a) penicillin G-streptomycin sulfate solution (100 units/ml and 10 µg/ml of medium, respectively; Grand Island Biological Co., Grand Island, N.Y.), b) HEPES buffer (for every liter of medium dissolve 5.96 g of HEPES in 10 ml of distilled water, Flow Labs, Rockville, Md.), c) $NaHCO_3$ (2 g/liter of medium). "Complete" medium had, in addition, 4×10^{-5} M mercaptoethanol and a 5% or 10% concentration of heat-inactivated fetal calf serum (FCS, Grand Island Biological Co., Grand Island, N.Y.). The medium was maintained at a pH of 7.4.

2. Animals

All mice were purchased at 6-8 weeks of age from Flow Laboratories, Dublin, Virginia. "Responder" (R) cells were obtained from mature Balb/c Dub male or female mice. "Stimulator" (S) cells were taken from the spleens of mature C_3H/He Dub male mice. Betel *et al.* (15) and Adler (1) have both shown that mice over six weeks of age are immunologically mature animals.

3. Fibrosarcoma Induction

To induce primary tumor growth, the method of Smith and Kondo (175) was followed. In this procedure, 0.25 mg of 2-methylcholanthrene (Eastman Kodak, Rochester, N.Y.) was suspended in five ml of olive oil. After

repeated mixing 0.1 ml portions of this preparation were injected into the left hind legs of mice. Tumors suitable for transplanting developed within 3 months.

Secondary passage of tumors was accomplished by following the protocol of Haskill et al. (73). Palpable tumors were excised and placed in plastic tissue culture plates containing 5 ml of complete medium at 37 C. Tissue was minced with scissors, the supernatant adsorbed with a sterile gauze pad and the minced tumor fragments placed in a 30 ml Erlenmyer flask containing 15 ml of a 0.25% trypsin solution. The preparation was agitated at room temperature by a magnetic stirrer. After 15 minutes, the solid tissue was allowed to settle out and the cell containing supernatant was removed with a pasteur pipette. To obtain a higher percentage of viable cells the first 15 ml of the trypsin solution was discarded and the trypsinization was repeated. The cell supernatants were placed in 15 ml conical centrifuge tubes and centrifuged at 400 x g for five minutes, washed twice with 10 ml of complete RPMI medium, and reconstituted to 6 ml in complete medium. Tumor cells were counted using a hemacytometer. A 0.025 ml portion of cells was suspended in 0.475 ml of Isoton and 0.1 ml of 0.4% trypan blue dye. After five minutes incubation at room temperature, viable cells were counted in a hemacytometer. An appropriate dilution of the tumor cell preparation was made so that 1×10^6 viable cells/0.1 ml were injected i.m. into the left hind leg of mice.

4. Preparation of Spleen Cells

To obtain pooled spleen cell preparations, the general procedures of Mardiney and Wren (131) were followed. One to 12 spleens was

removed aseptically and placed into # 50 wire mesh cylinders made of stainless steel. Spleens were pressed through cylinder screens with a 10 ml glass syringe plunger. Lymphocyte suspensions were placed in 15 ml of chilled (4 C) phosphate buffer saline (PBS) or pH 7.4 Hanks Balanced Salt Solution (HBSS, Grand Island Biological Co., Grand Island, N.Y.) containing 0.35 g $NaHCO_3$ per liter. Packed spleen cells were obtained by centrifuging for five minutes at 400 x g. Centrifugation at higher speeds caused excessive lymphocyte clumping (122). Packed spleen cells were diluted in 1-3 ml of HBSS or PBS.

Red blood cell (RBC) lysis (70) was accomplished using a 0.85% solution of NH_4Cl. For every volume of packed cells, four volumes of chilled (4 C) lysing agent were added. The preparation was incubated at 4 C for 5 minutes with frequent agitation. Following RBC lysis, lymphocytes were centrifuged at 400 x g, washed twice with HBSS and diluted in 3-6 ml of cold (4 C) serum free RPMI medium.

5. Counting Procedure

To determine the number of lymphocytes in suspension, 0.05 ml of cells were removed with a 50 lambda-Centaur pipetter (Microbiological Assoc., Bethesda, Md.) and added to 0.95 ml of Isoton (Coulter Diagnostics, Inc., Hialeah, Fla.). An automatic Cordis 205 diluter (Cordis Co., Miami, Fla.) aspirated 0.04 ml of the 1:20 dilution and mixed it with 19.96 ml of isoton, delivering into counting vials (Coulter Diagnostics, Inc., Hialeah, Fla.) a 1:10,000 dilution of lymphocytes. Six drops of "Zap-isoton" (Coulter Diagnostics, Inc., Hialeah, Fla.) were then added to insure total lysis of any residual RBC's. A Model D2 Coulter Counter (Coulter Diagnostics, Inc., Hialeah, Fla.) counted 0.5

ml of the 1:10,000 lymphocyte dilution three times.

6. Inactivation with Mitomycin C

Because of its ability to halt DNA synthesis and prevent blastogenesis, the antibiotic Mitomycin C (111) was used on stimulator populations (S_m) in the MLR. Maintenance of uniform cell density per culture well was crucial in running the MLR. Therefore, control populations used to assess non-specific stimulation, though they lack allogenic stimulator cells, contained an equal number of inactivated syngeneic responder (R_m) cells. Mitomycin C inactivation was according to Phillips et al. (151). Forty µl of Mitomycin C (Sigma Chemical Co., St. Louis, Mo.) were added to each ml of culture containing 10^7 cells in complete RPMI medium. Lymphocytes were incubated in a 37 C water bath for 45 minutes. The preparation was then centrifuged at 400 x g, washed twice with 15 ml of HBSS and resuspended to 1.0 ml in complete RPMI medium. The inactivated population was recounted and assessed for viability. Though Mitomycin C does not adversely affect viability (111), repeated cell washes caused 20% to 50% loss of the original population.

7. Diluting Cells

Dilution procedures generally followed those described by Thurman et al. (182) and Hartzman et al. (72). An average of the Coulter counts were taken and multiplied by 20,000 to give cells per ml in the original spleen cell preparation. "Responder" spleen cells were then mixed with a Vortex Genie (Scientific Industries, Springfield, Mass.) and diluted in complete RPMI medium to 4 x 10^6 cells/ml. Dilutions were made using 25, 50 or 100 lambda micropipetters. Inactivated "stimulator" (S_m) cells were made up to a concentration of 8 x 10^6 cells/ml. Hamilton

syringes (Hamilton Co., Reno, Nev.) or centaur pipetters were used to deliver 0.05 ml of responder or stimulator cells into wells of "U" bottom micro tissue culture plates (Linbro Scientific, Inc., Hamden, Conn.). Complete medium was added with a 5 ml Hamilton syringe so that each well contained 0.2 ml of cells in complete RPMI medium. Macro assays using flat bottom macro tissue culture plates (Linbro Scientific, Inc., Hamden, Conn.) were conducted in exactly the same fashion except all concentrations and volumes were increased five-fold. Before dispensing, a 0.025 ml sample of each cell preparation was taken and assayed for cell viability. Cells were incubated for 5 minutes at room temperature with 0.475 ml of isoton and 0.1 ml of a 0.4% trypan blue solution (Grand Island Biological Co., Grand Island, N.Y.). The preparation was pipetted onto a hemacytometer and assessed for viability by observing active trypan blue exclusion. Viability was consistently greater than 95%.

8. Incubation

Using the methodology of Thurman et al. (182) and Hartzman et al. (72), cultures were incubated for five days at 37 C in a humidified CO_2 (5%) incubator. Eighteen hours prior to assay termination, cultures were pulsed with 1 µCi of methyl ^{3}H-thymidine (Spec. activity 1.9 Ci/mM; Swartz/Mann, Becton Dickenson, Orangeburg, N.Y.) delivered in a 0.05 ml volume of RPMI medium by a 50 lambda pipetter. Macro cultures received a 2 µCi/0.05 ml pulse of ^{3}H-thymidine. "Counts per minute" represents DNA synthesis as measured by incorporation of ^{3}H-thymidine. All subsequent references to activity are measured in CPM.

9. Termination

To terminate the micro assay, a "Multiple Automated Sample Harves-

ter" (72, 182) (Microbiological Assoc., Bethesda, Md.) was used to wash the wells with distilled water and simultaneously to aspirate cells onto strips of Whatman 934 glass fiber filter paper (Microbiological Assoc., Bethesda, Md.). After drying for 45 minutes in 75 C hot air oven, filter disks containing radioactive DNA were removed from the glass fiber strip with forceps and placed in "mini" scintillation counting vials (A. H. Thomas Co., Phila., Pa.) along with 0.3 ml of methyl benzethonium hydroxide (131) (Hyamine, Sigma Chemical Co., St. Louis, Mo.). Vials were incubated overnight at 4 C, allowing Hyamine to solublize cells. The following day 1.5 ml of Econofluor (New England Nuclear, Boston, Mass.) was added to the vials. Before counting, the vials were incubated in the dark for 24 hours to eliminate chemiluminescence and equilibrated at 4 C for 15 minutes before placement into a scintillation counter.

Cells from macro plates were aspirated with a pasteur pipette onto Gelman 0.45 μm filters (A. H. Thomas Co., Phila., Pa.) held by a Millipore manifold (Millipore Corp., Bedford, Mass.). The wells were washed vigorously three times with 0.15 M saline, twice with 5% tricholoracetic acid (TCA, Sigma Chemical Co., St. Louis, Mo.) and twice with 95% ethyl alcohol. Filters, with precipitated DNA, were placed directly into "mini" vials and dried for 45 minutes at 75 C. Econofluor was added and the vials were incubated, equilibrated and counted.

10. Counting

Both micro and macro filters were counted for one minute on a Packard Tri-Carb liquid scintillation counter (Packard Inc., Downey Grove, Ill.). The counter was set at 60% gain; minimum and maximum

window settings were 20 to 1000, respectively. Background subtraction was automatically made by the counter.

11. Statistical Analysis

Samples of pooled male or female spleen cells were run in replicates of six. After the high and low CPM values were eliminated the mean and standard error of the remaining values were calculated. Data were recorded after the fashion of Ling and Kay (122), as an increment: stimulated counts ($R+S_m$) minus unstimulated counts ($R+R_m$).

B. Acquisition of Leucocyte Sub-Populations

1. Non Adherent Populations

a. General Procedures. Any procedures involving nylon wool purification of spleen cell preparations were done according to Julius et al. (91) with minor modifications.

To remove toxic factors, nylon wool (Fenwal Laboratories, Morton Grove, Ill.) was incubated in saline for two hours at 37 C. It was then rinsed three times in doubly distilled water. Subsequent soaking at 37 C for one week in frequently changed distilled water removed all toxic factors. The wool was dryed in a 75 C hot air oven for two days and packed into six or 12 ml syringes (Monojet-Medical Industries, Deland, Fla.). The 12 ml syringes contained 1.2 grams of wool packed to the 9 ml mark while the 6 ml syringes contained 0.6 grams up to the 5 ml mark. The plunger was replaced, the syringes fitted with 18 gauge needles (Monojet-Medical Industries, Deland, Fla.) and autoclaved.

Before use, columns were rinsed three times with 15 ml of HBSS and incubated at 37 C for 45 minutes with 3.0 ml of complete RPMI medium. RPMI medium was then removed, the needle plugged and 2.0 ml of cells

added. The large column received no more than 3 x 10^8 cells/2.0 ml and the small column, 1.5 x 10^8 cells/2.0 ml. Cells were washed into the wool with 1.0 ml of warmed complete medium and incubated at 37 C for 45 minutes. NA cells were obtained by gently washing each column with 25 ml of warmed (37 C) HBSS. NA cells were placed in 15 ml centrifuge tubes, centrifuged at 400 x g and reconstituted to 3.0 ml with complete medium. NA cells were then placed on 60 x 15 mm plastic Falcon tissue culture plates (Falcon, Becton-Dickinson, Cal.) to remove any remaining adherent macrophages. After incubating for 45 minutes at 37 C, the supernatants containing NA cells were removed and re-plated. Following this final re-plating incubation, NA cells were removed with a pasteur pipette, washed gently with 5 ml of complete medium. The NA cells were then centrifuged for five minutes at 400 x g. Cells were reconstituted to 1.0 ml, counted, and assessed for viability. To minimize cell loss, care was taken to use warm, complete medium whenever possible. During a lengthy assay, complete medium was substituted for HBSS or PBS, since, over the period of the assay lymphocyte viability could decrease by as much as 50%. All concentrations were adjusted so that 10^5 viable cells were placed in microplate wells. Even when viability was greater than 90%, coulter counts indicated a 10-fold loss of cells during purification procedure (i.e., if 3 x 10^8 whole spleen cells were placed over a column, 3 x 10^7 NA cells were recovered).

The general procedures needed to obtain purified B and T cell for use in the MLR are shown schematically in Figure 1.

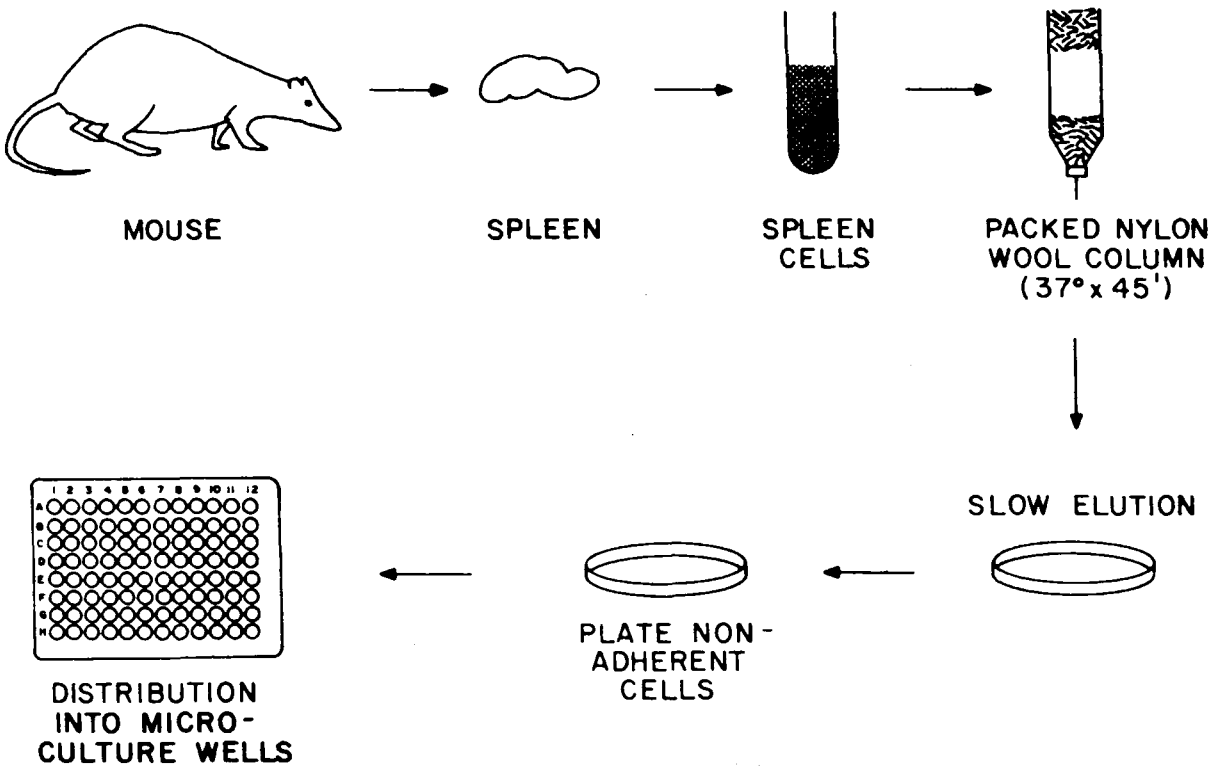


Figure 1. Techniques for Preparation of Purified B and T Mouse Lymphocytes by Nylon Wool Column Purification.

b. B Cell Contamination of NA Cell Populations.

(1) Response to Specific Mitogenic Stimulants. Specific mitogenic properties of NA cells were used to assess the purity of the NA cell preparation (15). A dose response experiment was set up, the optimal mitogenic concentrations found, and subsequently used to determine purity of the NA population. PHA-P (Difco, Detroit, Mich.), Con A. LPS (Sigma Chemical Co., St. Louis, Mo.), and pokeweed mitogen (PW) (Grand Island Biological Co., Grand Island, N.Y.) were made up in concentrations ranging from 0.5 µl of mitogen to 8.0 µl per well of RPMI medium. .05 ml aliquotes of these dilutions were added to flat bottom micro plates containing 2 x 10^5 cells in 0.15 ml of complete medium (10% FCS). Except for a 72 instead of a 120 hour incubation, the mitogen assay was identical to the MLR.

(2) Jerne Plaque Assay. Kappler's (95) micro plaque assay was used to determine degree of contamination by antibody-producing B cells. Sheep red blood cells (SRBC) were centrifuged at 500 x g for five minutes, washed twice in HBSS and resuspended in HBSS to a 2% concentration (10^8 cells). A 0.1 ml aliquot of this solution was injected i.v. or i.p. into mice using a 27 gauge needle and a 1 ml tuberculin syringe. Four to six days later, 1 x 10^5/0.025 ml purified NA cells were added to wells of flat bottom micro culture plates containing 0.025 ml of 25% guinea pig complement (Grand Island Biological Co., Grand Island, N.Y.) and 0.1 ml of SRBC solution. Microculture plates were incubated at 4 C for two hours, equilibrated to room temperature for 15 minutes and reincubated at 37 C for an additional 1.5 hours. At the end of the final incubation period, they were removed from the CO_2 incubator and examined

under the stereoscopic microscope for signs of plaque formation. If any difficulty was encountered in reading the micro plaque assay, results were confirmed by running a slide Jerne plaque assay (91).

This assay (91) involves suspension of SRBC antigen in a solid agarose matrix, allowing easy plaque identification. Microscope slides were coated with a 0.1% solution of hot agarose (Baker Chemical Co., Phillipsburg, N.J.). A 1.25% solution of hot agarose was cooled to 45 C and added to an equal volume of 2X Minimum Essential Media (MEM, Grand Island Biological Co., Grand Island, N.Y.). One-half ml of this agarose solution was dispensed into 10 X 75 mm test tubes and kept at 45 C. Five percent SRBC in 50 lambda portions were added to the tubes. Finally, 10^6 NA cells/0.05 ml from suitable immunized mice were added to the tube of agar and SRBC's. The preparation was mixed, poured onto the agarose-coated slides and allowed to harden. The slides were inverted on racks and placed in a humidified 37 C CO_2 incubator. After two hours, the slide rack was filled to capacity with a 1:25 dilution of guinea pig complement and re-incubated at 37 C for one hour. At the end of that time, slides were removed, washed twice in saline, and observed under a stereoscopic microscope for plaque formation.

(3) Fluorescent Antibody Studies. Fluorescent antibody directed against immunoglobulins (Ig) selectively confined to the membranes of B cells, was also used to determine purity of the NA cell preparation (22, 34, 35). Fluoroscein-conjugated IgG rabbit anti-mouse gamma globulin (Cappel Laboratories, Downingtown, Pa.) was reconstituted with HBSS to a concentration of 2 mg/ml. NA cells (107) were delivered in a 0.05 ml volume and mixed with 0.25 ml of the anti-Ig solution. The

cell solution was incubated for 20 minutes at 4 C. At the end of that time, 0.5 ml of inactivated FCS was added to the tube, the cells were mixed and the preparation pelleted by centrifugation at 400 x g for five minutes. The button was washed in complete RPMI medium, resuspended in 0.1 ml of inactivated FCS and pipetted onto a microscope slide. The slide was air dried and fixed for 15 minutes in 95% ethanol. It was allowed to air dry before addition of 9:1 solution of glycerol-phosphate buffered saline and coverslip. Lymphocytes were then observed under a fluorescent microscope (Leitz, Wetzler, Germany) for evidence of a green corona or "cap" of fluorescence, indicating presence of contaminating B cells. The microscope itself utilized an HBO 200 watt mercury lamp, Leitz, blue absorbing and interference exciting filters and a 95X fluorite darkfield objective lens.

c. Macrophage Contamination of NA Population. Macrophage uptake of latex particles (34, 61) was used to determine degrees of macrophage contamination in the NA preparation. A portion of 0.8 μ Bacto-Latex (Difco, Detroit, Mich.) diluted 1:2, was removed with a 25 lambda pipetter and added to a test tube containing 0.450 ml of isoton, 0.1 ml of a 0.4% trypan blue solution, and 0.025 ml of NA cells. After incubating at 37 C for 45 minutes, a drop of the mixture was placed in a hemacytometer and examined under the microscope for cell phagocytosis of three or more latex particles. Invariably, the number of such cells was negligible.

A more reliable way to determine the degree of macrophage contamination was made using a leucocyte stain specific for esterases found in macrophages (94, 118, 169). To prepare the fixative, four grams of

paraformaldehyde (Baker Chemical Co., Phillipsburg, N.J.) were dissolved in 55 ml of distilled water and agitated for 30 minutes at 60 C. The pH of the solution was adjusted to 7.1 with NaOH. Twenty mg of Na_2HPO_4 and 100 mg of KH_2PO_4 were added to the solution along with 45 ml of acetone. The fixative was adjusted to a pH of 6.6 with 1 N HCl, placed on ice, and used immediately. The stain itself was a colorless liquid composed of: a) 10 mg of a-naphthyl butyrate (Sigma Chemical Co., St. Louis, Mo.) in 0.5 ml ethylene glycol monomethyl ether (Baker Chemical Co., Phillipsburg, N.J.), b) 9.5 ml M/15 phosphate (Sorenson's buffer pH 6.3), c) 0.05 ml hexazotized paranosanilin. The a-naphthyl butyrate came in a liquid form, 1 µl being equivalent to 1.2 mg. Sorenson's buffer was made to the exact pH using 77 ml of a KH_2PO_4 solution and 23 ml of a Na_2HPO_4 solution. To obtain a hexazotized pararosanilin solution, 20 ml of distilled water was mixed with 5 ml of concentrated HCl and one gram of pararosanilin hydrochloride (Sigma Chemical Co., St. Louis, Mo.). To increase solubility the pararosanilin was warmed gently and agitated continuously. It was then mixed for one minute with an equal volume of a freshly prepared 4% solution of sodium nitrate.

Leucocyte smears could be stored before fixation for as long as two weeks, but after fixing at 4 C for 30 seconds, slides were washed gently, air dried, and immediately incubated for 45 minutes in a room temperature solution of stain. After incubating, washing and air drying, slides were counter-stained for 1-2 minutes with a 1% solution of methyl green. Again, slides were gently washed, air dried and mounted in preparation for observation under an oil immersion lens. Macrophages appeared pink or red against a blue background of lymphocytes.

An alternate assay used for detection of NA cell contamination by macrophages was the scanning of tissue culture plates for the presence of adherent cells. Microscopic examination of the second plating detected almost no adherent cells, indicating that the previous plating had efficiently removed most of the contaminating macrophages.

d. Lymphocyte Activity in Splenic Subpopulations from Normal and Tumor-Bearing Animals. Using the MLR and cell separation techniques described previously, an experiment was designed to compare the degree of reactivity in adherent, nonadherent and whole spleen cell populations from normal animals and those with a one or two week old palpable tumor.

2. Macrophages

a. Preparation of Macrophages. Following the procedure of Folch et al. (54), mice were injected I.P. with one ml of thioglycolate broth. This served as a non-immunogenic irritant which attracted macrophages to the peritoneal cavity but did not "arm" them or activate release of LAF (42, 63). However, thioglycolate stimulated macrophage lysosomal activity and enhanced their viability.

Four days after thioglycolate injection, mice were exsanguinated and macrophages removed by peritoneal lavage using a pasteur pipette and 5 ml of warmed (37 C) complete medium. Macrophages were centrifuged at 400 x g for five minutes, and resuspended in 3.0 ml of complete medium. These solutions were pipetted onto plastic tissue culture plates and incubated at 37 C for 55 minutes. At the end of this time, plates were vigorously washed with 25 ml of HBSS to remove contaminating T cells from the adherent macrophages. The macrophages were dislodged with a rubber policeman and suspended in 3.0 ml of complete medium. Cells

were then centrifuged for 5 minutes at 400 x g and pellets resuspended in 0.8 ml of complete medium. To inactivate macrophages, all tubes received 0.1 ml of Mitomycin C (400 µg/ml); half received, in addition, 0.05 ml of guinea pig complement and 0.05 ml of anti-theta serum (20 mg/ml) (Microbiological Assoc., Bethesda, Md.). All tubes were then incubated in a 37 C water bath for 45 minutes, centrifuged at 400 x g, washed twice with 15 ml of HBSS, and resuspended in 1.0 ml complete RPMI medium.

Counting was done with a Coulter counter, but because of the small number of cells involved, the initial 1:20 dilution was omitted. Instead, the diluter aspirated cells directly from the "stock" preparation. Therefore, after Coulter counting, calculation of cell concentration/ml was made by multiplying the average Coulter count value by 1000 instead of 20,000. After counting, macrophages were diluted to concentrations ranging from 2×10^3 cells/0.05 ml (1% of lymphocyte population) to 1.6×10^4 cells/0.05 ml (8% of lymphocyte population). Prior to dispensing macrophages into microculture plates, viability was assessed using the trypan blue dye exclusion test.

b. Macrophage Mediated Non-Adherent Cell Response to Allogeneic Cells. Having obtained macrophages and NA cells from normal animals, a dose response experiment was set up to determine the relationship between macrophage concentration and the ability to modulate normal NA cell reactivity.

c. Macrophage Addition to NA Cells from Normal and One Week Palpable Tumor-Bearing Mice. Kinetic experiments were undertaken to determine the effects of tumorigenesis on the CMI response, as

measured by MLR reactivity. The assay was designed to compare degree of NA cell activity in normal and one week palpable tumor-bearing animals. Macrophages from normal and one week palpable tumor-bearers were assessed for their ability to modulate reactivity in the NA cell population.

Prior to incubation with NA cells, half the macrophages were exposed to anti-theta serum plus complement, in an attempt to determine if the antiserum affects the stimulatory ability of macrophages.

d. Normal Macrophage Addition to Lymphocytes from Normal and Two Week Palpable Tumor-Bearers. This experiment compared the lymphocyte reactivity of normal animals to that of two week palpable tumor-bearing animals. No comparisons were made between macrophages from normal and two week palpable tumor-bearing mice, though a dose response curve was set up to compare "normal" and "tumor" lymphocyte reactivity upon exposure to varying concentrations of macrophages.

e. Normal and Tumor Macrophage Addition to NA Cells from Two Week Palpable Tumor-Bearers. This experiment's comparison was not between "normal" and "tumor" NA cells, but between macrophages from normal or two week palpable tumor-bearers. Differing macrophage concentrations were used to stimulate (or depress) NA cell response in two week palpable tumor-bearers.

f. Obtaining Macrophage Supernatants. The protocol for obtaining supernatants was done according to the method of Erb and Feldmann (42). A 3.2×10^6 concentration of macrophages (treated or untreated with anti-theta) was suspended in 6 or 9 ml of complete medium. Half the plates were incubated with 3.2×10^6 inactivated C_3H cells (S_m) while the

remaining plates received an equal number of inactivated Balb/c lymphocytes (R_m). In addition to using macrophages from tumor-bearing and normal mice, macrophage cultures were also prepared using allogeneic cells from C_3H mice. Table 1 on the following page shows the various combinations.

Incubation of cultures with FCS, though stimulating lysosomal activity, did not activate LAF production (63). Erb and Feldmann (42) showed no difference in supernatants taken from macrophages cultured with or without serum. Addition of FCS, however, enhanced viability over the four day incubation period. Following incubation in humidified 37 C, 5% CO_2 incubator, supernatants were harvested with a pasteur pipette, and centrifuged for 15 minutes at 20,000 x g. The supernatants were poured into the barrels of 12 ml syringes and sterilized by passage through 0.45 μ (Gelman) filters (A. H. Thomas Co., Phila., Pa.). To assay for bacterial contamination, one drop of each supernatant was incubated for 24 hours in thioglycolate broth. Contaminated supernatants were discarded. Fifty μl samples of the sterilized supernatants, concentrated or diluted 1:1 in complete media, were immediately dispensed into microculture plates.

The entire procedure used to obtain macrophages and macrophage supernatants is schematically diagrammed in Figure 2. A typical distribution of responder cells, stimulator cells and macrophages into the wells of a microculture plate is depicted in Figure 3.

g. Addition of Normal and Tumor Macrophage Supernatants to Lymphocytes from Normal and One Week Palpable Tumor-Bearing Animals. This experiment compared the activity of macrophage supernatants (not whole

TABLE I

DERIVATION OF MACROPHAGE SUPERNATANTS

"Tumor" macrophages + R_m	"Normal" macrophages + R_m
"Tumor" macrophages + anti-theta serum + R_m	"Normal" macrophages + anti-theta serum + R_m
"Tumor" macrophages + anti-theta serum + S_m	"Normal" macrophages + anti-theta serum + S_m
"Tumor" macrophages + S_m	"Normal" macrophages + S_m
S_m macrophages	S_m macrophages + anti-theta serum
Control (medium minus cells)	

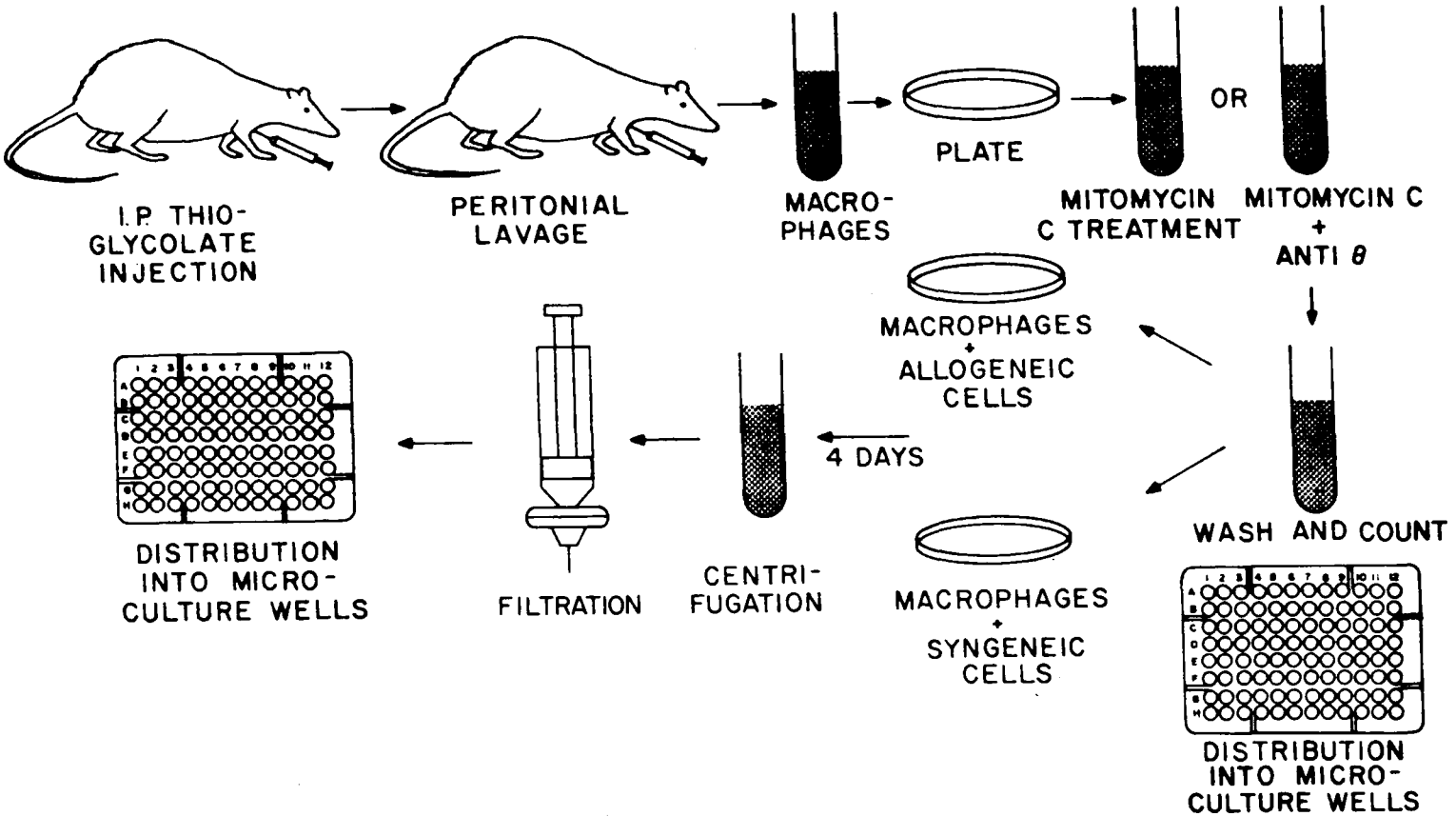
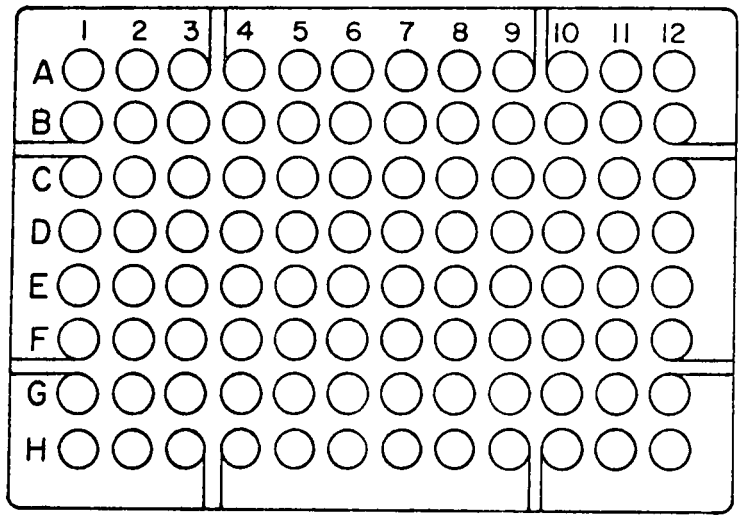


Figure 2. Protocol for Obtaining Macrophages and Macrophage Supernatants Used in Mixed Lymphocyte Reactivity Experiments.



Columns 1-6 Responders plus allogeneic cells ($R + S_m$)

Columns 7-12 Responders plus syngeneic cells ($R + R_m$)

Row A - "R" = Normal whole spleen cells before separation (BS)

Row B - "R" = Tumor whole spleen cells before separation (TuBS)

Row C - "R" = Nonadherent normal lymphocytes (NA)

Row D - "R" = Nonadherent tumor lymphocytes (Tu.NA)

Row E - "R" = Nonadherent normals plus normal macrophages (Φ)

Fow F - "R" = Nonadherent tumor plus normal macrophages (Φ)

Row G - "R" = Nonadherent normal plus tumor macrophages (Tu Φ)

Row H - "R" = Nonadherent tumor plus tumor macrophages (Tu Φ)

Figure 3. Distribution of Cells in Microculture Plate.

macrophages) derived from peritoneal exudates in normal and one week palpable tumor-bearing mice.

The NA cell reactivity was measured in both normal and one week palpable tumor-bearing populations. Measurements were also taken to show affects of anti-theta serum and/or allogeneic cells on the macrophage's ability to excrete stimulatory (or inhibitory) factors. The lymphocytes were exposed to concentrated supernatant and supernatant diluted 1:1 with complete medium.

h. Addition of Normal Macrophage Supernatants to Cells from Normal and Two Week Palpable Tumor-Bearers. A dose response experiment was done to determine how lymphocytes from normal and two week palpable tumor-bearing animals responded to normal macrophage supernatants. Comparisons were also made between reactivity in whole spleen cells and NA cells. This supernatant was derived from macrophages incubated in 9.0 ml of complete media, not 6.0 ml as used in the previous supernatant experiment.

i. Addition of Normal Macrophage Supernatants to NA Cells from Two Week Palpable Tumor Bearers. The following experiment was similar to the one just described. Various doses of macrophage supernatant were added to NA cells from two week palpable tumor-bearers to assess the ability of the NA cells to recover from in vivo exposure to a two week old tumor.

IV. RESULTS

A. Verification of Nonadherent Cell Purity

1. Lymphocyte Stimulation by PHA.

Because of its properties as a specific T cell mitogen, a PHA dose response experiment was run to determine the degree of T cell homogeneity in the NA population. The dose response curve for PHA is shown in Figure 4. Stimulation was recorded as: CPM's of responder cells plus PHA - CPM's of control . At optimal PHA concentration (.5 µl/well), NA cell stimulation was more than four times that of the whole spleen cell preparation. Nylon adherent cells showed negligible stimulation at .5 µl concentration and inhibition at higher concentration ranging from 1 µl to 4 µl. Whole spleen cells, as well as NA cells, exhibited a decreased stimulation as PHA concentration increased. At a concentration of 4 µl per well PHA totally inhibited whole spleen cell reactivity while causing a decrease in NA cell activity from a high of 12 x 10^3 to 3.2 x 10^3 CPM's.

2. B Cell Contamination.

Levels of B cell and macrophage contamination in the NA population are summarized in Table 2.

a. Lymphocyte Stimulation by LPS. To ascertain the purity of the NA cell preparation, a dose response experiment was set up, using the specific B cell mitogen, Lipopolysaccharide from E. coli 026:B6 (LPS). An optimal dose of 10 µg LPS per well registered 15,114 counts for the adherent B cell population. This is a 650% increase over whole spleen cell counts. After purification NA cell count fell to zero indicating a NA cell population, free from B cell contamination.

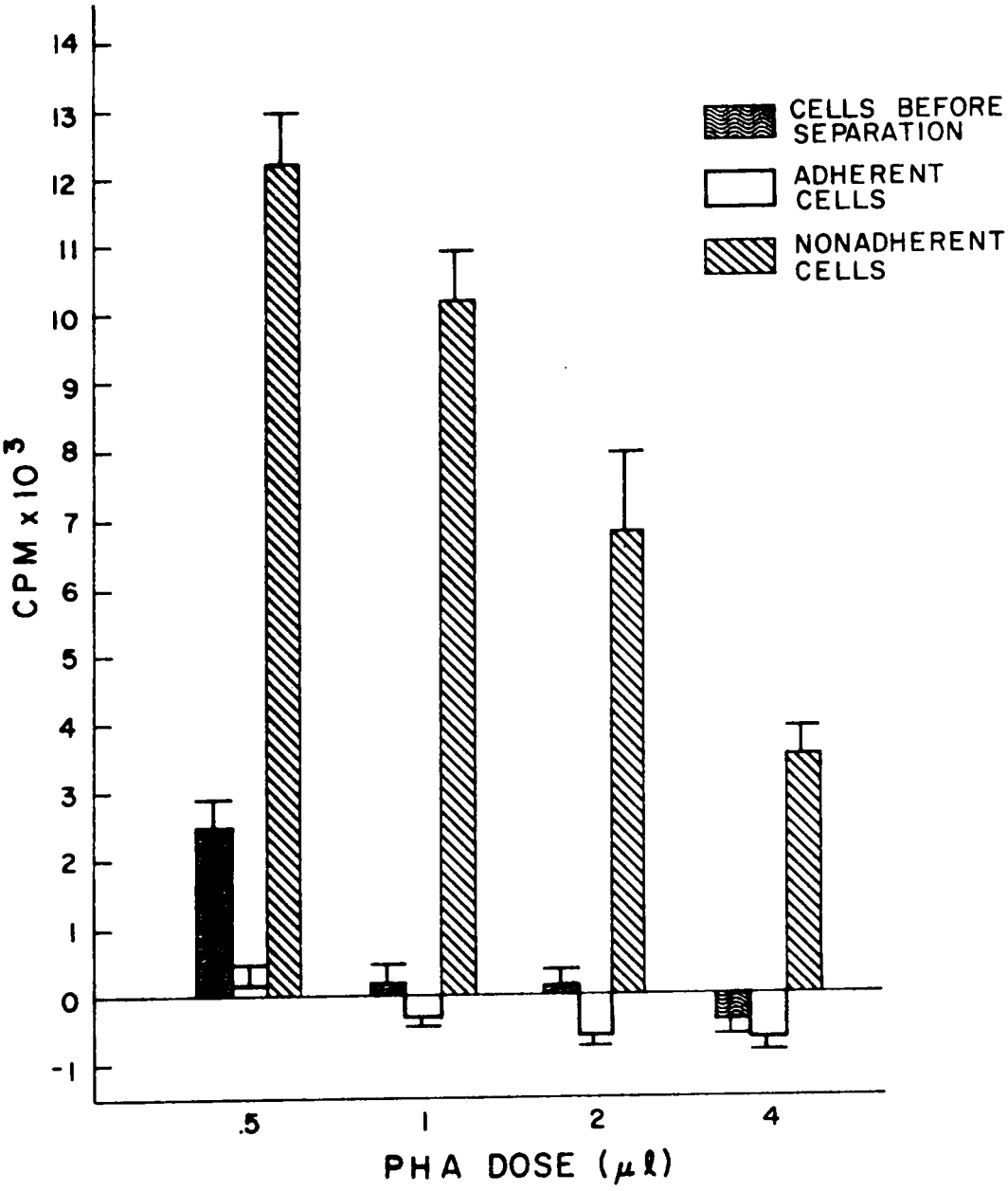


Figure 4. PHA dose response graph measuring the reactivity of normal lymphocyte subpopulations to a T cell mitogen. Whole spleen cells were denoted as cells "before separation." Effluent cell population washed through the nylon wool column was designated as the "nonadherent" population. Cells retained on nylon wool, after washing, were removed by mechanical agitation and were labeled "adherent" cells. PHA dosage was in "μl per microculture well." Activity was defined as CPM of cells plus mitogen - CPM of cell control. Six replicates were run on each sample. The standard error of the mean was calculated after discarding high and low values of each sample.

TABLE II

CHARACTERIZATION OF SPLEEN CELL SUBPOPULATIONS

Assay	Before Separation	Adherent Population	Nonadherent Population
PHA Stimulation[a] (CPM)	2440 ± 440	220 ± 140 (91% decrease)	12,300 ± 84 (404% increase)
LPS Stimulation[a] (CPM)	2014 ± 12	15114 ± 572 (650% increase)	0 (100% decrease)
Micro Jerne Plaque[b] ($PFC/10^5$ cells)	140	230 (64% increase)	0 (100% decrease)
Fluorescent Antibody[c] (Anti-mouse gamma globulin)	249:445 (56%)	31:37 (83%)	5:58 (6.9%)
Latex Particle[d] Uptake (2×10^6 cells)	112,500 (5.6%)	379,687 (18.9%)	2000 (>99.5% removed)
Esterase Staining[e]			
"Normal" Macrophages	19:937 (2%)	N.D.	2:415 (.48%)
"Tumor" Macrophages	180:658 (27%)	N.D.	4:332 (1.2%)

[a]Mitogen assays were conducted as previously described.

[b]Micro Jerne plaque assays contained 10^5 cells per microculture well. Total well was scanned to determine $PFC/10^5$ lymphocytes.

[c]Numbers in fluorescent antibody test represent ratio of fluorescent cells to total cells in a given microscope field. Ratio was calculated as the mean of three counted fields.

[d]Latex particle uptake was observed by counting a dilute concentration of cells on a hemacytometer.

[e]Esterase stain selectively colored macrophages red against a background of blue lymphocytes. Six microscope fields on each slide were observed to determine the mean ratio between macrophages and total cells in the field. All results are of triplicate experiments.

b. Jerne Plaque Assay. The micro Jerne plaque assay, registering B cell contamination in plaque forming cells (PFC) per 10^5 cells, detected no plaques in the NA cell preparation. Whole spleen cell plates contained 140 PFC/10^6 cells compared to the 230 PFC/10^6 cells in the adherent population. The adherent population showed a 64% increase in the number as FPC's when compared with the whole spleen cell preparation.

c. Fluorescent Antibody Study. As measured by the number of fluorescent cells per total number of cells, NA cell contamination by B cells was 6.9% as compared with 56% and 83% fluorescing cells present in whole spleen and adherent cell preparations.

3. Macrophage Contamination

a. Latex Particle Uptake. Incubation of latex particles with 2 x 10^6 cells resulted in less than 1% particle uptake in the NA cell population, as compared to an 18.9% and a 5.6% macrophage contamination in the respective adherent and whole spleen cell preparations.

b. Esterase Staining. Macrophage contamination of lymphocyte preparation from normal and tumor-bearing animals was assessed using a specific macrophage stain. Samples containing whole spleen and NA cell preparations were fixed onto slides and exposed to the stain. Six fields on each microscope slide were examined and the average ratio of macrophages to total cells was calculated. Normal whole spleen cell preparation contained a 2% concentration of macrophages while macrophages from a two week palpable tumor-bearer made up 27% of the leucocyte population. After purification macrophage contamination of the NA cell population fell to 0.48% for normal animals and 1.2% for tumor-bearing animals.

B. Lymphocyte Activity in Splenic Subpopulations From Normal and Tumor-Bearing Animals.

Figure 5 indicates that patterns of MLR activity differ significantly, depending on the source of the splenic subpopulations. Stimulation was expressed as a difference:

responder cells	+	inactivated allogeneic cells	-	responder cells	+	inactivated syngeneic cells

Whole spleen cell suspensions from animals with one week old palpable tumors had an 80% increase in activity over whole spleen cells from normal animals. Whole spleen cell activity in two week palpable tumor-bearers fell almost to zero.

Adherent populations from tumor and non-tumor-bearing animals exhibited no significant activity as did the NA cells from normal and two week palpable tumor-bearers. NA cells from one week palpable tumor-bearers showed an activity 25% higher than normal whole spleen cells but 33% less than the whole spleen cell activity from one week palpable tumor-bearing hosts. Activity in NA cells from one week palpable tumor-bearers was more than double that exhibited by whole spleen cells from animals with a two week palpable tumor.

C. Macrophage Mediated Non-Adherent Cell Response to Allogeneic Cells

As seen in Figure 6, without macrophage addition, normal NA cell response showed an activity of less than 500 CPM. Upon addition of an optimal 2% concentration of normal macrophages, the response rose to 5800 CPM, almost triple the recorded response for normal whole spleen cell suspensions. As the percentage of macrophages increased, the level of NA cell activity decreased, until, at 8% macrophage concentration,

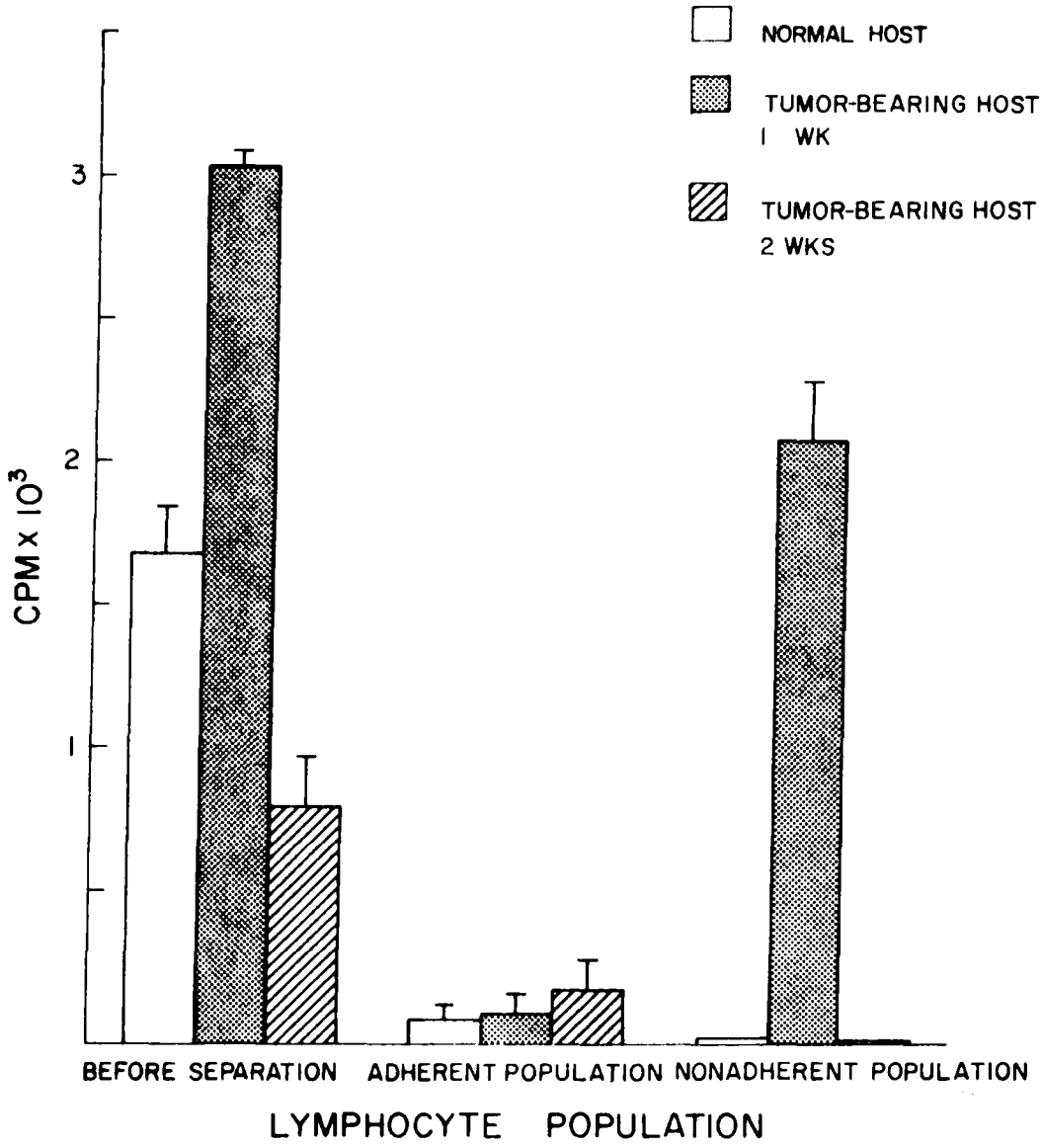


Figure 5. Lymphocyte activity in splenic subpopulations from tumor and and non-tumor-bearing animals. Animals designated as "one week or two-week nontumor-bearing hosts" were sacrificed no sooner than 7 or 14 days after detection of a palpable tumor. Activity was defined as CPM of 2×10^5 responder (R) cells + 4×10^5 Mitomycin C treated stimulator (S_m) cells - 2×10^5 R cells + 4×10^5 Mitomycin C treated R cells (R_m) .

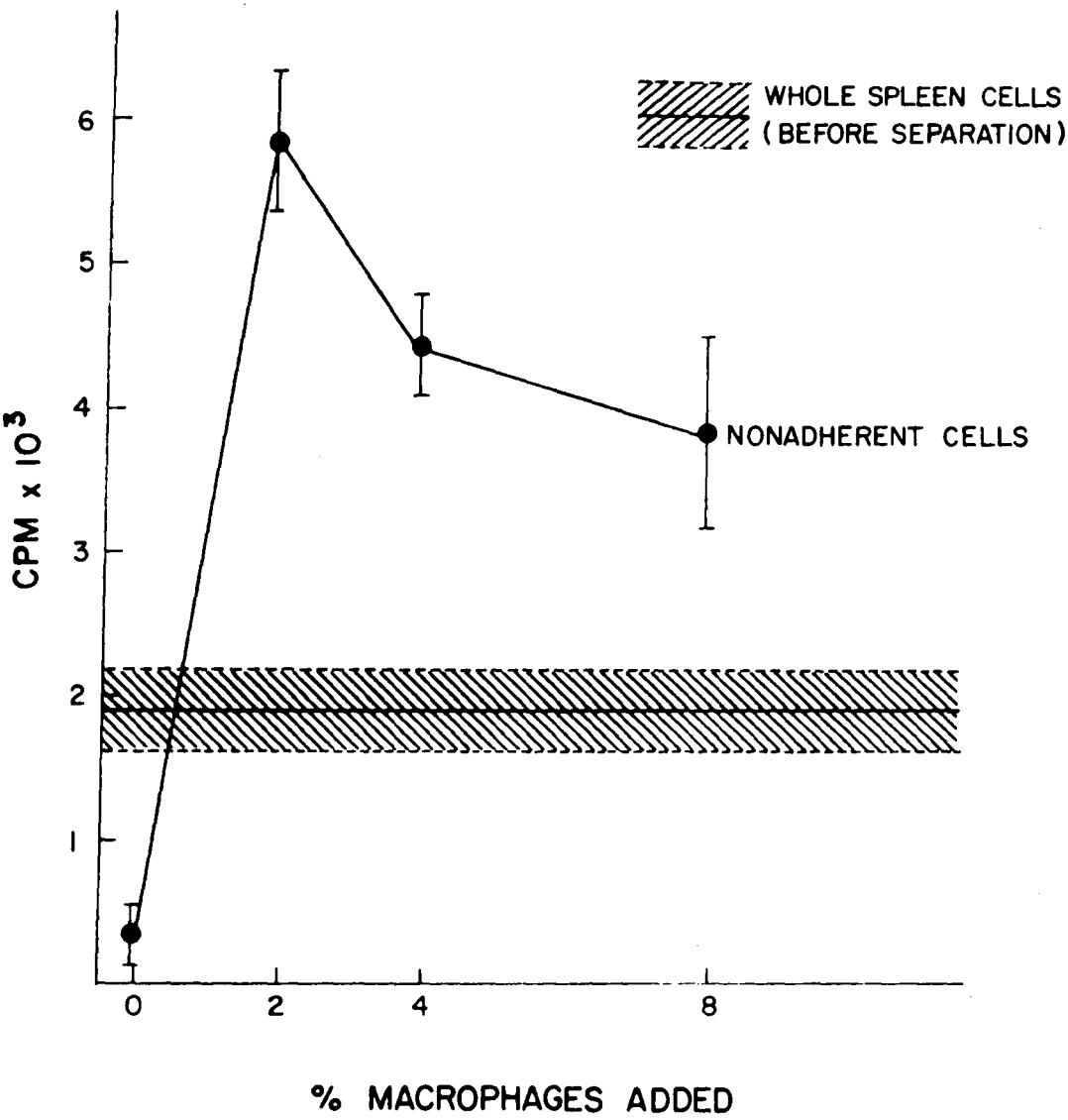


Figure 6. Macrophage mediated nonadherent cell response to allogeneic cells. Cross hatched bar represents the level of stimulation in normal whole spleen cell preparations, prior to treatment over a nylon wool column. Nonadherent cell activity (NA) was measured after addition of various concentrations of normal macrophages (Φ).

the NA cell activity decreased to 3900 CPM, a 30% decrease in reactivity.

D. Macrophage Addition to NA Cells from Normal and One Week Palpable Tumor-Bearing Mice

In Figure 7, 3% concentration of macrophages from normal and one week palpable tumor-bearers was added to NA cells from nontumor-bearing mice and those with one week old tumors. Addition of macrophages to purified NA cell populations when compared to whole spleen cell preparations increased MLR activity 2.5 times in cells from both normal and tumor-bearing animals response. Purified NA cells from tumor-bearers, when compared to tumor-bearing whole spleen cells, had only a 20% decrease in response whereas normal whole spleen cells suffered a 60% loss of activity after purification. This data is supported by results from the macro MLR assay.

Macrophage enhancing activity was not dependent on the presence of complement and anti-theta serum, nor did macrophage source, whether it be from normal or one week palpable tumor-bearers, significantly affect the enhancing ability of macrophages.

E. Normal Macrophage Addition to Lymphocytes from Normal and Two Week Palpable Tumor-Bearers

Purified NA cells exhibited no response to allogeneic cells. The Eighth Figure showed that addition of an optimal 2% concentration of macrophages doubled the NA tumor-bearing response and increased NA nontumor-bearing response two and half times when compared to counts from their original whole spleen cell preparations. Addition of macrophages to original whole spleen cell preparations did not enhance MLR activity, and in the case of normal animals, activity was totally repressed at a

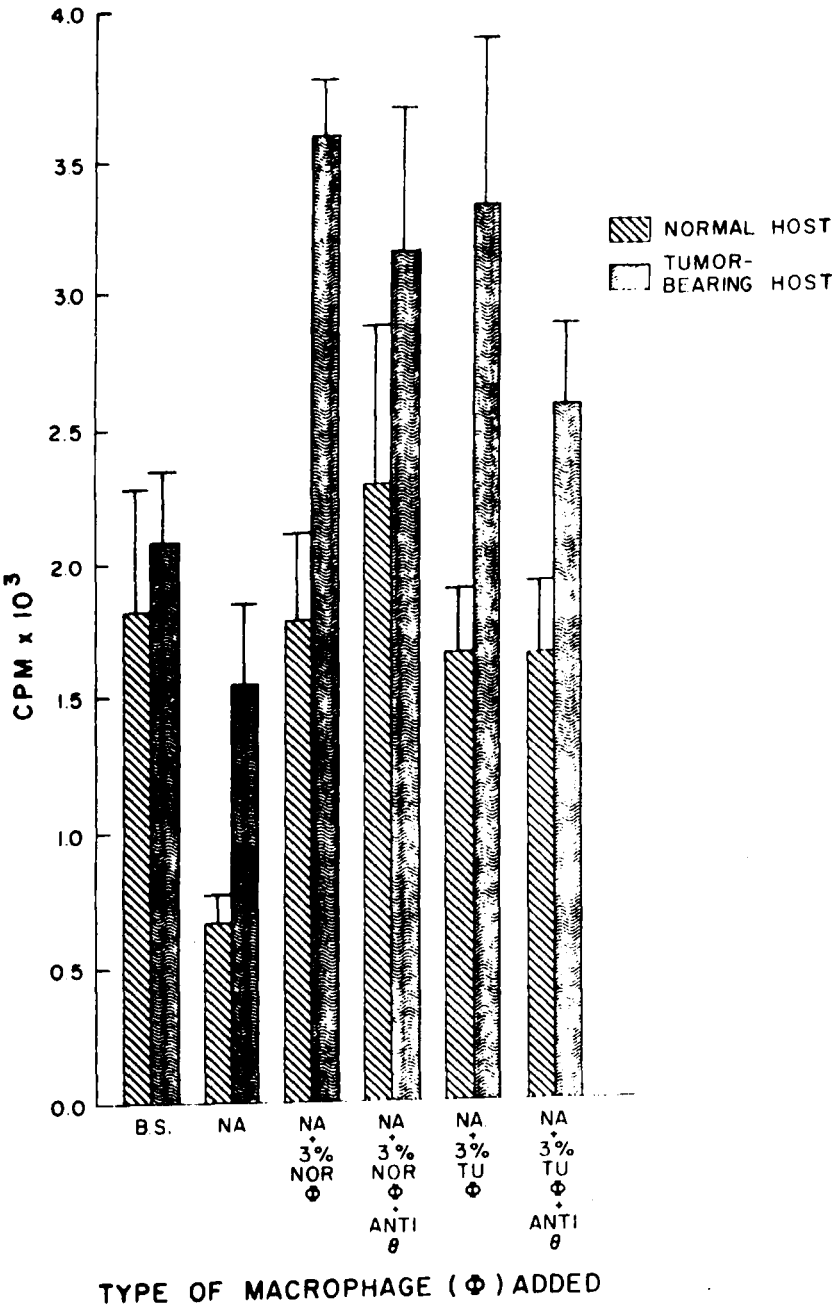


Figure 7. Macrophage addition to NA cells from normal and one week palpable tumor-bearing mice. Whole spleen cells before separation (B.S.) were not exposed to any additional in vitro concentration of macrophages (Φ). Nonadherent (NA) cells, without additional macrophages, served as a control group. All macrophages were treated with Mitomycin C. Half were exposed for 30 minutes to anti-theta serum plus complement. All spleen cells and macrophages were taken from normal (NOR) animals or those with a one week palpable tumor (TU).

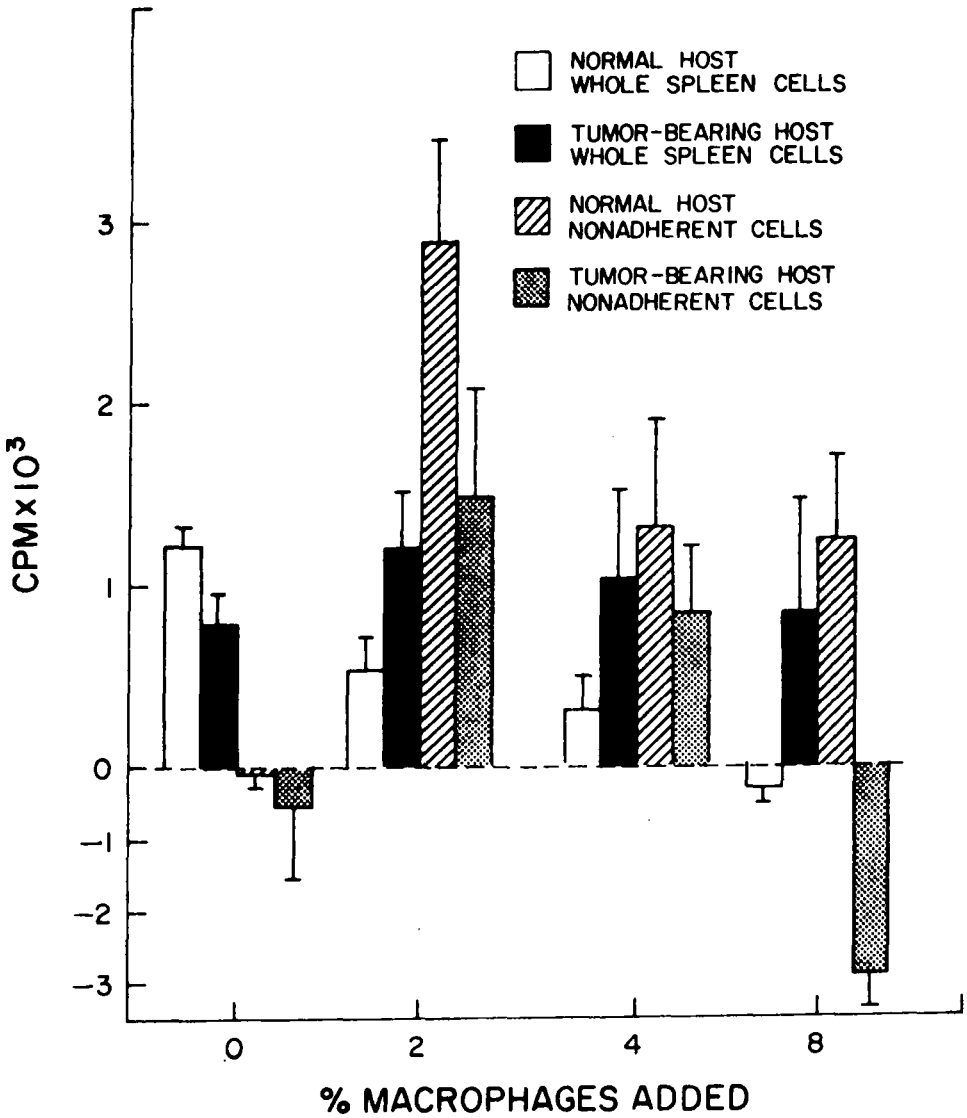


Figure 8. Normal macrophage addition to lymphocytes from normal and two week palpable tumor-bearers. Whole spleen cells from normal and tumor-bearing animals were exposed to various concentrations of normal macrophages. Negative CPM denotes more activity in the control group ($R + R_m$) than in the experimental group ($R + S_m$)

high macrophage dose. While not completely inhibiting normal NA activity, high doses (8%) of macrophages completely inhibited MLR activity in the NA tumor-bearing population and caused normal NA cell activity to fall 50% from the peak activity registered at a 2% concentration of macrophages.

F. Normal and Tumor-Bearing Macrophage Addition to NA Cells from Two Week Palpable Tumor-Bearers

Without macrophage addition, NA cells from two week palpable tumor-bearing animals showed no MLR activity. The graph shown in Figure 9 indicates that an optimal 2% dose of normal macrophages increased the NA cell response to 2100 CPM, while the same macrophage concentration from tumor-bearers increased counts only to 1300 CPM's. When 8% "normal" macrophages were added to NA cells from two week palpable tumor-bearers the allogeneic response was one-third of that elicited by 2% "normal" macrophages. An 8% concentration of "tumor" macrophages completely repressed NA cell response in two week palpable tumor-bearers.

G. Addition of Normal and Tumor Macrophage Supernatants to Lymphocytes from Normal and One Week Palpable Tumor-Bearing Animals

Contrary to previous experiments, results given in Figure 10 and 11 show that lymphocytes from normal animals exhibited significant activity even after removal of 99.5% of the macrophages. This was attributed to non-specific stimulation from a new lot of fetal calf serum. When the concentration of FCS in the media was changed from 10% to 5%, this non-specific stimulation was reduced, as shown in Figure 7. As in previous graphs, NA cell activity was noticeable in the one week palpable tumor-bearers. In this assay, supernatants were derived from macrophages in both normal and one week palpable tumor-bearing hosts. Concentrations of 1.6×10^6 macrophages per 6 ml were exposed to various combinations of

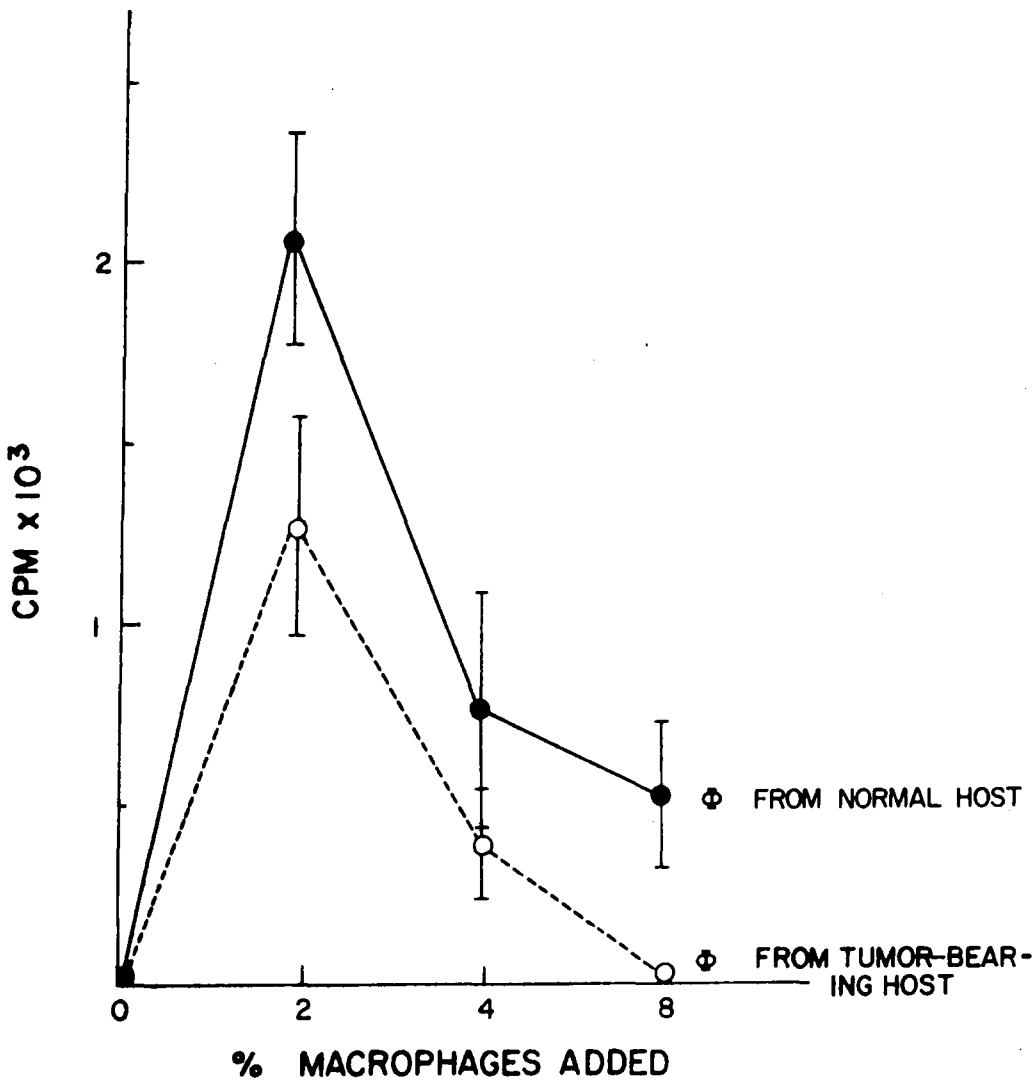


Figure 9. Normal and tumor macrophage addition to NA cells from two week palpable tumor-bearing animals.

●— Activity of NA cells after addition of macrophages (Φ) from normal host.

o--- Activity of NA cells after addition of macrophages from a host with a two week palpable tumor.

Figure 10. Addition of normal and tumor macrophage supernatants to lymphocytes from normal and one week palpable tumor-bearing animals. All spleen cells and macrophages (Φ) were taken from normal (NOR) or those with a one week palpable tumor (TU).

B.S. = Whole spleen cells unexposed to additional macrophage supernatants

N.A. = 2×10^5/.2 ml nonadherent cells unexposed to additional macrophage supernatants

NA + NORΦ or TUΦ = 2×10^5/.15 ml nonadherent cells plus .05 ml of macrophage supernatants

NA + NORΦ or TUΦ + Antiθ = 2×10^5/.15 ml nonadherent cells plus .05 ml of supernatants from macrophages exposed for 30 minutes to anti-theta serum plus complement.

NA + NORΦ or TUΦ + antiθ + C3H = 2×10^5/.15 ml nonadherent cells plus .05 ml of supernatants from macrophages non only exposed to anti-theta serum but incubated for four days with allogeneic whole spleen cells.

NA + NORΦ or TUΦ + C3H = 2×10^5/.15 ml nonadherent cells plus .05 ml supernatant from macrophages incubated with allogeneic C3H lymphocytes but unexposed to anti-theta serum

In all cases supernatants were derived from 3.2×10^6 Mitomycin C treated stimulator (S_m) or responder (R_m) cells. All cultures in microculture wells also contained 4×10^5 S_m cells. Fig. 10 depicts results using undiluted concentrations of supernatant while Fig. 11 shows results using supernatants diluted 1:1 with complete RPMI media.

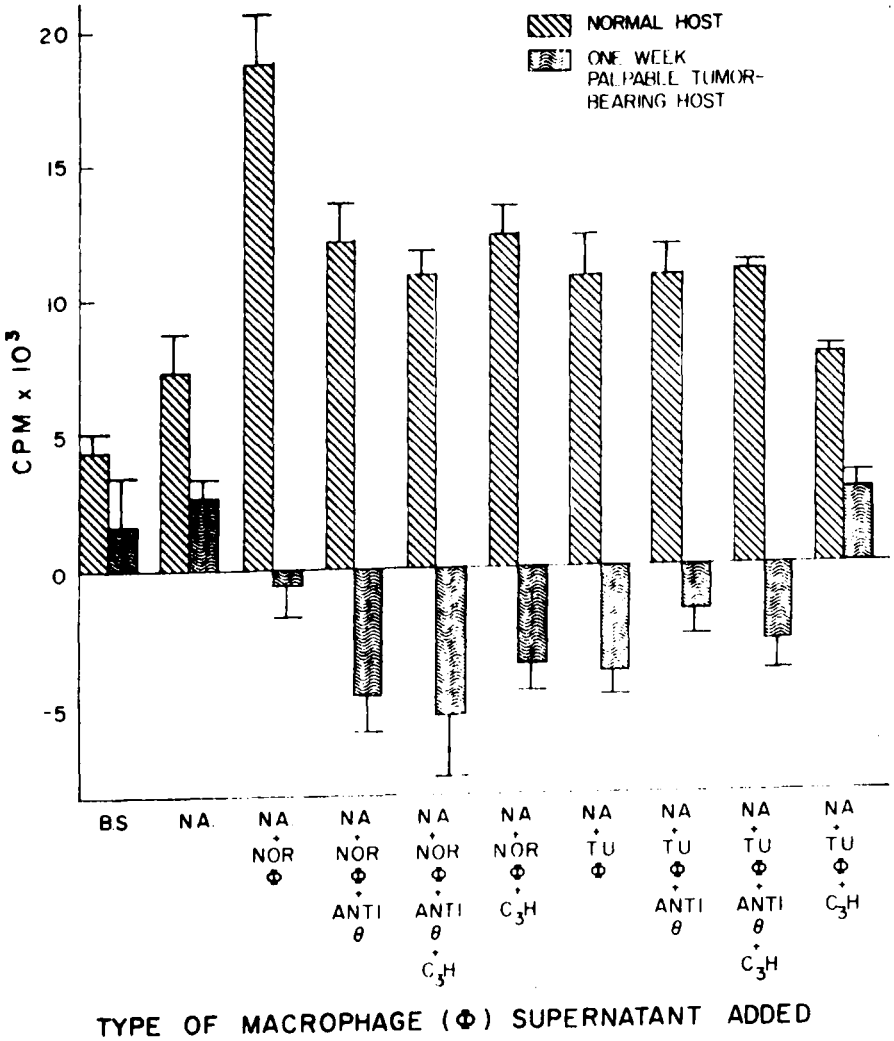

NORMAL HOST
ONE WEEK PALPABLE TUMOR-BEARING HOST
CPM x 10³
20
15
10
5
0
-5
BS
NA
NA + NOR Φ
NA + NOR Φ + ANTI θ
NA + NOR Φ + ANTI θ + C3H
NA + NOR Φ + C3H
NA + TU Φ
NA + TU Φ + ANTI θ
NA + TU Φ + ANTI θ + C3H
NA + TU Φ + C3H
TYPE OF MACROPHAGE (Φ) SUPERNATANT ADDED

Figure 10.

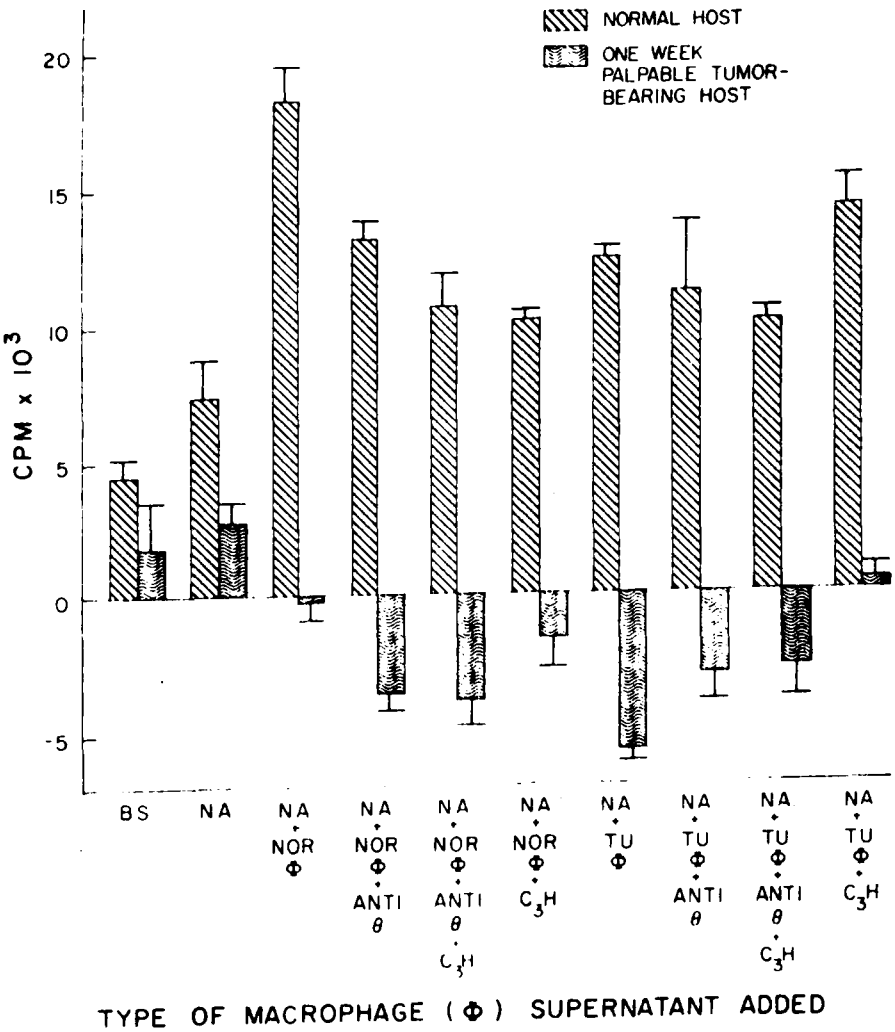


Figure 11. Legend as in Figure 10.

serum and allogeneic C_3H cells. The high levels of non-specific stimulation did nothing to obscure the twofold enhancement of normal NA cell activity upon addition of normal and tumor macrophage supernatants. Supernatants had just the opposite effect on NA cells from tumor-bearers. In all cases but one, supernatant addition depressed NA cell response to allogeneic cells. Supernatants from "tumor" macrophages plus C_3H cells, added to tumor NA cells, caused neither enhancement or depression of the allogeneic response. When normal NA cells were exposed to normal macrophages, supernatant lymphocyte activity increased appreciably over activity due to other macrophage preparations. Enhancement of normal NA cell response and depression of NA tumor-bearing cell response to allo-antigens did not depend on (a) source of macrophage (normal or one week palpable tumor-bearers), (b) exposure to complement plus anti-theta serum, (c) contact with allogeneic C_3H cells, (d) dilution of supernatant (concentrated or 50%).

H. Addition of Normal Macrophage Supernatants to Cells from Normal and Two Week Palpable Tumor-Bearers

Figure 12 demonstrates that nonadherent cell MLR activity without macrophages was very low, though whole spleen cell populations from normal and tumor-bearing animals had counts of 2900 and 1600 CPM's, respectively. Supernatants were derived from 1.6×10^6 macrophages per 9.0 ml. At 100% supernatant concentration, normal cells had an optimal activity of 4500 CPM while the tumor-bearing cells showed activity of only 500 CPM. At optimal supernatant concentration, normal NA cell counts were 50% higher than whole spleen cell counts. This contrasted sharply with the NA cell counts from two week palpable tumor-bearers

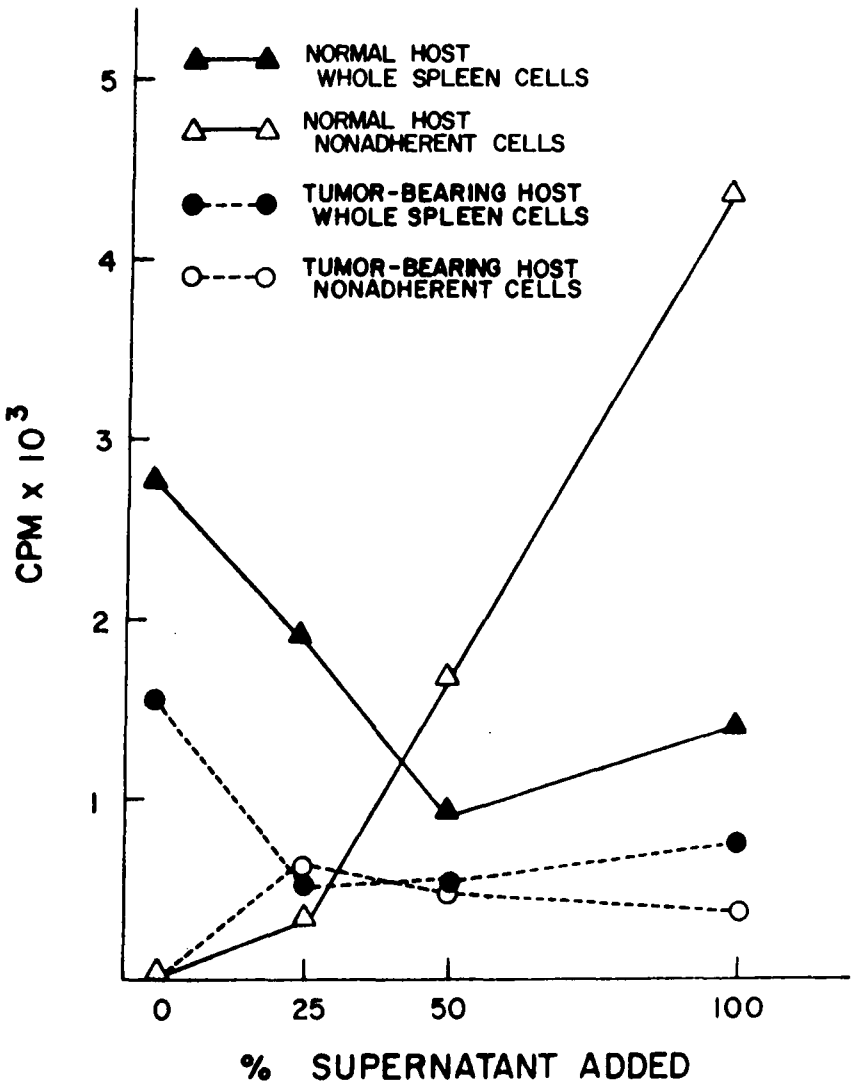


Figure 12. Addition of normal macrophage supernatants to cells from normal and two week palpable tumor-bearers. All normal macrophage supernatants were derived from a four day incubation of 3.2×10^6 macrophages in 9 ml of complete media. Dilutions of supernatant were also made with complete media. Macrophages were not incubated with additional allogeneic or syngeneic cells or anti-theta serum plus complement. Standard error is not shown in the graph but was less than 15%.

which, at optimum stimulation, were 50% lower than their corresponding whole spleen cell counts. At all supernatant concentrations, any addition of supernatant to normal or tumor-bearing whole spleen cell preparations depressed spleen cell activity.

I. Addition of Normal Macrophage Supernatants to NA Cells from Two Week Palpable Tumor-Bearers

In Figure 13, NA cells, free from macrophages, registered 50% less activity than whole spleen cells. Upon addition of an optimum 100% concentration of normal macrophage supernatant (1.6×10^6 macrophage/9 ml), "tumor" NA cells doubled their activity, resulting in counts approaching those of whole spleen cell preparations.

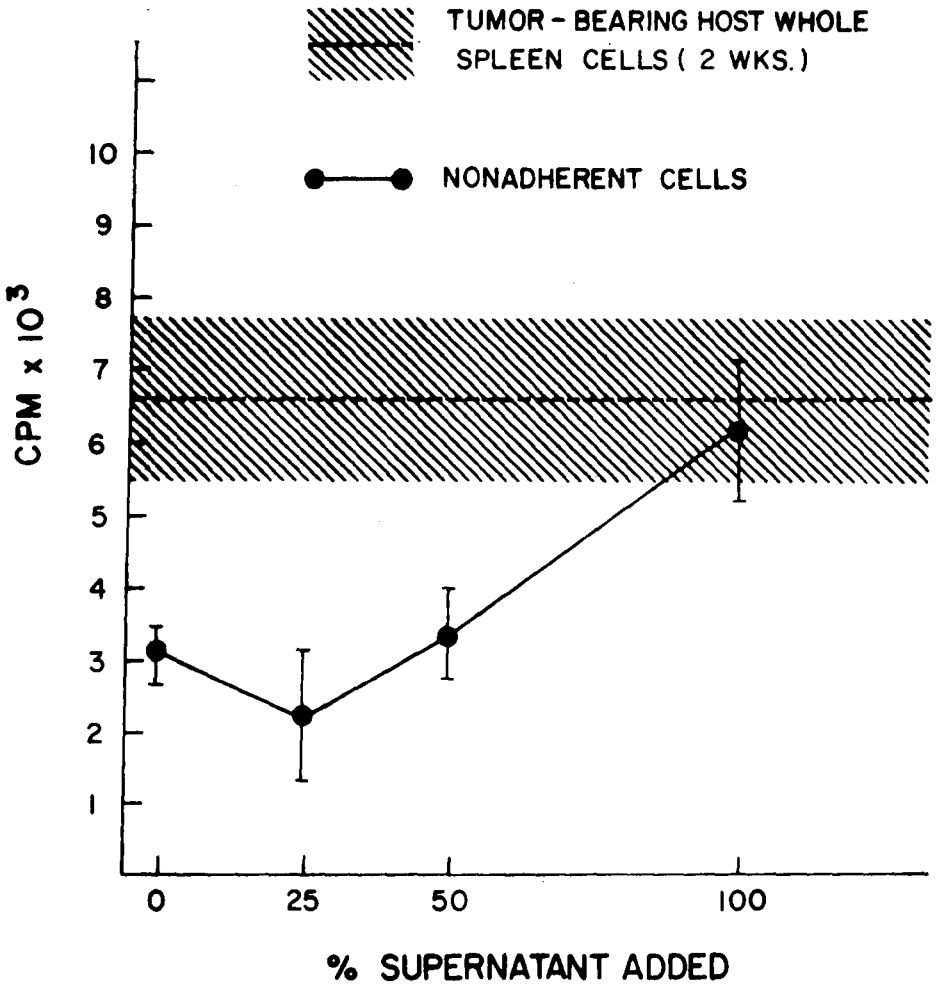


Figure 13. Addition of normal macrophage supernatants to NA cells from two week palpable tumor-bearers. Normal macrophages were incubated in 9 ml of lymphocyte-free complete RPMI media. Shaded bar represents activity of whole spleen cells from mice with two week palpable tumors.

V. DISCUSSION

The MLR assay and accompanying column purification techniques were used to assess "normal" and "tumor" T cell modulation by differing concentrations of macrophages and macrophage supernatants. To draw conclusions concerning purified T cell eluants, it was necessary to establish proof that the NA population was of T cell composition.

PHA is a mitogen which, when mixed with a heterogenous whole spleen cell population, stimulates T cells (36, 37). Controversy has arisen over PHA stimulation of macrophage free, purified T cell suspensions. Working with guinea pig lymph node cells, Rosenstreich et al. (160) felt there was an "absolute macrophage dependence of T lymphocyte activation by mitogens." Oppenheim et al. (147), Levis and Robbins (114), Gery et al. (62) felt that lack of macrophages diminished the T cell response to suboptimal PHA concentrations but did not entirely alleviate the response. At optimal PHA concentrations, maximum T cell stimulation was macrophage independent (136, 147) though excessive PHA concentrations produced T cell inhibition (136). Our studies with mice showed a higher level of PHA stimulation in T cells than in whole spleen cell preparations, an indication that column purified eluants were T cell enriched. Rosenstreich et al. (160) attributed PHA stimulation of T cell populations to "incomplete macrophage depletion." Latex particle uptake (70), esterase staining (169), and tissue culture plate counting revealed a macrophage contamination of less than .5%.

Use of fluorescent antibodies directed against B cell immunoglobulins (Ig) revealed a 6.9% B cell contamination of T cell preparations,

correlating well with the 5% literature value (70, 91, 176). The micro Jerne plaque assay revealed no B cell contamination.

A small degree of B cell contamination does not affect T cell MLR activity since allogeneic recognition is a cell-mediated response (3) and studies showed that addition of anti-Ig plus complement did not depress T cell response to alloantigens.

Having assured that the NA eluants were relatively free from contaminating B cells and macrophages, the T cell response to histoincompatibility antigens was tested. Lack of NA cell response in normal animals indicated a necessity for the presence of an adherent population retained on the nylon wool column. This adherent cell was not a B cell since B cell enriched adherent populations showed uniformly low levels of stimulation. Preliminary tests on whole spleen cell preparations indicated no loss of stimulation when exposed to anti-Ig plus complement, though total inactivation occurred upon administration of anti-theta serum plus complement. The synergistic adherent cell in the whole spleen cell preparation was a non-lymphoid leucocyte generally categorized as a macrophage, since Levy and Wheelock (117) and Erb et al. (42) have shown that selective macrophage removal with carageenan or iron fillings resulted in loss of whole spleen cell reactivity.

In contrast to normal T cell inactivity, cells from animals with one week palpable tumors exhibited hyper-reactivity to allogeneic cells. This is in agreement with the work of Paranjpe and Boone (149), Bhatnagar et al. (16) and Jurin and Suit (92). This could be attributed to direct in vivo T cell activation by the tumor or indirect T cell stimulation through LAF release from tumor activated macrophages. Inactivation of NA

cells from two week palpable tumor-bearers could signify direct "tumor" T cell cytotoxicity or inactivation mediated by tumor stimulated macrophages.

Normal T cell allogeneic response is fully restored by addition of 2% concentration of normal macrophages. This supported previous drawn conclusions concerning T cell-macrophage synergy and indicated that early tumor cell activation of T cells may be mediated by macrophages. T cells showed depression at excessive concentrations of macrophages, an indication that late tumor cell deactivation of T cells may also be mediated by an excessive concentration of macrophages. At this point, two questions can be posed: (a) How can one cell both depress and enhance lymphocyte reactivity? (b) What is the relationship between macrophage concentration and tumor growth?

Macrophages have the ability to enhance T cell activity in both normal and one week palpable tumor-bearers, though normal T cells without macrophages showed far less MLR activity than those from one week palpable tumor-bearers. It could be postulated that macrophages from one week tumor-bearers were releasing *in vivo* a greater quantity of LAF than macrophages from nontumor-bearing mice. There is indirect evidence supporting this conclusion. Holden *et al.* (82) has established a direct correlation between types of leucocytes found in the spleen and the types found in the tumor itself. Russell *et al.* (164) found a high concentration of macrophages in regressing tumors induced with Maloney sarcoma virus. The higher T cell proliferation in methycholanthrene induced tumor-bearers noted by Konda *et al.* (107) and Smith *et al.* (174, 175), could be attributed to triggering by the high number of macro-

phages present in the spleen of the tumor-bearing mouse. Initially this increased concentration of splenic macrophages releases large amounts of LAF which trigger the splenic T cell and causes them to proliferate. Using the esterase staining procedure, 14 times more macrophages were observed in spleen preparation from two week palpable tumor-bearers than in spleen preparations from normal animals. Qualitatively, there is no difference in the type or amount of soluble factor released by macrophages from normal mice and one week palpable tumor-bearers, for a 3% concentration of normal macrophages is no more or less inhibitory than a 3% concentration of macrophages from one week palpable tumor-bearers. T cell activity is not dependent on a separate T cell-macrophage complex since exposure to complement plus anti-theta serum does not affect the macrophages regulation of the T cell response to histoincompatibility antigens.

T cells from both normal and two week palpable tumor-bearers displayed a depressed MLR response. MLR activity was restored by addition of an optimal 2% concentration of normal macrophages. However, levels of T cell activity were far higher in normal animals than in two week palpable tumor-bearing animals. Though an excessive concentration of macrophages depressed normal T cell activity, it did not totally negate it, as in the case of T cells from animals with two week old palpable tumors.

What is the immunological difference between an animal bearing a one week old or two week old tumor? In the former case <u>in vitro</u> T cell response to allo-antigens was macrophage independent; in the latter case, MLR activity occurred only in the presence of macrophages. How-

ever, in contrast to macrophages from one week palpable tumor-bearers, macrophages from two week palpable tumor-bearers showed an increased ability to inhibit the T cell allogeneic response, when compared with macrophages from normal animals. Therefore, the tumor in its late stages may influence the type, as well as the amount, of soluble factors released by macrophages from two week tumor-bearing hosts. As in previous experiments, excessive concentrations of macrophages depressed the T cell response in both normal and two week palpable tumor-bearers. Macrophage activation of T cells was not contact dependent but could be mediated by cell free supernatants. Supernatants from both normal and one week palpable tumor-bearers inhibited tumor-bearers allogeneic response and enhanced MLR activity in normal animals. Furthermore, macrophage regulation was not influenced by presence or absence of allogeneic cells or anti-theta serum. The supernatant exhibited saturation kinetics since a 50% dilution of supernatant when compared with concentrated supernatant did not significantly alter the degree of enhancement or inhibition in the NA cell populations from normal or one week palpable tumor-bearers.

Can one cell both enhance and depress T cell reactivity? One answer to this is yes, because a single macrophage may contain not one, but two, supernatant factors (43, 188, 189), a T cell activating factor (43, 188, 189); and a T cell inhibition factor (37, 82, 101, 102, 103, 139, 146).

Why must the suppressor cell be a macrophage; why not an adherent T cell suppressor as in the guinea pig model observed by Folch and Waksman (52)? Anti-theta serum and complement did not affect the ability

of the adherent cells to cause T cell activation (or inhibition), nor did addition of anti-Ig serum and complement. But T cell contact with greater than a 3% concentration of macrophages did cause inhibition of the allogeneic response. It was therefore concluded that the activating (or inhibiting) supernatant originated in the macrophage (a loose term for any radiation resistant, adherent, phagocytic leucocyte). Immediately the next question is raised, "why not have two subpopulations of macrophages, each releasing a single type of supernatant?" As the tumor progresses, the inhibition population proliferates, becomes dominant, and depresses T cell activity. This theory works well when comparing macrophages from normal animals to ones from two week palpable tumor-bearers, since in vitro tests with macrophages from animals with two week tumors did not enhance T cell activity to the degree that an equal concentration of normal macrophages did. The macrophage subpopulation theory is less able to explain why T cells from one week palpable tumor-bearers, unlike normal T cells, exhibit macrophage-free MLR activity. It cannot be due to an in vivo shift in the ratio of activator cell to inhibitor cell, because such a shift should make in vitro concentrations of macrophages from one week palpable tumor-bearers more able to enhance T cell activity than the same concentrations of normal macrophages. This does not occur--there is no significant difference in the enhancing ability of macrophages from normal and one week palpable tumor-bearing animals. The only way to keep the macrophage subpopulation theory is to postulate that one week tumorigenesis causes equal proliferation of the two subpopulations. In this way, ratios between "normal" and "tumor" macrophages remain the same, and an in vitro test detects no difference

between macrophages derived from normal and tumor-bearing hosts. The difference, lies in the in vivo numbers of macrophages present in the spleens of normal and one week palpable tumor-bearers. As macrophage proliferation proceeds, the activator population releases enough LAF to trigger in vivo T cell stimulation which carries over into the in vitro MLR test. Though still proliferating, the suppressor cell, after one week of tumorigenesis is not present in sufficient concentrations to counteract the activating factor released by the stimulator macrophage. This mechanism explaining macrophage activation of T cells in normal and tumor-bearing animals is no better or worse than the "single macrophage, dual soluble factor" theory. In this theory each factor is released simultaneously by macrophages but each one has a threshold level at which T cell activation or deactivation occurs. The threshold level for inhibition is higher than the threshold level for activation. In the normal animal, in vivo macrophage concentrations are too low to trigger either lymphocyte activation or depression. When artificially concentrated in vitro, macrophage levels of factor rise high enough to trigger lymphocyte stimulation upon exposure to allo-antigens. At even higher macrophage concentrations, the threshold of inhibition is reached and T cells are inhibited by the presence of macrophages and soluble inhibition factor. The mechanisms of macrophage, T cell interaction during tumorigenesis could also be explained by hypothesizing a single macrophage, secretory factor, activating at a low dose concentration and inhibiting at high dose. Initially this theory appears plausible, since various mitogens stimulate at low concentrations and repress at high

concentrations. However, current theories hold that mitogens do not directly depress receptor cells; they inactivate indirectly by triggering a suppressor cell, sensitive only to mitogens at high concentrations (54). The most concrete evidence against existence of a single activation-inhibition factor is Unanue and Calderon's (188, 189) work in the biochemical characterization of two distinct macrophage supernatants, one activating and one inhibiting T cell response to antigens.

Though there is no difference between macrophages from one week palpable tumor-bearers and those from normal animals, there is a definite macrophage distinction between animals with one week old palpable tumors and those with two week old tumors.

When comparing populations in the spleens of normal and tumor-bearing animals initially, the difference is solely one of relative numbers of macrophages. As a foreign antigen, the tumor causes macrophage proliferation (164) and an initial T cell activation due to high concentrations of activating factor. If the tumor persists, the concentration of macrophages continues to rise, resulting in threshold levels of inhibition factor. As the tumor progresses, macrophages secrete a greater percentage of inhibition factor; really an anti-proliferation factor designed to halt tumor proliferation (82, 101-103). Unfortunately, it also non-specifically inhibits lymphocyte recognition of allo-antigens. Lymphocytes have receptors for both activation and inhibition factor. Acquisition of macrophage factors is cumulative. T cells from one week palpable tumor-bearers, when compared with normal cells, possess higher levels of both activation and inhibition factor coating their cell receptors. Therefore, they are more rapidly activated by <u>in vitro</u>

exposure to additional macrophages, or macrophage supernatants. But, because of their high, but sub-threshold levels of inhibition factor, they are inhibited by macrophage supernatant concentrations which stimulate normal T cells not exposed *in vivo* to inhibition factor. Because whole spleen cell preparations are forced into *in vitro* contact with high concentrations of macrophages, their threshold for inactivation is approached (and sometimes surpassed) by *in vitro* incubation. Addition of even dilute concentration of macrophage supernatant causes the concentration of inhibition factor to surpass threshold levels and depress whole spleen cell preparations even though macrophage free T cells are activated by the same concentration of supernatant. Because inhibition is not an easily reversible phenomena, T cells from two week palpable tumor-bearers show no great enhancement of allogeneic activity upon *in vitro* exposure to any concentration of macrophage activation factor.

VI. PROPOSED MODEL FOR T CELL MACROPHAGE INTERACTION DURING TUMORIGENESIS

1. Macrophages contain not one but two supernatant factors, a T cell activation factor and a T cell inhibition factor.

2. Normal T cells are exposed *in vivo* to sub-threshold levels of both factors. They therefore exhibit no *in vitro* activation or inhibition until macrophages are added.

3. Both factors are released simultaneously by the macrophage. Activation factor is released at a greater concentration than inhibition factor.

4. Initially, *in vitro* addition of low concentrations of macrophages enhance T cells activity since inhibition factor is still at a "sub-threshold" concentration.

5. At high concentrations of macrophages, effects of activating factor are negated by the high levels of inhibition factor and the cell ceases to respond to an allogeneic stimulus.

6. As the tumor progresses macrophage concentrations in the spleen increase.

7. T cells from one week palpable tumor-bearers are activated *in vivo* by increased concentrations of macrophages in the spleen. They therefore need no *in vitro* stimulation in order to respond to allogeneic cells.

8. Macrophages from one week palpable tumor-bearers do not physiologically differ from those in normal animals. They are just present in a higher concentration.

9. Macrophages from two week palpable tumor-bearers do not activate

T cells as well as normal macrophages. They have shifted to a greater production of inhibition factor, though activating factor is still being produced to a lesser degree.

10. T cells from two week palpable tumor-bearers have been exposed to a high *in vivo* level of inhibition factor. The effects of inhibition factor are not easily reversed. They cannot easily be activated *in vitro* once they have been inhibited *in vivo*.

11. T cells from one week palpable tumor-bearers, when compared with normal T cells, have been exposed to a higher (but still sub-threshold) *in vivo* level of inhibition factor. Therefore, *in vitro* T cells from one week palpable tumor-bearers are inhibited by macrophage supernatant concentrations which activate normal T cells.

VII. CONCLUSION

1. Macrophages possess the capacity to either activate or inhibit T cell activity.

2. Activation or inhibition is due to relative concentrations of supernatant factors given off by the macrophages.

3. Tumor stimulation or repression of T cell response is due in large measure to *in vivo* concentrations of macrophage activation factor and macrophage inhibition factor.

4. Supernatant factors may arise from a single type of macrophage or be released from physiologically distinct subpopulations of macrophages.

5. Inhibition factor influencing T cells in the mouse MLR assay does not originate solely from an adherent T cell.

6. Release of macrophage factors is not affected by *in vitro* presence or absence of allogeneic cells or anti-theta serum.

7. Both factors have threshold as well as saturation levels of activation (or inhibition).

8. T cell inhibition by release of macrophage inhibition factor cannot be easily reversed.

--We at least have kept our vulgar sense of wonder.
--Steinbeck

BIBLIOGRAPHY

1. Adler, W. H., H. T. Takiguchi, B. Marsh and R. T. Smith. 1970. Cellular recognition by mouse lymphocytes in vitro. J. Immunol. 105:984-1000.

2. Alexander, P., R. Evans and C. K. Grant. 1972. The interplay of lymphoid cells and macrophages in tumor immunity. Ann. Inst. Pasteur. 122:645-658.

3. Alter, B. J. and F. H. Bach. 1970. Cellular reconstitution of purified lymphocyte response. Cell. Immunol. 1:207-227.

4. Alter, B. J. and F. H. Bach. 1970. Lymphocyte reactivity in vitro. Cell. Immunol. 1:207-227.

5. Anderson, L. C. and P. Hayry. 1974. Primary and secondary responses in mouse MLC. in "Proceeding of the eighth leucocyte culture conference." ed. Lindahl-Kiessling, K. and Osaba, D. Academic Press. New York. pp. 297-302.

6. Astrom, K. and B. H. Waksman. 1962. The passive transfer of experimental allergic encephalomyelitis and neuritis with living lymphoid cells. J. Path. Bact. 83:89 as cited in Folia Biol. 10: 164-174.

7. Bach, F. H., B. J. Alter, S. Solliday, D. C. Zosch and M. Janis. 1970. Lymphocyte reactivity in vitro. Cell. Immunol. 1:219-227.

8. Bach, F. H., M. L. Bach, B. J. Alter, F. Lindahl, D. J. Schendel and P. M. Sandel. 1975. Recognition in MLC and CML. The LD SD dichotomy. in "Immune recognition." ed. Rosenthal, A. Academic Press. New York. pp. 173-199.

9. Bach, F. H., M. L. Bach, P. M. Sandel and Sundharadasis. 1972. Genetic control of mixed leucocyte culture response. Transplant. Rev. 12:30 as cited by "Lymphocyte stimulation" pp. 124-156.

10. Bach, F. H., M. Widmer, M. L. Bach and J. Klein. 1972. Serologically defined and lymphocyte defined components of the major histocompatibility complex in the mouse. J. Exp. Med. 136:1430.

11. Bain, B., M. Vas and L. Lowenstein. 1964. The development of large immature mononuclear cells in mixed leucocyte cultures. Blood. 23:108-116.

12. Baird, L. G. and A. M. Kaplan. 1975. Evidence for direct stimulation of T-lymphocytes and macrophages. Cell. Immunol. 20:167-176.

13. Baldwin, R. W. 1975. Immunological aspects of chemical carcinogenesis. Adv. Cancer Res. 18:1-75.

14. Berke, G. A., H. Ginsberg, and M. Feldman. 1969. Graft reaction in tissue culture. Quantitation of the lytic action on mouse fibroblasts by rat lymphocytes sensitized on mouse embryo monolayers. Immunol. 16:643. as cited in "Lymphocyte stimulation," pp. 124-152.

15. Betel, I. K., K. J. van der Berg, J. Martijnse and R. van der Berg. 1974. Modification of the mitogenic response of thymus and spleen by adherent cells and mercaptoethanol. in "Proceedings of eighth leucocyte culture conference." ed. Lendahl-Kressling. Academic Press, New York. pp. 639-644.

16. Bhatnagar, R. M., Zabriskie, J. B. and A. R. Rausen. 1975. Cellular immune responses to methylcholanthrene-induced fibrosarcomas in Balb/c mice. J. Exp. Med. 142:839-855.

17. Bloom, B. R. 1971. Mechanisms of cell mediated immune reactions. Adv. Immunol. 13:101-111.

18. Bondevik, H. and E. Thorsby. 1974. Still a role for HL-A in the MLC interaction. in "Proceedings from eighth leucocyte conference." ed. Lindahl-Kressling, K. and Osaba, D. Academic Press. New York. pp. 255-266.

19. Brent, L. 1959. Transplantation immunity and hypersensitivity. in "Mechanisms of hypersensitivity." Boston, Mass. p. 555, as cited in Folia Biol. 10:164-174.

20. Brent, L. and P. B. Medawar. 1963. Nature of the normal lymphocyte transfer reaction. Nature 204:90 as cited in "Lymphocyte stimulation," pp. 124-152.

21. Brondz, B. D. 1964. Interaction of immune lymphocytes in vitro with normal and neoplastic tissue cells. Folia Biol. 10:164-174.

22. Calderon, J., R. Williams, and E. R. Unanue. 1975. An inhibitor of cell proliferation released by cultures of macrophages. Proc. Nat. Acad. Sci. 71:4273 as cited in J. Immunol. 117:646-654.

23. Cantor, H. and R. Asofsky. 1970. Synergy among lymphoid cells mediating the graft-vs.-host response. II Synergy in graft-vs-host reactions produced by Balb/c lymphoid cells of differing anatomic origin. J. Exp. Med. 131:235. as cited in J. Exp. Med. 140: 1588-1633.

24. Cantor, H. and R. Asofsky. 1972. Synergy among lymphoid cells and mediating the graft-vs-host response. Evidence for interaction

between two types of thymus derived cells. J. Exper. Med. 135: 764 as cited in J. Exp. Med. 140:1588-1633.

25. Cerottini, J. C. and K. T. Brunner. 1974. Cell mediated cytotoxicity, allograft, rejection and tumor immunity. Adv. Immunol. 18:67-122.

26. Cerottini, J. C., A. Nordin and K. T. Brunner. 1970. In vitro cytotoxic activity of thymus cells sensitized to allo-antigens. Nature (Lond.) 227:72 as cited in J. Nat. Cancer Inst. 54:387-392.

27. Chapman, N. D. and R. W. Dutton. 1965. The stimulation of DNA synthesis in cultures of rabbit lymph node and spleen cell suspensions by homologous cells. J. Exp. Med. 121: 85-99.

28. Cohen, L. and M. Howe. 1973. Synergism between subpopulations of thymus derived cells mediating the proliferative and effector phases of the mixed lymphocyte reaction. Proc. Natl. Acad. Sci. 70:2707. as cited in J. Exp. Med. 140:1588-1603.

29. Cooper, M. D., R. Peterson, M. South, and R. Good. 1966. Effect of neonatal bursectomy and thymectomy on the immunological competence in the chicken. J. Exp. Med. 123:75-102.

30. Christie, G. H. and R. Bomford. 1975. Mechanisms of macrophage activation by Corynebacterium parvum. Cell. Immunol. 17:141-149.

31. Cunningham, A. J. 1975. Why do so many cells take part in mixed lymphocyte reactions? Cell. Immunol. 19:368-370.

32. Daguillard, F. 1973. The differing responses of lymphocytes to nitogens. in "Proceedings from seventh leucocyte culture conference." ed. Daguillard, F. Academic Press, New York. pp 571-587.

33. David, J. R. 1975. Macrophage activation by lymphocyte mediators. Fed. Proc. 34:1730-1736.

34. Decker, P., R. Davis, G. Parker, and J. Mannick. 1973. The effect of tumor size on concomitant immunity. Cancer Res. 33:33 as cited in Int. J. Cancer 13:187-195.

35. Dutton, R. W. 1965. Spleen cell proliferation in response to homologous antigens studied in congenic resistant strains of mice. J. Exp. Med. 122:665-671.

36. Eardley, D. O. and R. K. Gershon. 1976. Induction of specific suppressor T cells in vitro. J. Immunol. 117:313-318.

37. Eggers, A. E. and J. R. Wunderlich. 1975. Suppressor cells in tumor bearing mice capable of nonspecific blocking of in vitro immunization against transplantation antigens. J. Immunol. 114: 1554 as cited in J. Immunol. 117:646-654.

38. Eijsvoogel, V. P., P. T. Schellekens, B. Breur-Vriesendorp, L. Konig, C. Kock, A. Van Leewan and J. J. Van Rood. 1971. Mixed lymphocyte cultures and HL-A. Transplantation Proceedings. III:85 as cited in "Lymphocyte stimulation." pp. 124-156.

39. Elves, M. E. 1969. Comparison of mitomycin C and x-ray for the production of one way stimulation in mixed leucocyte cultures. Nature 223:90-91.

40. Erb, P. and M. Feldman. 1973. The genetic control of the macrophage-T cell interaction for helper cell induction with soluble antigens. J. Exp. Med. 142:460-472.

41. Erb, P. and M. Feldman. 1975. Influence of macrophage-derived factors in helper cell induction. Eur. J. Immunol. 5:759-766.

42. Erb, P. and M. Feldman. 1975. The role of macrophages in the generation of T-helper cells. Cell. Immunol. 19:356-367.

43. Evans, R. 1972. Macrophages in syngeneic animal tumors. Transplantation 14:206 as cited in J. Immunol. 117:440-467.

44. Evans, R. 1973. Macrophages and the tumor bearing host. British J. Cancer 26:19-25.

45. Feldman, M. 1972. The requirement for macrophages in lymphoid cell collaboration. J. Exp. Med. 135:1049-1057.

46. Feldman, M. 1974. Specific T cell suppression of T and B cell functions in vitro. in "Proceedings from eighth leucocyte culture conference." Lindahl-Kressling, K. and Osaba, D. Academic Press, New York. 605-610.

47. Feldman, M. and A. Basten. 1972. Specific collaboration across a cell impermeable membrane. J. Exp. Med. 136:49-67.

48. Feldman, M. and S. Kontiainen. 1976. Cellular requirements of superior cell induction. Eur. J. Immunol. 6:302-307.

49. Feldman, M. and J. Schrader. 1974. Induction by specific T cell products. Cell. Immunol. 11:255-269.

50. Festenstein, H. 1966. Antigenic strength investigated by mixed cultures of allogeneic mouse spleen cells. Ann. N.Y. Acad. Sci. 129:567 as cited in "Lymphocyte stimulation." pp. 124-156.

51. Fitch, F. W., H. D. Engers, J. C. Cerottini, and K. T. Brunner. 1976. Suppressive effect of irradiated MLC cells on CTL response. J. Immunol. 116:716-723.

52. Folch, H., and B. H. Waksman. 1972. Suppressor activity of adherent and non adherent rat lymphoid cells. Cell. Immunol. 9:12-24.

53. Folch, H., and B. H. Waksman. 1973. Regulation of lymphocyte response in vitro. Cell. Immunol. 9:12-24.

54. Folch, H., M. Yoshenaga, and B. H. Waksman. 1972. Inhibition by adherent cells of the T lymphocyte response to phytohemoglutinin. J. Immunol. 110:835-839.

55. Fowler, A. R., A. Hellman, H. G. Steinman and A. C. Quatrale. 1971. Studies on blastogenic response of murine lymphocytes. Proc. Soc. Exp. Biol. Med. 138:345-349.

56. Fujimoto, S., M. Greene, and A. H. Sehon. 1975. Immunosuppressor T cells in tumor-bearing hosts. Immunol. Com. 4:201-217.

57. Fujimoto, S., M. Greene, and A. H. Sehon. 1976. Immunosuppressor cells in tumor-bearing hosts. J. Immunol. 116:791-799.

58. Fujimoto, S., M. Greene, and A. H. Sehon. 1976. The nature of immunosuppressor cells in tumor-bearing hosts. J. Immunol. 116: 800-806.

59. Gershon, R. K., R. Carter, and K. Kondo. 1967. On concomitant immunity in tumor bearing hamsters. Nature (Lond.) 313:674 as cited in Int. J. Cancer 13:187-195.

60. Gershon, R. K., P. Cohen, R. Hencin, and S. Liebhaber. 1971. Suppressor T cells. J. Immunol. 108:586-590.

61. Gershon, R. K., S. A. Liebhaber, and S. Riju. 1974. T cell regulation of T cell response to antigens. Immunology 26:904-923.

62. Gery, I., R. K. Gershon, B. H. Waksman. 1972. Potentiation of the T-lymphocyte response to mitogens. J. Exp. Med. 136:128-155.

63. Gery, I., and E. Wiener. 1975. Potentiation of the T lymphocyte response to mitogens: relationship between LAF production and activation of macrophages. J. Reticulo. Soc. 18:257-259.

64. Ghaffer, A., R. T. Cullen, and M. L. Woodruff. 1975. Further analysis of the anti-tumor effect in vitro of peritoneal exudate cells from mice treated with Corynebacterium parvum. British J. Cancer 31:2-24.

65. Gillette, R. W., and A. Fox. 1975. The effect of T lymphocyte deficiency on tumor induction and growth. Cell. Immunol. 19: 328-335.

66. Gorczynski, R. M. and C. Norbury. 1974. Immunity to murine sarcoma virus induced tumours. Analysis of the cell population involved in protection from lethal tumor progression of sublethally irradiated, MSV inoculated mice. British J. Cancer 30:118-128.

67. Gordon, J. 1967. Role of monocytes in mixed leucocyte culture reactions. Proc. Soc. Exp. Biol. Med. 127:31-33.

68. Gordon, J. and I. MacLean. 1965. A lymphocyte stimulating factor produced in vitro. Nature 208:795 as cited in "Lymphocyte stimulation." 1975, 124-152.

69. Gowans, J. L., D. D. MaCregor, D. M. Cowen and C. E. Ford. 1962. Initiation of immune response by small lymphocytes. Nature 196: 651-655.

70. Handwerger, B. S. and R. H. Schwartz. 1974. Separation of murine lymphoid cells using nylon wool columns. Transplantation 18: 544-548.

71. Hartman, K., R. W. Dutton, M. M. McCarthy and R. I. Mishell. 1970. Cell components of the immune response. Cell. Immunol. 1:182-189.

72. Hartzman, R. J., M. L. Bach, and F. H. Bach. 1972. Precipitation of radioactively labeled samples: a semi-automatic multiple sample processor. Cell. Immunol. 4:182-186.

73. Haskill, J., J. W. Proctor and Y. Yamamura. 1975. Host response within solid tumors. monocytic effector cells within rat sarcomas. J. Nat. Cancer Inst. 54:387-392.

74. Havemann, K. and W. Schmidt. 1974. Potentiating effect of adherent cell supernatants on lymphocyte proliferation. in "Proceedings from eighth leucocyte culture conference" ed. Lindahl-Kiessling, K. and Osaba, D. Academic Press, New York. pp. 181-185.

75. Heber-Katz, E. and R. E. Click. 1972. Role of mercaptoethanol in the mixed-leucocyte reaction. Cell. Immunol. 5:410-428.

76. Heilman, D. H. and M. R. Gambril. 1974. Requirement of the macrophage or its products for the transformation of cortisol-resistant human lymphocytes by phytohemagglutinin. Immunol. Commun. 3:97-107.

77. Hellstrom, K. E. and I. Hellstrom. 1974. Lymphocyte mediated cytotoxicity and blocking serum activity to tumor antigens. Adv. Immunol. 18:209-269.

78. Hellstrom, K. E. and I. Hellstrom. 1974. The role of cell mediated immunity in control and growth of tumors. Clin. Immunol. 2:233-264.

79. Hersh, E. M. 1971. Blastogenic response of human lymphocytes to xenogeneic cells. Transplantation. 12:287, as cited in "Lymphocyte stimulation." pp. 124-152.

80. Hersh, E. M. and J. E. Harris. 1968. Macrophage-lymphocyte interaction in the antigen-induced blastogenic response of human peripheral blood leukocytes. J. Immunol. 100:1184-1194.

81. Hirano, T. and A. A. Nordin. 1976. The development of suppressor cells and cytotoxic lymphocytes in mixed lymphocyte cultures. J. Immunol. 116:1115-1122.

82. Holden, H. T., J. S. Haskill, H. Kirchner, and R. B. Herberman. 1976. Two functionally distinct anti-tumor effector cells isolated from primary murine sarcoma virus-induced tumors. J. Immunol. 117:440-445.

83. Howe, M. B. 1973. Selective elimination of PHA-responsive lymphocytes from murine spleen cell suspensions. in "Proceedings of 7th leucocyte culture conference," ed. Daguillard, F. Academic Press, New York. pp. 523-533.

84. Howe, M. G. and L. Cohen. 1974. Participation of lymphoid cell subpopulations in the mixed lymphocyte interaction. in "Proceedings from eighth leucocyte culture conference," ed. Lindahl-Kiessling, K. and Osaba, D. Academic Press, New York. pp. 611-616.

85. Howe, M. B., B. Manziello and A. Goldstein. 1972. Studies on the nature of the stimulator cell in the mixed lymphocyte interaction. in "Proceedings of the sixth leucocyte culture conference," ed. M. R. Schwartz. Academic Press, New York. pp. 599-607.

86. Ishizaka, K. and T. Adachi. 1976. Generation of specific helper cells and suppressor cells in vitro for IgE and IgG antibody responses. J. Immunol. 117:40-47.

87. Jakway, J. D., D. W. Thomas and D. W. Talmage. 1975. Activation of T lymphocyte helper function by brief exposure to antigen-pulsed macrophages. Proc. Soc. Exp. Biol. Med. 150:791-795.

88. Jeffes, E. W. and G. A. Granger. 1976. Relationship of CIF, LT and PIF released in vitro by activated human lymphocytes. J. Immunol. 117:1-6.

89. Johnson, R. A., T. K. Smith, and C. H. Kirkpatrick. 1972. Augmentation of PHA mitogenic activity by erythrocyte membranes. Cell. Immunol. 3:186 as cited in "Lymphocyte stimulation." pp. 253-293.

90. Jones, A. L. 1966. The effect of polymorphonuclear leucocytes on the blastoid transformation of lymphocytes in mixed leucocyte cultures. Transplantation 4:337-343.

91. Julius, M. H., E. Simpson, and L. A. Herzenberg. 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. Eur. J. Immunol. 3:645-649.

92. Jurin, M. and H. D. Suit. 1974. In vitro activity of lymphocytes and serum of C_3Hf/Bu mice during the growth of methylcholanthrene-induced tumor and its regression following local irradiation. Cancer Res. 34:672-678.

93. Kaliss, N. and N. Molomut. 1952. The effect of prior injections on tissue antiserums on the survival of cancer homografts in mice. Cancer Res. 12:110 as cited in Immunology 26:623-637.

94. Kaplan, A. M. (personal communication).

95. Kappler, J. W. 1974. A micro-technique for hemolytic plaque assays. J. Immunol. 112:1271-1274.

96. Kappler, J. W. and P. C. Marrack. 1974. Helper and suppressor T cells activated by concanavalin A. Cell. Immunol. 18:9-20.

97. Kasahara, T. and K. Shiori-Nakano. 1976. Splenic suppressing factor. Purification and characterization of a factor suppressing thymidine incorporation into activated lymphocytes. J. Immunol. 116:1251-1256.

98. Katz-Heber, E., A. B. Peck, and R. E. Click. 1973. Mixed leukocyte interaction in a protein-free medium. Eur. J. Immunol. 3: 379-385.

99. Kearney, R., A. Basten and D. S. Nelson. 1974. Cellular basis for the immune response to methylcholanthrene-induced tumors in mice heterogeneity of effector cells. Int. J. Cancer 15:438-450.

100. Keller, R. 1974. Major changes in lymphocyte proliferation evoked by activated macrophages. Cell. Immunol. 17:542-551.

101. Kirchner, H., T. M. Chused, R. Herberman, H. T. Holden and D. Lavin. 1974. Evidence of suppressor cell activity in spleens of mice bearing primary tumors induced by Maloney sarcoma virus. J. Exp. Med. 139:1473 as cited in J. Immunol. 117:440-446.

102. Kirchner, H., R. Herberman, M. Glaser, and D. Lavin. 1974. Suppression of in vitro lymphocyte stimulation in mice bearing primary Maloney sarcoma virus-induced tumors. Cell. Immunol. 13:32 as cited in J. Immunol. 117:646-654.

103. Kirchner, H., A. V. Muchmore, T. M. Chused, H. T. Holden and R. B. Herberman. 1975. Inhibition of proliferation of lymphoma cells and T lymhpocytes by suppressor cells from spleens of tumor bearing mice. J. Immunol. 114:206 as cited in J. Immunol. 117: 440-446.

104. Klein, G. 1972. Tumor immunology. Clin. Immunobiol. 1:219-240.

105. Klein, G. and E. Klein. 1975. Are methyl-cholanthrene-induced sarcoma-associated, rejection-inducing (TSTA) antigens, modified forms of H-2 or linked determinants. Int. J. Cancer 15:879-887.

106. Koch, C. T., E. Frederiks, V. P. Eijsvoogel and J. J. Van Rood. 1971. Mixed lymphocyte culture and skin graft data in unrelated HL-A identical individuals. Lancet. 2:1334 as cited in "Lymphocyte stimulation," pp. 124-156.

107. Konda, S., Y. Nakao, and R. T. Smith. 1973. The stimulatory effect of tumor bearing upon the T and B cell subpopulations of the mouse spleen. Cancer Res. 33:2247-2256.

108. Kontiainen, S. and M. Feldmann. 1976. Kinetics of induction of antigen-specific suppressor cells. Eur. J. Immunol. 6:296-301.

109. Kountz, S. L., K. C. Cochrum and R. K. Main. 1972. Fine discrimination of antigenicity at the AgB locus by the mixed skin cell-leukocyte reaction. "Proceedings of the sixth leucocyte culture conference," ed. M. R. Schwartz. Academic Press, New York. pp. 609-621.

110. Kuhner, A. L. and J. R. David. 1976. Partial characterization of murine migration inhibitory factor. J. Immunol. 116:140-145.

111. Lebrun, A. 1971. Effects of mitomycin C dosage on phytohemagglutinin stimulation and on the one way mixed leukocyte reaction. Transplantation 11:567-569.

112. Lemke, H. and H. G. Opitz. 1976. Function of 2-mercaptoethanol as a macrophage substitute in the primary immune response in vitro. J. Immunol. 117:388-395.

113. Levis, W. R. and J. H. Robbins. 1968. Function of glass adherent cells in lymphocyte cultures. Fed. Proc. 28:566.

114. Levis, W. R. and J. H. Robbins. 1970. Effect of glass adherent cells on the blastogenic response of purified lymphocytes to phytohemagglutinin. Exptl. Cell. Res. 61:153-158.

115. Levis, W. R. and J. H. Robbins. 1970. Function of glass adherent cells in human mixed lymphocyte cultures. Transplantation 9: 515-518.

116. Levis, W. R. and J. H. Robbins. 1972. Methods for obtaining purified lymphocytes, glass-adherent mononuclear cells, and a population containing both cell types from human peripheral blood. Blood 40:77-89.

117. Levy, M. H. and E. F. Wheelock. 1974. The role of macrophages in defense against neoplastic disease. Adv. Cancer Res. 20:131-163.

118. Li, C. Y., K. W. Lam, L. T. Yam. 1973. Esterases in human leukocytes. J. Histochem. Cytochem. 21:1-12.

119. Li, C. Y., L. Yam, and W. H. Crosby. 1972. Histochemical characterization of cellular and structural elements of the human spleen. J. Histochem. Cytochem. 20:1049-1058.

120. Ling, N. R. and D. A. Hardy. 1974. Human mixed cell reactions in relation to surveillance of lymphoid tissue. in "Proceedings from eighth leucocyte conference," ed. Lindahl-Kiessling, K. and Osaba, D. Academic Press, New York. pp. 235-241.

121. Ling, N. R. and J. E. Kay. 1975. The properties of lymphocytes from various lymphoid tissues in "Lymphocyte stimulation." American Elsevier Publishing Co., Inc. New York. pp. 9-22.

122. Ling, N. R. and J. E. Kay. 1975. Methods of culturing lymphocytes in "Lymphocyte stimulation." American Elsevier Publishing Co., Inc. New York. pp. 25-67.

123. Ling, N. R. and J. E. Kay. 1975. Activation of human and animal lymphocytes by common stimulants. in "Lymphocyte stimulation." American Elsevier Publishing Co., Inc. New York. pp. 69-90.

124. Ling, N. R. and J. E. Kay. 1975. The activation of lymphocytes by foreign cells. in "Lymphocyte stimulation." American Elsevier Pub. Co., Inc. New York. pp. 123-158.

125. Ling, N. R. and J. E. Kay. 1975. The mechanism of lymphocyte activation. in "Lymphocyte stimulation." American Elsevier Publishing Co., Inc. New York. pp. 253-302.

126. Lipsky, P. E., J. J. Ellner and A. S. Rosenthal. 1976. Phytohemagglutinin-induced proliferation of guinea pig thymus derived lymphocytes. J. Immunol. 116:868-875.

127. Lonai, P., A. Eliraz, H. Wekerle and M. Feldmann. 1973. Depletion of specific GVH activity following adsorbtion of non-sensitized lymphocytes on allogeneic fibroblasts. Transplantation 15: 368 as cited in "Lymphocyte stimulation." pp. 124-156.

128. Luzzate, A. L., L. Lafleur. 1976. Suppressor cells in rabbit peripheral blood. Eur. J. Immunol. 6:125-129.

129. Main, R. K., K. C. Cochrum, M. J. Jones and S. L. Kountz. 1971. Immunological potential of the in vitro mixed skin cell-leukocyte reaction. Nature New Biol. 229:89 as cited in "Lymphocyte stimulation." pp. 124-152.

130. Mangi, R. J. and M. R. Mardiney. 1970. Transformation of mouse lymphocytes to allogeneic lymphocytes and phytohemagglutinin. J. Immunol. 105:90-97.

131. Mardiney, M. R. and R. E. Wren. 1973. A current methodology for performing murine mixed lymphocyte reactions. Biomedicine 19: 331-334.

132. Martinez, J. P. and H. Festenstein. 1975. New mixed lymphocyte micro-culture test harvested by a simple device developed for this process. Transplantation 20:26-37.

133. Meltzer, M. S., R. W. Tucker, and A. C. Breuer. 1975. Interaction of BCG-activated macrophage with neoplastic and nonneoplastic cell lines in vitro: Cinemacrographic analysis. Cell. Immunol. 17: 30-42.

134. Miller, J. P., K. Brunner and J. Sprent. 1971. Thymus derived cells as killer cells in cell mediated immunity. Transplant Proc. 3:915 as cited in J. Nat. Cancer Inst. 54:387-392.

135. Miller, G., and J. Mitchell. 1968. Bone marrow origin of antibody forming cells. J. Exp. Med. 128:821-837.

136. Moller, G. 1969. Induction of DNA synthesis in human lymphocytes: interaction between non-specific mitogens and antigens. Immunology 19:583-598.

137. Mugraby, L., I. Gery and D. Sulitzeanu. 1974. The participation of thymus derived and bone marrow derived lymphocytes of sensitized mice in the proliferative response to specific antigen, in vitro. Eur. J. Immunol. 4:402-405.

138. Namba, Y. and B. H. Waksman. 1976. Regulatory substances produced by lymphocytes. J. Immunol. 116:1140-1144.

139. Nelson, D. A. 1973. Production by stimulated macrophages of factors depressing lymphocyte transformation. Nature 246:306 as cited in J. Immunol. 117:646-654.

140. Nelson, D. A. 1976. "Immunobiology of the macrophage." Academic Press. New York. p. 45-46.

141. Nelson, D. A. and C. N. Shneider. 1974. Effect of normal mouse serum on mouse lymphocyte transformation in vitro. Eur. J. Immunol. 4:74-86.

142. Nguyen, B. T. and S. Stadtsbaeder. 1976. Spontaneous interaction in vitro between lymphocytes and syngeneic peritoneal macrophages of mice. Infect. Immun. 13:884-889.

143. Nisbet, N. W., M. Simonsen, and M. Zaleski. 1969. The frequency of antigen-sensitive cells in tissue transplantation. J. Exper. Med. 129:759 as cited in "Lymphocyte stimulation." pp. 124-156.

144. Nisbet, N. W. 1969. Induction of specific immunological tolerance by parabiosis and antilymphocyte serum. Transplantation 8:356-368.

145. Opitz, H. G., D. Niethammer, H. Lemke, H. Flad and R. Huget. 1975. Biochemical characterization of a factor released by macrophages. Cell. Immunol. 18:70-75.

146. Opitz, H. G., D. Niethammer, R. C. Jackson, H. Lemke, H. Flad and R. Huget. 1975. Inhibition of ^{3}H-thymidine incorporation of lymphocytes by a soluble factor from macrophages. Cell. Immunol. 16:379 as cited in J. Immunol. 117:646-654.

147. Oppenheim, J. J., B. G. Leventhal, and E. M. Hersh. 1968. The transformation of column-purified lymphocytes with nonspecific and specific antigenic stimuli. J. Immunol. 101:262-270.

148. Oppenheim, J. J., A. Shneyour and A. Kook. 1976. Enhancement of DNA synthesis and cAMP content of mouse thymocytes by mediators derived from adherent cells. J. Immunol. 116:1466-1471.

149. Paranjpe, M. S. and C. W. Boone. 1974. Kinetics of the anti-tumor delayed hypersensitivity response in mice with progressively growing tumors: stimulation followed by specific suppression. Int. J. Cancer 3:179-186.

150. Peck, A. B. and R. E. Click. 1973. Enhancement of the mouse mixed lymphocyte interaction by isologous and homologous sera. Eur. J. Immunol. 3:385-392.

151. Phillips, S. M., C. B. Carpenter and J. P. Merrill. 1972. Cellular immunity in the mouse. Cell. Immunol. 5:235-248.

152. Piper, C. E. and K. L. McIvor. 1974. Alloimmune peritoneal macrophages as specific effector cells: characterization of specific macrophage cytotoxin. Cell. Immunol. 14:423-430.

153. Polet, H. 1972. The mixed lymphocyte culture as a tool for the quantitative determination of HL-A difference. Transplantation 13:472.

154. Resch, K., E. Ferber, M. Prester, and E. W. Gelfand. 1976. Mitogen-induced membrane changes and cell proliferation in T lymphocyte subpopulations. Eur. J. Immunol. 6:168-173.

155. Rich, S. S. and R. R. Rich. 1974. Regulation of mixed lymphocyte reaction by alloantigen-activated thymus derived lymphocytes. J. Exp. Med. 140:1588-1603.

156. Rich, S. S. and R. R. Rich. 1975. A genetically restricted suppressor of mixed lymphocyte reactions released by allo-antigen activated spleen cells. J. Exp. Med. 142:1391-1402.

157. Rode, H. N. and J. Gordon. 1970. The mixed leukocyte culture: a three component system. J. Immunol. 104:1403-1409.

158. Rosenthal, A. S., P. E. Lipsky and E. M. Shevach. 1975. Macrophage-lymphocyte interaction and antigen recognition. Fed. Proc. 34:1743-1748.

159. Rosenthal, A. S., D. L. Rosenstreich, J. T. Blake, P. E. Lipsky and J. A. Waldron. 1973. Mechanisms of antigen recognition by T lymphocytes. in "Proceedings of the seventh leucocyte culture conference." ed. Daguillard, F. Academic Press. New York. pp. 201-216.

160. Rosenstreich, D. L., J. J. Farrar and S. Dougherty. 1976. Absolute macrophage dependence of T lymphocyte activation by mitogens. J. Immunol. 116:131-139.

161. Rouse, B., M. Rollinghoff and N. Warner. 1973. Tumor immunity to murine plasma cell tumors. Essential role of T lymphocytes in immune response. Eur. J. Immunol. 3:218 as cited in J. Nat. Cancer Inst. 54:387-392.

162. Rubin, H. 1962. The immunological basis for noninfective tumors and regressions in Rous sarcoma. Ann. N.Y. Acad. Sci. 101:181 as cited in Folia Biol. 10:164-174.

163. Ruhl, H., W. Vogot, G. Bochert, S. Schmid and T. Deamantstein. 1974. Stimulation of mouse lymphoid cells by polyanions *in vitro* in "Proc. eighth leucocyte culture conference." ed. Lindahl-Kressling, K. and Osaba, D. Academic Press. New York. pp. 31-37.

164. Russell, S. W., W. F. Doe and C. G. Cochrane. 1976. Number of macrophages and distribution of meitotic activity in regressing and progressing Moloney sarcomas. J. Immunol. 116:164-166.

165. Russell, S. W., W. F. Doe and A. Tozier. 1975. The relative occurrences of macrophages in regressing and progressing Maloney sarcoma. Fed. Proc. 34:268 as cited in J. Immunol. 117:440-446.

166. Schaeffer, A. E., M. Fishman and L. T. Adler. 1976. Requirements for the generation and transfer of suppressor cell activity. J. Immunol. 116:1157-1162.

167. Schmidtke, J. R. and S. Hatfield. 1976. Activation of purified human T cells by mitogens. Diminished mitogen-induced deoxyribonucleic acid synthesis in human T cells compared with autologous peripheral blood lymphocytes. Infect. Immun. 13:1061-1067.

168. Schmidtke, J. R. and S. Hatfield. 1976. Activation of purified human thymus-derived T cells by mitogens. J. Immunol. 116:357-362.

169. Schellekens, P. T. and V. P. Eysvoogel. 1970. Lymphocyte transformation in vitro. Mechanism of stimulation in the mixed lymphocyte culture. Clin. Exptl. Immunol. 7:229 as cited in "Lymphocyte stimulation." 1975. pp. 124-152.

170. Shevach, E. 1976. The function of macrophages in antigen recognition by guinea pig T lymphocytes. J. Immunol. 116:1482-1489.

171. Shortman, K. and J. Palmer. 1971. The requirement for macrophages in the in vitro immune response. Cell. Immunol. 2:399-410.

172. Simes, R. J., R. Kearney, and D. S. Nelson. 1975. Role of a non-committed accessory cell in the in vivo suppression of a syngeneic tumor by immune lymphocytes. Immunology 29:343-351.

173. Small, M. and N. Trainin. 1976. Separation of populations of sensitized lymphoid cells into fractions inhibiting and fractions enhancing syngeneic tumor growth in vivo. J. Immunol. 117:292-297.

174. Smith, R. T., J. T. Forbes, Y. Nakao and S. Kondo. 1973. Cell subpopulation analysis in the MCA tumor-bearing mouse: changes in class and functions of spleen, thymus and lymph node cells. "Proceedings from eighth leucocyte culture conference." ed. Lindahl-Kiessling, K. and Osaba, D. Academic Press. New York. pp. 673-682.

175. Smith, R. T. and S. Konda. 1973. The stimulatory effects of bearing primary methyl-cholanthrene-induced tumors upon the murine lymphoreticular system. Int. J. Cancer 12:577-588.

176. Schwartz, R. H., L. Jackson and W. E. Paul. 1975. T lymphocyte enriched murine peritoneal exudate cells. J. Immunol. 115:1330-1338.

177. Tadakuma, T., A. L. Kuhner, R. R. Rich, J. R. David and C. W. Pierre. 1976. Characterization of a soluble immune response produced by concanavalin A activated spleen cells. J. Immunol. 117:323-330.

178. Tanaka, S., K. Orita and S. Kaneda. 1968. Demonstration of genetic by mixed cultures of allogeneic mouse lymph node cells. Transplantation 6:127 as cited in "Lymphocyte stimulation." pp. 124-156.

179. Tardieu, M., Y. Fradet and F. Daguillard. 1974. Isolation of suppressor cells of the mitogenic response of rat thoracic duct lymphocytes. Cell. Immunol. 17:123-130.

180. Tarnik, A. 1970. A role for red cells in PHA-induced lymphocyte stimulation acta. Pathol. Microbiol. Scand. 78:733 as cited in "Lymphocyte stimulation." pp. 253-293.

181. Thorsby, E. 1974. The major histocompatibility system and its relation to lymphocyte activation. in "Proceedings of eighth leucocyte culture conference." ed. Lindahl-Kiessling, K. and Osaba, D. Academic Press. New York. pp. 327-348.

182. Thurman, G. B., D. M. Strong, A. Ahmed, S. S. Green, K. W. Sell, R. J. Hartzman and F. H. Bach. 1973. Evaluation of a microculture technique utilizing the multiple automated sample harvester (MASH). Clin. Exptl. Immunol. 15:289-302.

183. Treves, A. J., C. Carnared, N. Trainin, M. Feldman and I. R. Cohen. 1974. Enhancing T lymphocytes from tumor-bearing mice suppress host resistance to a syngeneic tumor. Eur. J. Immunol. 4:722-727.

184. Treves, A. J., B. Schechter, I. R. Cohen and M. Feldmann. 1976. Sensitization of T lymphocytes in vitro by syngeneic macrophages fed with tumor antigen. J. Immunol. 116:1059-1064.

185. Tridente, G., G. M. Cappuzzo and L. Choco-Bianchi. 1967. Lymphocyte interaction in mixed mouse leukocyte cultures. Histocompatibility testing 1967 (Munksgaard, Copenhagen), p. 67 as cited in "Lymphocyte stimulation." pp. 124-156.

186. Trizio, D. and G. Cudkowicz. 1974. Separation of T and B lymphocytes by nylon wool columns-evaluation of efficacy by functional assays in vivo. J. Immunol. 113:1093-1097.

187. Umiel, T. and N. Trainin. 1974. Immunological enhancement of tumor growth by syngeneic thymus-derived lymphocytes. Transplantation 18:244-250.

188. Unanue, E. R. and J. Calderon. 1975. Evaluation of the role of macrophages in immune induction. Fed. Proc. 34:1737-1742.

189. Unanue, E. R., J. Calderon and J. Kiely. 1975. Secretion by macrophages of two molecules modulating cell proliferation. in "Immune Recognition." ed. Rosenthal, A. Academic Press, New York. pp. 555-561.

190. Vaage, J. 1971. Concomitant immunity and specific depression of immunity by residual or reinjected syngeneic tumor tissue. Cancer Res. 31:1655 as cited in Int. J. Cancer 13:187-195.

191. Van Leeuwen, A., J. van den Tweel and J. van Rood. 1974. Polymorphism at the MLC locus studied by MLC testing and serology in "Proceedings from eighth leucocyte culture conference." ed. Lundahl-Kiessling, K. and Osaba, D. Academic Press. New York. pp. 269-280.

192. Veit, B. C. and J. D. Feldmann. 1976. Altered lymphocyte functions in rats bearing syngeneic Moloney sarcoma tumors. J. Immunol. 117:646-654.

193. Veit, B. C. and J. D. Feldmann. 1976. Altered lymphocyte functions in rats bearing syngeneic Moloney sarcoma tumors. J. Immunol. 117:655-660.

194. Vischer, T. L. 1972. Culture of mouse lymphoid cells in serum free medium. J. Immunol. Met. 1:199-202.

195. Vischer, T. L. and C. Jaquet. 1972. The effect of antibodies against immunoglobulins and the theta antigen on the specific and nonspecific stimulation of mouse spleen cells in vitro. Immunology 22:259-266.

196. Wagner, H., A. Harris and M. Feldmann. 1972. Cell-mediated immune response in vitro. Role of the thymus derived lymphocytes. Cell. Immunol. 4:39 as cited in J. Nat. Cancer Inst. 54:387-392.

197. Waldron, J. A., R. G. Horn and A. S. Rosenthal. 1973. Antigen-induced proliferation of guinea pig lymphocytes in vitro: obligatory role of macrophages in the recognition of antigen by immune T lymphocytes. J. Immunol. 111:58-64.

198. Widmer, M. B., M. L. Bach, and F. H. Bach. 1973. Major histocompatibility complex control of mixed leucocyte culture in "Proceedings of seventh leucocyte culture conference." ed. Daguillard, F. Academic Press. New York. pp. 665-672.

199. Winn, H. 1961. Quantitative assay of the immunological activity of lymphoid cells stimulated by tumor homografts. J. Immunol. 86:228 as cited in Folia Biol. 10:164-174.

200. Yachnin, S., L. W. Allen, J. M. Baron and R. Svenson. 1971. Potentiation of lymphocyte transformation by membrane-membrane interaction. "Proceedings fourth leucocyte culture conference." ed. O. R. McIntyre, p. 37 as cited in "Lymphocyte stimulation." pp. 253-293.

201. Yam, L. T., C. Y. Li, and W. H. Crosby. 1970. Cytochemical identification of monocytes and granulocytes. Am. J. Clin. Pathol. 55:283-290.

202. Yoshinaga, M. S. Nakamura and H. Hayashi. 1975. Interaction between lymphocytes and inflammatory exudate cells. J. Immunol. 115:533-538.

203. Yutoku, M., H. Senoh, Y. Matsuoka and M. Kitogawa. 1974. Suppression and enhancement of allografted tumour. Immunology 26: 623-637.

204. Zembala, M. and G. L. Asherson. 1974. T cell suppression of contact sensitivity in the mouse. Eur. J. Immunol. 4:799-807.

The vita has been removed from the scanned document

MACROPHAGE REGULATION OF THE T CELL ALLOGENEIC RESPONSE DURING TUMORIGENESIS

By

Kevin Michael Connolly

(ABSTRACT)

One-way mixed-lymphocyte reactions (MLR) were performed to determine qualitatively and quantitatively how macrophages and macrophage-derived factors (MDF), interacting with mouse spleen nonadherent (NA) subpopulations, affected the ability of NA cells to respond to allogeneic lymphocytes. Comparative reactivity was measured using normal cells and cells from mice in various stages of tumorigenesis.

In MLR's using normal NA cells, recognition of allogeneic cells was dependent on addition of an optimal (2-3%) concentration of macrophages. Beyond this optimum concentration, macrophage addition became inhibitory to NA cell stimulation. Macrophage enhancement or inhibition was not contact dependent, since cell-free macrophage supernatants (depending on their concentration) also possessed a similar capacity for enhancement or depression of the thymus-derived (T) cell response to allo-antigens.

In the tumor-bearing host, initial phases of tumorigenesis caused *in vitro* NA cell activation in the absence of macrophages or MDF; however in advanced stages of tumorigenesis, NA cells required macrophages in order to respond to histoincompatibility antigens. Macrophages and MDF from normal or one week palpable tumor-bearers did not differ in their ability to enhance or depress *in vitro* T cell activity. Macrophages and MDF from two-week palpable tumor-bearing hosts exhibited a decreased

capacity to stimulate. As tumorigenesis progressed, MLR activity of the NA population decreased, in response to the suppressor action of macrophages and macrophage supernatants. To explain the dual role of the macrophage as both an enhancing cell and a repressor cell, possession of two macrophage supernatant factors has been proposed.