POSITIVE AND NEGATIVE IMMUNOREGULATION OF NORMAL AND TUMOR-BEARING MOUSE T CELL BLASTOGENESIS

by

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Conan Doyle, Edgar Allan Poe and *Yersinia pestis*, will always serve to remind and reassure me that the pedagogue will forever win out over the pedant. To Dr. Schurig, whose sympathetic reassurances, on the eve of my prelims, allowed me to venture the hope that I too just might come through my orals unscathed (relatively unscathed, anyway).

Though I'm most eager to start work at MCV, it would be a mistake to assume that I won't miss each one of you.

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DEDICATION

To the whole microbiology section -- a group of people who have been not only my teachers, but my friends. Thank you for not taking the "fun" out of serious science.

What good men are most biologists!
the tenors of the scientific world -
Tempermental, lecherous, loud-laughing
and healthy.
Once in a while one comes on the "other" kind -
what we called a "dry-ball".
Such men are not biologists.
they are the embalmers, the picklers.
They see only preserved forms of life -
failing to see its principles.
Out of their own crusted minds they
create a world wrinkled with formaldehyde.
The true biologist deals with teeming
boisterous life, and learns from it,
That the first rule of life is LIVING.
the dry-balls cannot possibly learn it.
Your true biologist will sing you a song
as loud and as off-key as any blacksmith.
He knows that so called "morals" are
too often diagnostic of prostatitis and ulcers.
The true biologist is very good company.

Steinbeck

PREFACE

In doing battle with anything as formidable as a Ph.D. dissertation, it is prudent for the writer (and the reader) to come to the combat armed with some foreknowledge of what is to come. For this reason (and because of gentle hints dropped by the Boss), I have provided an immunological road map, outlining quite briefly, the framework of my dissertation. Five immunoregulatory topics concerning positive and negative macrophage (Mϕ) and T cell regulation, have been listed in the Introduction. The remainder of the dissertation has been written around this order. In the Literature Review, I have taken each topic and presented other's research only as it directly applies to some aspect of my own work with regulators of lymphocyte blastogenesis in normal and tumor-bearing animals. The Results section has been organized around these topics, with an additional summary of negative Mϕ and T cell regulation, as well as a closing synopsis of both positive and negative aspects of Mϕ and T cell regulation. The Discussion, though not divided by formal sub-titles, has been patterned after the order first established in the Introduction. Information gleaned from the Literature Review, the Results and the Discussion has been synthesized into a Model section detailing a hypothetical model of homeostatic immunoregulation by populations of Mϕ, amplifier T cells and suppressor T cells. In the closing portion, unanswered questions, brought to the surface by my research, are dealt with in a "Future Directions" section, outlining the strategy and tactics of future experimentation.

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I. INTRODUCTION

Wiping out cancer "in our time" has become the rallying cry, not only of the public in general, but of countless immunologists. Despite concerted research efforts and some impressive strides in tumor therapy (182), the "cure" for cancer has proved to be frustratingly elusive. For the tumor immunologist the key to the problem lies in discovering how neoplastic tissue, possessing its own foreign tumor antigens, often escapes recognition and destruction at the hands of the body's immunosurveillance system, thereby facilitating its own proliferation and metastasis. Tumorigenesis, by itself, would be of less pathological significance were it not for the systemic immune debilitation often accompanying tumor growth (21, 121, 185). Though it may be argued that tumorigenesis is the result, not the cause of immune debilitation, all normal animals receiving our tumor cell inoculum, invariably manifest rapid immunological degeneration (58, 59, 68). Thus there appears to be a direct cause and effect relationship between tumor presence and immune dysfunction.

Therefore the general question addressed in this dissertation is:

"What mechanism(s) lies behind successful tumor cell proliferation and the concomitant debilitation of the host animal's immune response?"

In more specific terms:

"Using the mixed lymphocyte reaction (MLR) as a model, what factors govern positive and negative blastogenic immunoregulation of spleen cells from normal and fibrosarcoma-bearing mice?"

Shorn of its jargon this translates as:

"Why are lymphocytes from tumor-bearing mice (TBM) incapable of reacting to foreign lymphocytes, and by what means can this immunologically regretable state of affairs be reversed?"

Using the MLR to explore the mechanism of tumor-induced suppressor activity requires some further explanation. It was necessary to choose an assay system which measured the competency of the cell-mediated immune (CMI) system as opposed to the humoral system. Emphasis on the CMI response dates from Winn's experiments (248) in the early sixties when he established that tumor cell recognition and destruction was due, not to B cell antibody production, but to an immunosurveillance system composed of macrophages (Mϕ) and thymus-derived lymphocytes (T cells).

Since actively proliferating, T cells represent a necessary (but not sufficient) component in cell-mediated destruction of tumor cells (5, 109, 216), an assay system was chosen in which T cell blastogenesis (proliferation) could be easily assessed as uptake of tridiated thymidine (^{3}H-TdR) into precipitable DNA (106, 221).

A variety of <u>in vitro</u> methods are used to stimulate normal spleen cells (a rich source of T lymphocytes) into a state of proliferation (23, 98). These range from addition of mitogens (20, 89, 183) to the chosen method -- contact with a foreign (allogeneic) lymphocyte (14, 50). Such <u>in vitro</u> recognition of foreign lymphocytes, correlates closely with the <u>in vivo</u> ability to reject foreign (histoincompatible) tissue (11, 12, 13, 25, 188). The recognition of histocompatibility antigens is, in turn, closely linked to detection of tumor-associated

antigens (139). In short it is felt that the in vitro T cell ability to recognize a foreign antigen on the surface of an allogeneic lymphocyte may correlate with its in vivo ability to react against tumor antigens on the surface of neoplastic cells (27). Thus by examining the regulatory factors controlling expression of 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 cell presence.

At this point, "regulatory factors" have been discussed only in the most general of terms. My initial prejudice, guided by past research (58), led me to believe that Mϕ dominated the immunoregulatory system in normal and TBM. It is admirably suited for such a role since, as Zanvil Cohn points out in his "Saga of the Macrophage" (38), "it helps the less fortunate in its micro-environment...it schleps, it ingests, it secretes, it binds, it stimulates, it inhibits...it activates, it modulates, it differentiates...it is a healer of wounds." However, my coworker, Bill Farrar, minimized the role playing ability of the Mϕ, concentrating his immunoregulatory studies around the function of a suppressor T (T_s) cell found in the spleens of TBM (60, 70). In the end, our research efforts reveal a dualistic overlapping regulatory mechanism (47, 59, 69) which, as our work continued to dovetail, forced us to concede that T cell blastogenesis was regulated both positively (42) and negatively (43, 60) by the presence of Mϕ (44) and/or TBM T cells (43, 70). Though the bulk of this dissertation will deal with the immunoregulatory properties of normal and TBM Mϕ, to view the immunoregulatory picture in its entirety, it was necessary to include TBM T cell data.

Finally, all *in vitro* systems, to be of clinical value in the treatment of cancer, must at some point demonstrate *in vivo* biological relevance. Though the confines of this dissertation do not extend past *in vitro* immunoregulatory mechanisms, our adoptive transfer studies (185) and Dr. Elgert's *in vivo* delayed hypersensitivity experiments (accepted for publication) bear out many of the conclusions drawn from these *in vitro* experiments. In addition, results from other studies of CMI regulation in animal models, have already led to their application, in a limited degree, to the treatment of human neoplasia (182). Hopefully, continued *in vivo* and *in vitro* studies will silence Zanvil Cohn's lament that "we know little about the *in vivo* role of macrophages in controlling the immune response" (38).

This study summarizes research which I feel will serve as the necessary foundation for further investigation of *in vivo* as well as *in vitro* CMI regulation. It represents 3 years of investigation of T cell blastogenic immunoregulation in normal and TBM. The areas of interest, examined most closely are listed below:

i) Characterization of enhancing and inhibitor factors in Mϕ supernatants.

ii) Concentration dependent role of Mϕ in immunoregulation.

iii) Demonstration of enhancing and inhibitory activity in TBM Mϕ supernatants.

iv) Reversal of Mϕ augmented MLR reactivity by tumor-induced splenic suppressor T cells.

v) Positive blastogenic regulation by TBM T cells.

II. LITERATURE REVIEW

In the immunological triumvirate of macrophage (Mϕ), T cell and B cell, the Mϕ bears the distinction of being the first cell type thought to protect the host from foreign intruders (210). Metchnikoff, in 1882, noticed that transparent starfish larvae, possessed motile cells which surrounded and attempted to engulf foreign material (a rose thorn) introduced into the host. He called these cells mononuclear phagocytes or Mϕ (99).

The 100 years since Metchnikoff first proposed his theory of cellular immunity, has seen the Mϕ grow in immunological stature, rising from its humble role as cellular garbage man, to its current premier position as immunoregulator of humoral and cell-mediated immunity (225).

As with any leucocyte, the Mϕ traces its origin to bone marrow stem cells. It develops through the monoblast, promonocyte, monocyte stages, arriving finally at its mature end cell stage -- the macrophage. It then takes up residence in a variety of tissues, i) connective tissue (histiocytes), ii) liver (Kupffer cell), iii) lung, (alveolar Mϕ), iv) serous cavities (peritoneal Mϕ) (232).

The actual definition of Mϕ was established most recently at the 1973 Leiden conference on "Mononuclear Phagocytes in Immunity, Infection and Pathology" (232). As discussed at that meeting, Mϕ i) are mononuclear cells, ii) possess peroxidase and esterase activity, iii) bear specific surface antigen receptors for antibody and complement, iv) exhibit phagocytic and pinocytic abilities, v) may be uniquely stimulated into a state of "activation" (defined as increased

phagocytosis, increased quantity of acid hydrolases, more active metabolism, and increased bacteriocidal capacity).

To this definition, Unanue has added a sixth characteristic -- a varied and prolific secretory ability (225). Now recognized as one of the body's primary secretory cells, Mϕ have been seen to release i) a variety of enzymes (lypozyme, plasminogen activators, collagenase, elastase), ii) a number of complement proteins, iii) various interferons, and iv) a multitude of immunoregulatory factors (lymphostimulatory factors, inhibitors of DNA synthesis, cellular toxins). It is this fourth category of secretory products which holds my interest. Analysis of these factors (and those secreted by T cells) is the central topic of my dissertation. As outlined below, the first topic of this literature review is positive Mϕ regulation of the lymphocyte response to antigen.

A. Positive or Negative Immunoregulation by Normal Macrophages

1. Positive Regulation

Under the heading of cell-mediated immunity (CMI), comes a variety of T lymphocyte activities, ranging from T helper cell induction of antibody (51) to cytotoxic T cell destruction of foreign tissue (204). The many facets of the CMI response, varied though they may be, bear at least one common denominator -- they all require the presence of an actively dividing, blastogenic T cell population and some foreign stimulus to trigger that activity (5, 164). Because of its central role in the CMI response, T cell proliferation, is used by us (43, 44, 58, 59) and others (14, 89, 183) as a measure of T cell immunocompentency.

As stated in the Introduction, a number of antigenic stimuli can induce T cell blastogenesis, which in all cases, is enhanced by Mϕ presence.

Mitogens, plant glycoproteins inducing polyclonal blastogenesis of the entire T lymphocyte population, have long been a favored method of lymphocyte activation because the high percentage of DNA synthesizing cells, facilitates determination of lymphocyte blastogenic rate (as measured by ^{3}H-TdR uptake). T cells from guinea pigs (201), mice (89) and humans (151, 183), when exposed to the specific T cell mitogen phytohemagglutinin (PHA), show an enhancement of activity in the presence of Mϕ. Though the MLR was used as my primary proliferative model, the PHA assay was often used as a secondary means of verifying Mϕ regulation of T cell blastogenesis. My major interest in mitogen related studies was not so much their results, but the materials and methods used to obtain purified lymphocytes. Combining protocols followed by Oppenheim et al. (183), Julius et al. (120) and Gery et al. (89) I passed lymphocytes through a nylon wool column and incubated nonadherent eluates on tissue culture plates. This separated nonadherent T cells from adherent Mϕ populations (55). Recently, noting Rosenstreich's emphasis on the necessity for a rigorously purified T cell population (201), we have, in lieu of T cell plating, substituted passaging through a glass bead and Sephadex G-10 column.

As mentioned earlier, antigen-induced lymphocyte proliferation (in this case purified protein derivative from Mycobacteria) is also augmented by the in vitro presence of Mϕ (210, 240). The importance of this assay system, to me, lies in the use of peritoneal exudate cells

as a source of Mϕ. Results also indicate that mitomycin C-treated Mϕ retained their enhancing properties, in agreement with our results (58).

Most pertinent to my own research were those papers showing Mϕ enhancement of MLR reactivity (149, 150, 198, 199). They (like us) show that Mϕ-depleted T cell cultures responded maximally to allogeneic lymphocytes only when glass adherent, phagocytic cells were added back to MLR cultures.

It is satisfying to note that others (157, 162, 223) have verified our findings (40) showing augmentation of lymphocyte reactivity not only by addition of syngeneic, but also allogeneic or xenogeneic Mϕ. In other words, in their capacity as accessory cell, Mϕ may cross the lymphocyte histocompatibility barrier and enhance T cell activity in both MLR (162, 223) and PHA (157, 208) reactivity. We (58) and others (102, 177) observe that allogeneic Mϕ may enhance blastogenesis simply because they possess a greater number of foreign histocompatibility surface antigens, and, compared to allogeneic target lymphocytes, are superior stimulators. It is true that enhanced lymphocyte proliferation may be partially due to the passive target cell role of allogeneic Mϕ. However, nowhere is it shown that soluble factors may substitute for viable intact target (stimulator) cells (97, 207), and, since we (45, 46) and others (4, 10, 230) describe enhancing Mϕ supernatant factors, it appears as though allogeneic (as well as syngeneic) Mϕ are capable of actively synthesizing soluble factors which enhance lymphocyte proliferation in response to target cell histocompatibility antigens.

Soluble MLR enhancing factors were easily obtained from supernatants of Mϕ, cultured for varying lengths of time (24 to 96 hr) in RPMI medium. As mentioned previously, we (58) and others (4) find that enhancing supernatants can be obtained from allogeneic or mitomycin C-treated Mϕ. In our system, in order to obtain such supernatants, it was not necessary to stimulate Mϕ by culturing them with allogeneic lymphocytes (40). In agreement with Unanue et al. (230), we do however feel that a superior quantity of enhancing factor(s) may be obtained from Mϕ if they are stimulated by addition of lipopolysaccharide or activated immune T cells.

Blastogenic enhancement by Mϕ supernatants, has at times been decried as an in vitro artifact due to Mϕ release of reducing agents, which, by enhancing in vitro T cell viability, gives the false impression of actively augmenting MLR reactivity (148). Some feel that the in vitro addition of 2-mercaptoethanol (2-ME), a strong reducing agent, would substitute for Mϕ supernatant presence (96, 110). However in our hands, Mϕ-depleted T cells invariably exhibit a low level of MLR reactivity, even though we routinely use an optimal 10^{-5}M concentration of 2-ME in all cultures (47, 60). We therefore concur with Ellner et al. (62) that "2-ME can support a Mϕ-depleted population of resting lymphocytes so that the latent biologic activity is maintained, but it is not itself sufficient to allow the induction of lymphocyte proliferation."

A strong argument in defense of the biological validity of Mϕ enhancing supernatants lies in the rigorous biochemical characterization of a lymphocyte activating factor (LAF) derived from Mϕ

supernatants. Originally defined functionally by Oppenheim as the ability to induce PHA stimulation in normally unresponsive thymocytes (184), LAF has now been characterized by a variety of biochemical procedures, and its purification will probably set the precedent for further "lymphokinology" studies. By means of ultra-filtration (145) and Sephadex gel filtration chromatography (90) LAF is judged to be approximately 15,000 daltons in molecular weight. Ion exchange chromatography (24), gel electrophoresis (144) and iso-electric focusing (145) further separate LAF from other contaminating proteins. Though our own enhancing factor is not LAF (it fails to induce PHA stimulus in thymocytes) we are currently using a similar biochemical purification scheme to characterize our factors. A major problem I have encountered in our biochemical studies is the lack of an appropriately large quantity of starting material. In the case of LAF this problem is circumvented in two ways i) adding agents to Mϕ cultures which artificially induce Mϕ to release a greater quantity of LAF (91, 224), and ii) use of a Mϕ cell line which can be maintained indefinitely in vitro (163). However, a sizeable portion of my research is devoted to comparing the relative regulatory activity of normal and TBM Mϕ supernatant (44). Therefore, I have attempted to minimize in vitro manipulations which might alter the status of the normal or TBM Mϕ under examination.

The heterogeneous composition of Mϕ supernatants contains a variety of enhancing factors less well characterized than LAF. Proteolytic enzymes are isolated (39) possessing enhancing activity (108) or an ability to enhance MLR reactivity indirectly by degrading soluble

inhibitor factors (see Results section). The transient appearance of a mitogenic protein is also noted (229). In addition, Mϕ supernatants are seen to contain a plasminogen activator which possibly regulates differentiation and normal cell proliferation (231, 234). Finally a variety of factors are isolated which enhance various aspects of antibody production (63-65).

2. Negative Regulation

The immunoregulatory capacity of Mϕ is not limited entirely to their positive, enhancing role in T cell blastogenesis. Under various circumstances Mϕ may also inhibit T cell proliferation.

The effects of Mϕ-induced CMI suppression are most easily seen when Mϕ have been "activated" -- an ambiguous term generally defined as Mϕ stimulation into a metabolically active state resulting in an enhanced, phagocytic, cytostatic, and cytocidal activity (174). The most commonly used stimulatory agents are lipopolysaccharide (171), Corynebacterium parvum (137), pyran copolymer (15, 16) and Bacillus Calmette Guerin (BCG) (141). Though Mϕ activation undoubtedly increases the yield of inhibitory factors (130, 132, 133, 135), the use of artificial stimulants is a laboratory manipulation which I was willing to sacrifice for the sake of a more valid comparison of normal and TBM Mϕ-mediated immunoregulation.

A great deal of energy has been expended at the National Cancer Institute on comparative normal and TBM Mϕ experiments, in an attempt to prove that TBM T cells are hyporesponsive due to the presence of unique tumor-induced suppressor Mϕ (134, 138). In extensive depletion

experiments, they show that the removal of splenic Mϕ from TBM T lymphocyte populations partially restored T cell activity -- proof, they say, of the existence of a tumor-induced inhibitor Mϕ (138). Our evidence shows that TBM Mϕ are not uniquely suppressive; rather they augment MLR reactivity more than their normal counterparts (57). Any inhibition is probably the result of the quantitative increase in number of Mϕ in a TBM spleen rather than a qualitative difference in their immunoregulatory activity (44).

There is one point of agreement in most all Mϕ immunoregulatory studies -- the mechanism of T cell suppression is not contact dependent but rather mediated by release of soluble inhibitor factors (178, 181, 225, 226, 228, 237, 238). Researchers involved with Mϕ suppressor factors, generally polarize into the low molecular weight suppressor (LMWS) camp (31, 160, 205) or the high molecular weight school of immunoregulation (37, 222). Our inhibitor factor is greater than 12,000 daltons, as shown by dialysis. It therefore qualifies as a high molecular weight inhibitor (42). However, supernatant dialysis does decrease, somewhat the degree of inhibition. Therefore, we find ourselves sitting on the fence with Nelson et al. (173) who stated that, "Mϕ produce both a dialyzable inhibitor...and a macromolecular inhibitor."

Though Calderon et al. (31) describes no high molecular weight suppressor factor, we at least agree with him that the inhibitor is heat stable (100° C for 10 min). Like Toh (222) we have seen that DEAE (Diethyaminoethyl) anion exchange chromatography of whole supernatant produced multiple peaks of inhibitor activity -- some inhibitor

fractions positively charged, while others possessing a strong negative charge. Since the inhibitor factor, in many assay systems, is destroyed by prolonged incubation with pronase (31, 222), it is possible that the high levels of proteolytic enzymes present in Mϕ supernatants (48), may partially degrade the inhibitor factor, changing its net charge, while not totally abrogating its suppressor qualities. This would result in spurious multiple peaks of inhibitor activity. The case for partial in vitro inhibitor factor degradation is strengthened by our recent results showing that Mϕ incubation with phenylmethylsulfonyl-fluoride (PMSF), a potent proteolytic enzyme inhibitor, increased the suppressive capacity of high dose Mϕ supernatants.

Another question often raised by immunological gadflies, is one concerning the biological relevance of inhibitory Mϕ supernatants. Opitz et al. (179, 180) and others (215) felt that blastogenic inhibition, as measured by decrease in the uptake of ^{3}H-thymidine (^{3}H-TdR), is an artifact due to high levels of extracellular thymidine in Mϕ supernatants -- competition with the ^{3}H-thymidine producing a reduction in cpm, and giving the false impression that proliferation was inhibited. Like Hoessli et al. (113), we avoid this controversy altogether by analyzing in detail, only those supernatants, previously dialyzed to remove any small inhibitor molecules (including thymidine) (42). However, others show the non-thymidine nature of Mϕ inhibitor factors by documenting suppression, using uptake of ^{32}P (247), ^{3}H-leucine (252), ^{3}H-uridine (127) and ^{14}C (239). In addition, Fernbach et al. by correlating ^{3}H-TdR incorporation and lymphoblasts counts,

showed a "real" visible abrogation of T cell proliferation upon exposure to Mϕ supernatants (72).

Thymidine is not the only small molecule thought, by some, to be responsible for the inhibitor qualities of Mϕ supernatant. Prostaglandins heighten intracellular levels of cyclic AMP (242, 243) thus inhibiting DNA synthesis (26, 191). Because Mϕ are known to release prostaglandin (103), some researchers feel that prostaglandin in Mϕ supernatants is the causative agent of Mϕ-induced inhibition (175). However, in our system, Mϕ incubation with indomethacin, a prostaglandin inhibitor, did not abrogate the suppressor activity of our dialyzed concentrated supernatants. I therefore eliminated another small molecule from the list of possible inhibitor candidates.

3. Dual Positive and Negative Immunoregulation

Thus far, discussion of monokines has centered around isolated examination of either enhancing factors or inhibitor supernatants -- as if positive and negative immunoregulatory activity were mutually exclusive Mϕ functions. Obviously, because of the heterogeneous nature of Mϕ supernatants, it is important not only to purify and isolate, but to examine how these factors interact. We (42) and others (29, 30, 173, 214, 227) see that Mϕ supernatants, derived from a single culture, can, with certain manipulations, manifest either inhibitor or enhancing activity. Unanue et al. (227) is able to unmask enhancing factor in Mϕ supernatants after dialyzing away a small but potent inhibitor molecule. Our inhibitor, like that from Unanue's laboratory (30), was heat stable -- but, unlike his, was nondialyzable. In our hands, at low supernatant

concentrations, it was the enhancing factor which masked inhibitor activity. To separate the two factors, whole supernatants were boiled -- thus inducing loss of heat labile enhancing factor, while retaining the inhibitory activity (61). In high supernatant concentrations however, our inhibitor factor, like that of Sredni et al. (214), invariably overruled any activity of enhancing factor. To separate our high dose supernatants into their component enhancing and inhibitory parts required application of more rigorous biochemical techniques (i.e., anion exchange chromatography).

B. Macrophage Immunoregulation: A Concentration Dependent Phenomenon

So far, it appears as though a single heterogeneous Mϕ supernatant preparation contains both inhibitor and enhancing factors. These supernatants, by artificial manipulations (i.e., dialysis, heat treatment) can be induced to exhibit a single immunoregulatory property. However, the following papers indicate that, under more natural circumstances, manifestation of enhancement or inhibition is simply a concentration dependent phenomenon -- that is, low doses of Mϕ, enhance T cell proliferation, while high doses inhibit blastogenesis. This characteristic dose dependency extends to any blastogenic assay system, whether it be mitogens (76, 200), soluble antigen (239) or alloantigen (19, 131) stimulation. This pattern of concentration dependent Mϕ regulation extends not only to T cell blastogenesis but to T cell cytolytic activity as well (159) -- thus establishing even further, the link between T cell proliferation and cytotoxicity. Like whole Mϕ, Mϕ supernatants also express dose dependent immunoregulation, as is seen by us (42) and others (113, 251).

In general, all of these concentration dependent, titration experiments reinforce results of various aspects from our own research (42, 44):

a) activated Mϕ supernatants possess a higher level of immunoregulatory factors than their normal counterparts (200);

b) Mϕ sonicates yield no inhibitory factors (239);

c) thymidine release may constitute part of the inhibitory picture but is not exclusively responsible for suppression of T cell blastogenesis (113);

d) Mϕ immunoregulation crosses histocompatibility barriers -- allogeneic as well as syngeneic cell Mϕ are capable of exerting positive or negative immunoregulation (247); and

e) mitomycin C treatment does not abrogate Mϕ immunoregulatory properties (260).

C. Positive and Negative Immunoregulation by Tumor-Bearing Mouse Macrophages

1. Positive Regulation

Clearly, normal Mϕ possess the ability to enhance lymphocyte proliferation. In view of *in vitro* TBM T cell hyporeactivity (21, 121, 185), the positive immunoregulatory capacity of TBM Mϕ is a far more controversal question. However, Nelson and Kearney (172) showed that a tumor-bearing animal receiving a second tumor cell inoculation at a site other than that of the primary tumor inoculation, will suppress tumor growth at that second (but not the initial) site. This phenomenon of "concomitant immunity" indicates that there is at least some remaining activity in the CMI system; though the animal can reject a

secondary tumor transplant it invariably succumbs to the primary inoculation (88, 128). Rejection of this second tumor cell inoculation was hastened if TBM (but not normal) Mϕ were injected along with the tumor cells. However, if the host animal is thymectomized and irradiated before the second tumor injection, no secondary tumor cell rejection would occur, no matter how many TBM Mϕ were injected into the second site (211). This indicates that TBM Mϕ were not the cells directly responsible for tumor cell cytolysis, but acted to enhance T cell blastogenic and cytolytic activity in the host animal (2).

In in vitro experiments we (44) and others (152) have also examined the ability of TBM Mϕ and TBM Mϕ supernatants to enhance (or inhibit) normal T cell proliferation. The results showed that, far from being uniquely inhibitory, TBM Mϕ supernatants, on a volume to volume basis, possessed a greater (not lesser) ability to enhance normal proliferation. Whether this was due to i) greater production of enhancing factor, ii) reduced synthesis of inhibitor factor, iii) elaboration of a more potent enhancing factor, or iv) secretion of a less potent inhibitor factor, is the subject of current biochemical investigations. This in vitro work of ours, demonstrating enhanced T cell blastogenesis in the presence of TBM Mϕ, correlates well with other cytotoxicity studies showing that TBM Mϕ also amplify the cytolytic, tumorcidal activity of T effector cells (67, 158).

2. Negative Regulation

Despite concomitant immunity experiments pointing to some level of immunocompetency, the tumor-bearing host is immunologically

debilitated to a degree which renders him incapable of destroying the primary tumor graft. Extensive in vitro experiments with whole leucocyte populations indicate that tumor-bearing host populations, prior to Mϕ depletion, invariably manifested an inability to respond, blastogenically, to concanavalin A (136), PHA (252), allogeneic lymphocytes (72, 236) or syngeneic tumor cells (94, 176). Whole lymphocyte populations were taken from a variety of tumor systems: 1) Moloney sarcoma (136), 2) methylcholanthrene-induced fibrosarcoma (190), 3) human lymphoma (252), 4) virus-induced leukemia (94). In addition to blastogenic hyporeactivity, loss of TBM T cell cytotoxicity is also noted in a Mϕ enriched TBM lymphocyte population (56, 235).

The evidence indicting TBM Mϕ as tumor-induced suppressor cells seems unassailable, and unequivocably damning. However, Mϕ suppressor activity was usually measured by methods of Mϕ depletion. Using a variety of purification techniques including rayon wool (136), nylon wool (236), tissue culture plating (190) or iron filing phagocytosis (94), hyporeactive TBM lymphocytes were depleted of Mϕ, whereupon some (but not all) blastogenic reactivity was restored to the previously recalcitrant TBM whole lymphocyte population. Thus the TBM Mϕ was judged to possess a unique suppressor factor, the removal of which reversed T cell hyporeactivity.

We do not contest the fact that high numbers of TBM Mϕ inhibit T cell proliferation. The difference of opinion arises when this suppression is attributed to a unique Mϕ inhibitor factor elaborated only by TBM Mϕ. We commonly note (58, 59) that TBM spleens contain up to 10 times more Mϕ than their normal counterparts. Coupled with the high

dose inhibition pattern of Mϕ regulation, I feel that Mϕ-induced TBM T cell inhibition could be explained by quantitative increase in Mϕ numbers rather than a qualitative alteration in Mϕ suppressive capacity. Our "add back" (as opposed to depletion) studies, comparing the function of normal and TBM Mϕ on a cell to cell basis, bear out my conclusion -- TBM Mϕ are no more inherently inhibitory than normal Mϕ.

D. Suppression of Lymphocyte Blastogenesis by Tumor-Induced Suppressor T Cells

In our hands, TBM T cells, even after Mϕ depletion, fail to proliferate normally in response to antigenic stimulus (58, 59). In view of the finding that TBM Mϕ are no more suppressive (indeed, perhaps less inhibitory) than equal numbers of normal Mϕ (44, 211), the question arises as to why Mϕ-depleted TBM T cells remain immunologically hyporeactive. Fujimoto et al. (81-83) suggested that the causative agent in tumor-induced CMI debilitation was a population of suppressor T (T_s) cells. Detection of T_s cells in mastocytoma-bearing animals (7, 218) further establishes the connection between T_s cell induction and tumor presence; a connection we (47) and others (100, 101, 186) see using the methylcholanthrene-induced fibrosarcoma model. In agreement with our research (46, 59), T_s cells in other laboratories, are most often derived from thymic (125) and splenic (52, 71, 85, 86, 125) lymphocyte populations, or, less frequently, from thorasic duct lymphocytes (219) -- but almost never from lymph node cells (59). We (47) and others, find that T_s cells suppressed proliferation induced by mitogen (219), soluble antigen (8, 9) and allogeneic cells (193, 195-197) as well as inhibiting T cell cytolytic activity (74, 112).

As with Mϕ immunoregulation, our results, in agreement with data from other laboratories, indicate that T_s cell inhibition was mediated by soluble factors inhibiting CMI responses ranging from T helper cell function (217), to PHA responsiveness (206), to MLR reactivity (194). Our T_s cells (43, 47, 59, 68) also possess certain other characteristics held in common by T_s cells isolated by other researchers: i) they are mildly adherent to nylon wool (76, 77, 79). (Thus, they could be separated from nonadherent responder cells by passing whole TBM splenic lymphocyte populations over columns of nylon wool and collecting the nonadherent, T_s cell-free, population of responder T cells.) ii) Their inhibitory ability is not completely abrogated by treatment with mitomycin C (78); signifying that inhibitor activity is not totally dependent upon DNA synthesis. iii) The T_s cell is sensitive to the presence of hydrocortisone (168, 169, 176, 177), though the remaining TBM responder cells were not. iv) Their inhibitory effect differs from Mϕ inhibitor mechanisms in that T_s cell suppression inhibited T cell blastogenesis by inactivating DNA polymerases in the responder population (147).

E. Dualistic Suppressor Cell Regulation: Macrophage and Tumor-Induced Suppressor T Cell Inhibition

The above analysis of the literature indicates that suppression of T cell blastogenesis and cytotoxicity is under multicellular immunoregulation -- basically governed by sub-populations of Mϕ and T cells. The complexities of immunoregulation can be summarized in the following models.

Pope et al. has detailed a dual suppressor cell system in the spleens of TBM (189). Like ours, their tumor model is a methylcholanthrene-induced fibrosarcoma. Hyporeactivity is caused by release of nondialyzable soluble factors from splenic T_s cells or high numbers of Mϕ. Macrophage suppressor cells, are adherent to nylon wool, phagocytic for iron filings and unaffected by mitomycin C treatment. Suppressor T cells are sensitive to anti-thy 1 serum plus complement but, unlike our T_s cells, are also sensitive to mitomycin C.

In the non-tumor model developed in Waksman's laboratory (17, 76) suppressor cells generated by heavy injections of ovalbumin, produce high dose tolerance in rats. Like our suppressor populations, both T cell and Mϕ inhibitory cells were adherent to nylon wool. Incubation of whole spleen cells with carragheenan (a Mϕ cytolytic agent) or cyclophosphamide (toxic for T_s cells) caused partial, but not total, reversal of T cell blastogenic hyporeactivity. It was concluded that high dose tolerance was the result of Mϕ-T_s cell inhibitor factors acting synergistically.

Klimpel and Henney (141) focused, not on blastogenic regulation, but the T cell cytolytic mechanism. By comparing both Mϕ and T_s cell-induced suppression they conclude that the T cell cytolytic mechanism was as vulnerable to immunosuppression as the blastogenic stage of T cell differentiation.

Rice et al. (192) have evidence of an immunosuppressive triangle composed of two Mϕ sub-populations and a T_s cell population. The three populations interact normally in humans to hold immunological activity in check. One Mϕ population is adherent, radio-resistant and produces

soluble suppressor factors even when cultured with indomethacin. The other Mϕ population is less phagocytic, less glass adherent and (unlike) our Mϕ population) elaborated large quantities of prostaglandin. The T_s cell is anti-Thy 1 serum sensitive but differed from our observed T_s cell population by its hydrocortisone insensitivity. Apparently, it is important as a fail safe, back-up immunoregulatory cell, since its suppressor effects are most notable only after the host had been depleted of monocytic immunoregulators (185).

The elucidication of these other multicellular immunoregulatory models can not help but reinforce our confidence in our own dualistic suppressor cell model, described in brief at the 12th Annual Leucocyte Culture Conference (47, 61) and in more detail in this dissertation.

F. Positive Blastogenic Regulation by Tumor-Induced T Cells

Heretofore, examination of the immunoregulatory role of TBM T cells has been confined to analysis of their inhibitory properties. Though a concerted effort was made to establish the role of Mϕ in positive and negative immunoregulation, I took for granted that TBM T cells were suppressive. In fact, positive TBM T cell regulation was only recognized after reexamining a serendipitous set of experiments (originally discounted as unexplainable failures), showing that tumor-induced T cells, could in fact, enhance responder cell blastogenesis -- but only if the original background level of DNA synthesis in the responder population was quite low. We therefore abandoned the "T_s cell" designation as a misnomer, adopting "tumor-induced T cell" as a more accurate description of a TBM T cell which enhanced activity of

slowly proliferating responder cells but inhibited rapidly proliferating lymphocytes.

Gershon et al. (85) were among the first to recognize that this positive and negative cycle of T cell regulation could be a type of homeostatic, feedback control mechanism, boosting low levels of responder cell DNA synthesis, but checking high levels of T cell blastogenesis. In in vitro (52) and in vivo (35, 86) studies with mouse thymocytes, Gershon and co-workers noted a direct correlation between a high rate of host cell proliferation and a vulnerability to immunosuppression. Using the graft-vs-host reaction, as opposed to a tumor model like ours (41), they found that when DNA synthesis in parental thymocytes was high, the effect of F_1 thymocyte addition was suppressive; when it was low, the same F_1 thymocytes augmented activity. They attributed this homeostatic blastogenic balance to the presence of a suppressor-helper T cell, dubbed by them, "the hermaphrocyte" (34). Recently these hermophrocytic cells have been identified by a characteristic cell surface phenotype designated as Ly $1^+,2,3^+$. Depending on the blastogenic state of the surrounding responder cells, Ly $1^+,2,3^+$ will differentiate further into Ly $1^+,2,3^-$ helper cells (if responder cell blastogenesis is low) or Ly $1^-,2,3^+$ inhibitor cells (if responder cell blastogenesis is high) (53, 209). From Gershon's proposed model, it appears that the hermaphrocytic cell takes no active part in immunoregulation. Rather, it is the common precursor cell; its differentiation into an inhibitor or enhancing cell being dependent upon the type of blastogenic feedback signal it receives from responder cells. The immunoregulatory end cells differ not only in their phenotypic cell markers (87) but also

their half-live (125), radio-sensitivity (36), bouyant density (244) and susceptibility to small doses of anti-thymocyte serum (125). Basically, helper cells (Ly 1^{+},2,3^{-}) are long lived (15 wk), radio-resistant, low density, anti-thymocyte serum sensitive T lymphocytes while inhibitor T cells (Ly 1^{-},2,3^{+}) are short lived (2 to 3 wk), radiosensitive, high density, anti-thymocyte serum resistant, lymphocytes. Though we have not as yet characterized in detail, the tumor-induced T regulator cell, it is heartening to note that our phenomenological experiments were in agreement with others describing T cell regulation from the standpoint of responder cell blastogenic activity.

When analyzing the various control mechanisms governing the CMI response, there is a tendency to "home in" on some hapless immunological tree, missing the ever-growing regulatory forest. Nowhere is this more apparent than in the field of lymphokinology. One has only to peruse a lymphokine review article (239), or better yet to read the minutes of "First International Workshop on Mechanism of Action and Characterization of Lymphocyte Mediators (181). If the reader escapes being LAFed, GATed, BAFed or MAFed to death he is certain to stumble upon lurking LMWS, SIRS or AEF's. The point is, a great deal of work has been done with a large number of very specific immunoregulatory models. Basically, almost all of these models can be conveniently placed in one of four immunoregulatory categories: i) Mϕ enhancement of a certain leucocyte response (LAF, BAF), ii) Mϕ inhibition of a given response (LMWS), iii) T cell enhancement (AEF, MAF) or iv) T cell inhibition (GAT, SIRS). Examined in this fashion, immunoregulatory processes appear very ordered and balanced. In fact, an "immunological

balance" is an apt description of the way in which Mϕ and T cells hold responder cell activity to a effective but also efficient level (244). This homeostatic mechanism is described in terms of Mϕ immunoregulation by Nelson (170) and in terms of T cells by Jerne (119). Their (and my) analysis of immunoregulation is but an overview designed to restore order to a confusing array of interactions. However, if there is one point of emphasis especially highlighted in this literature review it is the complexity of the various immunoregulatory pathways. In the face of such intricacies, it would be presumptuous to assume an overly dogmatic attitude. However, to the best of my ability, I will present evidence which I feel points to concentration dependent Mϕ regulation and blastogenic feedback TBM T cell immunoregulation.

III. MATERIALS AND METHODS

A. General Protocol

1. Medium Preparation

For preparing medium, procedures described by Ling and Kay (154) 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 10.0 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 2-mercaptoethanol and a 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 to 8 weeks of age from Flow Laboratories, Dublin, Virginia. "Responder" (R) cells were obtained from mature BALB/c Dub male mice. "Stimulator" (S) cells were taken from the spleens of mature C3H/He Dub male mice. Betel *et al.* (20) and Adler (1) have both shown that mice over 6 weeks have reached immunological maturity.

3. Fibrosarcoma Induction

To induce primary tumor growth, the method of Smith and Kondo (212) was followed. In this procedure, 0.25 mg of 2-methylcholanthrene (Eastman Kodak, Rochester, N.Y.) was suspended in 5.0 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*. (107). Palpable tumors were excised and placed in plastic tissue culture plates containing 5.0 ml of complete medium at 37°C. Tissue was minced with scissors, the supernatant absorbed with a sterile gauze pad and the minced tumor fragments placed in a 30 ml Erlenmyer flask containing 15.0 ml of a 0.25% trypsin solution. The preparation was agitated at room temperature by a magnetic stirrer. After 15 min, 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 15.0 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 5 min, washed twice with 10.0 ml of complete RPMI medium, and reconstituted to 6.0 ml in complete medium. Tumor cells were counted using a hemocytometer. 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 5 min incubation at room temperature, viable cells were counted in a hemocytometer. An appropriate

dilution of the tumor cell preparation was made so that 10^6 viable cells/0.1 ml were injected i.m. into the left hind leg of mice.

4. Spleen Cell Preparation

To obtain pooled spleen cell preparations, the general procedures of Mardiney and Wren (161) were followed. Six to 12 spleens were removed aseptically and placed into # 50 wire mesh stainless steel cylinders. Spleens were pressed through cylinder screens with a 10 ml glass syringe plunger. Lymphocyte suspensions were placed in 15.0 ml of chilled (4°C) phosphate buffered saline (pH 7.4) 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 centrifugation for 5 min at 400 x g. Packed spleen cells were diluted in 1 to 3 ml of HBSS or PBS.

Red blood cell (RBC) lysis (104) was accomplished using a 0.85% solution of NH_4Cl. For every volume of packed cells, 4 volumes of chilled (4°C) lysing agent were added. The preparation was incubated at 4°C for 5 min with frequent agitation. Following RBC lysis, lymphocytes were centrifuged at 400 x g, washed twice with HBSS and diluted in 3 to 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 3 times.

6. Mitomycin C Inactivation

Because of its ability to halt DNA synthesis and prevent blastogenesis, the antibiotic mitomycin C (146) 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 nonspecific 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. (187). 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 min. The preparation was then centrifuged for 5 min 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.

7. Cell Dilution

Dilution procedures generally followed those described by Thurman et al. (221) and Hartzman et al. (106). 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 mixed 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. Oxford syringes 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 multiple dispensing Oxford syringe so that each well contained 0.2 ml of cells in complete RPMI medium. Before dispensing, a 0.025 ml sample of each cell preparation was taken and assayed for cell viability. Cells were incubated for 5 min 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 hemocytometer 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. (221) and Hartzman et al. (106), cultures were incubated for 4 days at 37°C in a humidified CO_2 (5%) incubator. Six hours before assay termination, cultures were pulsed with 1 µCi of methyl ^{3}H-thymidine (^{3}H-TdR, Spec. act 6.0 Ci/mM; Swartz/Mann, Becton Dickenson, Orangeburg, N.Y.) delivered in a 0.05 ml volume of RPMI medium by a 50 lambda pipetter. "Counts per minute" (cpm) represents DNA synthesis as measured by incorporation of ^{3}H-TdR. All subsequent references to activity are reported in cpm.

9. Assay Termination

To terminate the micro assay, a "Multiple Automated Sample Harvester" (106, 221) (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 min in 75°C hot air oven, filter disks containing radioactive DNA were removed from the glass fiber strip with forceps and placed in scintillation counting vials (A.H. Thomas Co., Phila., Pa.) along with 0.3 ml of methyl benzethonium hydroxide (166) (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.

10. Counting

Filters were counted for 1 min on a Beckman LS-230 liquid scintillation counter (Beckman Instruments, Irvine, Calif.). The counter was set at 32% gain and a full ^{3}H window.

Data Presentation. All experiments were conducted using pooled spleen cells from 6 to 12 mice. Each data point represents the mean of 6 replicate samples $\pm$ S.E. Results were recorded as: i) the difference (Δ cpm) between cpm of Mϕ supplemented and unsupplemented populations ($[R + S_m + M\phi] - [R + R_m]$); or ii) stimulation index, cpm of Mϕ supplemented responder population divided by unsupplemented population ($[R + S_m + M\phi]/[R + R_m]$). Activity was determined by the incorporation of ^{3}H-TdR and expressed as cpm. $R + R_m$ populations were

chosen as the appropriate control only after determining that supernatant addition to syngeneic R + R_m controls, caused no significant difference in uptake of ^{3}H-TdR when compared to unsupplemented R + R_m populations (R + R_m + high dose Mϕ supernatant = 2329 ± 278 cpm; R + R_m + low dose Mϕ supernatant = 2008 ± 77 cpm; R + R_m = 2172 ± 273 cpm). Thus we felt justified in using R + R_m populations as background controls, since Mϕ supernatants, while significantly enhancing or inhibiting R + S_m alloreactivity, apparently did not significantly alter background R + R_m syngeneic activity. In the following experiments uninhibited R + S_m populations commonly exhibited a cpm value 2 to 7 times higher than that of the syngeneic R + R_m control.

B. Preparation of Leucocyte Sub-Populations

1. Nonadherent T Cell Population

a. Nylon Wool Purification. Any procedures involving nylon wool purification of spleen cell preparations were done according to Julius et al. (120) with minor modifications.

To remove toxic factors, nylon wool (Fenwal Laboratories, Morton Grove, Ill.) was incubated in saline for 2 hr at 37°C. It was then rinsed 3 times in doubly distilled water. Subsequent soaking at 37°C for 1 wk in frequently changed distilled water removed all toxic factors. The wool was dried in a 75°C hot air oven for 2 days and packed into 20 ml syringes (Monojet-Medical Industries, Deland, Fla.). The 20 ml syringes contained 2.0 g of wool packed to the 15 ml mark. The plunger was then replaced, the syringes autoclaved and fitted with 18 gauge needles (Monojet-Medical Industries, Deland, Fla.).

Before use, columns were rinsed 3 times with 15 ml of HBSS and incubated at 37°C for 45 min with 3.0 ml of complete RPMI medium. RPMI medium was then removed, the needle plugged and 3.0 ml of cells added. Each column received no more than 3×10^8 cells/3.0 ml. Cells were washed into the wool with 1.0 ml of warmed complete medium and incubated at 37°C for 45 min. Nonadherent T cells were obtained by gently washing each column with 25.0 ml of warmed (37°C) RPMI medium. T cells were placed in 15 ml centrifuge tubes, centrifuged at 400 x g and reconstituted to 10.0 ml with complete medium. T cells were then placed on 150 x 15 mm plastic tissue culture plates (Lux, Scientific Corporation, Newberry Park, Calif.) to remove any remaining adherent Mϕ. After incubating for 45 min at 37°C, the nonadherent T cells were removed and re-plated. Following this final re-plating incubation, T cells were removed with a pasteur pipette, by washing plates gently with 5 ml of warmed medium. The T cells were then centrifuged for 5 min 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 medium whenever possible. All concentrations were adjusted so that 2×10^5 viable responder cells (or 4×10^5 stimulator cells) were placed in microplate wells. Though viability was invariably greater than 90%, coulter counts indicated a 10-fold loss of cells during purification procedure (i.e., if 3×10^8 whole spleen cells were placed over a column, 3×10^7 T 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.

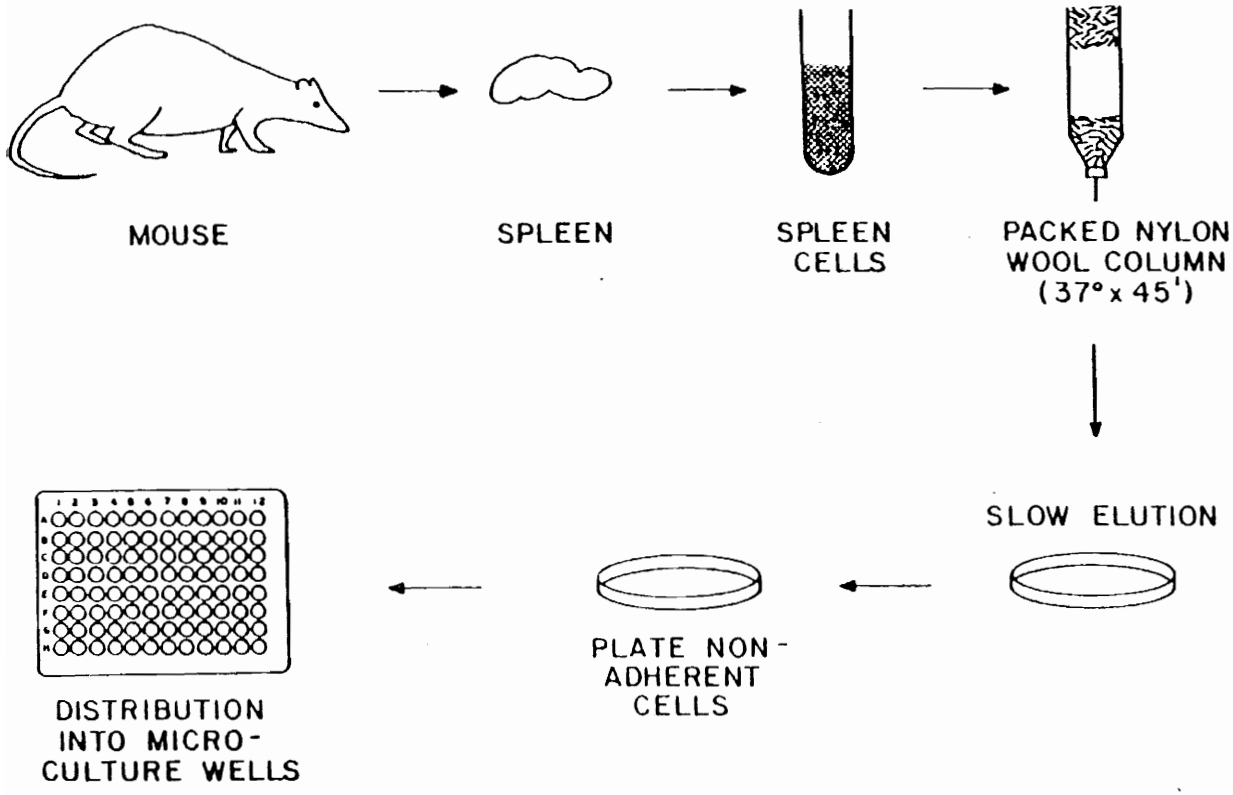


Figure 1. Techniques for Preparation of Purified B and T Mouse Lymphocytes by Nylon Wool Column Purification.

b. Sephadex G-10 Purification. An alternate procedure, employing a column of Sephadex G-10 (Sigma Chem. Co., St. Louis, Mo.) and glass microbeads (Ferro Chemicals, Cataphote Division, Jackson, Miss.) was also used to derive Mϕ-depleted lymphocyte populations.

To remove any contaminating material, glass beads were washed with concentrated HCl once or twice over a 24 hr period. Beads were then thoroughly rinsed in distilled H_2O (24 to 48 hr) until a pH reading indicated that all acid had been removed. After addition of a small layer of nylon wool to prevent glass bead seepage, each 60 ml syringe (Monojet Medical Industries, Deland, Fla.), filled to the 15 ml mark with 27 grams of glass beads, was capped and autoclaved.

Sephadex G-10, layered over glass beads, was prepared in the following manner. Each column was alotted 10 g of unswelled Sephadex. After swelling overnight in physiological saline, and changing saline 2 to 3 times, the Sephadex was finally diluted 1:1 with saline, and the slurry dispensed in 50 ml aliquots into serum vials for autoclaving and storage (4°C).

To use the G-10 technique in purification assays, 10.0 ml of warmed complete medium was added to stoppered column of glass beads. The column was unstoppered and the saline, G-10 slurry poured over the column. After the G-10 packed, the column was flushed with 75 ml of warmed, serum-free RPMI medium, followed by 40 ml of warmed complete medium. Spleen cell preparations (1.5×10^8 cells/ml) in 5.0 ml of complete medium were then allowed to run into the column. Purified lymphocytes (B and T cells) depleted of Mϕ were eluted from the column with 25.0 ml of warm complete medium.

If lymphocytes were to be used as responder cells, the eluate from the G-10 column was incubated on a nylon wool column and T cells obtained as described previously.

If lymphocytes were to be used as stimulator (S_m) cells, whole spleen cells were first incubated on a nylon wool column. The non-adherent T cell eluate from this column was discarded and the adherent population removed by soaking, and vigorously agitating the nylon wool. The resulting B cell-Mϕ population was passed successively over two G-10 columns to remove contaminating Mϕ. The purified B cell eluate was collected, mitomycin C-treated, and used as a stimulator population in the MLR. Heretofore, with the nylon wool plating technique we were confined to using T cells as allogeneic stimulators, for any attempt to make use of the superior B cell stimulators resulted in an unacceptably high percentage of contaminating Mϕ.

c. B Cell Contamination of T Cell Populations

(1) Response to Specific Mitogenic Stimulants. Specific mitogenic properties of T cells were used to assess the purity of the T cell preparation. A dose response experiment was conducted, the optimal mitogenic concentrations found, and subsequently used to determine purity of the T cell 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. The 0.05 ml aliquotes of these dilutions were added to flat bottom micro plates containing 2×10^5 cells in 0.15 ml of complete

medium (10% FCS). Except for a 72 instead of a 120 hr incubation, the mitogen assay was identical to the MLR.

(2) Jerne Plaque Assay. Kappler's (124) 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 5 min, 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 6 days later, mice were sacrificed and 1×10^5/0.025 ml purified T 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 2 hr, equilibrated to room temperature for 15 min and reincubated at 37°C for an additional 1.5 hr. At the end of the final incubation period, they were removed from the CO_2 incubator and examined under the dissecting 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 (120).

This assay (120) 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 0.05 ml portions were added to the tubes. Finally, 10^6 T cells/0.05 ml from suitable immunized mice were added to the tube of agar and SRBC. 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 2 hr, the slide rack was filled to capacity with a 1:25 dilution of guinea pig complement and re-incubated at 37°C for 1 hr. At the end of that time, slides were removed, washed twice in saline, and observed under a dissecting 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 T cell preparation (50, 120). Fluorescein-conjugated IgG rabbit anti-mouse gamma globulin (Cappel Laboratories, Downingtown, Pa.) was reconstituted with HBSS to a concentration of 2 mg/ml. T cells (142) 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 min 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 5 min. 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 min 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.

d. Macrophage Contamination of T Cell Population. Macrophage uptake of latex particles (49, 86) 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 T cells. After incubating at 37°C for 45 min, a drop of the mixture was placed in a hemocytometer and examined under the microscope for cell phagocytosis of 3 or more latex particles. Invariably, the number of such cells was negligible.

A more reliable way to determine the degree of Mϕ contamination was made using a leucocyte stain specific for esterases found in Mϕ (153, 250). To prepare the fixative, 4 grams of paraformaldehyde (Baker Chemical Co., Phillipsburg, N.J.) were dissolved in 55.0 ml of distilled water and agitated for 30 min 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 45.0 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 α-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 α-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 77.0 ml of a KH_2PO_4 solution and 23.0 ml of a Na_2HPO_4 solution. To obtain a hexazotized pararosanilin solution, 20.0 ml of distilled water was mixed with 5.0 ml of concentrated HCl and 1 g 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 1 min with an equal volume of a freshly prepared 4% solution of sodium nitrate.

Leucocyte smears could be stored before fixation for as long as 2 wk, but after fixing at 4°C for 30 sec, slides were washed gently, air dried, and immediately incubated for 45 min in a room temperature solution of stain. After incubating, washing and air drying, slides were counter-stained for 1 to 2 min 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.

2. Tumor-Bearing Mouse T Cell Population

Spleens from TBM were excised and prepared as described in Spleen Cell Preparation. As outlined elsewhere (43), whole spleen cells were plated for 1 hr. The plates were gently washed to remove nonadherent cells. The nonadherent cells were replated to remove any

remaining Mϕ. After 1 hr, these nonadherent cells were harvested, analyzed for Mϕ contamination using esterase staining techniques and judged to be >96% Mϕ-free. The small remaining number of Mϕ could not account for the inhibitory properties of these cells since there was no inhibitory activity upon treatment with anti-Thy 1 serum plus complement.

3. Macrophage Population

a. Preparation of Macrophages. Following the procedure of Folch et al. (76), mice were injected i.p. with 2.0 ml of thioglycolate broth. This served as a non-immunogenic irritant which attracted Mϕ to the peritoneal cavity but did not "arm" them or activate release of LAF (92). However, thioglycolate stimulated Mϕ lysosomal activity and enhanced their viability.

Four days after thioglycolate injection, mice were exsanguinated and Mϕ removed by peritoneal lavage using a pasteur pipette and 5 ml of warmed (37°C) medium. Macrophages were centrifuged at 400 x g for 5 min, and resuspended in 10.0 ml of complete medium. These solutions were pipetted onto plastic tissue culture plates and incubated at 37°C for 1 hr. At the end of this time, plates were vigorously washed with 25 ml of warm RPMI to remove contaminating T cells from the adherent Mϕ. The Mϕ were dislodged with a rubber policeman and suspended in 3.0 ml of complete medium. Cells were then centrifuged for 5 min at 400 x g and pellets resuspended in 0.8 ml of complete medium. To inactivate Mϕ, all tubes received 0.1 ml of mitomycin C (40.0 μg/ml); 0.05 ml of guinea pig complement and 0.05 ml of anti-Thy 1 serum (20

mg/ml) (Microbiological Assoc., Bethesda, Md.). All tubes were then incubated in a 37°C water bath for 45 min, centrifuged at 400 x g for 5 min, washed twice with 15 ml of RPMI medium and resuspended in 1.0 ml complete RPMI medium.

To verify antiserum potency, treatment of purified T cells abolished their response to PHA and caused cytolysis in >95% of the T cell population (though having no cytolytic effect on Mϕ). Such procedures yielded a 97% pure Mϕ population, as shown by esterase staining procedures.

After counting on a hemocytometer, Mϕ were diluted to concentrations ranging from 2 x 10^3 cells/0.05 ml (1% of lymphocyte population) to 1.6 x 10^4 cells/0.05 ml (8% of lymphocyte population). Prior to dispensing Mϕ into microculture plates, viability was assessed using the trypan blue dye exclusion test.

b. Supernatant Preparation. Supernatants were obtained by culturing BALB/c Mϕ (normal or TBM) or T_s cells in 3.0 ml of serum-free medium at a concentration of 1.2 x 10^7 cells. After a 2 to 4 day incubation period, supernatants were harvested, centrifuged for 10 min at 800 x g, passed through a 0.45 micron filter, diluted and immediately dispensed into microculture wells (use in biochemical assays is described later) in 0.05 ml aliquots bringing cultures up to a total volume of 0.2 ml (Figure 2). Some supernatants were dialyzed (pore size of 12,000 daltons) against RPMI medium for 24 hr while others were placed for 10 min in a boiling water bath before addition to wells. Appropriate controls were maintained to establish that results of heat

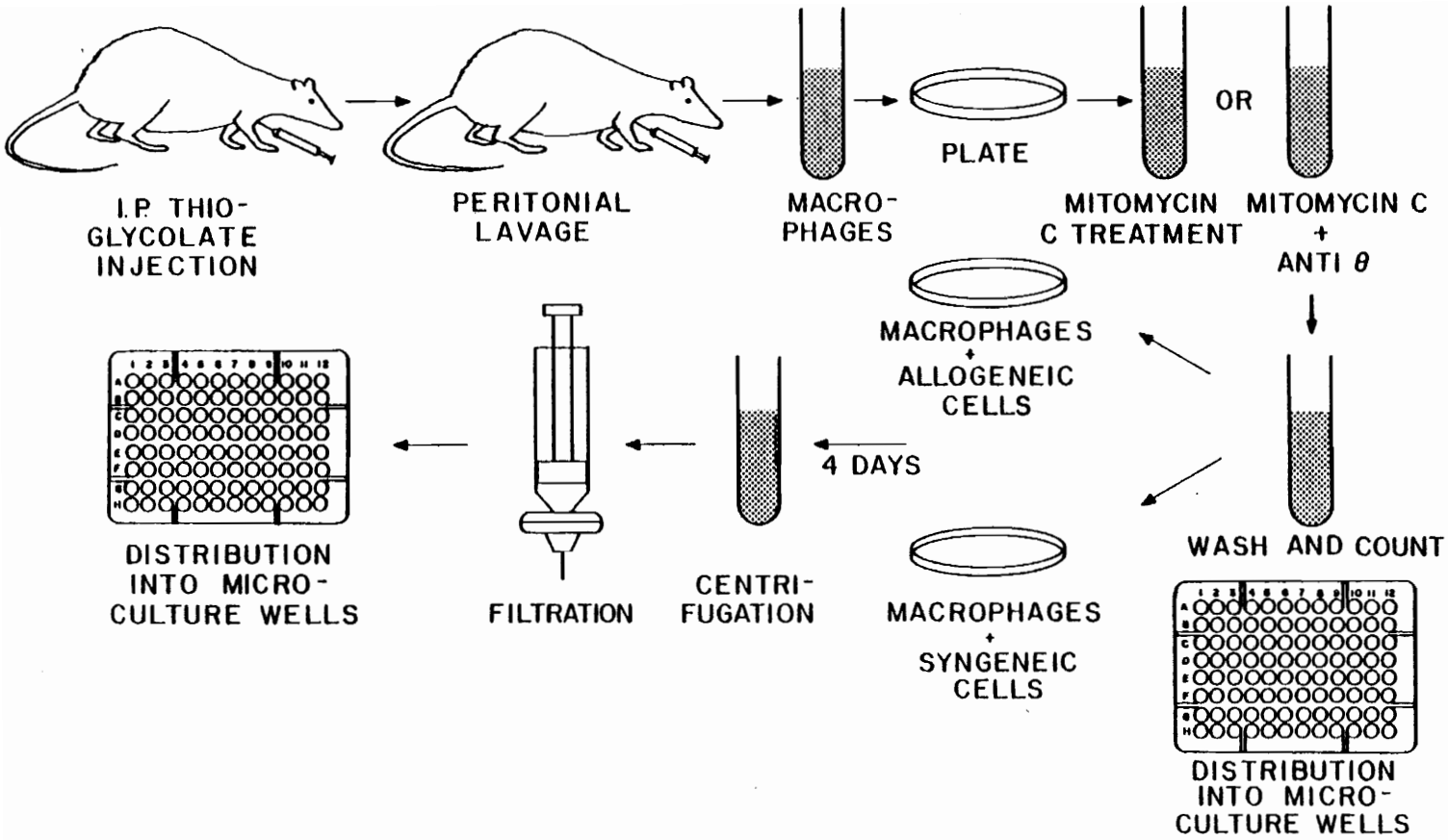


Figure 2. Protocol for Obtaining Macrophages and Macrophage Supernatants Used in Mixed Lymphocyte Reactivity Experiments.

treatment and/or dialysis were not due to artifacts introduced by alternation of RPMI medium.

In some cases Mϕ were incubated with 10^{-5} M concentrations of indomethacin (Sigma Chemical Co., St. Louis, Mo.) or proteolytic enzyme inhibitors such as benzidine dihydrochloride or PMSF (Fisher Scientific Company, Fair Lawn, N.J.). Before dispensing into MLR wells, all such treated Mϕ supernatants were dialyzed overnight in RPMI medium. While benzidine could be dissolved in distilled water, indomethacin was solubilized in isopropyl alcohol and PMSF in 95% ethyl alcohol. For stock solutions of 10^{-2} M, a 10.0 ml volume was used to dissolve 0.035 g of indomethacin, 0.025 g of benzidine or 0.0174 g of PMSF.

C. Cytoxicity (^{51}Cr) Assay

To establish the non-toxicity of Mϕ inhibitor factor the following assay was performed. Responder and stimulator T cells were purified in the previously described manner. Normal or TBM responder cells were incubated for 72 hr with mitomycin C-treated stimulator cells at a ratio of 1:2. The R + S_m population was then harvested, washed, resuspended in 1.0 ml of fresh complete medium and incubated for 2 hr with 0.4 mCi ^{51}Cr (1 mCi/ml, Amersham Corp., Arlington Heights, IL). At the end of that period, cells were centrifuged, washed twice, counted and dispensed into microculture wells at a final concentration of 6 x 10^5 cells/0.25 ml. Within this volume, "treated" cells contained 0.05 ml of Mϕ inhibitor supernatant ("untreated" cells received only complete RPMI medium). After 18 hr, a 0.1 ml volume of cell supernatant was carefully removed and assayed for ^{51}Cr release in

a gamma counter. Cytolysis was calculated as cpm of treated wells/ total (Triton X) lysis. The percentage lysis of treated and untreated cells was easily compared using the formula

$$\% \text{ lysis} = \frac{[R + S_m + M\phi + \text{target}] - [R + R_m + M\phi + \text{target}]}{\text{Total lysis}} \times 100$$

In all assays, controls lacking Mϕ supernatants (or purified factors) were run to obtain a background level of reactivity.

D. Biochemical Procedures

1. General Biochemical Purification Scheme

A sequential biochemical purification scheme has been devised based on procedures outlined in part by Lee and Lucas (147). Cell-derived supernatants were/will be subjected to successive purification by Sephadex gel-filtration, ion exchange chromatography, and slab gel electrophoresis. Results from these experiments represent only initial data in an ongoing series of biochemical characterizations. Results presented here should be considered as a springboard for further research into supernatant purification.

2. Supernatant Concentration

a. Ammonium Sulfate Precipitations. After obtaining supernatants, regulatory factors were precipitated by saturating the volume of supernatant (15 to 25 ml) with ammonium sulfate. The final concentration of ammonium sulfate was approximately 70%, or 7 g per 10.0 ml of supernatant. Precipitates were then centrifuged at 12,000 x g for 1 hr. Supernatants left after precipitation were discarded, since MLR assays showed them lacking either enhancing or inhibitory activity.

The precipitates were resuspended in 1 ml of RPMI medium, dialyzed over night against two 250 ml volumes of medium or buffer, and either used in the various cellular and biochemical assays, or stored at -196°C.

b. Ultrafiltration. Mϕ supernatants were pipetted into a 10 ml or 50 ml Amicon filtration chamber (Amicon Corporation, Lexington, Mass.). Using 20 to 30 lbs of pressure (nitrogen source) supernatants were forced through a 25 or 43 mm UM2 ultrafiltration membrane, pre-coated with bovine serum albumin (1 mg/ml) to prevent nonspecific absorption of supernatant protein to the filter membrane. After approximately 4 hr, the volume of supernatant dropped from approximately 15 ml to 1 ml. At this point, the concentrated preparation was removed and used, or stored in liquid nitrogen.

c. Lyophilization. Normal and TBM Mϕ supernatants were dialyzed against a phosphate buffer of low molarity (0.05 M) with 12,000 dalton exclusion dialysis tubing for 48 hr at 4°C. The total volume was then frozen by agitation in a 95% ethanol-dry ice mixture. The beaker containing supernatant was covered and placed in a freeze-dry apparatus model 5-7800 (American Instrument Co., Silver Spring, MD) until complete dryness was observed. The dry material was stored at 4°C until used. Supernatants prepared in this manner generally have yielded 5 to 10 mg dry weight containing 0.1 to 0.5 mg protein.

3. Sephadex Gel Filtration

G-200 Sephadex beads (Sigma Chemical Co., St. Louis, Mo.) were rehydrated and equilibrated overnight with 0.2 M Tris-HCl buffer pH 7.2. Following equilibration, the resin was degassed for 1 hr and poured into a 2 x 100 cm column. Concentrated supernatant containing 10% sucrose (2.0 ml total volume) was carefully layered onto column and allowed to flow at a rate of 20 ml/hr. Two ml fractions were collected, scanned at 280 nm and, after 24 hr dialysis in RPMI medium, used in the MLR.

4. Anion Exchange Chromatography

Concentrated dialyzed supernatant (2 to 3 ml) was applied to a 0.5 x 9 cm column of DEAE Sephadex (Sigma Chemical Co., St. Louis, Mo.), equilibrated in 60 mM phosphate buffer pH 7.9. Equilibration was established by incubating beads (27°C) for 24 hr with the chosen buffer. The swelled beads were degassed for 30 min and packed in the DEAE column. Buffer was washed through the resin until the pH of the eluting buffer was unaltered (pH 8.0). Addition of Mϕ supernatant was followed by 3 void volumes (approximately 40 ml) of starting buffer before initiation of a linear 0.0 to 0.5 M NaCl gradient (total gradient volume 50 ml). In some experiments, the 0.0 to 0.5 M gradient was immediately followed by a 0.5 to 1.0 M NaCl gradient made up in starting buffer. One ml fractions, collected at a rate of 40 ml an hr, were scanned at 280 nm and assayed for activity following addition of 0.1 ml of a 1:10 concentration of gentamicin in RPMI medium.

5. Polyacrylamide Gel Electrophoresis

The following solutions were used in the characterization of supernatants by slab gel electrophoresis. The separating gel was made in a 30.0 ml volume by adding: 7.5 ml 30% acrylamide (0.8% bis acrylamide), 7.5 ml of 1.4 M Tris-HCl pH 8.9, 0.01 ml TEMED, 14.7 ml water and 0.3 ml of a 5% ammonium persulfate solution. The stacking gel was made in a 10 ml volume by adding 1.0 ml 30% acylamide 0.8% bis acrylamide), 0.5 ml 1.4 M Tris HCl pH 8.9 0.005 ml TEMED, 8.5 ml water and 0.5 ml of a 5% ammonium persulfate solution. The electrode buffer was composed of 3.0 g of Tris and 14.4 g of glycine dissolved in 1.1 of water. The staining solution was prepared by adding 0.3 g of coomassie brilliant blue to 250 ml methanol, 35 ml of glacial acetic acid and 215 ml of water. To destain, the gel was placed in a solution containing 70 ml of acetic acid, 50 ml of methanol and 880 ml of water.

To run the sample, 180 ml of concentrated supernatant, 0.04 ml of buffer and 0.020 ml of bromophenol blue tracking die was mixed and layered onto the stacking gel. A constant current of 20 mA was maintained throughout the run. At the end of the experiment a small portion of the slab gel was fixed and stained. The remaining portion of the gel (at least 90%) was sliced at 3 mm intervals, placed in 1.0 ml of RPMI medium, agitated and material eluted from gels for 24 hr at 4°C (see Figure 4). The soluble material was then tested for enhancing or inhibitory activity.

IV. RESULTS

A. Characterization of Enhancing and Inhibitor Factors in Macrophage Supernatants

1. Properties of Enhancing Supernatants

a. Derivation of Enhancing Supernatants from Anti-Thy 1 Serum Treated Mϕ.

Soluble mediators found in supernatants from Mϕ cultures were the result of the activity of a synergistic Mϕ-T cell complex (211). To determine whether Mϕ would continue to elaborate regulatory factors in the absence of T cells, Mϕ were incubated for 45 min with anti-Thy 1 serum (2.0 mg/ml) plus complement (1:10 dilution) before culturing for supernatants. Results in Table I showed that supernatants derived from low concentrations of Mϕ (1.6×10^6 Mϕ/3.0 ml), whether treated or untreated with anti-Thy 1 serum, did not vary appreciably in their ability to enhance responder [$R + S_m$] cell MLR reactivity. When standard error was taken into consideration, the Student's *t* test showed no significant difference in enhancing activity of undiluted (i.e., untreated Mϕ Δ cpm = 4026 ± 823 *vs* anti-Thy 1 treated Mϕ Δ cpm = 5398 ± 687) or 50% diluted supernatants (untreated = 4843 ± 396 *vs* anti-Thy 1 treated = 3851 ± 889 Δ cpm). In both treated and untreated Mϕ cultures, supernatant enhancing activity was lost by diluting 1:4 with RPMI medium (i.e., control population = 2491 ± 235 Δ cpm; untreated 1:4 dilution = 2336 ± 274 Δ cpm; anti-Thy 1 treated 1:4 dilution = 2156 ± 281).

b. Derivation of Enhancing Supernatants from Trypsinized Macrophages.

There has also been some speculation that the immunoregulatory activity of Mϕ supernatants might be due to presence

TABLE I

DERIVATION OF ENHANCING SUPERNATANTS FROM ANTI-THY 1 SERUM TREATED Mϕ

Mϕ[a] Supernatant (1.6×10^6 Mϕ/3.0 ml)	Treatment[b]	T Cell DNA Synthesis	
		^{3}H-TdR Incorporation[c] (Δ cpm ± S.E.)	% Activity[d] Over T Cell Control
None	None	2491 ± 235	100
Undiluted	None	4026 ± 823	161 ± 32
Undiluted	Anti-Thy 1 Serum	5398 ± 687[e]	216 ± 27
50%	None	4843 ± 396	194 ± 7
50%	Anti-Thy 1 Serum	3851 ± 889[e]	154 ± 35
25%	None	2336 ± 274	93 ± 10
25%	Anti-Thy 1 Serum	2156 ± 281[e]	86 ± 11

[a]Macrophages were obtained by peritoneal lavage, 4 days after i.p. injection of 2.0 ml of thioglycolate into host mice. Dilution of supernatant was made with RPMI medium plus 10% heat inactivated fetal calf serum. Undiluted supernatants were obtained after a 4 day Mϕ incubation in RPMI medium at 37°C in a 5% CO_2, humidified atmosphere.

[b]Anti-Thy 1 serum treatment consisted of a 30 min exposure (37°C) to 2.0 mg/ml of anti-Thy 1 serum plus a 1:10 dilution of guinea pig complement. Serum potency was established by exposing T cells to the serum, and performing subsequent mitogen stimulation and viability assays. Treatment of T cell population resulted in >95% cell lysis and a background level of PHA stimulation.

[c]Each well was pulsed with 1 μCi/0.05 ml (Spec. Act. 6 Ci/mM) 6 hr before termination of the 96 hr MLR assay. Δ cpm = $[R + S_m] - [R + R_m]$.

Table I (continued)

[d]Counts derived from control T cells unexposed to supernatants represent 100% activity. Activity of the treated cells was calculated as

$$\frac{\text{cpm treated cells}}{\text{cpm control}} \times 100.$$

[e]As shown by Student's *t* test, not significantly different from Mϕ untreated with anti-Thy 1 serum.

of membrane antigens shed by Mϕ into the culture medium. By incubating Mϕ for 30 min with a 0.25% concentration of trypsin, I wished to see if Mϕ, stripped temporarily of trypsin sensitive membrane antigens, were any less capable of producing active, immunoregulatory supernatants. Data in Table II indicated that, far from abrogating positive activity of low dose supernatants (1.6 to 6.4 x 10^6 Mϕ in 3.0 ml), trypsinization of Mϕ further increased supernatant enhancing activity (untreated Mϕ = 3820 Δ cpm vs trypsinized Mϕ = 5960 Δ cpm). Though large standard errors made this increase statistically insignificant as shown by the Student's t test, comparison of ^{3}H-TdR incorporation in all supernatant concentrations showed that, enhancing activity of trysinized Mϕ supernatants, invariably resulted in Δ cpm values 1200 to 2000 counts higher than those from untrypsinized Mϕ supernatants. At a concentration of 3.2 x 10^6 Mϕ per 3.0 ml for example, untreated Mϕ supernatant raised MLR counts from a background of 401 to 6208 Δ cpm. Supernatants from trypsinized Mϕ raised the same background level to 7796 Δ cpm, a 1588 Δ cpm increase over untreated Mϕ supernatant activity, made insignificant only by the large 1762 Δ cpm standard error.

At this time there is no concrete explanation for increased activity in the supernatant from trypsinized Mϕ. I can only state that failure of Mϕ trypsinization to abrogate positive immunoregulatory activity indicates that enhance-activity is probably not mediated by shedding of trypsin-sensitive Mϕ surface antigens.

TABLE II

DERIVATION OF ENHANCING SUPERNATANTS FROM TRYPSINIZED Mϕ

Concentration of Mϕ Supernatant[a]	Trypsinization Treatment[b]	T Cell DNA Synthesis ^{3}H-TdR Incorporation (Δ cpm ± S.E.)[c]
None	None	401 ± 183
	-	3820 ± 709
1.6×10^6 Mϕ/3.0 ml		
	+	5960 ± 1508[d]
	-	6208 ± 872
3.2×10^6 Mϕ/3.0 ml		
	+	7796 ± 1762[d]
	-	8404 ± 493
6.4×10^6 Mϕ/3.0 ml		
	+	9625 ± 455[d]

[a] Macrophage supernatants were obtained as described in Table I.

[b] Trypsinized Mϕ, in Hanks Balanced Salt Solution were incubated, at room temperature, for 30 min with a 0.25% concentration of trypsin. The cells were then washed twice, resuspending in 3.0 ml RPMI medium and incubated on tissue culture plates for 4 days. Supernatants from the incubation were centrifuged at 400 x g for 10 min to remove cellular debris. They were then passed through a 0.45 μ millipore filter and immediately dispensed in 0.05 ml aliquotes.

[c] Δ cpm values represent responder counts ($R + S_m$) minus control counts ($R + R_m$).

[d] As shown by Student's t test, not significantly different from un-trypsinized Mϕ.

c. Thymocyte PHA Reactivity Following Addition of Normal or TBM Mϕ Supernatant. Table III represents results from another assay designed to further determine the nature of our enhancing factor(s). One of the few well characterized enhancing factors released by Mϕ is "lymphocyte activating factor" (LAF) (66-69). To insure that this research was original and not merely an exercise in redundancy, I felt it desireable to establish a clear difference between LAF and our enhancing factor(s) (42). By definition, LAF presence permits normally inactive thymocytes to undergo blastogenesis in response to PHA stimulus (184). The distinction between our enhancing factor(s) and LAF was brought out by its inability to induce thymocyte proliferation upon addition of PHA. Thymocyte PHA stimulation in the untreated control was recorded as 920 $\pm$ 215 Δ cpm. Addition of normal or TBM Mϕ supernatants did not significantly raise this level of activity (or, more appropriately this level of inactivity), and, in the case of normal Mϕ supernatants, actually lowered the degree of reactivity quite slightly (i.e., normal Mϕ supernatant = 448 $\pm$ 115 Δ cpm). Mϕ supernatants themselves were not mitotic stimulators, since, in the absence of PHA, thymocytes also failed to undergo blastogenesis (Δ cpm = 304, data not shown).

d. Enhancing Supernatants Derived from One or Five Day Mϕ Incubation. I wished not only to biophysically and biochemically characterize Mϕ enhancing factor(s), but also to determine the kinetics of their release. In most assays, following the methodology of Folch et al. (76), Mϕ were routinely incubated for 4 days.

TABLE III

THYMOCYTE PHA REACTIVITY FOLLOWING ADDITION OF NORMAL OR TBM Mϕ SUPERNATANT

Derivation of Host Mϕ Supernatants[b]	^{3}H-TdR Incorporation PHA Stimulation[d] (responders) - (control)
-	Δ cpm
None	920 ± 215
Normal	448 ± 115
TBM[c]	1071 ± 620

[a] To obtain thymocytes, the thymus from BALB/c mice were removed and a thymocyte suspension prepared following the methods used for spleen cell preparation (see Material and Methods). Due to the lack of erythrocyte contamination no NH_4Cl treatment was used.

[b] 3.2×10^6 Mϕ in 3.0 ml of serum-free medium were incubated for 4 days and harvested in the previously described manner (Fig. 1).

[c] TBM Mϕ were obtained from BALB/c mice with a 2 to 3 week palpable tumor.

[d] 2×10^5 thymocytes were suspended in microculture wells with 0.5 µl of PHA. After addition of 0.05 ml of Mϕ supernatant, well volume was brought up to 0.2 ml.

In the following experiment (Table IV), by bracketing this normal time interval with a 1 and 5 day incubation period, I hoped to see: a) if there was a necessary lag period preceding Mϕ elaboration of enhancing factor(s), and b) whether production of enhancing factor was a cumulative process. As shown in Table IV, any lag period between Mϕ incubation and release of enhancing factor into supernatant, was considerably less than 24 hr, since 1-day supernatants added to MLR cultures nearly doubled the responder rate of ^{3}H-TdR incorporation, raising counts from 2951 to 4928 Δ cpm. This enhancement was even more dramatic when 5-day Mϕ supernatants were tested (Δ cpm of 14,432). Thus, it appears that a direct relationship exists between length of Mϕ culturing period and enhancing activity of Mϕ supernatant. It is unlikely that this relationship was due to release of enhancing factor by gradually dying Mϕ, since after 5 days, 80% of the Mϕ population still remained viable. It is much more likely that Mϕ, actively and continuously synthesize quantities of relatively stable enhancing factor(s) which accumulate in culture supernatants. In the interest of time, the incubation period was limited to 4 days, sacrificing the small increase in activity afforded by a longer incubation period.

Satisfied that elaboration of Mϕ enhancing factor was a continuous process, I decided to see if this enhancing factor was inherently present in the intracellular Mϕ milieu. Towards this end, I sonicated 1.2×10^{7} Mϕ in 3.0 ml of RPMI complete medium, and immediately assayed the sonicates for enhancing activity (Figure 3).

TABLE IV

ENHANCING SUPERNATANTS DERIVED FROM ONE OR FIVE DAY Mϕ INCUBATION

Low Dose Mϕ Supernatant[a]	Incubation Period[b]	T Cell DNA Synthesis	
		^{3}H-TdR Incorporation (Δ cpm ± S.E.)	% Activity[b] Over T Cell Control
None	None	2951 ± 360	100
3.2×10^{6} Mϕ/3.0 ml	1	4928 ± 1625	190 ± 62
3.2×10^{6} Mϕ/3.0 ml	5	14,432 ± 1693	556 ± 65

[a]Macrophages were incubated at 37°C in a humidified CO_2 atmosphere for the designated time.

[b]As described in Table I.

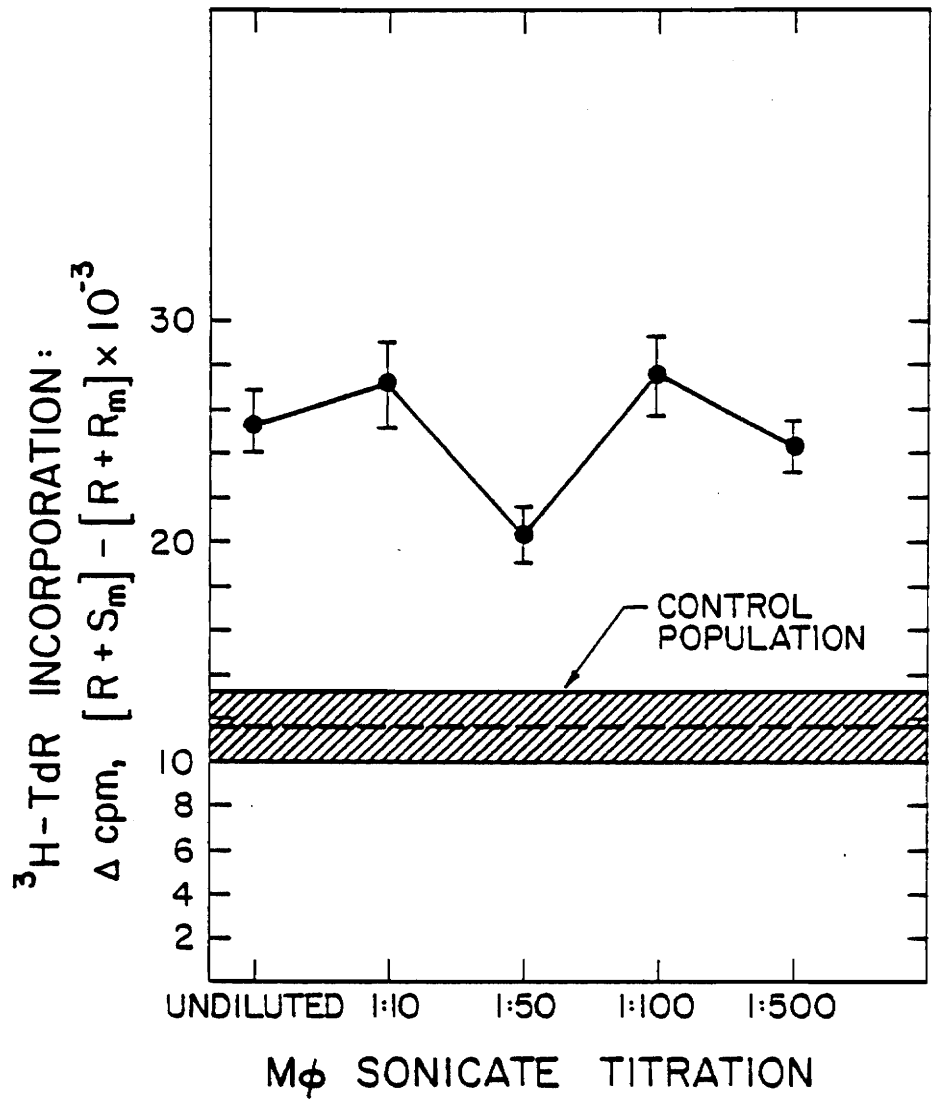


Figure 3. Enhancing activity of normal Mϕ sonicates. 1.2×10^7 Mϕ, suspended in 3.0 ml of complete RPMI medium received 2, twenty sec bursts from a cell sonicator. Microscopic examination revealed total cell lysis. Sonicates, centrifuged at 12,000 x g for 20 min to remove cell debris, were diluted to the appropriate concentration with complete RPMI medium and immediately dispensed in 0.05 ml aliquotes. Dashed bar represents Δ cpm ± S.E. of populations receiving no sonicates.

e. Enhancing Activity of Normal Mϕ Sonicates. As results in Figure 3 indicate, Mϕ sonicates, titrated to a dilution of 1:500, still possessed the ability to double background levels of MLR reactivity. The enhancing curve was biphasic in nature, possessing two peaks of maximum activity. The shape of this curve may be due to the presence of 2 distinct enhancing factors, differing in their optimum enhancing dilution.

Though sonication afforded a rapid means of obtaining enhancing factor(s), it also released into the sonicate an abundance of enzymes which may otherwise have been contained (at least to some degree) intracellularly. For this reason I again decided in favor of the established 4-day incubation period.

f. Summary. Mϕ inherently possessed at least 1 (possibly more) MLR enhancing factors accounting for the enhancing activity of supernatants removed from low concentrations of cultured Mϕ. Enhancing activity was not dependent on a) Mϕ DNA synthesis, b) T cell presence, c) trypsin sensitive Mϕ membrane Ag, or d) LAF presence.

2. Properties of Inhibitor Supernatants

a. Nondialyzable Inhibitor Factor Manifested in High Dose Mϕ Supernatants. Enhancing factors are not the only monokines released into Mϕ supernatants. When Mϕ were grown in high concentrations (1.2×10^7 Mϕ in 3.0 ml of medium), their supernatants inhibited MLR reactivity. Many of these inhibitors have been characterized by others (100, 107-109) as small, dialyzable, heat stable factors. I therefore wished to examine the inhibitory capacity of our normal

high dose Mϕ inhibitor supernatants a) before and after dialysis, and b) before and after heat treatment (10 min at 100°C). As depicted in Table V, addition of undialyzed high dose Mϕ supernatant caused responder activity to fall below controls to a S.I. of 0.51. After dialysis (pore size of 12,000 daltons), high dose inhibitory activity was still present in the dialyzed supernatant, reducing responder population activity to control levels (1.40 S.I.). Addition of heat treated dialyzed supernatant lowered responder activity from an S.I. of 5.85 to 1.12. In all heat treatment experiments the untreated responders which received no supernatants were exposed to 0.05 ml of heated serum-free medium. As can be seen from the high Δ cpm and S.I., inhibition was not a result of cytotoxic properties inherent in heat treated medium. Also, had inhibitor supernatants been composed entirely of thymidine, dialysis would have resulted in a loss of the small thymidine molecule and an abrogation of all inhibitory properties of the supernatant.

b. Non-thymidine nature of Mϕ Inhibitor Supernatants. Much controversy has been generated by the possibility that inhibition of ^{3}H-TdR incorporation may be due to the presence of "cold" thymidine (179, 180). Further experiments were conducted to determine the extent of thymidine participation in Mϕ supernatant inhibition. Inhibitor supernatants were added to (R + S_m) cultures at the initiation or termination of the MLR assay. Had inhibition been due to thymidine competition, the time of supernatant addition should not have changed the degree of inhibition. However, when inhibitor was added with

TABLE V

NONDIALYZABLE INHIBITOR FACTOR MANIFESTED IN HIGH DOSE Mϕ SUPERNATANTS

Assay Number	Mϕ Addition to Responder Population[a]	^{3}H-TdR Incorporated: Responders R + S_m	Control R + R_m	(Responders)-(Control)	Stimulation Index (Responders)/(Control)
				Δ cpm	
1	None	5763 ± 913	1724 ± 338	5039 ± 913	3.92
	High Dose, Undialyzed Supernatant	899 ± 44		-825 ± 338	0.51
2	None	13,154 ± 1087	2935 ± 1686	10,219 ± 1686	4.48
	High Dose, Dialyzed Supernatant	5530 ± 815		1595 ± 1686	1.40
3	None	11,755 ± 2077	2009 ± 256	9746 ± 2077	5.85
	High Dose, Heat Treated Undialyzed Supernatant	2518 ± 408		509 ± 409	1.25
4	None	8429 ± 1350	2022 ± 620	6407 ± 1350	4.17
	High Dose, Heat Treated Dialyzed Supernatant	2269 ± 523		247 ± 620	1.12

[a]Normal Mϕ supernatants were placed in dialysis bags (pore size 12,000 daltons) and dialyzed against 4 liters of distilled water. Forty-eight hr (and 2 changes of water) later, supernatants were collected and dispensed. Those receiving heat treatment were boiled for 10 min, cooled and dispensed. Control wells in dialysis experiments received 0.05 ml of distilled water rather than dialyzed supernatant. Control wells in heat treated undialyzed supernatant experiments received 0.05 ml of boiled RPMI medium rather than heat treated Mϕ supernatant

^{3}H-TdR (1 μCi/well, sp. act. 6 Ci/mM), 6 hr before assay termination (Table VI), Δ cpm fell only 2000 to 3000 counts below those obtained from cells receiving ^{3}H-TdR suspended only in RPMI medium. Compare this degree of inhibition (15 and 25%) with the nearly 100% inhibition achieved when inhibitor supernatant was added at the initiation of the 96 hr assay (Table VII). Thus the presence of thymidine could not totally account for Mϕ inhibition of the T cell MLR response.

c. T Cell Viability Following Addition of High Dose Inhibitory Supernatant. Apart from its non-thymidine properties, it was necessary to show that inhibition of MLR reactivity was not due merely to a cytotoxic property of high dose Mϕ supernatants. As shown in Table VIII inhibitor concentrations of normal or TBM Mϕ supernatant were added to wells containing 4 x 10^{5} C3H T cells and 2 x 10^{5} BALB/c responder T cells from normal or TBM. After 24 hr, trypan blue was added, cells were counted on a hemocytometer and viable cell numbers calculated. In no case did viability of the supernatant treated T cells, significantly differ from the 80% ± 1% value of the untreated responder population (i.e., R + normal Mϕ supernatant = 74% ± 4.33%; R + TBM Mϕ supernatant = 83% ± .16%).

However trypan blue viability studies were conducted only after an elapsed period of 24 hr. The possibility existed that, after only 24 hr, T cells were not yet fully stimulated by allogeneic lymphocytes. Perhaps, proliferating lymphocytes were more susceptible to the proposed cytotoxic activity of inhibitor supernatants. To cover this possibility, cytotoxicity experiments (Table IX) were conducted using

TABLE VI

INHIBITION DUE TO COMPETITION BY THYMIDINE

Mϕ Addition[a]	Dilution of ^{3}H-TdR[b] in	T Cell MLR Reactivity (^{3}H-TdR Incorporated, [R + S_m + Mϕ] - [R + R_m])	Stimulation Index (R + S_m + Mϕ)/ (R + R_m)
		Δ cpm	
3%	RPMI Medium	12,456 ± 1632	10.43 ± 1.36
	Mϕ Inhibitory Supernatant	10,736 ± 448[c]	8.99 ± .37
10%	RPMI Medium	13,026 ± 1756	10.90 ± 1.46
	Mϕ Inhibitory Supernatant	9239 ± 881	8.15 ± .73

[a]Syngeneic peritoneal exudate Mϕ were added, in varying concentrations to 2 x 10^5 normal responder (R) BALB/c splenic T cells. Peritoneal exudates were obtained after peritoneal lavage of BALB/c mice, injected i.p. 4 days previously, with 2.0 ml of thioglycolate. Exudates were plated (2 hr), mitomycin C treated (40 μg/ml) and subjected to anti-Thy 1 plus complement treatment. Such purification procedures yielded a viable T cell-depleted population >97% Mϕ as shown by esterase staining techniques. Responder (BALB/c) and stimulator (C3H) populations consisted of nonadherent (T cell) nylon wool column eluates, which were doubly plated to remove Mϕ. Viability and T cell purity exceeded 95% as demonstrated by trypan blue exclusion, latex particle uptake, esterase staining procedures, and responsiveness to PHA. In no case did (R + R_m) populations receive additional Mϕ or supernatants.

[b]Six hr before assay termination ^{3}H-TdR (1 μCi/well) was dispensed either in RPMI medium or inhibitory concentrations of Mϕ supernatant (supernatant, derived from 1.2 x 10^7 Mϕ/3.0 ml).

[c]Not significantly different from ^{3}H-TdR in RPMI medium, as shown by Student's *t* test.

TABLE VII

ADDITION OF Mϕ INHIBITOR SUPERNATANT AT THE INITIATION OR TERMINATION OF ASSAY: NORMAL T CELL MLR REACTIVITY

Mϕ Inhibitory Supernatant Addition	Time of Addition (hr)	T Cell MLR Reactivity (^{3}H-TdR Incorporated, $[R + S_m] - [R + R_m]$)	Stimulation Index[a] $(R + S_m)/(R + R_m)$
		Δ cpm	
None		14,394 ± 2468	5.78 ± 1.15
+	0	1564 ± 32	0.52 ± 0.02
+	90	11,991 ± 625	5.16 ± 0.20

[a]Assay protocol similar to that described in Table VI. The comparison has been made between untreated controls and $(R + S_m)$ treated with inhibitory supernatants (derived from 1.2×10^7 Mϕ incubated in 3.0 ml serum-free medium), either at the initiation or 6 hr before (initiation of ^{3}H-TdR pulse) termination of the MLR assay.

TABLE VIII

T CELL VIABILITY FOLLOWING INCUBATION WITH INHIBITORY CONCENTRATIONS OF Mϕ SUPERNATANTS

Responder T Cell Type[a]	Inhibitor Mϕ Supernatant Concentration	Viable Cells After 24 hr[b] (x 10^5)	% Viability Final Cell Number / Initial Cell Number (6 x 10^5)
Normal	None	4.85 ± .06	80 ± .99
	Normal	4.43 ± .26	74 ± 4.33
	TBM	5.04 ± .01	83 ± .16
TBM	None	4.81 ± .30	80 ± 5.02
	Normal	4.79 ± .27	79 ± 3.95
	TBM	4.81 ± .44	80 ± 7.38

[a] Into the wells of microculture plates were placed 4 x 10^5 C3H T cells and 2 x 10^5 BALB/c T cells from normal or TBM. A 0.05 ml volume of Mϕ inhibitor supernatant was added, the wells brought to a volume of 0.2 ml and incubated for 24 hr.

[b] After 24 hr 0.05 ml of trypan blue was added to each well. Cells were aspirated, placed on a hemacytometer and accessed for viability based on trypan blue exclusion.

TABLE IX

MEASUREMENT OF LYMPHOCYTE CYTOLYSIS FOLLOWING INCUBATION WITH HIGH DOSE INHIBITORY NORMAL OR TBM Mϕ SUPERNATANTS

Assay Number[a]	Responder Cell Type[b]	Inhibitor Supernatant Addition[c]	^{51}Cr Release (cpm)	% Lysis $\frac{\text{Test}}{\text{Total Lysis}} \times 100$[d]
1	Normal	None	4078 ± 105	27.30 ± 2.34
		Normal	3979 ± 59	26.64 ± 2.38
		TBM	4114 ± 104	27.54 ± 2.34
		Triton X	14,935 ± 1282	100.00 ± 8.25
2	TBM	None	3591 ± 48	23.84 ± 0.32
		Normal	3440 ± 48	22.84 ± 0.32
		TBM	4541 ± 319	30.15 ± 2.12
		Triton X	15,060 ± 331	100.00 ± 2.19

[a] Experiments were conducted to determine the cytoxicity of Mϕ supernatants upon addition to normal or TBM T cells.

[b] Responder (R) cells were taken from normal or tumor-bearing BALB/c mice. Responder cells were incubated at a 1:2 ratio with mitomycin C treated C3H T cells. After 72 hr, cells were harvested, resuspended in 1.0 ml of complete medium and incubated for 2 hr with 0.4 mCi ^{51}Cr. Cells were then washed twice, resuspended in complete medium, counted and dispensed into microculture wells so that each well received 6 x 10^5 cells/0.25 ml. Controls received only RPMI medium as opposed to the "treated" populations which received 0.05 ml of Mϕ inhibitor supernatant. After 18 hr, 0.1 ml of supernatant was carefully asperated and assayed on a gamma counter. Cytotoxicity was judged by release of ^{51}Cr into cell medium.

Table IX (continued)

[c]For derivation of supernatants see Table I. An 0.05 ml volume of Triton X was added to "100% lysis" wells to induce total ^{51}Cr release.

[d]As shown by Student's *t* test there exists no significant difference in the amount of cell lysis before and after Mϕ supernatant treatment.

responder populations previously incubated 72 hr with mitomycin C treated allogeneic cells. The responder population was then harvested, incubated with ^{51}Cr, washed, and dispensed into microculture wells at a concentration of 6 x 10^5 cells/0.2 ml. "Treated" responder populations received in addition, 0.05 ml of high dose inhibitor supernatant. After 18 hr, supernatants were collected and cytolysis determined by measuring the amount of ^{51}Cr released into culture medium. When compared to ^{51}Cr release in the untreated responder wells, responder populations treated with supernatant, exhibited no appreciable increase in degree of cytolysis. The greatest cytolytic variance, was a 6% difference between untreated TBM T cells and those treated with TBM Mϕ supernatants. However when the 2% standard error was taken into consideration, the Student's t test showed no significant difference between the two groups. Since treated and untreated, 72 hr cultures of responder cells failed to differ in their degree of cytolysis, there remained little possibility that supernatants were selectively cytolytic for a stimulated population of T cell which subsequently disintegrated.

d. Mϕ Supernatant Inhibitor Activity Following Mϕ Incubation With Indomethacin, PMSF or Benzidine. Thymidine is just one of many nontoxic factors thought to be present in Mϕ inhibitory supernatants. Prostaglandins, potent inhibitors of DNA synthesis (26, 191) have also been isolated from Mϕ supernatants (103, 175). By incubating Mϕ with indomethacin, (10^{-5} M), a prostaglandin antagonist, I wished to determine if prostaglandin-free supernatants retained inhibitory activity. Results shown in Figure 4 indicated that

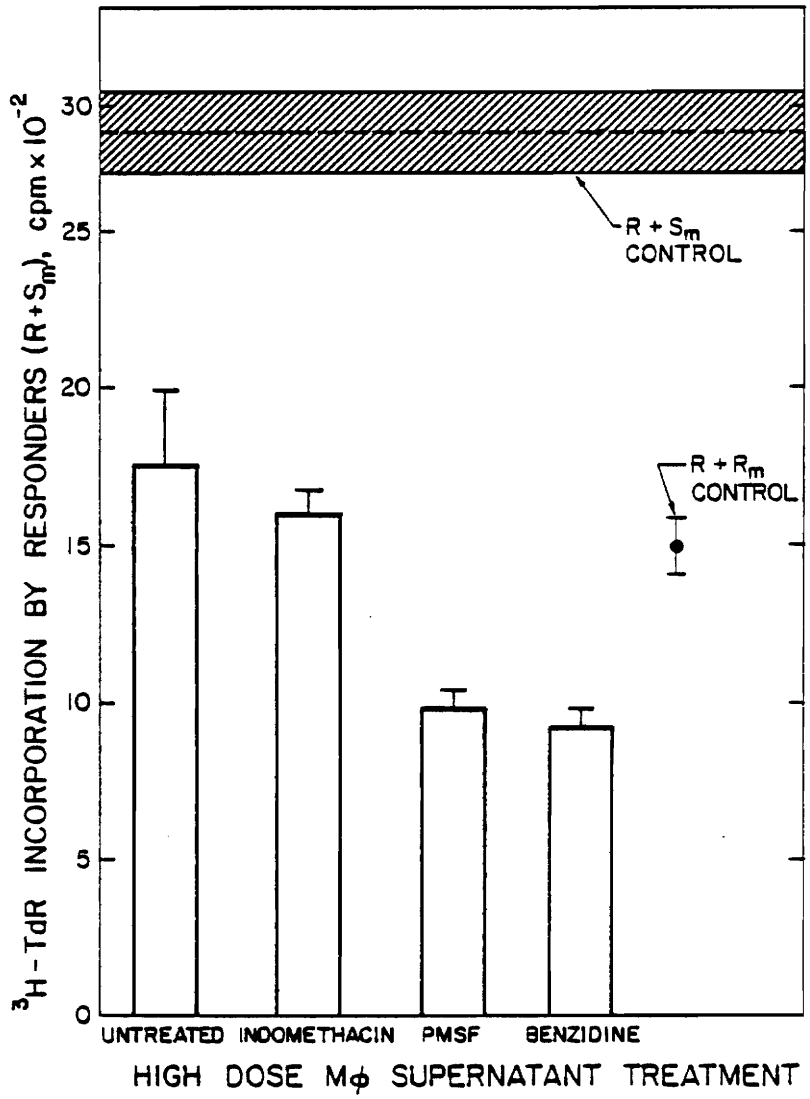


Figure 4. Mϕ supernatant inhibitor activity following Mϕ incubation with indomethacin, PMSF or benzidine. 1.2 x 10^7 Mϕ in 3.0 ml of serum-free medium were incubated with 10^{-5} M indomethacin, PMSF or benzidine. "Untreated" Mϕ were used to obtain the appropriate supernatant control. After 4 days all supernatants were harvested and dialyzed for 24 to 48 hr against RPMI medium and dispensed immediately into microculture wells. Dashed bar represents cpm of the untreated responder population.

dialyzed, high dose (1.2×10^7 Mϕ/3.0 ml) Mϕ supernatants lowered MLR counts from 3000 to 1700 cpm. Dialyzed supernatants, derived from Mϕ incubated with indomethacin, continued to exhibit inhibitor activity (1550 cpm) -- establishing the non-prostaglandin nature of our supernatants.

In this experiment only a part of the total Mϕ population was incubated with indomethacin. Other Mϕ fractions were incubated with proteolytic enzyme inhibitors (PMSF or benzidine) to see if abrogation of proteolytic enzyme activity, would enhance the inhibitory potency of high dose Mϕ supernatant. In comparison with untreated Mϕ supernatants, the data in Figure 4 shows a significant increase in the inhibitor capacity of dialyzed supernatants from Mϕ incubated with PMSF or benzidine. It is possible that these proteolytic enzyme inhibitors halted the degradative activity of extracellular Mϕ enzymes. Thus, Mϕ incubation with PMSF allowed a greater quantity of inhibitor factor to accumulate in high dose Mϕ supernatants.

e. Ammonium Sulfate Precipitation of Mϕ Supernatants: Inhibitor Activity Following Incubation With Indomethacin or PMSF. Since inhibitor factor appeared vulnerable to the activity of Mϕ-derived proteolytic enzymes, it must, at least in part, be composed, of protein, and therefore susceptible to ammonium sulfate precipitation. From the results in Table X it can be seen that Mϕ supernatants, saturated with ammonium sulfate, yielded precipitates, which, when reconstituted in 1.0 ml of RPMI medium and dialyzed for 24 hr, inhibited MLR reactivity. In agreement with Mϕ supernatant

TABLE X

AMMONIUM SULFATE PRECIPITATION OF Mϕ SUPERNATANTS: INHIBITOR ACTIVITY FOLLOWING INCUBATION WITH INDOMETHACIN OR PMSF

Mϕ Supernatant Addition	3H-TdR Incorporation $[R + S_m] - [R + R_m]$
	Δ cpm
None	2679 ± 692
Untreated	1816 ± 587
Indomethacin-treated	-76 ± 178
PMSF-treated	-391 ± 436

[a]Methodology identical to that described in Figure 4 except supernatant precipitates (rather than whole supernatants) were assessed for inhibitor activity. Precipitates were obtained by saturating supernatants with ammonium sulfate to a final concentration of 70%. Supernatants were centrifuged at 12,000 x g for 30 min. Solid material was resuspended in 2.0 ml of RPMI medium and dialyzed overnight in two 500 ml volumes of medium.

inhibitor ability shown in Figure 4, supernatant precipitates from Mϕ cultures treated with indomethacin and proteolytic enzyme inhibitors, retained MLR suppressor activity, and, in the case of PMSF-treated Mϕ, far exceeded the suppressive ability of their untreated Mϕ counterparts. In calculating activity based on the Δ cpm value of [responders] - [controls], both indomethacin and PMSF-treated Mϕ supernatants inhibited [R + S_m] activity below that of [R + R_m] controls -- thus resulting in a negative Δ cpm value (-76 and -391 Δ cpm, respectively). To insure that all immunoregulatory factors were contained in the precipitatable Mϕ supernatant fraction, the non-precipitable fraction was dialyzed and assessed for enhancing or inhibitory effects. The results (not shown) showed no immunoregulatory activity in the non-precipitable fraction.

f. Titration of PMSF Treated Mϕ Supernatant Precipitates. From data presented in Figure 4 and Table X increased inhibitor activity in supernatants from Mϕ incubated with PMSF may have resulted from inactivation of extracellular, proteolytic enzymes, normally capable of degrading Mϕ inhibitor factor(s). However, in those experiments, there was no certainty that PMSF was not merely inactivating Mϕ enhancing factors, making it appear as though supernatants contained more inhibitory factor(s), when in fact they contained less enhancing factor(s). To show that increased inhibitory activity at high dose concentration was not made at the expense of low dose enhancing activity, normal and PMSF-treated supernatants were titrated for inhibitor and enhancing activity (Figure 5). When compared to untreated supernatants, results showed that inhibitor activity of the concentrated

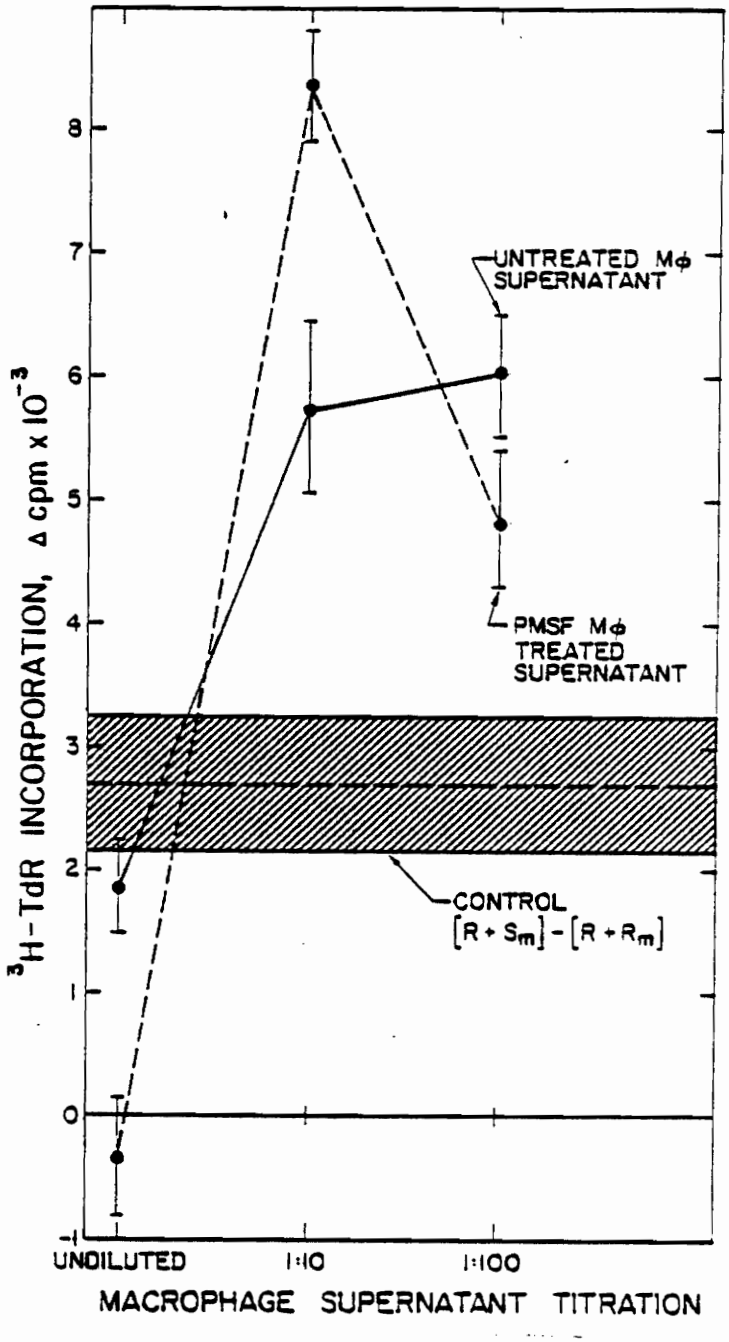


Figure 5. Titration of PMSF treated Mϕ supernatant precipitates. Protocols were followed as described in Table VIII and Figure 5. All dilutions were made with RPMI complete medium. Dashed bar represents Δ cpm counts of control population. Solid line (—) represents Δ cpm of T cells exposed supernatant precipitates derived from Mϕ not incubated with PMSF. Dashed line (--) represents Δ cpm after addition of PMSF treated Mϕ supernatant precipitates.

PMSF-treated supernatant precipitate was significantly greater (by Δ cpm of 2000), while enhancing capabilities at dilute concentrations remained undiminished, and, in fact at a 1:10 dilution, possessed a greater enhancing activity (i.e., 8250 Δ cpm vs non-enhancing activity of 5750 Δ cpm).

g. Polyacrylamide Gel Electrophoresis of Supernatants From Mϕ Incubated in the Presence or Absence of Indomethacin, PMSF or Benzidine. Using slab gel electrophoresis techniques (Figure 6), normal supernatants from Mϕ incubated with proteolytic enzyme inhibitors, exhibited a dark staining band of protein not readily apparent in untreated or indomethacin-treated Mϕ supernatants. Though this band has not as yet been assayed for inhibitor activity, its very appearance lends credence to the possibility that PMSF allows for greater accumulation of inhibitor factor(s) by inactivating degradative extracellular proteolytic enzymes. Obviously, however, this assay can only be proved conclusive upon completion of future experiments testing and comparing the various immunoregulatories activities of each gel fraction.

h. Control [R + R_m] Activity Before and After Addition of Enhancing and Inhibitor Supernatants. In the preceding experiments (and indeed in all the experiments to come), "MLR" reactivity was judged as the ability of responder T cells (R) to proliferate upon contact with mitomycin C treated allogeneic (foreign) stimulator cells (S_m). The combined [R + S_m] population has been designated as the "responder" population. As already seen, Mϕ

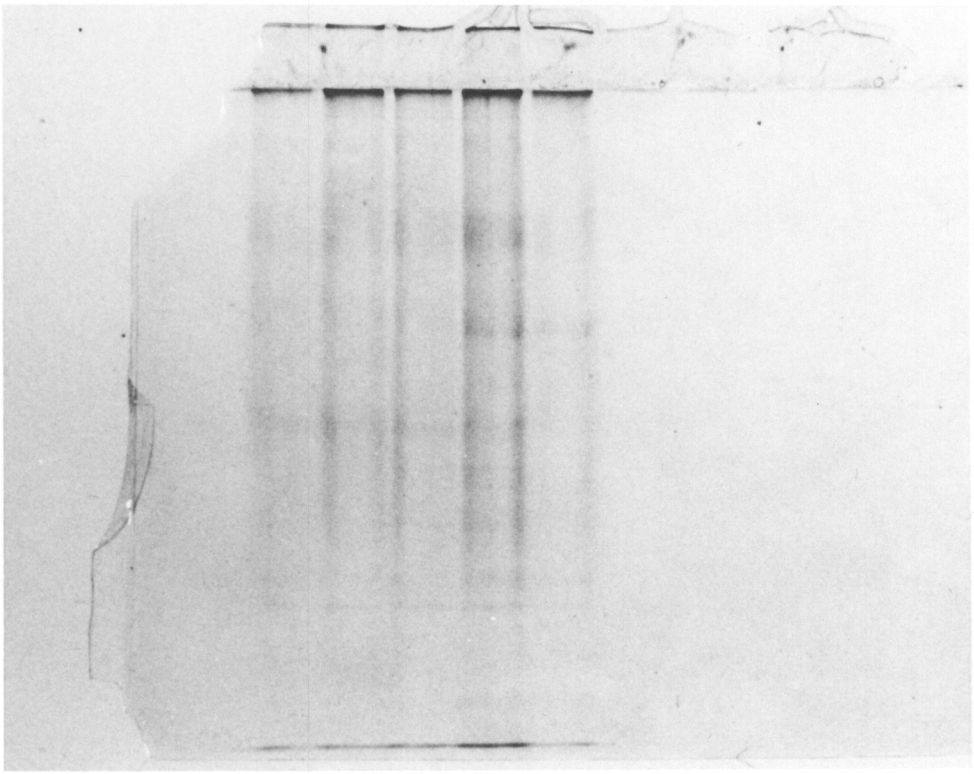


Figure 6. Polyacryalmide gel electrophoresis of high dose normal supernatants from Mϕ incubated in the presence or absence of indomethacin, PMSF, and benzidine. Identification of the supernatants, reading left to right, is as follows: i) first normal untreated Mϕ supernatant; ii) supernatant from indomethacin treated Mϕ; iii) second normal untreated Mϕ supernatant; iv) supernatant from PMSF treated Mϕ; and v) supernatant from benzidine treated Mϕ.

Methods of obtaining supernatants are described in Figure 4 and Table VIII. For detailed description of electrophoresis procedures see Material and Methods. Briefly, supernatants were lyophilized and reconstituted in 1.5 ml of phosphate buffer (pH 8.0). After phosphate buffer dialysis, 0.180 ml of sample was mixed with 0.05 ml of a Tris buffer sucrose solution and 0.015 ml of tracking dye. The samples were placed on a discontinuous polyacrylamide slab gel (7% running gel, 3% stacking gel) run at a constant current of 20 milliamps. Upon completion of the assay, gels were stained and fixed with a Coomassie blue-methanol-acetic acid solution.

supernatants possess a spectrum of immunoregulatory activities enabling them to enhance or inhibit responder cell blastogenesis. This assay (Table XI) however, points out the singular inability of Mϕ supernatants to significantly alter syngeneic background activity in "R + R_m" control populations. When adding Mϕ supernatants to "R + S_m" populations it was necessary to establish whether the enhanced R + S_m activity was due to i) direct nonspecific stimulation of syngeneic lymphocytes by Mϕ supernatants or ii) augmentation of T cell response to specific allo-antigen. When compared to ^{3}H-TdR incorporation in untreated R + R_m populations, addition of low or high dose normal and TBM Mϕ supernatants caused a slight but not significant fluctuation in R + R_m activity. This lack of appreciable nonspecific R + R_m stimulation from Mϕ supernatant, paralleled previous results obtained when enhancing concentrations of whole Mϕ were added to R + R_m populations (e.g., [R + S_m] = 21,949 $\pm$ 1299; [R + S_m + 6% Mϕ] = 56, 676 $\pm$ 2033; [R + R_m] = 7882 $\pm$ 392; [R + R_m + 6% Mϕ] = 6601 $\pm$ 710). Though Mϕ supernatants regulate T cell reactivity, they apparently have little affect upon normal R + R_m interactions. Therefore, it was felt that conclusions drawn from data using "untreated" R + R_m populations would not have been altered had addition of Mϕ supernatants to syngeneic R + R_m controls been made. So for economic reasons Mϕ or Mϕ supernatants were not added to R + R_m controls.

i. Summary. Nondialyzable, heat stable inhibitor factor(s) in Mϕ supernatants suppressed responder cell MLR reactivity in a nontoxic manner. In addition, this factor was neither thymidine or

TABLE XI

CONTROL [R + R_m] ACTIVITY BEFORE AND AFTER ADDITION OF ENHANCING AND INHIBITOR SUPERNATANTS[a]

Mϕ Supernatant Addition to R + R_m Controls[b]	^{3}H-TdR Incorporated[b]	Stimulation Index of Treated R + R_m Control $\left[\frac{R + R_m + M\phi \text{ Supernatants}}{R + R_m}\right]$[c]
	cpm	
None	2172 ± 273	1.00 ± .12
Low dose normal	2008 ± 77	.92 ± .03
High dose normal	2329 ± 278	1.07 ± .12
Low dose TBM	2011 ± 39	.92 ± .01
High dose TBM	2197 ± 276	1.01 ± .12

[a] "R + R_m" populations consisted of: 2×10^5 purified responder (R) T cells from tumor-bearing or (in this case) normal BALB/c mice; plus 4×10^5 purified BALB/c responder T cells (R_m) previously incubated for 45 min with mitomycin C at a 40 mg/ml concentration. All cells were finally placed in microculture wells in a total volume of 0.2 ml of RPMI complete medium.

[b] To obtain supernatants, Mϕ were incubated 4 days in 3.0 ml of serum-free RPMI medium at a high (1.2×10^7 Mϕ) or low (3.2×10^6 Mϕ) dose concentration. Supernatants were then harvested, centrifuged at 800 x g for 20 min, passed through a 0.45 μ filter and immediately dispensed in 0.05 ml aliquotes.

[c] Using the Student's t test, values for treated R + R_m controls did not significantly differ (at the 95% level) from those of the untreated R + R_m population.

prostaglandin, but rather, of protein origin, since it was ammonium sulfate precipitable, and apparently susceptibily to the degradative activity of extracellular Mϕ proteolytic enzymes.

3. Biochemical Differentiation of Whole Macrophage Supernatant Into Inhibitor and Enhancing Fractions by Anion Exchange Chromatography

a. MLR Reactivity Following Addition of Undialyzed DEAE Fractions From Normal Supernatant. Heretofore, no attempt had been made to biochemically separate heterogeneous Mϕ supernatants into distinct inhibitor and enhancing components. To more definitely characterize inhibitor and enhancing factors released by normal Mϕ, 4 day supernatants were fractionated on a DEAE Sephadex anion exchange column. Fractions, eluted using a linear NaCl gradient, were collected and immediately tested for regulatory activity in the MLR assay. In all anion exchange experiments, unfractionated concentrated Mϕ supernatant was invariably inhibitory.

When DEAE fractions were added to the MLR (Figure 7), inhibitor activity, as in the PHA assay (not shown) was noted in the fast eluting cation fraction and in all fractions collected beyond 0.12 M NaCl. It appeared that normal Mϕ supernatants contained a positively charged inhibitor molecule absorbing at 280 nm (fraction 10). Following elution of this molecule, 280 absorbance fell rapidly (fractions 13 to 33). Enhancing activity (fraction 6), was seen in 280 absorbing protein fraction immediately preceding the cationic inhibitor fraction (fraction 10). A broad double peak of enhancing activity was also noted in the non 280 nm absorbance fraction (fractions 13 to

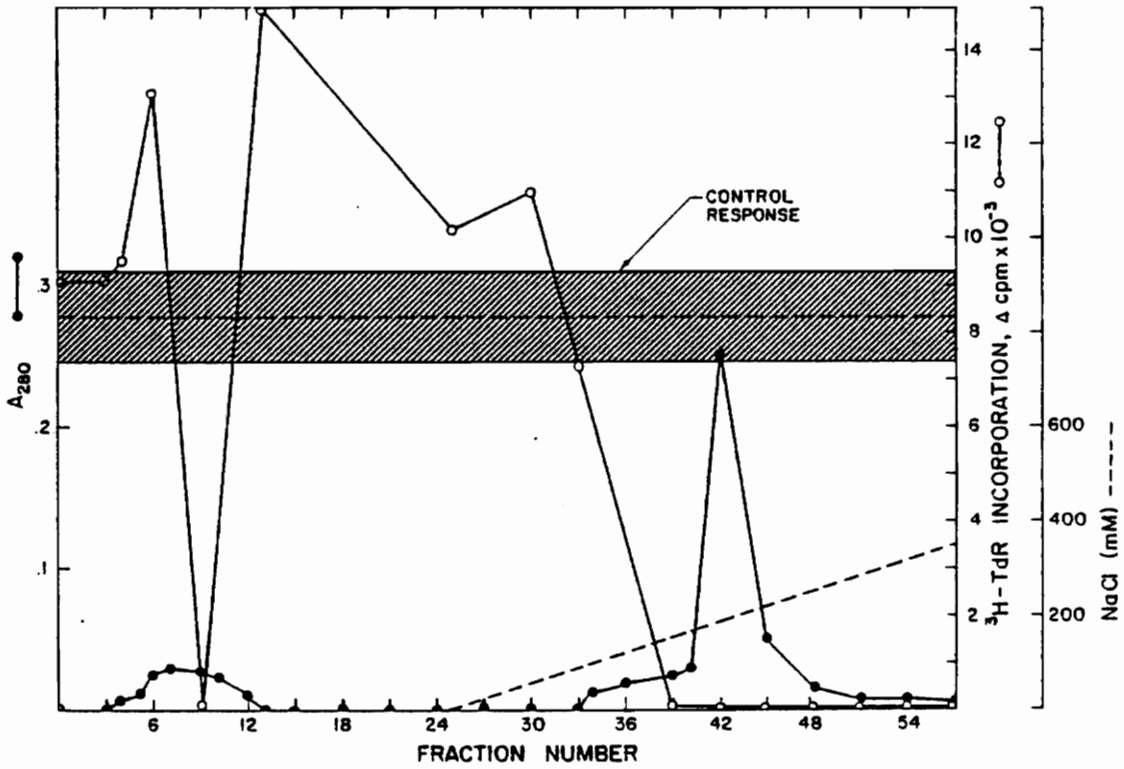


Figure 7. Activity of undialyzed DEAE fractions from Mϕ supernatant. A linear 0.5 M NaCl gradient was prepared in 50 ml of 0.066 M phosphate buffer (pH 7.9). Dashed line (--) represents NaCl concentration in mM. Solid circled line (●—) represents absorbance (O.D.) at 280 nm. Open circled line (○—) represents activity as measured by ^{3}H-TdR incorporation.

33). Activity then fell sharply, as the NaCl molarity reached 0.12 M (fraction 36). As the eluting salt concentrations rose to approximately 0.2 M, the major protein components were collected (fractions 39 to 48). However, no reverse in inhibitory activity could be detected.

b. MLR Reactivity Following Addition of Dialyzed DEAE Fractions From Normal Supernatant. To remove any possible artifactual inhibitor effects due to salt presence, the experiment outlined in Figure 7 was repeated with an added overnight dialysis step after supernatant fractionation. Following dialysis in a 0.066 M phosphate buffer, fractions were dispensed and tested for MLR reactivity.

In Figure 8 when examining the raw Δ cpm of this population (8800 Δ cpm) there does appear to be peaks of enhancing activity early in the breakthrough peak (fraction 2) as well as in the non 280 absorbance region (fraction 20) and in the fraction eluting around 0.4 M NaCl (fraction 68). Inhibitor activity continued to occur in the cationic protein peak (fraction 4) and around the 0.2 M NaCl region; a double peaked area where the bulk of the supernatant protein eluted (fractions 54 to 60). However, it appears that salt presence was responsible for much of the inhibition noted in the earlier experiments, since dialysis abrogated inhibitor activity of those fractions collected immediately before (fractions 52 to 56) and after (fractions 68 to 76) the major protein inhibitor peak.

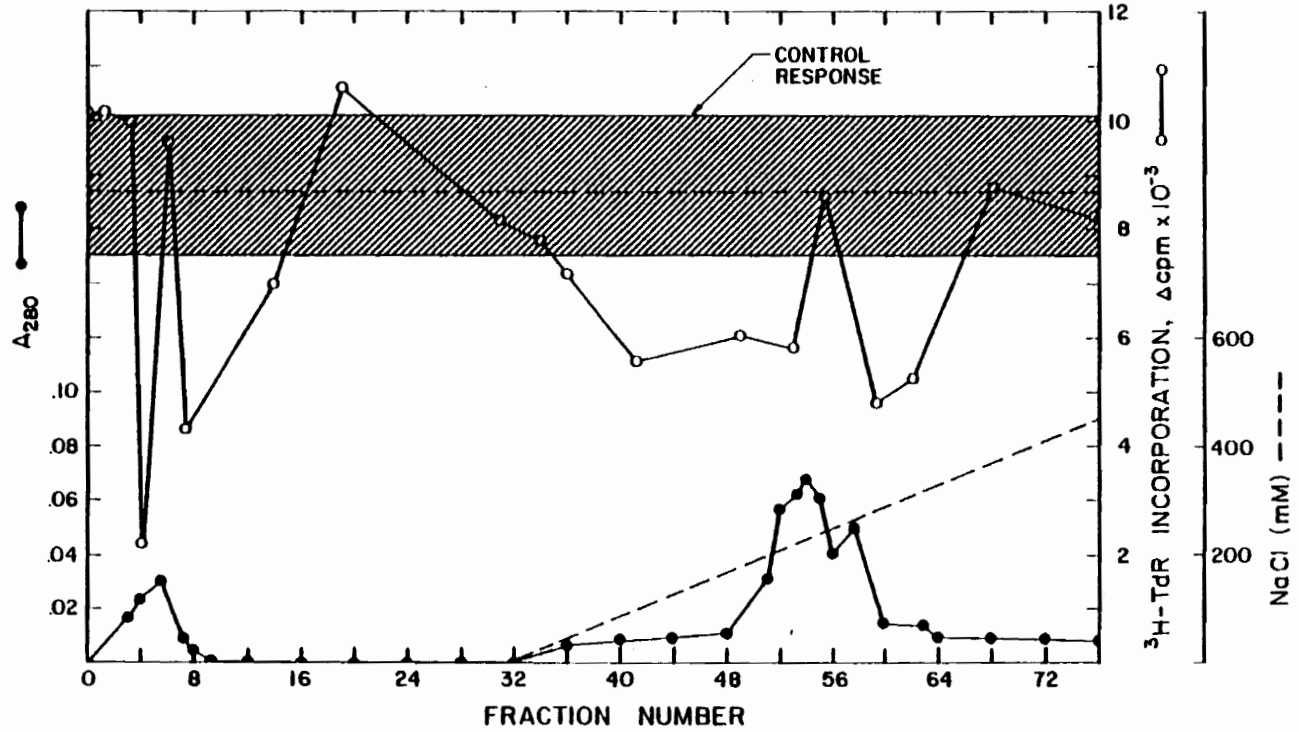


Figure 8. Activity of dialyzed DEAE fractions from Mϕ supernatant. For description and methodology see Figure 7. The only point of difference between this and the previously described assay, was the dialysis of supernatant fractions in RPMI medium prior to their addition into microculture wells. The assessment of activity (o—) is recorded as a Δ cpm value.

c. Normal Mϕ Supernatant DEAE Fractionation on a 1 M NaCl Gradient. To obtain a more complete biochemical profile of normal Mϕ supernatants the previous experiments were repeated and the gradient extended out to 1.0 M NaCl (Figure 9). The protein profile of this assay showed the small cationic peak (fractions 2 to 12) followed by the broad non 280 absorbance region (fractions 13 to 60). A large fraction of the protein again eluted around 0.2 M NaCl. The double protein peak noted in Figure 8 was present here; however, the second peak (fraction 83) was much broader than that seen in the previous assay. The area of slight 280 absorbance (fractions 60 to 66) immediately preceding the major protein 0.2 M region was slightly shorter here than in the previous two assays. The most dramatic addition to the protein profile in this assay was the detection of a broad double peak eluting between 0.62 and 0.8 M NaCl.

The characteristic cationic inhibitor was again evident in the MLR assay (fraction 8). The high level of enhancing activity in the non 280 absorbing region (fractions 16 to 66) was seen, as in the previous assays, as a double peak of MLR activity. The large protein component eluting around 0.2 M NaCl inhibited MLR stimulation (fraction 71), as in the previous assay. Some enhancing activity around 0.4 M NaCl (fractions 104 to 110) was also seen in this MLR assay.

d. Regulatory Activity of Normal Pooled Supernatant Fractions. In the previous experiments an activity profile of normal supernatant DEAE fractions was obtained by taking various single fractions, dialyzing them and adding them to the MLR assay. In this assay the

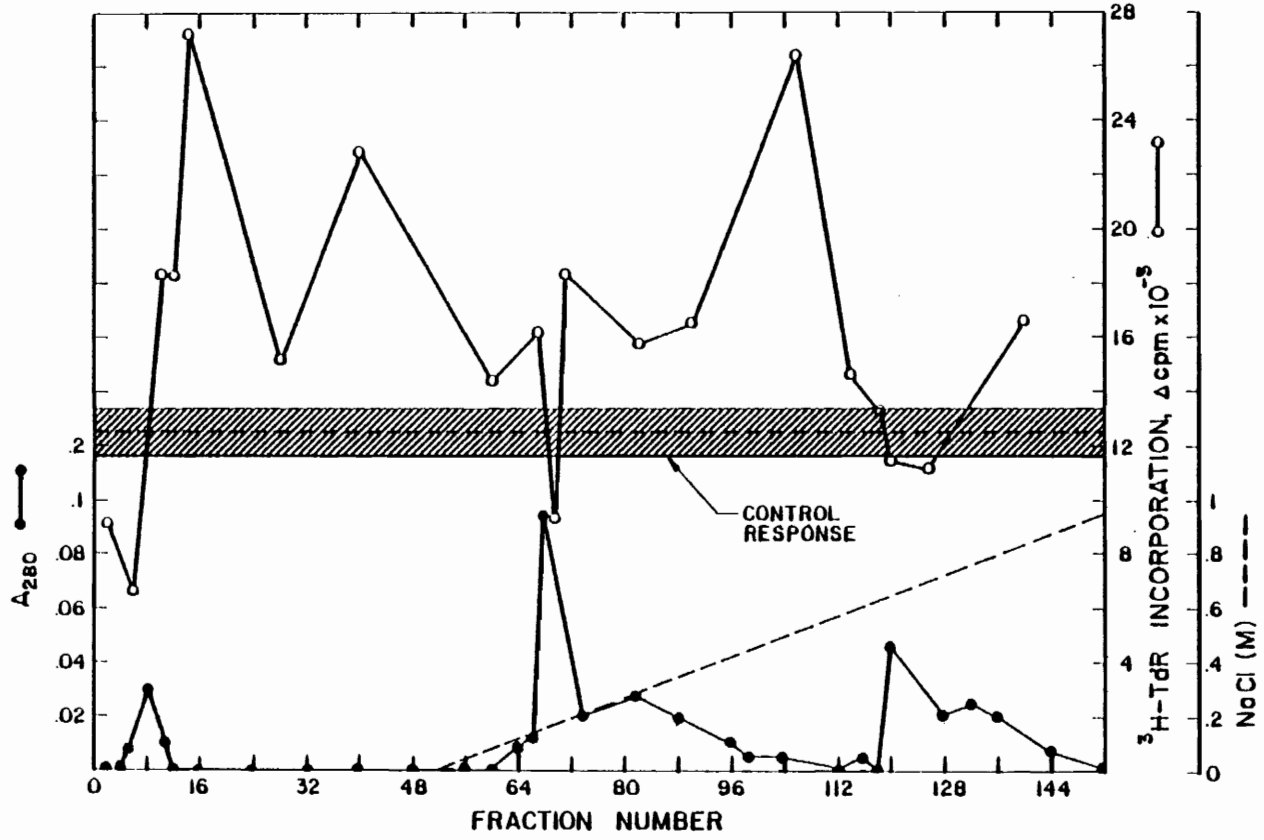


Figure 9. Activity of Mϕ supernatant DEAE fraction on a 1 M NaCl gradient. Procedures were identical to those outlined in Figure 8 except a linear 1 M NaCl gradient (in .06 M phosphate buffer) was used in place of the 0.5 M gradient.

remaining fractions, in groups of 8 to 12 were pooled. The pooled fractions totaling 10 to 20 ml, were dialyzed in 0.06 M phosphate buffer, lyophilyzed and reconstituted to 1.2 ml with distilled water. The concentrated pooled fractions were titrated and dispensed in 0.05 ml aliquots into microculture wells.

The concentration effect due to lyophilization also increased salt concentration to such an extent that untitrated pooled fractions inhibited all MLR activity. At a 1:10 dilution of supernatant, the salt content had been diluted out while some regulatory properties of the pooled fractions could still be detected (Figure 10). The cationic protein pooled fraction did not inhibit, but rather, enhanced MLR activity (fractions 1 to 10). Since the inhibitor activity noted in the previous assays was present only in a very narrow band of protein, it is possible that no residual inhibitor factor remained following assays of single DEAE fractions. The large protein peak eluting around 0.2 M NaCl continued to show inhibitor activity (fractions 42 to 56) lowering Δ cpm from 5800 to 2700.

In the succeeding assay (Figure 11) pooled fractions were collected in the previously described manner except fractions were brought to 1.2 ml and dialyzed after, rather than before, lyophilization. Thus a small concentrated volume of pooled supernatant could be obtained while the inhibitory salt presence could be removed. When this procedure was followed, the MLR assay showed enhancing activity in the cationic protein peak (fractions 1 to 12) as well as the characteristic double peak of enhancement (fractions 13 to 31 and 40 to 60) in the non 280 nm absorbance area. There was also some enhancement in the band

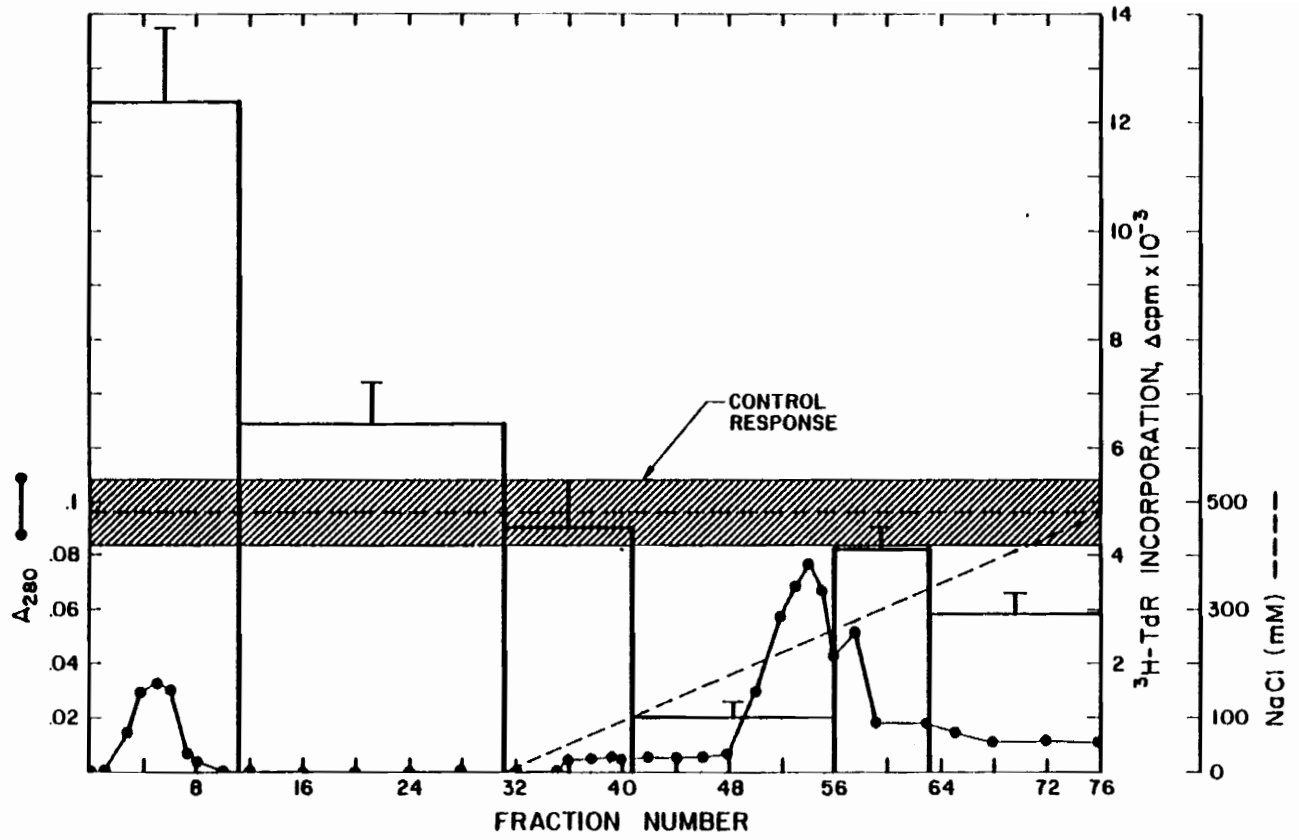


Figure 10. Activity of pooled DEAE fractions from Mϕ supernatant. Vertical bars represent pooled fractions, dialyzed against 0.066 M phosphate buffer, concentrated by lyophilization and reconstituted in 1.0 ml of RPMI complete medium. Pooled fractions were then diluted 1:10 in complete medium and assessed for activity.

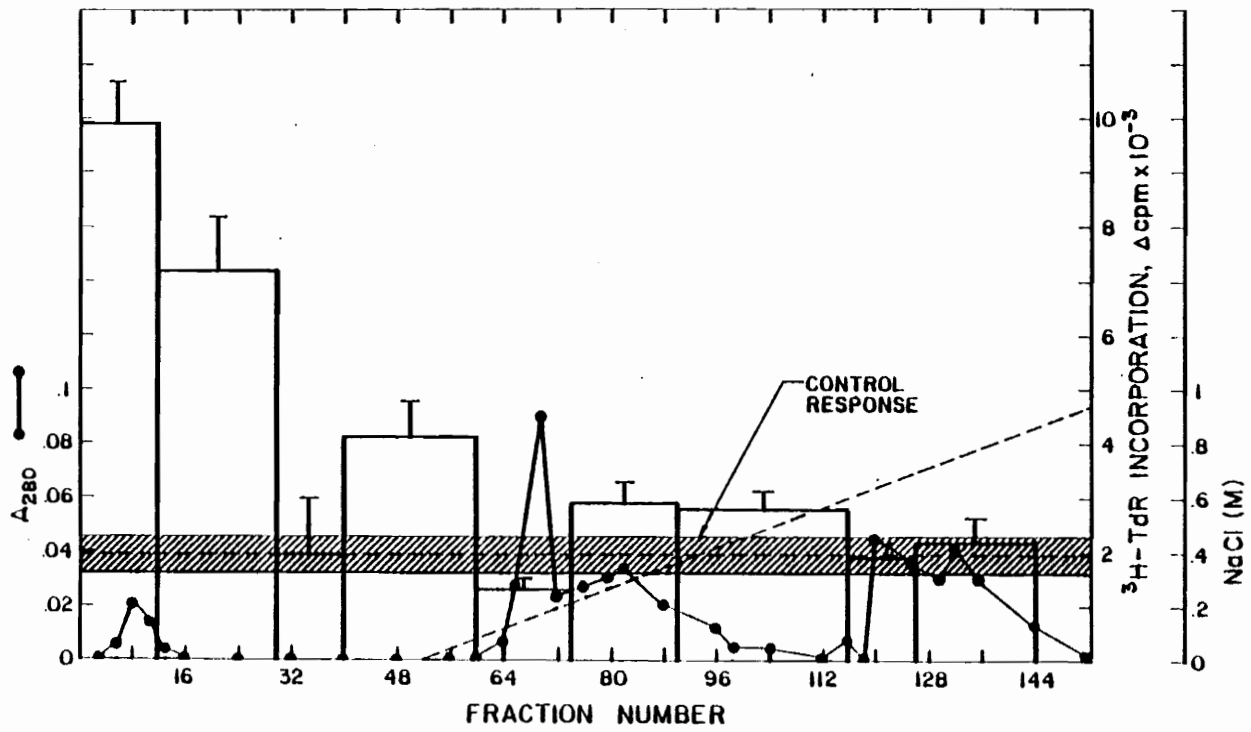


Figure 11. Activity of pooled dialyzed DEAE fractions from normal mouse Mϕ supernatant. Vertical bars represent pooled fractions, lyophilized, reconstituted in 1.0 ml medium and dialyzed overnight in RPMI medium.

eluting around 0.4 M NaCl (fractions 90 to 116). The sole fraction exhibiting inhibitory properties was the large pooled protein fraction collected around 0.2 M NaCl. The lack of inhibitor activity in the pooled cationic fractions may be due to a masking effect of enhancing factors contained in that pooled fraction; much as the 0.2 M NaCl inhibitor fraction masked all enhancing activity in unfractionated high dose Mϕ supernatants.

e. Summary. Whole Mϕ supernatants, possessed multiple peaks of enhancing and inhibitory activity, distinguishable by their characteristic elution profile on DEAE anion exchange columns. One inhibitor was a cation and was eluted prior to addition of NaCl gradient. Another inhibitor was negatively charged and eluted, with the majority of protein, around 0.2 M NaCl. There also seemed to be a number of enhancing factors; one eluting in the cationic protein fraction, and a second eluting immediately after the cationic inhibitor fraction and showing a double peak of enhancing activity but no measurable 280 absorbance.

B. Concentration Dependent Role of Macrophages in Immunoregulation

The following experiments were conducted to determine if the checks and balances of positive and negative immunoregulation were governed by the relative concentration of Mϕ and their soluble factors.

1. Dose Dependent Mϕ Inhibition or Enhancement of Normal T Cell MLR Reactivity

Figure 12 depicts the results from 2 dose response experiments. In this, and the 5 following figures the slashed bar, designated as

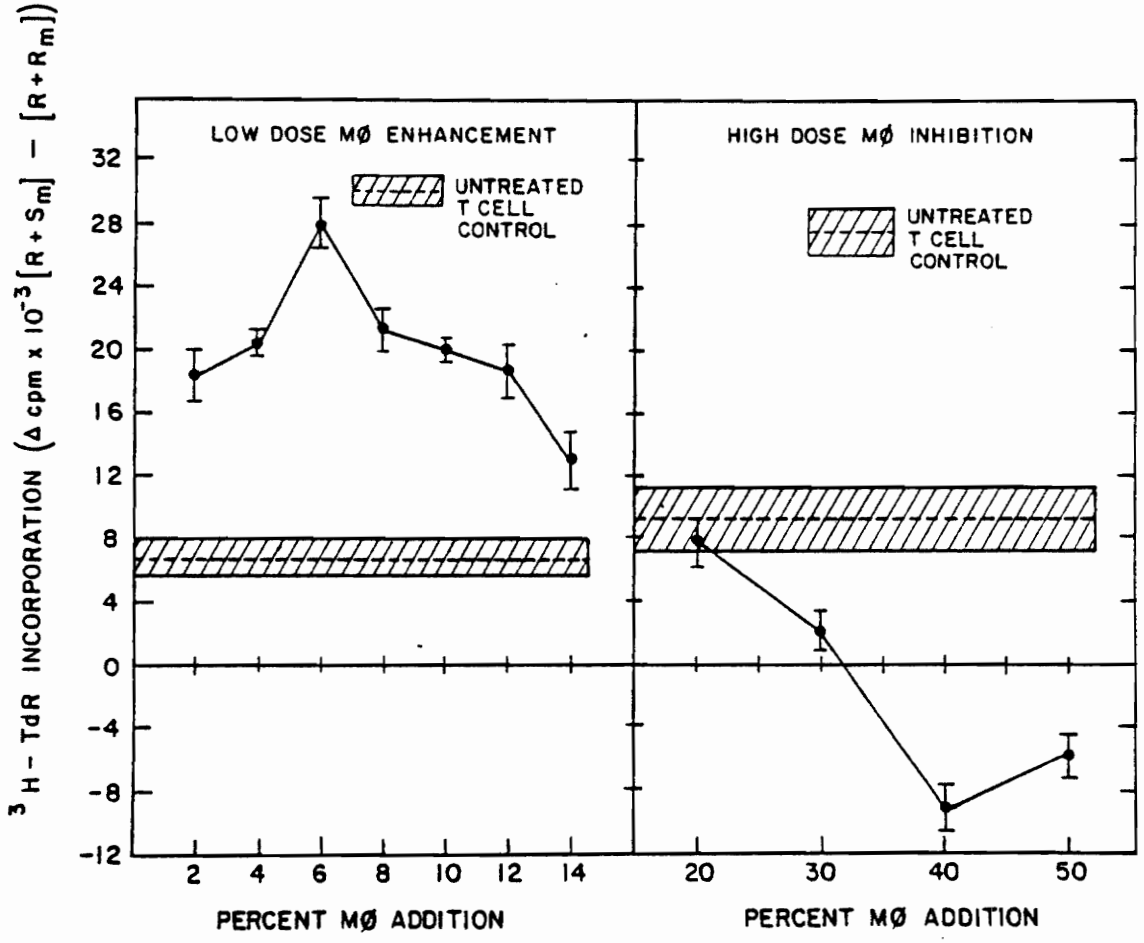


Figure 12. Dose dependent Mϕ regulation T cell MLR reactivity. Mϕ were derived from normal BALB/c mice sacrificed 4 days after a 2.0 ml i.p. thioglycolate injection. Peritoneal exudates were plated to remove contaminating nonadherent cells. After plating, adherent cells (Mϕ) were incubated for 1 hr with mitomycin C, and anti-Thy 1 serum (20 mg/ml) plus complement (1:10 dilution). As judged by latex particle uptake and esterase staining, peritoneal cells contained >96% Mϕ.

T cells were obtained by incubating BALB/c spleen cells on a nylon wool column, gently eluting nonadherent cells and plating to remove remaining Mϕ. As previously described, T cell population contained less than 0.5% Mϕ and 7% adherent (B) cells. Slashed bar represents Δ cpm of responders. Solid line (●—) represents Δ cpm of responders following addition of varying concentrations of Mϕ.

"untreated T cell control" represents Δ cpm value of unsupplemented $R + S_m$ populations after subtraction of the $R + R_m$ value (i.e., Δ cpm of T cell control = $[R + S_m] - [R + R_m]$). All other points represent cpm value of Mϕ supplemented $R + S_m$ population after subtraction of the $R + R_m$ value (i.e., Δ cpm = $[R + S_m + M\phi] - [R + R_m]$). In this set of experiments syngeneic peritoneal Mϕ, added to wells containing 2×10^5 BALB/c responder T cells (R) and 4×10^5 mitomycin C-treated (40 μg/ml) C3H stimulator T cells (S_m), exhibited a characteristic low dose enhancement and high dose inhibition of T cell MLR reactivity. Experiments were undertaken to investigate the mechanism of this dose dependent effect and to determine what part soluble factors played in the concentration dependent Mϕ regulation of T cell MLR reactivity.

2. Titration of Mϕ Enhancing and Inhibitory Supernatants

Results shown in Tables XII and XIII represent data from assays conducted to determine i) at what dilution inhibitor activity was superceded by enhancing activity and ii) at what dilution enhancing activity was reduced to levels of the untreated responder population.

Titration curves were developed for supernatants derived from high dose inhibitory (1.2×10^7 Mϕ cultured in 3.0 ml) (Table XII) and low dose enhancing (3.2×10^6 Mϕ cultured in 3.0 ml) Mϕ supernatants (Table XIII). Upon addition of Mϕ supernatants at a 100% inhibitor concentration (Table XII), normal T cell reactivity was reduced approximately 10-fold when compared to untreated responders (Δ cpm drop from 8168 to 925). When Mϕ inhibitor supernatant was reduced to a 40% concentration, enhancement rather than inhibition of MLR activity

TABLE XII

TITRATION OF HIGH DOSE Mϕ INHIBITORY SUPERNATANTS

% Concentration of Normal High Dose Mϕ Inhibitor Supernatant[a]	^{3}H-TdR Incorporated		
	Responders[b]	Control	(Responders) - (Control)
			Δ cpm
0	10,849 ± 809	2681 ± 444	8168 ± 809
100	3606 ± 96		925 ± 444
80	7562 ± 132		4881 ± 444
60	9473 ± 277		6792 ± 444
40	15,788 ± 585		13,107 ± 585
20	20,632 ±1165		17,942 ± 1165

[a] To derive inhibitor supernatants 1.2 x 10^7 Mϕ were incubated in 3.0 ml of serum-free medium as described in Table I. All supernatants were dispensed in 0.05 ml aliquots bringing the final well volume to 0.2 ml. Supernatants at 100% concentration were dispensed directly into culture wells. Inhibitor supernatants diluted 1:5 in serum-free medium were designated "20%" and dispensed in 0.05 ml volumes.

[b] In all experiments, allogeneic C3H purified splenic T cells were treated with mitomycin C and used as stimulator (S_m) cells at a concentration of 4 x 10^5 cells/well. To derive a purified stimulator population C3H whole spleen cells were incubated for 45 min on a nylon wool column. The eluates were then plated twice to remove any remaining Mϕ, and judged to be >99% Mϕ-depleted. The stimulator cells (S_m) were added to 2 x 10^5 R cells to arrive at the proper R + S_m responder population.

TABLE XIII

TITRATION OF LOW DOSE Mϕ ENHANCING SUPERNATANTS

% Concentration of Normal Low Dose Mϕ Enhancing Supernatant[a]	^{3}H-TdR Incorporated		
	Responders[b]	Control	(Responders) - (Control)
			Δ cpm
0	4007 ± 303	1072 ± 46	2935 ± 303
100	9861 ± 823		8789 ± 823
50	7337 ± 396		6265 ± 396
25	3867 ± 274		2795 ± 274

[a]For supernatant derivation see Table I. Enhancing supernatants were derived from 3.2×10^6 Mϕ cultured in 3.0 ml of serum-free medium.

[b]Responder T cell populations were taken from purified normal BALB/c splenic lymphocytes as described in Table III and initially shown to be 95% viable by trypan blue exclusion.

occurred (Δ cpm rise to 13, 107). Enhancement was even more pronounced at a 20% concentration of the original inhibitor supernatant (Δ cpm 17,942). Enhancement at a 20% concentration of inhibitor supernatant was to be expected since a 20% concentration of the 1.2×10^7 Mϕ used in the derivation of inhibitor supernatants, yielded 2.4×10^6 Mϕ, a number, slightly less than the 3.2×10^6 Mϕ used to derive enhancing supernatants. Indeed, in later experiments (Figures 13 to 16) a single dilution curve from inhibitor supernatants showed initial Mϕ inhibitor activity, subsequent enhancing activity and finally (at a sufficiently high dilution), a reduction in activity to levels of the untreated responder population. As shown here, in Table XIII, low dose enhancing Mϕ supernatants tripled MLR activity in the normal T cell responder population (i.e., responders = 2935 Δ cpm; responders + Mϕ supernatants = 8789 Δ cpm). As enhancing Mϕ supernatant was diluted, MLR activity also fell, till at a 25% concentration of Mϕ enhancing supernatant, T cell incorporation of ^{3}H-TdR did not appreciably differ from that of the untreated responders.

3. Titration of Low and High Dose Normal Mϕ Supernatants

Table XIV, depicting results of a single assay, reinforced conclusions drawn from results of the past two individual high dose and low dose Mϕ supernatant experiments (Table XII and XIII).

Undiluted concentrations of low dose supernatant more than doubled MLR reactivity (216% over untreated responders). However as low dose supernatants were diluted, enhancing activity dropped off,

TABLE XIV

TITRATION OF LOW AND HIGH DOSE NORMAL Mϕ SUPERNATANT

Mϕ Supernatant	Dilution	DNA Synthesis: % Activity of T Cell Control[a]
1.6×10^6 Mϕ/3.0 ml	Undiluted	216 ± 27
1.2×10^7 Mϕ/3.0 ml		48 ± 4
1.6×10^6 Mϕ/3.0 ml	50%	154 ± 35
1.2×10^7 Mϕ/3.0 ml		131 ± 9
1.6×10^6 Mϕ/3.0 ml	25%	86 ± 13
1.2×10^7 Mϕ/3.0 ml		181 ± 13

[a] DNA synthesis represents ^{3}H-TdR uptake by normal BALB/c mouse T cells purified by eluting over nylon wool columns and plating to remove remaining Mϕ. Activity of the treated cells was calculated as $\frac{\text{cpm treated cells}}{\text{cpm control}} \times 100$.

until at a 1:4 dilution, no enhancement of MLR reactivity could be detected.

Inhibitor activity, originally suppressing responder cell proliferation by 52%, exhibited enhancing activity at the 1:2 and 1:4 dilution.

A series of experiments were then conducted in which concentrated inhibitor supernatants were titrated past their enhancing dilution and out to a final dilution exhibiting no immunoregulatory activity.

4. Macrophage Elaboration of a Heat Stable Inhibitor and a Heat Labile Enhancing Factor

Purified peritoneal Mϕ were cultured 4 days at a concentration of 1.2×10^7 Mϕ per 3.0 ml of serum-free medium. Supernatants were then harvested and processed as described in Material and Methods, and immediately dispensed in 0.05 ml aliquots. As in the Mϕ cellular titration (Figure 12), high concentration of supernatant caused responder culture ($R + S_m$) reactivity to fall below background counts of unsupplemented controls ($R + R_m$) (Figure 13) -- hence the negative Δ cpm value. Dilute supernatant raised ($R + S_m$) counts from 4000 to 12,000 Δ cpm. At first it appeared as if inhibition and enhancement were two faces of the same coin -- inhibitory factor, nothing more than excess enhancing factor acting as too much of a good thing. However, results in Figure 14, showed that 10 min heat treatment at 100°C, failed to abolish inhibitory activity at high dose concentrations, while completely abrogating low dose Mϕ supernatant enhancement. If inhibition were due merely to an excess of heat labile enhancing factor, heat treatment would nullify the inhibitory effects of high

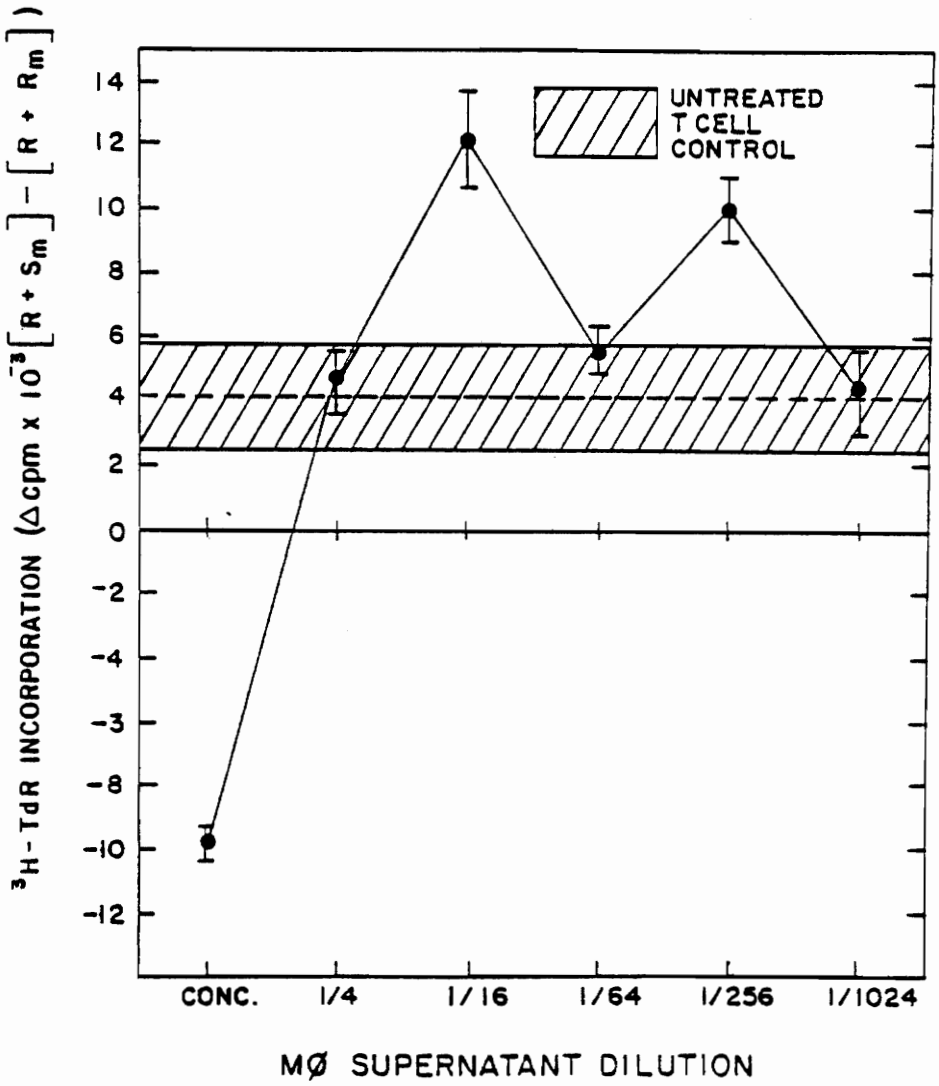


Figure 13. T cell MLR reactivity following Mϕ supernatant addition. Purified T cells (●—) were exposed to decreasing concentrations of Mϕ supernatant. Purified peritoneal exudate Mϕ (as described in Table VI) were cultured in serum-free RPMI medium for 4 days at a concentration of 1.2 x 10^7 Mϕ/3.0 ml. Supernatants were harvested, centrifuged for 10 min at 400 x g, passaged through a 0.45 μ filter, diluted in serum-free medium and immediately dispensed in 0.05 ml aliquots bringing well volume up to 0.2 ml. T cell controls represented Δ cpm of responder populations ([R + S_m] - [R + R_m]) untreated with Mϕ supernatant. Reactivity was measured by the incorporation of ^{3}H-TdR expressed in the cpm difference

Figure 13 (continued)

between the supernatant treated ($R + S_m$) population and the untreated ($R + R_m$) population. At enhancing concentrations of Mϕ supernatant (1:4 dilution), stimulation indices $(R + S_m)/(R + R_m)$ commonly varied between 4 and 10.

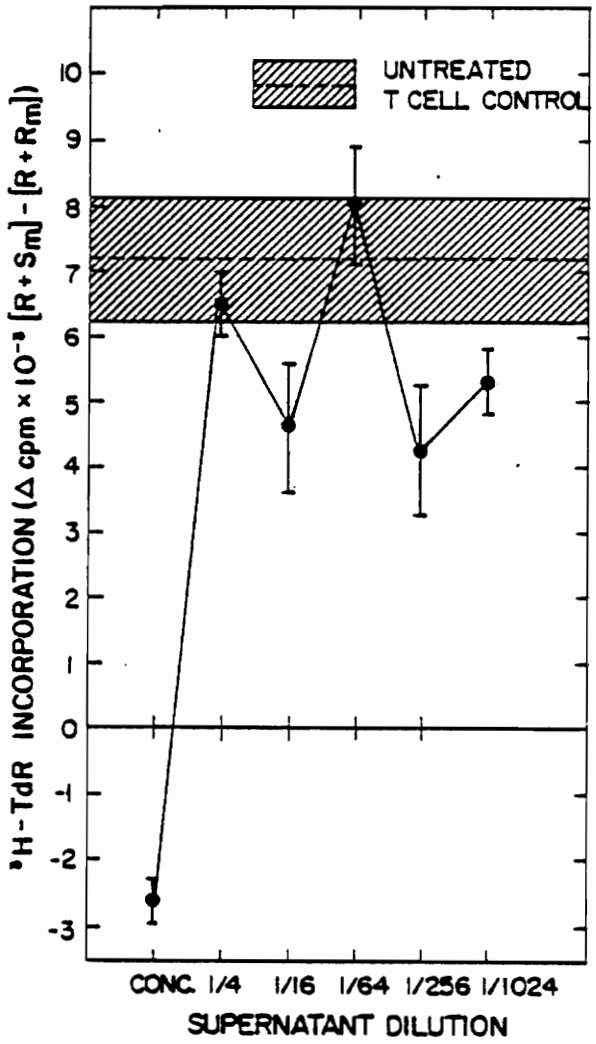


Figure 14. Activity of a heat stable inhibitory Mϕ-derived factor. Serum-free syngeneic Mϕ supernatants (as previously defined in Figure 13) were heat treated for 10 min at 100°C. Dilutions were immediately dispensed into wells containing 2 x 10^5 column purified BALB/c T Cells (●—) and 4 x 10^5 purified C3H T cells. At all times, controls were run to confirm that inhibition, present after heat treatment, was due to a heat resistant Mϕ inhibitor factor and not merely in vitro artifact due to heat treated RPMI medium. Trypan blue exclusion tests also showed that Mϕ inhibitor factor, while blocking DNA synthesis, was not cytotoxic to T cells.

dose Mϕ supernatant. At all times controls were run to insure that inhibition was due to Mϕ soluble factors not simply boiled RPMI medium. These results suggested that Mϕ regulation occurred via release of a heat stable noncytotoxic inhibitory factor(s) and a heat labile enhancing factor(s). From T cell viability and cytotoxicity studies, Table VIII and IX, it was also determined that Mϕ inhibitor supernatants, while blocking DNA synthesis in R + S_m populations was not cytolytic to the responder T cells.

5. Macrophage Regulation Via Nondialyzable Enhancing and Inhibiting Factors

A great deal of controversy has arisen over the question of artifactual supernatant inhibition due to "cold" thymidine release by Mϕ. To settle such questions, Mϕ supernatants, obtained in the previously described manner were dialyzed (pore size of 12,000 daltons) against distilled water for 24 hr. At that time, supernatants were diluted and immediately dispensed in 0.05 ml aliquots thus bringing well volume up to 0.2 ml (Figure 15). Had Mϕ inhibition been totally due to thymidine presence, dialysis of Mϕ inhibitor supernatants, would have largely removed low molecular weight thymidine, resulting in a drastic loss of inhibitor activity. However, titration of supernatants before and after dialysis did not appreciably differ -- unboiled supernatants inhibited ^{3}H-TdR uptake at high concentrations and exhibited the biphasic enhancement of MLR activity at low concentration (Figure 13); boiled dialyzed supernatant again showed high dose inhibitor activity but no enhancing capabilities (Figure 16). These titration assays further substantiated results from the high dose dialysis experiments

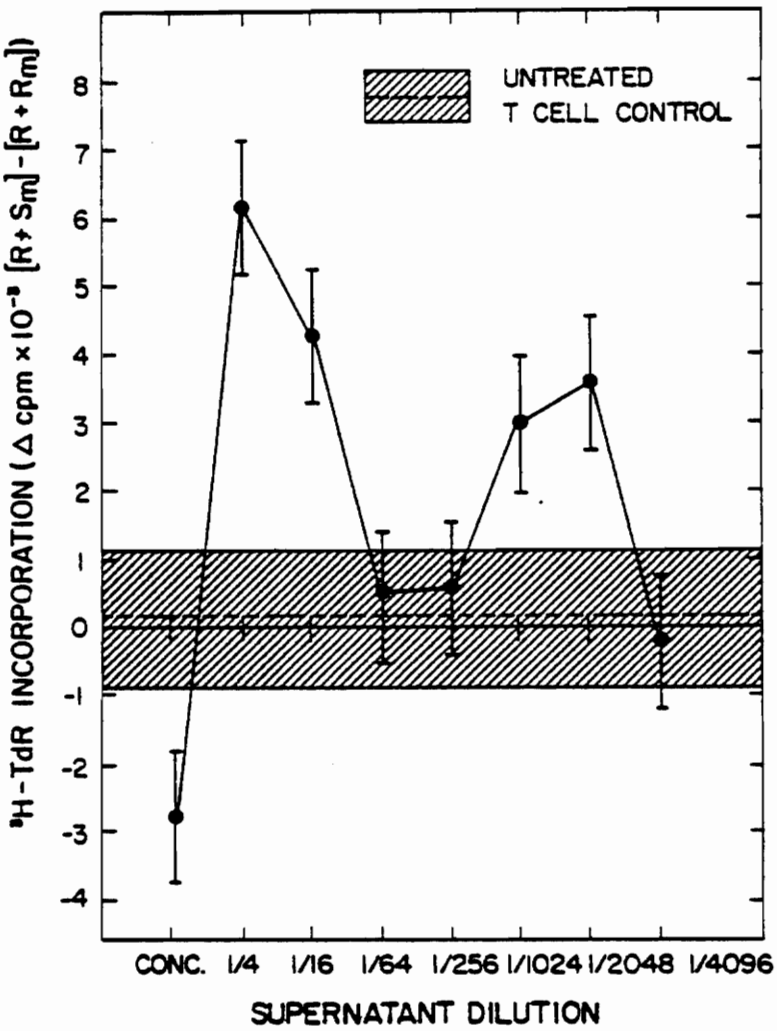


Figure 15. Titration of a nondialyzable enhancing Mϕ-derived factor. Serum-free BALB/c Mϕ supernatants were dialyzed (pore size of 12,000 daltons) against distilled water for 24 hr. After diluting, supernatants were dispensed in 0.05 ml aliquots into wells containing BALB/c responder cells (●—) and allogeneic C3H cells. Negative Δ cpm values represented complete abrogation of MLR reactivity in the (R + S_m) supernatant treated population. Raw cpm values falling below those of the untreated (R + R_m) population yielded a negative Δ cpm value.

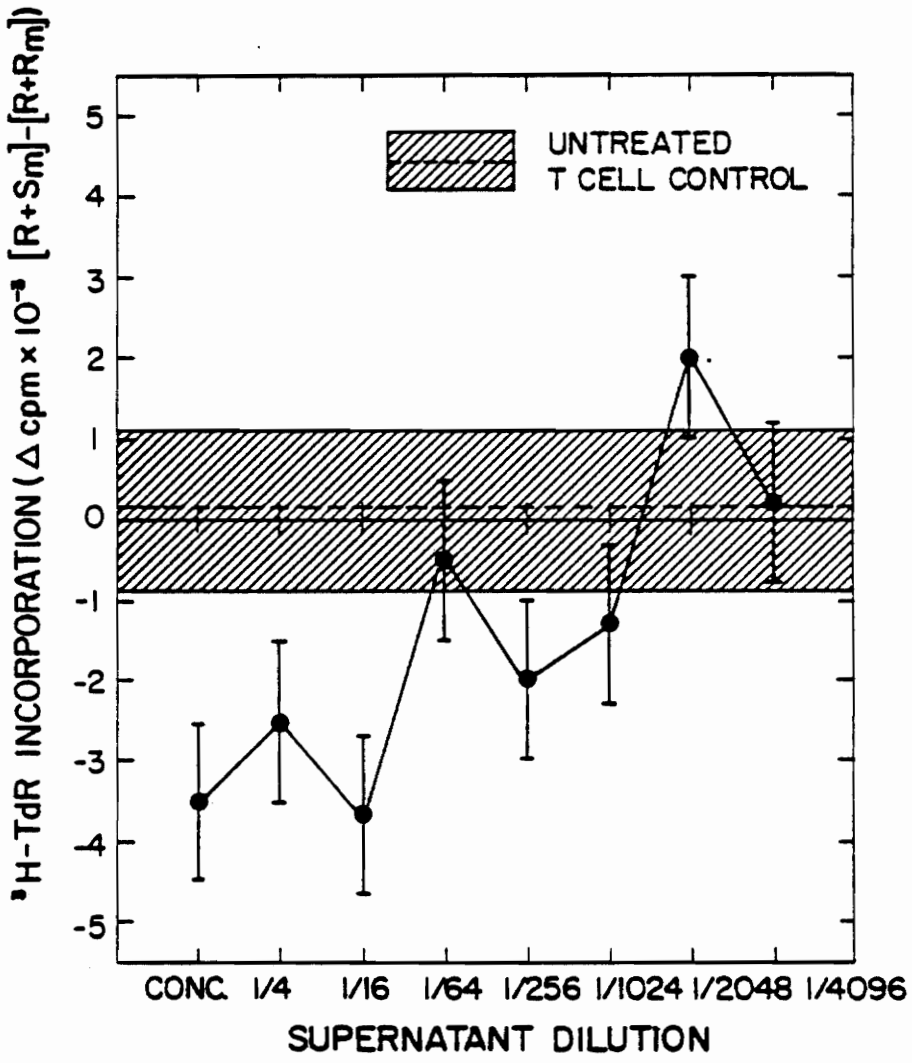


Figure 16. Addition of a heat stable nondialyzable inhibitory Mϕ-derived factor. Macrophage supernatant derivation, treatment and dilution mirrored that outlined in Figure 15 with the addition of a 10 min 100°C heat treatment before delivery of supernatant into culture wells.

(Table V) indicating the nondialyzable, heat stable nature of Mϕ inhibitor supernatants.

6. Summary

Mϕ supernatant immunoregulation was a concentration dependent phenomena, correlating well with the high dose inhibition and low dose enhancement seen in whole Mϕ titrations. Inhibitor factor(s) was nondialyzable and heat stable, while enhancing concentrations of supernatant exhibited a biphasic double peak of enhancing activity abrogated by boiling, but not dialysis.

C. Demonstration of Enhancing and Inhibitory Activity in Tumor-Bearing Mouse Mϕ Supernatants

The detection of inhibitor activity in normal Mϕ supernatant has led some researchers to investigate inhibitory properties of Mϕ in the tumor-bearing host (94, 176). Experimentation with various tumor models caused some to conclude that tumor-induced hyporeactivity was due to the action of a suppressor Mϕ found only in the tumor-bearing host and its release of a unique suppressor factor. The following experiments were undertaken to compare the immunoregulatory effects of normal and TBM Mϕ supernatant.

1. Addition of Low Dose Normal and TBM Mϕ Supernatants to Normal and TBM MLR Cultures

Table XV represents results from an experiment designed to see whether the low level of TBM T cell MLR reactivity was due to the presence of inhibitor factors released by tumor-induced Mϕ. TBM T cells showed a low MLR response not totally reversible by addition of normal

TABLE XV

ADDITION OF LOW DOSE NORMAL AND TBM Mϕ SUPERNATANTS TO NORMAL AND TBM MLR CULTURES

Source of T Cells	Source of Mϕ[a] Supernatant (3.2×10^6 Mϕ/3.0 ml)	MLR Reactivity ^{3}H-TdR Incorporation (Responder) - (Controls)	Stimulation Index (Responders)/(Controls)
		Δ cpm	
Normal	None	3652 ± 369	3.16 ± .42
	Normal	9841 ± 542	8.52 ± .46
	TBM	14,084 ± 529[b]	12.20 ± .45
TBM	None	3546 ± 692	1.79 ± .34
	Normal	1960 ± 350	1.43 ± .25
	TBM	2565 ± 261	1.56 ± .10

[a]Macrophages were counted on a hemocytometer, assessed for viability and homogeneity using trypan blue exclusion and esterase staining, respectively. Cell suspension contained a 95% pure population of viable Mϕ. Macrophage supernatants from both normal and TBM were obtained in the manner previously described in Table I.

[b]Using Student's t test, activity was significantly higher ($p < 0.05$) than produced by normal Mϕ supernatants.

Mϕ supernatant (TBM T cells + normal Mϕ supernatant = 1960 Δ cpm; normal T cells + normal Mϕ supernatant = 9841 Δ cpm). However, the hyporeactivity seen in TBM T cells could not be attributed to TBM inhibitor Mϕ since TBM Mϕ supernatants enhanced, rather than inhibited, normal MLR reactivity (normal T cells = 3652 Δ cpm; normal T cells + TBM Mϕ supernatants 14,084 Δ cpm). In fact, when compared to their normal counterparts, TBM Mϕ supernatants showed a significantly greater degree of enhancing activity (normal T cells + normal Mϕ supernatant = 9841 Δ cpm; normal T cells + TBM Mϕ supernatant = 14,084 Δ cpm).

2. Addition of High Dose Normal and TBM Mϕ Supernatants to Normal MLR and PHA Cultures

Since it was apparent that normal Mϕ supernatants contained inhibitor as well as enhancing factors, I decided to again test the hypothesis that T cell hyporeactivity evidenced by TBM in PHA and MLR assays might be due to the preponderance of a potent inhibitor factor found in TBM Mϕ supernatants. To compare the regulatory properties of normal and TBM Mϕ, 4 day supernatants were collected, lyophilized, reconstituted to 2.0 ml with distilled water and dialyzed successively in low molar phosphate buffer and RPMI medium. Following dialysis the supernatants were dispensed in 0.05 ml aliquots into microculture wells, bringing the final volume up to 0.2 ml. In agreement with experiments reported previously (Figures 13 to 16), concentrated normal supernatants in this experiment (Table XVI), severely inhibited both MLR (100% loss) and PHA activity (70% loss). However, TBM Mϕ supernatants not only failed to inhibit PHA activity (16% increase) but enhanced MLR reactivity more than 3-fold (5482 cpm up to 20,092

TABLE XVI

ADDITION OF HIGH DOSE NORMAL AND TBM Mϕ SUPERNATANTS TO NORMAL MLR AND PHA CULTURES

Mϕ Derivation	High Dose Supernatant Addition[a]	PHA Stimulation[b] [Responder] - [Control]	MLR Stimulation [Responder] - [Control]
		Δ cpm	Δ cpm
Normal	-	12,071 ± 1132	8016 ± 2461
	+	3613 ± 1011 (-70)[c]	-903 ± 305 (0)
TBM	-	9919 ± 1182	5482 ± 549
	+	11,545 ± 983 (+116)	20,042 ± 3654 (+365)

[a] Supernatants were derived from 1.2×10^7 Mϕ cultured in 3.0 ml of serum-free medium.

[b] PHA stimulation as was conducted as described in Table III with the difference that purified splenic T cells were used in place of thymocytes.

[c] Number in parentheses represents percentage of inhibition (or enhancement, +) compared to untreated populations.

cpm), again indicating the superior enhancing properties of TBM Mϕ supernatants.

3. Normal and TBM Mϕ Supernatant Addition to Normal or TBM T Cells

Results from previous experiments (Table XV) indicated MLR hyporeactivity in TBM T cell populations. The data in Table XVII and XVIII depict results from experiments attempting to answer the following questions: i) how do T cells from normal or TBM differ in their ability to respond to various concentrations of Mϕ supernatant; ii) how do Mϕ supernatants from normal or TBM differ in their ability to enhance or inhibit MLR reactivity; iii) to what extent is Mϕ activator or inhibitor activity a concentration dependent phenomenon; and iv) to what extent is Mϕ activator or inhibitor activity affected by heat treatment?

Low dose TBM Mϕ supernatants (3.2×10^6 Mϕ cultured in 3.0 ml), when added in 0.05 ml volumes to normal responder cultures (Table XVII), caused a higher (not lower) degree of augmentation, when compared to the augmenting effect of low dose normal Mϕ supernatants (Δ cpm of 13,084 ± 529 vs 9841 ± 542). In addition, high dose supernatants (1.2×10^7 Mϕ cultured in 3.0 ml) from TBM Mϕ (Table XVII) continued to elicit the enhancing response not seen when high dose normal Mϕ supernatants were added to responder populations (Δ cpm of 6301 ± 943 vs 3042 ± 187).

When low dose (enhancing concentrations of Mϕ supernatant) were subjected to a 10 min 100°C heat treatment (Table XVII), enhancing activity was reduced in both normal and TBM Mϕ supernatants. However, heat treated TBM Mϕ supernatants retained a greater degree of activity

TABLE XVII

ADDITION OF HEAT TREATED, LOW AND HIGH DOSE SUPERNATANT FROM NORMAL AND TBM Mϕ TO NORMAL MLR CULTURES

Characterization of Mϕ Supernatant[a]	Heat Treatment[b]	^{3}H-TdR Incorporated			
		Responders[c]	Control	(Responders)-(Control)	Stimulation Index (Responders)/(Control)
				Δ cpm	
None	37°C	4806 ± 369	1154 ± 187	3652 ± 369	4.16
Normal		10,995 ± 542		9841 ± 542	9.52
Low Dose	37°C				
TBM		14,238 ± 529		13,084 ± 529[d]	12.33
Normal		4196 ± 105		3042 ± 187	3.63
High Dose	37°C				
TBM		7455 ± 256		6301 ± 256[d]	6.46
Normal		4099 ± 268		2945 ± 268	3.55
Low Dose	100°C				
TBM		10,123 ± 943		8969 ± 943[d]	8.77
Normal		2684 ± 308		1530 ± 308	2.32
High Dose	100°C				
TBM		2910 ± 231		1756 ± 231[e]	2.52

Table XVII (continued)

[a]Mϕ supernatants were derived as described in Table I. "Low dose" supernatants refer to supernatants obtained from Mϕ cultured at a concentration of 3.2×10^6 Mϕ in 3.0 ml. "High dose" supernatants refer to supernatants obtained from Mϕ suspended at a concentration of 1.2×10^7 Mϕ in 3.0 ml.

[b]Supernatants dispensed directly into microculture wells were given the heat treatment designation "37°C". Those supernatants boiled for 10 min before dispensing were given the designation "100°C". All supernatants were obtained from Mϕ cultured in serum-free medium and dispensed in 0.05 ml aliquots. In all cases it was shown that boiled RPMI serum-free medium was not cytotoxic to cell structures.

[c]"Responders" or "Control" represent R + S_m and R + R_m cultures, respectively.

[d]Using the Student's *t* test these values differed significantly (95% level) from corresponding normal supernatant value.

[e]No significant difference at the 95% level when compared with its corresponding normal supernatant value.

[f]All responder T cells were taken from BALB/c mice, while C3H mice served as a source of stimulator T cells.

than that shown by similarly treated normal Mϕ supernatants (Δ cpm of 8969 vs 2945). It had been determined in earlier experiments that T cell inhibition was not caused by cytotoxic action of boiled serum-free medium, since at no time did ^{3}H-TdR incorporation diminish upon addition of boiled medium to T cell populations (see Table V). In addition, to allay fears that inhibition might be due to cytotoxicity of heat-treated denatured supernatant proteins, T cell viability, as assessed by trypan blue exclusion, was not significantly diminished by exposure to heat-treated Mϕ supernatants.

Though addition of low dose normal or TBM Mϕ supernatants caused a dramatic rise in normal T cell MLR reactivity (Table XVII), no manipulation involving Mϕ supernatants could enhance activity in the TBM responder population (Table XVIII). It should be noted that in a comparison of normal and TBM MLR reactivity it is necessary to examine not raw cpm responder values but the Δ cpm value. We (57) have shown that TBM T cells possess a high degree of background (basal level) proliferation. This high level of nonspecific background activity should not be confused with allogeneic recognition of foreign lymphocytes. Tumor-bearing mice T cell hyporeactivity became strikingly evident when stimulation indices (S.I.) (responders divided by control) as well as Δ cpm values were examined. Normal T cells (Table XVIII), exposed to low dose, untreated normal Mϕ supernatant had a S.I. of 9.52 while the TBM T cells (Table III) under identical conditions exhibited a S.I. of 1.43.

The data presented in Tables XVII and XVIII can be summarized as follows: i) TBM T cells failed to respond to enhancing concentrations

TABLE XVIII

ADDITION OF HEAT TREATED LOW AND HIGH DOSE SUPERNATANTS FROM NORMAL AND TBM Mϕ TO TBM MLR CULTURES

Characterization of Mϕ Supernatant		Heat Treatment	^{3}H-TdR Incorporated			
			Responders[b]	Control	(Responders)-(Control)	Stimulation Index (Responders)/(Control)
					Δ cpm	
None		37°C	8047 ± 692	4501 ± 205	3546 ± 692	1.78
	Normal		6461 ± 350		1960 ± 350	1.43
Low dose		37°C				
	TBM[a]		7066 ± 261		2565 ± 261	1.56
	Normal		3220 ± 98		-1281 ± 205	.71
High dose		37°C				
	TBM		5193 ± 108		692 ± 205	1.15
	Normal		6369 ± 287		1868 ± 287	1.41
Low dose		100°C				
	TBM		6213 ± 380		1712 ± 380	1.38
	Normal		3550 ± 584		-951 ± 584	.78
High dose		100°C				
	TBM		5248 ± 439		747 ± 439	1.16

Table XVIII (continued)

[a]To obtain tumor bearers, BALB/c mice were injected i.m. with 10^6 tumor cells originally derived from a methylcholanthrene-induced fibrosarcoma.

[b]All responder T cells were taken from BALB/c mice and judged to be >99% Mϕ-free by esterase staining. The purified T cell population was obtained by passing splenic lymphocytes over a nylon wool column and plating the nonadherent column eluates twice to remove any remaining Mϕ.

of Mϕ supernatant; ii) this refractory response could not be attributed to the action of TBM Mϕ, since low dose Mϕ supernatants from TBM as well as normal animals appreciably enhanced normal MLR reactivity; iii) as the Mϕ supernatant dose was increased T cell MLR reactivity diminished significantly, there being a greater decline in T cell activity when high dose normal (as opposed to TBM) Mϕ supernatants were used; iv) though heat treatment diminished enhancing activity in normal Mϕ supernatants, it was less effective in abrogating stimulatory activity of TBM Mϕ supernatants; and v) based on concentration and heat treatment experiments, it appears that TBM Mϕ supernatants, when compared to normal Mϕ supernatants, possess a greater ability to enhance MLR reactivity.

4. Addition of Freeze Thawed Normal and TBM Macrophage Supernatants to Normal MLR Cultures

In the previous set of experiments (Table XVII and XVIII) the heat stability of normal and TBM immunoregulatory factors were compared by boiling them and testing for activity. In this experiment (Table XIX) I did a 180° turn (a -20° turn to be more precise) and froze supernatants to determine if thawed low dose supernatants retained their enhancing capabilities. As can be seen, freeze thawing did not significantly decrease the ability of low dose (1.6×10^6 Mϕ/3.0 ml) Mϕ supernatants to enhance MLR reactivity. However, when compared to normal supernatants on a volume to volume basis, the TBM Mϕ supernatants possessed a significantly higher degree of enhancing activity.

TABLE XIX

ADDITION OF FREEZE THAWED NORMAL AND TBM
Mϕ SUPERNATANTS TO NORMAL MLR CULTURES

Source of Mϕ Supernatant[a]	Treatment of Mϕ Supernatant[b]	Stimulation Index (S.I.) [Responders] / [Control]
Normal	None	5.04 ± .68
	Freeze-thawed	5.20 ± .84
TBM	None	8.66 ± .37
	Freeze-thawed	7.60 ± .76

[a] To obtain supernatants, Mϕ from normal or TBM were cultured for 4 days with complete medium in a humidified CO_2 incubator. The supernatants were harvested, centrifuged at 400 x g for 10 min and passed through a 0.45 μ filter.

[b] Supernatants were harvested from Mϕ at a concentration of 3.2×10^6 cells/6.0 ml. Supernatant derivation was as outlined in Table I. Frozen supernatants were stored at -20°C for 7 days, thawed and dispensed into microculture wells.

5. Differences in the Electrophoretic Pattern of Normal and TBM Mϕ Supernatants

The past section of experiments all held, as their common theme, "the comparison of immunoregulatory properties in normal and TBM Mϕ supernatants."

Based on assays of supernatant activity, TBM Mϕ supernatants enhanced responder cell blastogenesis, to a greater (not lesser) extent than normal Mϕ supernatants. In Figure 17 and 18, we have taken the first step in the comparison of supernatants on a biochemical level. Using slab gel electrophoresis techniques described in Figure 6, the electrophoretic protein banding patterns of concentrated normal and TBM Mϕ supernatants were compared. As seen in the schematic diagram of Figure 18, TBM Mϕ supernatants differ in protein composition compared to normal Mϕ supernatants. Of course, this is hardly conclusive proof of biochemically distinct TBM Mϕ enhancing factors. However, it does not exclude that possibility. In future experiments, gels will be sliced and assayed for activity. In this way it can be determined whether the characteristic TBM Mϕ supernatant bands are unique TBM Mϕ enhancing factors or merely irrelevant proteins contained in the heterogeneous TBM Mϕ supernatant.

6. Summary

Based on MLR and PHA activity assays, TBM Mϕ supernatant, when compared to normal Mϕ supernatants, possessed a greater degree of enhancing activity when tested under a variety of conditions involving immunoregulatory comparisons: a) between low dose supernatants; b) between high dose supernatants; c) between heat treated supernatants;

Figure 17. Differences in electrophoretic pattern of normal (left side) and TBM (right side) Mϕ supernatants on polyacrylamide gel. Slab gel electrophoresis run as described in Figure 6.

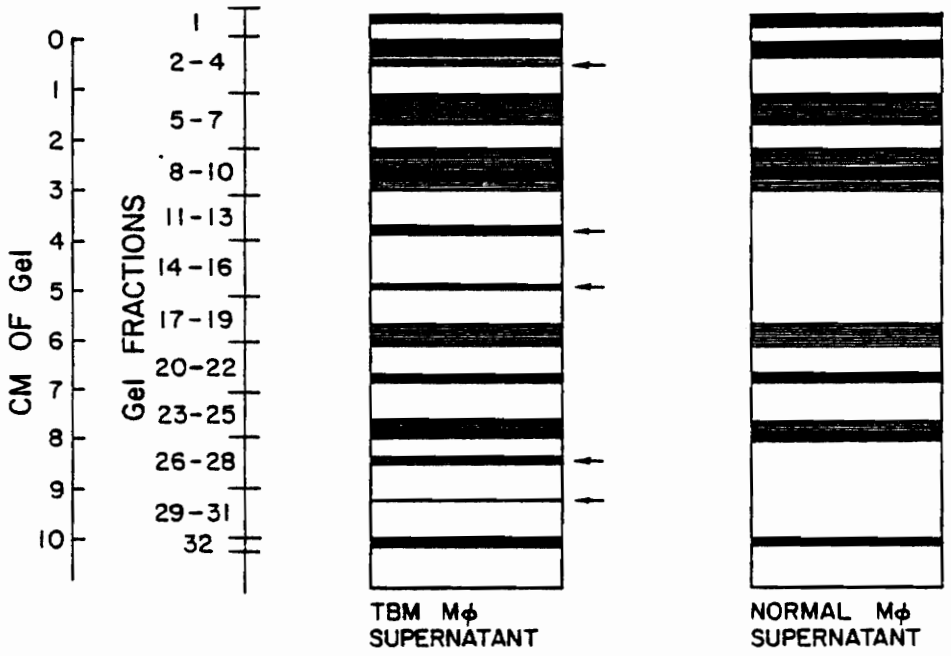


Figure 18. Schematic diagram showing the electrophoretic pattern of normal and TBM Mϕ supernatants on polyacrylamide gel. Arrows denote the protein bands unique to TBM Mϕ.

and d) between freeze thawed supernatants. It is possible that the superior positive regulatory activity of TBM Mϕ supernatants may be due to the possession of additional enhancing protein factors.

D. Reversal of Mϕ Augmented MLR Reactivity by Tumor-Induced Splenic Suppressor T Cells

Succinctly stated, "if TBM T cell hyporeactivity is not due to a unique TBM Mϕ suppressor factor, what then is the cause of blastogenic inhibition in Mϕ-depleted TBM T cells." The following experiments indicated that a tumor-induced splenic T cell was responsible for suppressing MLR reactivity in Mϕ-depleted TBM T cell populations.

1. Restoration of TBM T Cell Activity After Removal of Tumor-Induced Suppressor Cells

Tumor-bearing mice T cells, passaged over a single nylon wool column were unable to respond to allogeneic cells even upon addition of optimal concentrations of normal Mϕ (Table XX) or Mϕ supernatants (Table XXI). Though 99% TBM Mϕ-free, these T cells were further purified by passaging over a second nylon wool column. Upon exposure to normal Mϕ (Table XX), or Mϕ supernatants (Table XXI), purified TBM cell populations exhibited a significantly greater (2 to 10-fold) increase in MLR activity over their unpurified counterparts. Results from previous studies (Table XVII) also showed normal MLR enhancing activity using comparable concentrations of TBM Mϕ and supernatants. This indicated that MLR suppression was due to a non-phagocytic, mildly adherent cell, present in TBM cell eluates after a single column purification but removed by 2 successive passages over nylon

TABLE XX

REMOVAL OF TUMOR-INDUCED SUPPRESSOR CELLS: RESTORATION OF MLR ACTIVITY AFTER NORMAL Mϕ ADDITION

Mϕ Addition	Responder T Cell[a] Source	T Cell MLR Reactivity[c] (^{3}H-TdR Incorporated, [Responders] - [Controls])
		Δ cpm[c]
None	Normal[b]	4711 ± 690
	TBM, Unpurified	-16 ± 202
	TBM, Purified	5363 ± 747[d]
10%	Normal	19,749 ± 3086
	TBM, Unpurified	1122 ± 232
	TBM, Purified	11,054 ± 1584[d]

[a]Animals designated as TBM (tumor-bearing BALB/c mice) were sacrificed no earlier than 14 days after development of a palpable fibrosarcoma, derived from an i.m. inoculation of 10^6 viable transplanted methylcholanthrene-induced tumor cells.

[b]Six to 8-week old BALB/c mice were used as a source of all responder (R) T cells. "Normal" T cells were eluted from a nylon wool column and plated twice to remove Mϕ. "Unpurified T cells, from 2-week palpable TBM were passaged over a single nylon wool column and depleted of Mϕ by 2 one-hr platings. "Purified" T cells were obtained from 2-week palpable TBM, plated and successively passaged over nylon wool columns to remove mildly adherent T_s cells as well as Mϕ.

[c]"R + S_m" normal responders = 7720 ± 690 cpm, "R + S_m" unpurified TBM responders = 9304 ± 202 cpm, and "R + S_m" purified TBM responders = 16,011 ± 747 cpm.

[d]As shown by Student's *t* test these values are significantly higher than their unpurified counterparts.

TABLE XXI

REMOVAL OF TUMOR-INDUCED SUPPRESSOR CELLS: RESTORATION OF ACTIVITY AFTER NORMAL Mϕ SUPERNATANT ADDITION

TBM Responder[a] T Cell Source	Mϕ Supernatant[b] Addition	MLR Reactivity (^{3}H-TdR Incorporated, [Responders] - [Control])
		Δ cpm[c]
Unpurified	-	2834 ± 368
Purified	-	7375 ± 165[d]
Unpurified	+	3253 ± 181
Purified	+	9939 ± 138[d]

[a]For definition of "purified" and "unpurified" responders, see Table XX. All responder (R) populations (2 x 10^5 cells) and stimulator (S_m or R_m) populations (4 x 10^5 cells) were suspended at final concentrations of 0.02 ml/well.

[b]Normal syngeneic Mϕ enhancing supernatants were derived from 1:4 dilutions of "concentrated" supernatant, created by 4-day culturing of 1.2 x 10^7 Mϕ/3.0 ml in serum-free RPMI medium. All supernatants were harvested, centrifuged 10 min at 400 x g, passed through a 0.45 μ filter and immediately dispensed in 0.05 ml aliquots.

[c]"R + S_m" unpurified TBM responders = 4443 ± 368 cpm and "R + S_m" purified TBM responders = 9517 ± 165.

[d]Student's t test indicated that there was a significant increase in activity over unpurified TBM T cells.

wool. The following experiment further characterized tumor-induced suppressor cells by assaying sensitivity to anti-Thy 1 serum plus complement.

2. Abrogation of MLR Activity by Anti-Thy 1 Serum Sensitive Cells From TBM (T_s Cells)

Additional examination of the mildly nylon wool adherent suppressor cell was undertaken using Mϕ-depleted TBM spleen cells, half of which were subjected to anti-Thy 1 treatment (Table XXII). Untreated TBM spleen cells, added back to normal populations of T cells plus normal optimal concentrations of Mϕ, caused MLR reactivity to fall significantly from 19,749 to 6297 Δ cpm of ^{3}H-TdR incorporation. However, addition of a 10% concentration of anti-Thy 1 treated TBM spleen cells failed to suppress normal T cell MLR reactivity. Since, anti-Thy 1 serum plus complement abrogated TBM suppressor cell activity, the non-phagocytic, mildly nylon wool adherent, tumor-induced suppressor cell was judged to be a subpopulation of T cells.

3. Abrogation of Whole Spleen Cell MLR Reactivity by T_s Cell Addition

In add back experiments (Figure 19), as little as 5% T_s cells, completely inhibited MLR reactivity in normal whole spleen cell preparations. Uptake of ^{3}H-TdR in the responder populations fell from 1000 Δ cpm to -4200 Δ cpm in the presence of T_s cells. As stated earlier, a negative Δ cpm number represented a responder [R + S_m] level of activity below that of the control [R + R_m] value. Thus 5% to 50% T_s cell addition, consistently lowered responder activity 1500 to 4200 cpm below that of control populations.

TABLE XXII

ABROGATION OF MLR ACTIVITY BY ANTI-THY 1 SERUM SENSITIVE CELLS FROM TBM (T_s CELLS)

Admixtures		Normal T Cell MLR Reactivity (^{3}H-TdR Incorporated, [Responders] - [Control])
Mϕ[a]	T_s Cells[b]	
		Δ cpm[c]
None	None	4711 ± 690
10%	None	19,749 ± 3086
10%	10%	6297 ± 736[d]
10%	10%, Anti-Thy 1 treated	21,710 ± 1136

[a]Cells (Mϕ and/or T_s) were added back to 2×10^5 normal T cell responder populations. Cell percentages represent percent addition to 2×10^5 responder cells (i.e., R + S_m + 10% Mϕ = ([2×10^5 R] + [4×10^5 S_m] + [2×10^4 Mϕ]). In all admixture experiments, controls were maintained using R_m cells to insure that T cell regulation was not an *in vitro* artifact due only to cell concentration.

[b]To obtain suppressor T (T_s) cells, whole spleen cells from 2 week palpable BALB/c TBM were plated for 2 hr and, as assessed by esterase staining, were >99% Mϕ-depleted. Trypan blue viability studies exhibited that all cellular subpopulations (T cells, T_s cells and Mϕ) showed >99% viability at assay initiation, and >70% viability at assay termination. Anti-Thy 1 treatment consisted of a 45 min exposure to anti-serum (20 mg/0.1 ml) at 4°C followed by addition of a 1:10 dilution of complement during a 45 min incubation (37°C) period. Serum potency was established by exposing T cells to the antiserum and performing subsequent mitogen stimulation and viability assays. Treatment of T cells resulted in 95% lysis and no more than background levels of ^{3}H-TdR incorporation after PHA (0.5 µl) stimulation.

[c]"R + S_m" normal T cell responders = 7720 ± 690 cpm.

[d]Significantly lower than normal Mϕ augmented T cell activity.

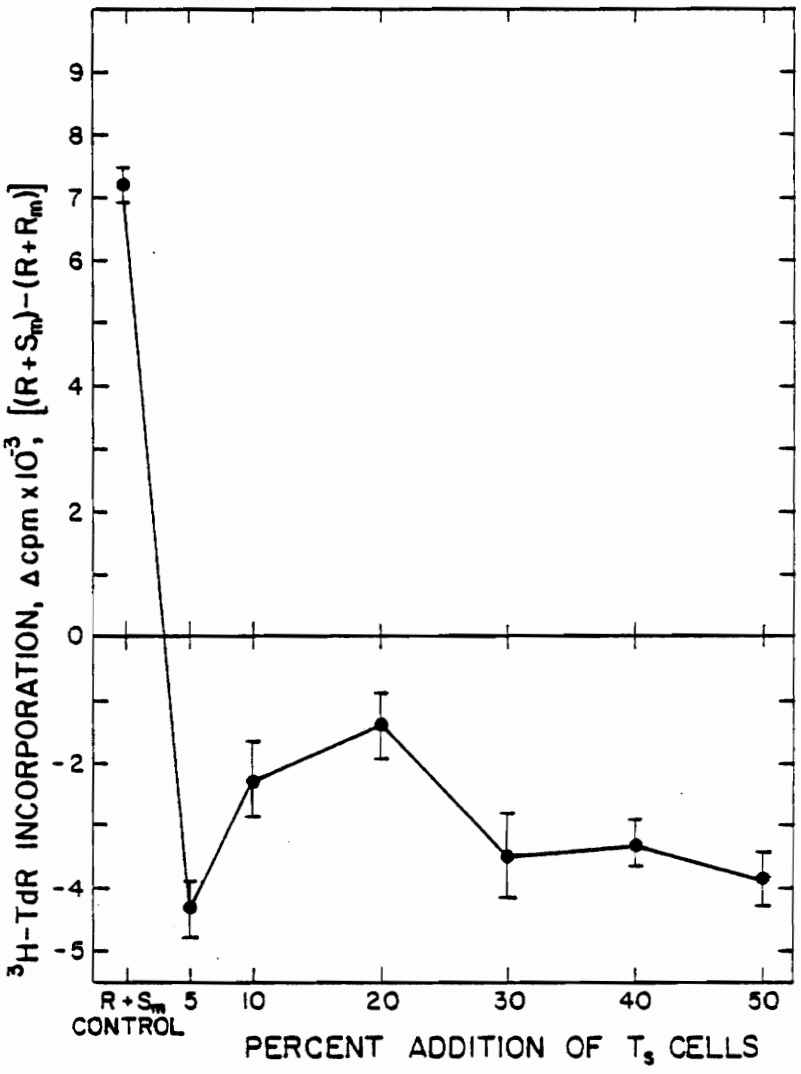


Figure 19. Abrogation of whole spleen cell MLR reactivity by T_s cell addition. Experimental protocols followed as described in Table XXI except whole normal splenic lymphocytes were used as responders in place of purified normal T cells. Negative Δ cpm values represent depression of responder population [R + S_m], below that of background [R + R_m] population. Δ cpm was calculated as [R + S_m] - [R + R_m], hence the negative value.

4. Abrogation of Mϕ Enhancing Activity by T_s Cell Addition to Normal Cultures

Purified T cell populations, by definition, have been depleted of Mϕ, and therefore possess a relatively low level of MLR reactivity (as compared to whole spleen cell populations which have not been depleted of Mϕ). To more clearly determine whether T_s cells can subvert MLR enhancing properties of Mϕ, a series of different add back experiments were conducted using purified T cells (as opposed to whole spleen cells) and various concentrations of Mϕ or their supernatants) and T_s cells (or their supernatants).

The presence of a mildly nylon wool adherent T_s cell in the spleens of TBM, was shown earlier in "depletion" experiments (Table XX and XXI). Cell mixing experiments also showed the suppressor cell to be of T cell origin (Table XXII). In this set of reconstitution experiments, a 50% concentration of T_s cells completely reversed the ability of normal T cells (Table XXIII) to respond to enhancing concentrations of syngeneic peritoneal Mϕ. Before T_s cell addition, Mϕ augmented T cell MLR reactivity, showing a Δ cpm value of 7729. Upon T_s cell addition, ^{3}H-TdR incorporation fell to background levels (813 ± 809 cpm). At all times, appropriate controls were run to insure that T_s cell inhibition was not an artifact due merely to high dose "*in vitro* crowding."

5. Abrogation of Enhancing Mϕ Supernatants by T_s Cell Addition to Normal MLR Cultures

This experiment (Table XXIV) is similar to the preceeding one (Table XXIII) except results showed T_s cell abrogation of enhancing

TABLE XXIII

ABROGATION OF Mϕ ENHANCING ACTIVITY BY T_s CELL ADDITION TO NORMAL T CELL CULTURES[a]

Admixtures[a]		MLR Reactivity (^{3}H-TdR Incorporated, [Responders] - [Control])
10% Mϕ Addition	10% T_s Cell Addition	
		Δ cpm[b]
-	-	1397 ± 301
+	-	7729 ± 1254
+	+	813 ± 809[c]

[a]All Mϕ were treated routinely with anti-Thy 1 to remove T cells. All T_s cells were routinely plated twice for 1 hr to remove Mϕ.

[b]Normal "R + S_m" responders = 7412 ± 301.

[c]Significantly lower than normal Mϕ augmented T cell activity.

TABLE XXIV

ABROGATION OF ENHANCING Mϕ SUPERNATANTS BY T_s CELL ADDITION TO NORMAL T CELL MLR CULTURES[a]

Admixtures[a]		MLR Reactivity (^{3}H-TdR Incorporated, [R + S_m + Treatment] - [R + R_m])
Mϕ Enhancing Supernatant	T_s Cells	
		Δ cpm[c]
-	-	1684 ± 809
+	-	10,701 ± 1261
+	50%	-8624 ± 280

[a]All Mϕ were routinely treated with anti-Thy 1 serum to remove T cells. All T_s cells were routinely plated for 2 hr to remove Mϕ. Enhancing Mϕ supernatants were derived from 3.2×10^6 Mϕ in 3.0 ml medium. "R + R_m" normal T cell control = 2009 ± 273 cpm. "Treatment of (R + S_m) population indicates addition of T_s cells and/or Mϕ supernatant.

activity of Mϕ supernatants rather than whole Mϕ. Mϕ supernatant addition to responder populations raised MLR reactivity from 1684 to 10,701 Δ cpm. Addition of T_s cells reversed this enhancement causing it to drop below control levels of activity (Δ cpm of -8624).

6. Abrogation of Enhancing Mϕ Supernatants by T_s Cell Addition to Purified TBM MLR Cultures

If MLR reactivity can be restored to TBM T cells by depleting them of T_s cells (Table XX and XXI); and if T_s cell addition to normal T cells can inhibit Mϕ supernatant enhancement, (Table XXIV) then T_s cell addition to purified TBM T cells plus Mϕ supernatants, should cause TBM cells to revert to their pre-purified state of hyporeactivity. Table XXV showed this to be the case. TBM T cells, originally unresponsive (-224 Δ cpm), were restored to activity (7754 Δ cpm) following nylon wool column purification. Addition of T_s cells subverted this trend, plunging Δ cpm values 8624 counts below $R + R_m$ controls.

7. Abrogation of Enhancing Mϕ Supernatants by T_s Soluble Factors

In the previous experiments (Table XXIV and XXV) it was determined that T_s cell addition could reverse the enhancing effects of Mϕ supernatants. The following assay was designed to determine whether such suppression was contact dependent (Table XXVI). Normal Mϕ and T_s cells were cultured at concentrations of 6.4×10^6 cells/3.0 ml of RPMI medium. After 4 days, supernatants were harvested, and dispensed into wells containing 2×10^5 BALB/c T cells and 4×10^5 C3H T cells. Those wells receiving only Mϕ enhancing supernatants, more than doubled MLR reactivity when compared to untreated ($R + S_m$) populations.

TABLE XXV

ABROGATION OF ENHANCING Mϕ SUPERNATANTS BY T_s CELL ADDITION TO PURIFIED TBM MLR CULTURES[a]

TBM Responder[a] Cell Type	Admixtures: Mϕ Supernatant	Admixtures: T_s Cells	MLR Reactivity (^{3}H-TdR Incorporated, [R + S_m + Treatment] - [R + R_m])
			Δ cpm[b]
Whole spleen	-	-	-955 ± 458
Unpurified T	+	-	-224 ± 326
Purified T	+	-	7754 ± 1064
Purified T	+	50%	-8624 ± 809

[a]Purification schemes and admixture preparations as described in Tables IV and V.

[b]"R + R_m" TBM whole spleen cell control = 2874 ± 458 cpm, "R + R_m" unpurified TBM T cell control = 2669 ± 278 cpm, and "R + R_m" purified TBM T cell control = 12,297 ± 447 cpm.

TABLE XXVI

ABROGATION OF ENHANCING Mϕ SUPERNATANTS BY T_s CELL SOLUBLE FACTOR(S)

Supernatant Addition[a]	T Cell MLR Reactivity[b] (^{3}H-TdR Incorporated, [Responders] - [Control])
	Δ cpm[c]
None	8517 ± 1089
Mϕ	20,209 ± 941
Mϕ + T_s cells	-150 ± 27[d]

[a] Mϕ-depleted T_s cells from TBM spleen were cultured at a concentration of 1.2×10^7 cells/3.0 ml serum-free RPMI medium. After 4 days, supernatants were processed as described in Table I.

[b] BALB/c responder (R) T cells were derived from purified normal splenic T cells. Stimulator (S_m) T cells were taken from the spleens of C3H mice, incubated 45 min (37°C) with 40 μg of mitomycin C (10^7 cells/ml) washed twice, resuspended in complete medium, and dispensed at a final concentration of 4×10^5 cells/0.05 ml. In all experiments responder (R) and stimulator (S_m) cells contained <1% Mϕ as shown by latex particle uptake and esterase staining techniques.

[c] "R + S_m" normal T cells responders = 9770 ± 1089 cpm.

[d] Significantly lower than normal T cells receiving no T_s cell supernatant.

However, wells receiving both Mϕ and T_s cells supernatants, suffered a loss in activity, Δ cpm of ^{3}H-TdR incorporation dropping below untreated (R + R_m) populations. Thus it was seen that T_s cell activity was mediated by a soluble suppressor factor(s).

8. Abrogation of Normal PHA Activity by T_s Cell Addition

To further demonstrate the ability of TBM T cells to abrogate a high rate of T cell proliferation, TBM T cells were added to PHA stimulated cultures of normal T cells (Figure 20). From a peak Δ cpm value of 50,569 cpm, normal T cell activity dropped to zero upon addition of 2 x 10^5 (100%) TBM T_s cells, while the addition of the same number of normal T cells, raised counts (raised to 62,681 cpm upon the addition of 2 x 10^5 normal T cells).

9. Lack of Inhibitor Activity in Syngeneic Control (R_m) Populations

To insure that T_s cell inhibition was not a function of "cell crowding", responder populations were divided into 2 groups, one half received a 10% concentration of T_s cells, the other a comparable number of mitomycin C treated syngeneic (R_m) cells (Table XXVII). Activity, as assessed by ^{3}H-TdR uptake, was measured in PHA and MLR assays. Using either normal or purified TBM T cells in the responder population, at no point did R_m cell addition ever inhibit ^{3}H-TdR incorporation. T_s cell presence, however, in every case reduced activity by nearly 100%.

10. Effects of Mitomycin C Treatment Upon T_s Cell Inhibitor Activity

To determine if this T_s inhibitor activity was dependent upon DNA synthesis, mitomycin C treated or untreated T_s cells were added to

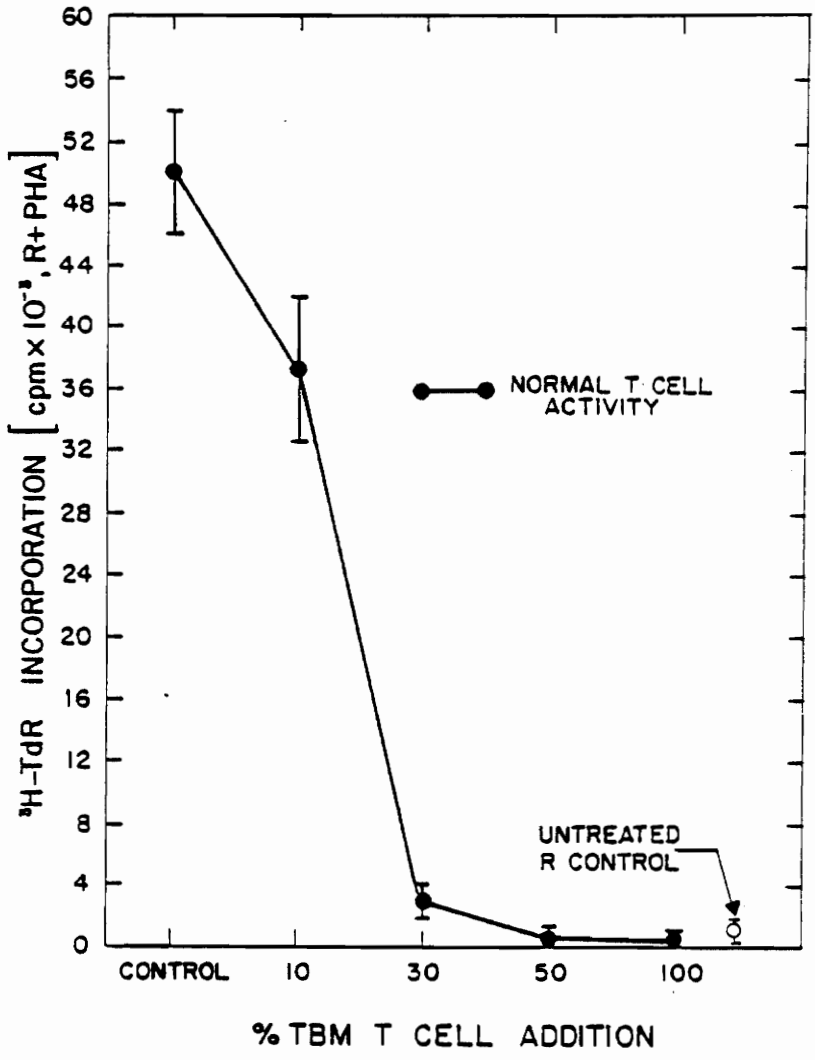


Figure 20. Abrogation of normal PHA activity by TBM T cell addition. Macrophage-depleted splenic T cells (2 x 10^5 R cells) were incubated with 0.5 µl of PHA in a total volume of 0.2 ml. Six hr before termination of the 3-day PHA assay, each well was pulsed with 1 µCi of ^{3}H-TdR. To obtain a purified population of TBM T cells, whole spleen cells from animals with 2-week palpable tumors were plated for 1 hr and gently washed to obtain nonadherent cells. The nonadherent cells were replated for 1 hr, harvested and analyzed for Mϕ contamination using esterase staining. The small remaining number of Mϕ (<2%) could not account for the immunoregulatory properties of these cells since treatment with anti-Thy 1 serum plus complement removed their immunoregulatory ability. This indicates that regulatory activity was dependent on the presence of TBM T cells.

TABLE XXVII

LACK OF INHIBITOR ACTIVITY IN SYNGENEIC CONTROL (R_m) POPULATIONS

Source of Responder (R) T Cells	Admixture Cell Population (T_s or R_m)[a]	^{3}H-TdR Incorporation	
		PHA Activity [Responder] - [Control]	MLR Activity [Responder] - [Control]
Normal	None	45,076 ± 1484	8046 ± 1169
	R_m	67,681 ± 1567	7692 ± 266
	T_s	-2815 ± 151	1897 ± 235
Purified TBM	None	95,071 ± 4362	13,538 ± 718
	R_m	117,702 ± 3115	18,933 ± 819
	T_s	-1314 ± 460	-1392 ± 577

[a] 2×10^4 T_s cells or mitomycin C treated BALB/c T cells (R_m) were added to PHA or MLR cultures.

populations of normal or purified TBM T cells (Table XXVIII). The ambiguous nature of the results made it impossible to unequivocaly state whether T_s inhibitor activity was, or was not, dependent upon DNA synthesis. Mitomycin C treated T_s cells significantly lowered the ^{3}H-TdR uptake of normal and TBM T cells in response to PHA and allogeneic lymphocyte stimulation. However, this level of inhibition was not as pronounced as the level of inhibition induced by untreated T_s cells. It can only be assumed at this point, that DNA synthesis plays some role in T_s cell inhibitor factor production, but that abrogation of DNA synthesis does not imply immediate cessation of T_s cell inhibitor activity.

11. Summary

In the absence of TBM Mϕ, TBM T cells exhibited blastogenic hyporeactivity due to the presence of a tumor-induced mildly nylon wool adherent, suppressor T cell. T_s cell suppression was mediated by soluble factors and could overcome the enhancing effect of Mϕ or their supernatants. Its inhibition was partially destroyed by incubation with mitomycin C.

E. Summarization of Tumor-Induced Dualistic Suppressor Cell Regulation by Mϕ and Suppressor T Cells

In emphasizing the inhibitor role of T_s cells it would be a mistake to minimize the negative immunoregulatory role of high concentrations of Mϕ (or their supernatant). To gain a better overall perception of dualistic Mϕ and T_s cell suppression, the following tables (abstracted from our presentation at the 12th International Leucocyte

TABLE XXVIII

EFFECTS OF MITOMYCIN C TREATMENT UPON T_s CELL INHIBITOR ACTIVITY

Responder Population	T_s Cell[a] Addition	Mitomycin C[b] Treatment of T_s Cells	PHA Reactivity	MLR Reactivity
Normal	-	-	45,076 ± 1484	9545 ± 1104
	+	-	-2815 ± 151[c]	-8624 ± 809[c]
	+	+	15,701 ± 1214[d]	5767 ± 480[d]
Purified TBM	-	-	95,071 ± 4362	7754 ± 1064
	+	-	-1314 ± 460[c]	-8220 ± 447[c]
	+	+	66,001 ± 4696[d]	4204 ± 241[d]

[a] 2×10^4 T_s cells were added to 2×10^5 normal responders assayed for PHA or MLR responsiveness as described in Tables III and VIII.

[b] T_s cells receiving mitomycin C treatment were incubated (37°C) for 45 min with 40 µg/ml of mitomycin C. Cells were washed twice and dispensed in culture wells.

[c] Significantly lower than mitomycin C treated T_s cells.

[d] Significantly lower than responder populations unexposed to T_s cells.

Culture Conference) (47) will hopefully summarize our laboratory's work in the area of immunosuppression. I conducted all MLR experiments while all of the soluble antigen (DNP-BSA) studies and the bulk of the PHA work was done by Bill Farrar. I feel that analysis of all 3 assay systems reinforces and enhances the immunoregulatory concepts brought out in my own MLR work.

1. Characterization of Tumor-Induced T_s Cells

Table XXIX depicts results from experiments designed to delineate the cell responsible for inhibiting TBM T cell blastogenesis. As shown in experiment sets 1 and 2, Mϕ-depleted spleen cells from TBM exhibited a much lower degree of activity than those from normal mice. Since addition of Mϕ-depleted TBM splenic T cells (set 4) or their supernatants (set 6) inhibited normal T cell proliferation, the suppressor cell was judged to be a subpopulation of splenic T cells. This was further confirmed by anti-Thy 1 serum treatment (set 7) of TBM spleen cells which reversed their inhibitory properties. In adoptive transfer experiments (set 9), intravenous injection of TBM T cells (or their supernatants, data not shown) into normal recipients abrogated subsequent splenic cell PHA or DNP responses. Though not shown here, at the molecular level, T_s cell supernatant contained an inhibitor of approximately 15,000 daltons which suppressed DNA polymerase α activity in syngeneic, allogeneic, and even xenogeneic cells.

2. Mϕ Regulation of T Cell Blastogenesis

Results illustrated in Table XXX showed that Mϕ, as well as T_s cells, regulated T cell blastogenesis. As depicted in set 1, removal

TABLE XXIX

CHARACTERIZATION OF TUMOR-INDUCED T_s CELLS

Expt. Set	Mϕ-Depleted Splenic Admixtures: Host Type	Treatment	% Inhibition of Normal[a] T Responder Cells, PHA, DNP-8SA, MLR (0 20 40 60 80 100)
1	No admixture		
2[a]	No admixture		
3	Normal	Whole cells	
4	Tumor	Whole cells	
5	Normal	T cell supernatants	
6	Tumor	T cell supernatants	
7	Tumor	Anti-Thy 1 treatment	
8	Normal	Adoptive transfer	
9	Tumor	Adoptive transfer	

[a]All responder cells were taken from normal animals with the exception of set 2 which shows the characteristic hyporeactivity of TBM spleen cells. In both normal and TBM, splenic T cell blastogenesis was assessed by a) PHA stimulation, b) dinitrophenylated bovine serum albumin (DNP-BSA) responsiveness (after contact sensitization with DNFB), and c) mixed lymphocyte reactivity (MLR). All experiments were conducted using normal or tumor-bearing BALB/c mice to obtain splenic and peritoneal Mϕ as well as T lymphocyte responder cells. In the case of the MLR, C3H mice were used to provide a Mϕ-depleted mitomycin C treated, allogeneic, stimulator population. To provide the necessary TBM, fibrosarcomas, originally induced with 2-methyl-cholanthrene, were transplanted into mice by intramuscular injection. To simplify presentation of results, all raw data (cpm of ^{3}H-TdR incorporated) has been converted to "% inhibition", as measured against the value for normal T cell reactivity. One hundred percent inhibition represents activity below background control levels of ^{3}H-TdR incorporation.

TABLE XXX

MACROPHAGE REGULATION OF T CELL BLASTOGENESIS[a]

Expt. Set	Mϕ Admixture: Mϕ Concentration	Mϕ Admixture: Mϕ Source	Responder T Cell Source	% Inhibition of Responder T Cells: PHA, DNP-BSA, MLR (0 20 40 60 80 100)
1	None	None	Normal	
			Tumor	
			Nylon wool purified tumor	
2	Low	Normal	Normal	
			Tumor	
3	High	Normal	Normal	
			Tumor	
4	Low	Tumor	Normal	
			Tumor	
5	High	Tumor	Normal	
			Tumor	
6	Adoptive transfer of high dose	Normal	Normal	
		Tumor	Normal	

[a]For protocols see Table XXVIII.

of mildly adherent T_s cells by sequential passaging over nylon wool columns, reversed hyporeactivity in Mϕ-depleted TBM splenic T cells. T cell reactivity was not inhibited by low concentrations of normal (set 2) or TBM (set 4) Mϕ, though high concentrations of normal (set 3) and TBM (set 5) Mϕ inhibited reactivity in adoptive transfer (set 6) as well as in vitro experiments.

3. T Cell Regulation by Mϕ-Derived Soluble Factors

Results from Mϕ supernatant add back experiments (Table XXXI) correlated well with cellular Mϕ results described in Table XXX; neither normal (set 1) nor TBM (set 2) Mϕ supernatants inhibited normal T cell activity when added back in low concentrations, though both supernatant types were suppressive at high concentrations. In addition, activator factor proved to be heat labile (10 min at 100°C) though inhibitor factor was stable after boiling and could manifest itself at even low dose supernatant concentrations if the supernatant was first boiled. Since supernatants demonstrated no diminution of inhibitory activity after dialysis (set 3), high dose Mϕ supernatant inhibition could not be totally explained by thymidine presence. This was confirmed by ^{3}H-TdR competition experiments. Further experimentation (data not shown) revealed that elaboration of Mϕ enhancing factor was not dependent on a Mϕ-T cell complex and could be partially absorbed out of solution by prior incubation (2 hr, 37°C) with normal T cells.

TABLE XXXI

REGULATION OF T CELL MLR REACTIVITY BY SOLUBLE Mϕ ENHANCING AND INHIBITOR FACTOR[a]

Expt. Set	Mϕ Supernatant Admixture: Mϕ Supernatant Derivation	Heat Treatment(C)	Concentration	% Inhibition of T Responder Cells: Normal, TBM Responders (0 20 40 60 80 100)
1	Normal	37	Low	
		100	Low	
		37	High	
		100	High	
2	Tumor	37	Low	
		100	Low	
		37	High	
		100	High	
3	Normal supernatants following dialysis	37	Low	
		100	Low	
		37	High	
		100	High	

[a]Experimental procedures outlined in Table XXVIII.

4. Summary

From the data presented, we have postulated dualistic regulation of TBM T cell blastogenesis. Negative immunoregulation was exerted by a TBM splenic T cell. Through elaboration of a soluble factor(s), it suppressed blastogenesis, most probably at the DNA polymerase level. Mϕ regulation appeared less clear. Elaboration of a heat labile activator and heat stable inhibitor factor was seen in the MLR model. Mϕ enhancing factor did not override the inhibitory role played by tumor-induced T_s cells/factor(s). When compared to normal Mϕ, TBM Mϕ did not possess unique suppressor qualities. It was postulated that TBM T cell hyporeactivity was due to a physiologically distinct T_s cell, while Mϕ regulation seems to be strictly a concentration dependent mechanism.

F. Positive Blastogenic Regulation by Tumor-Bearing Mouse T Cells

It was only recently that I realized that tumor-induced T cells possessed positive, as well as negative immunoregulatory activity. However, enhancement was only noted when responder cell blastogenesis was low. Thus, TBM T cell enhancement depended on the proliferative state of the responder cell rather than the quantity of regulatory cells present (as in Mϕ immunoregulation).

1. Enhancement of Mϕ-Depleted T Cell MLR Reactivity by TBM T Cells

As shown in Figure 21, Mϕ-depleted normal T cells responded poorly to allogeneic T cells -- showing less activity than syngeneic $R + R_m$ controls. When TBM T cells were added, responder cell activity was enhanced, raising cpm from 1400 to a final cpm value of 4500. The

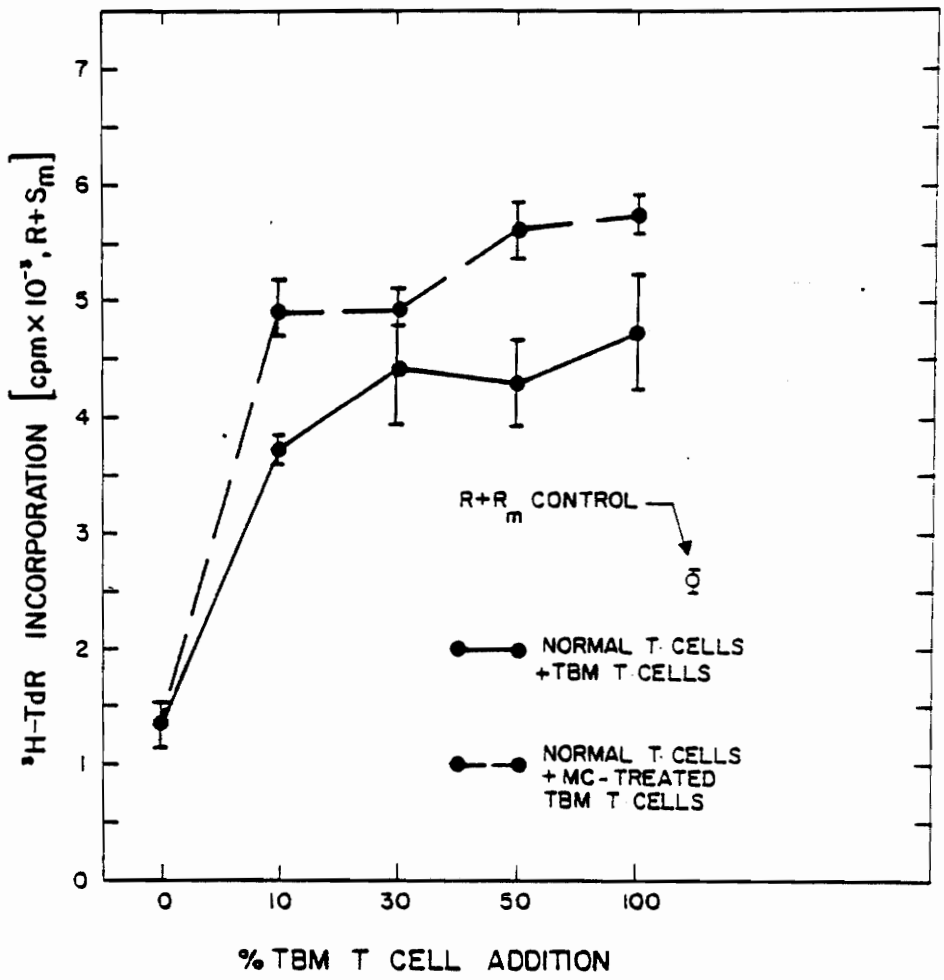


Figure 21. Enhancement of Mϕ-depleted T cell MLR reactivity by TBM T cells. Results are of representative experiments duplicated at least 3 times. Pooled spleens of 6 to 12 mice were used, each data point representing the mean of 6 replicate samples ± S.E. Activity was expressed as either raw R + S_m and R + R_m counts; or Δ cpm values, i.e., responders minus control ([R + S_m] - [R + R_m]).

increase in activity could not be attributed to blastogenesis in the TBM T cell population since mitomycin C treatment of that population did not affect its ability to enhance the low level MLR activity of normal T cells (if anything, it seemed to increase reactivity when compared to its non-mitomycin C treated counterparts).

2. TBM T Cell Enhancement of MLR Cultures Containing Suboptimal Concentrations of Mϕ

MLR reactivity is a Mϕ dependent reaction. The greater the degree of Mϕ depletion, the lower the level of MLR reactivity in the purified T cell population. In highly purified T cell populations, 5% addition of Mϕ does not fully restore MLR reactivity. Because the rate of T cell blastogenesis in Figure 22 was so low, TBM T cell addition, which normally inhibits MLR reactivity, in this case, enhanced it. Normal responders, containing suboptimal concentrations (5%) of normal or TBM Mϕ, showed a 3000 to 4000 cpm increase in activity upon addition of 30% TBM T cells.

3. Augmentation of Normal and Purified TBM T Cell Blastogenesis by Plated TBM T Cells

Mϕ-depleted TBM T cells were also capable of augmenting low rates of blastogenesis in MLR cultures with Mϕ supernatants. Table XXXII shows results from an experiment where Mϕ depletion of the purified normal and TBM T cell population was most thorough, reducing background Δ cpm values to less than 1000 counts. Due to the extensive degree of depletion, the enhancing effect of low dose Mϕ supernatants raised ^{3}H-TdR incorporation to only 3200 Δ cpm for normal T cells, and 1632 Δ cpm for purified TBM T cells. Addition of 10% TBM

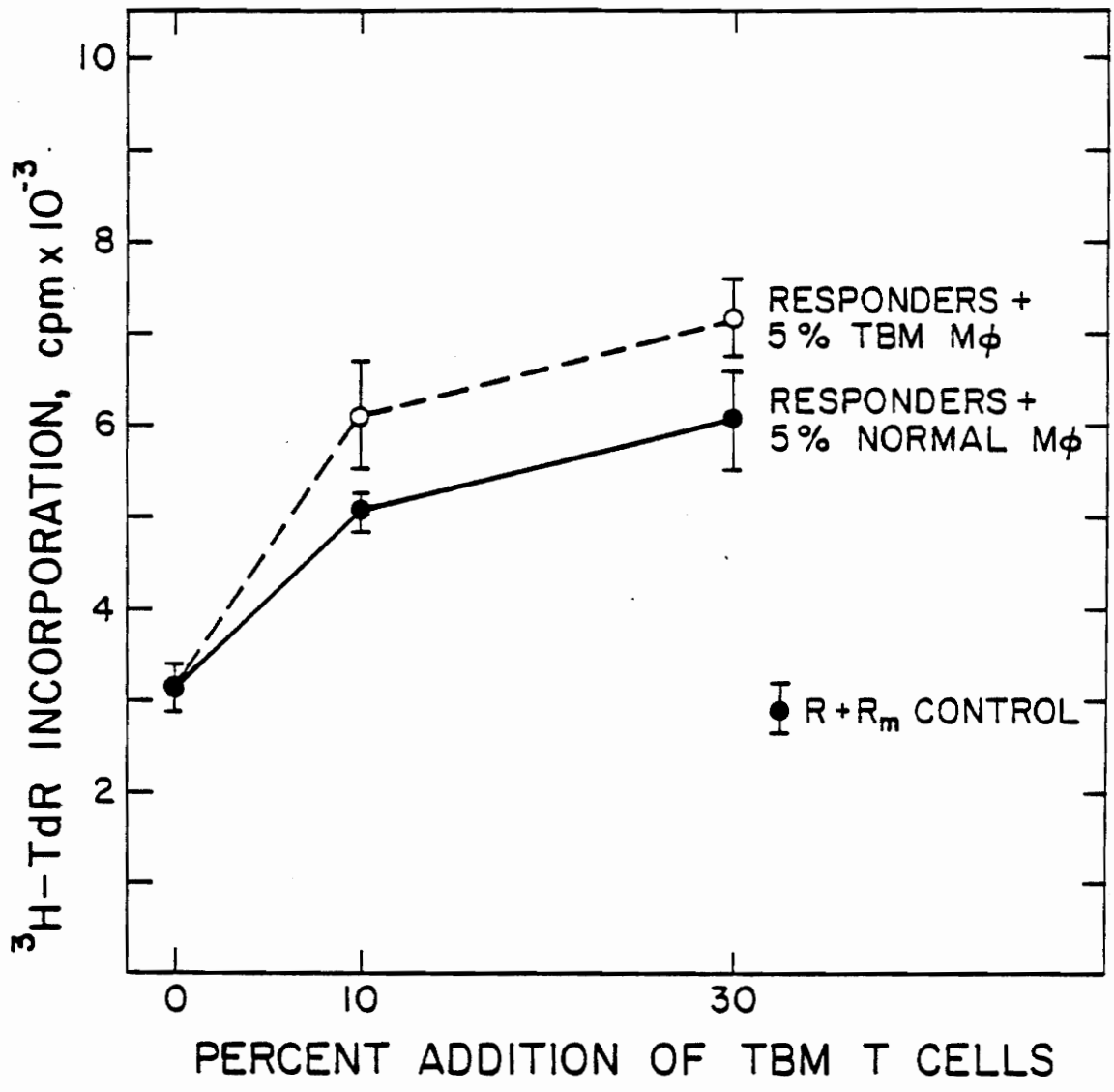


Figure 22. TBM T cell enhancement of MLR cultures containing suboptimal concentrations of Mϕ. Solid line (●—) represents normal responder activity in the presence of TBM T cells and/or 5% normal Mϕ. Broken line (○---) represents normal responder activity in the presence of TBM T cells and/or 5% TBM Mϕ.

TABLE XXXII

AUGMENTATION OF NORMAL AND PURIFIED TBM T CELL BLASTOGENESIS: BY PLATED TBM T CELLS

Responder Cell Source	Addition[a] Normal Mϕ Supernatant	Addition of 10% TBM T cells	^{3}H-TdR Incorporation (Responders) - (Control)
			Δ cpm
Normal	-	-	24 ± 99
	+	-	3200 ± 185[b]
	+	+	5419 ± 197
Purified TBM	-	-	281 ± 57
	+	-	1632 ± 203[b]
	+	+	3859 ± 496[c]

[a]Normal Mϕ supernatants were derived from 3.2×10^{6} Mϕ in 3.0 ml of serum-free medium.

[b]Significantly greater than Δ cpm of responder controls, as shown by Student's *t* test.

[c]Significantly greater than Δ cpm of responders treated only with Mϕ supernatants, as shown by Student's *t* test.

T cells further augmented this activity, raising counts to 5419 and 2759 Δ cpm, respectively.

4. Immunoregulation of Normal T Cell MLR Reactivity by TBM T Cells

In Table XXXIII, at a suboptimal concentration of Mϕ (5%), MLR activity showed a Δ cpm value of 4645. Addition of a 10% concentration of TBM T cells to the responder population ($R + S_m$ + 5% Mϕ) increased this activity to 13,670 Δ cpm. However, addition of the same number of TBM T cells to $R + S_m$ cultures containing 10% Mϕ did not enhance but rather inhibited the already high rate of responder cell DNA synthesis. In this case TBM T cell addition caused activity in the responder population ($R + S_m$ + 10% Mϕ) to drop from a Δ cpm value of 19,749 to 6297. Moreover, reconstitution experiments with mitomycin C-treated TBM T cells gave similar results (Figure 21). Appropriate controls were run with normal R_m cells to insure that inhibition was not an artifact due to an in vitro crowding effect (i.e., $R + S_m$ = 10,405 cpm; $R + S_m + R_m$ cells = 10,051 cpm).

5. Summary

It is possible that TBM T cell blastogenic regulation is the result of a feedback mechanism which directs a population of regulator cells to enhance low level T cell blastogenesis but inhibit high level proliferation. Macrophage-depleted splenic TBM T cells enhanced low level MLR activity in normal T cell populations containing a suboptimal concentration of Mϕ. However, in highly stimulated PHA cultures (Figure 20) or MLR cultures containing an optimum number of Mϕ, the high rate of normal T cell proliferation was abrogated by the

TABLE XXXIII

IMMUNOREGULATION OF NORMAL T CELL MLR REACTIVITY BY TBM T CELLS

Addition of 10% TBM T Cells	%Mϕ Addition[b]	^{3}H-TdR Incorporation (Responders) - (Control)	S/I (Responder)/(Control)	Blastogenesis Following TBM T Cell Addition
		Δ cpm[c]		
-	5%	4645 ± 662	2.54	Enhancement
+	5%	13,670 ± 1258[d]	5.54	
-	10%	19,749 ± 3605	7.56	Inhibition
+	10%	6297 ± 736[d]	3.09	

[a]TBM T cell derivation as described in Table XXII.

[b]Mϕ were derived from peritoneal lavage 3 days after i.p. injection of thioglycollate. Peritoneal exudates were first plated and then treated with anti-Thy 1 serum plus complement to remove contaminating nonadherent T cells. As judged by esterase staining, treated exudates yielded a 97% pure Mϕ population.

[c]$R + R_m$ control value = 3009 cpm. Basal levels of DNA synthesis as measured by (responding $R + S_m$) divided by (control $R + R_m$) fluctuated around an S.I. value of 1.2 ± .02.

[d]Significantly different from the population unexposed to TBM T cells (Student's t test).

same (formerly enhancing) population of TBM T cells. This dual regulatory role of Mϕ-depleted TBM T cells could not be duplicated using normal T cells. In addition, this feedback regulatory response exhibited by TBM T cells was not altered by mitomycin C treatment.

G. Conclusions

1. Positive and Negative Mϕ Immunoregulation

a. Mϕ regulation (either + or -) is mediated by release of soluble, ammonium sulfate precipitable, nondialyzable factors.

b. Though enhancing factor is heat labile, inhibitor factor retains activity following heat treatment (10 min at 100°C).

c. Mϕ (or their supernatants) augment MLR reactivity only at low concentrations, while a high concentration causes inhibition.

d. Mϕ elaboration of regulatory factors is not reversed by incubation with anti-Thy 1 serum.

e. Mϕ supernatants may be resolved into distinct enhancing and inhibitory immunoregulatory fractions using anion exchange chromatography.

f. TBM Mϕ supernatants, on a volume to volume basis, possess greater enhancing capabilities than normal supernatants.

2. Positive and Negative Tumor-Induced T Cell Immunoregulation

a. Immunoregulatory T cells are mildly nylon wool adherent, anti-Thy 1 serum sensitive, splenic T cells found in tumor-bearing (but not normal) mice.

b. TBM T cell regulation is dependent upon the blastogenic rate of the responder cell -- inhibiting high rates of proliferation and enhancing low rates.

V. DISCUSSION

An initial step in the characterization of any immunoregulatory factor is the determination of its cellular origin. In the case of enhancing factor derived from peritoneal exudate supernatants, anti-Thy-1 serum treatment of these peritoneal exudates, effectively removed T cell presence, but failed to abrogate supernatant enhancing activity (Table I). Esterase staining showed the remaining peritoneal exudate population to be composed almost entirely of Mϕ (58). Though production of enhancing factor was not the result of an in vitro Mϕ-T cell complex (17), results from Table I did not rule out the possibility that Mϕ elaboration of enhancing factor(s) was due to prior in vivo stimulation by T cells. This is a possibility, but a rather remote one, since a) thioglycolate is a poor stimulator of CMI immunogenic activity (64, 92), b) enhancing factors are obtained from a Mϕ cell line,free of any T cell contamination (166, 167), c) active Mϕ may be obtained from thymectomized, T cell-depleted host animals (123).

Since trypsinization of Mϕ also failed to suppress the release of enhancing factor into Mϕ supernatants (Table II), I concluded that enhancing activity was not due to a trypsin sensitive membrane surface antigen shed into Mϕ supernatants. Even if the 4-day Mϕ incubation period allowed for re-establishment of the Mϕ shedding process, trypsinized Mϕ supernatant should still have shown a diminished enhancing capacity, when compared to supernatants from untrypsinized Mϕ. It is possible that the enhancing factor does not arise from any Mϕ

membrane bound antigen (trypsin sensitive or not). Results showed that Mϕ sonicates still possessed enhancing activity following centrifugation (12,000 x g) and passaging through a 0.45 μ millpore filter -- procedures aiding in removal of Mϕ membrane fragments. Rather than decrease elaboration of enhancing factor, trypsinization of Mϕ increased the potency of enhancing supernatants. Perhaps the trypsinization process itself is responsible for nonspecific cellular stimulation (93).

In terms of stimulation, Mϕ were totally unsuccessful in stimulating blastogenesis in PHA exposed thymocytes. Though the DEAE chromatographic pattern was similar to that of LAF, our enhancing supernatant failed to meet the basic definition of LAF. Inability to stimulate thymocyte PHA reactivity (184), separated our factor from LAF (Table III), even though both monokines, in a state of partial purity, possess a neutral or slightly positive charge at pH 7.9 and a high degree of activity with little or no absorbance at 280 nm (166). It was not surprising that enhancing factor was not LAF, since "unactivated" Mϕ produce almost no LAF (167).

Even in its unactivated state, Mϕ possessed intracellular pools of enhancing factor which could be obtained by sonicating Mϕ (Figure 3) or by incubating them and allowing the factor to accumulate extracellularly in Mϕ supernatants (Table IV). Mϕ sonicates however showed only a positive immunoregulatory ability (Figure 3). That is not to say that intracellular pools of inhibitor factor did not exist. Other experiments indicated that inhibitor factor may be susceptible to the degradative action of proteolytic enzymes (Figure 4 and 5). Thus

release of free hydrolytic enzymes following sonication, may cause rapid inactivation of any inhibitor factor normally present in Mϕ (or) their supernatants). This is in keeping with results of others, showing inhibitor factors to be protease-sensitive (31).

The macromolecular nature of inhibitor factor (>12,000 d), while not definitely establishing it as protein, at least rules out its identification as dialyzable prostaglandin (Table V) or thymidine (Table VI and VII).

Other researchers, though not identifying inhibitor factor as thymidine (179, 180) or prostaglandin (103, 175), feel it to be a Mϕ altered component of the complement pathway (C3a) (122). However, in that system, the C3a inhibitor was cytocidal for all target cells, whereas, the cytostatic affect of our inhibitor factor produced no loss of viability (Table VIII) or cytolysis (Table IX).

Further evidence of the non-prostaglandin nature of our non-dialyzable inhibitor was established when dialyzed supernatant from indomethacin-treated Mϕ retained inhibitor activity (Figure 4). It is however still possible that inhibitor factor, though not itself a prostaglandin, triggers prostaglandin production in the responder population.

Table X reinforced the concept of an indomethacin resistant inhibitor factor. It also lent credence to the previously stated belief that inhibitor supernatant was at least partially protein, showing inhibitor activity to be a) ammonium sulfate precipitable and b) enhanced by addition of the proteolytic enzyme inhibitor PMSF.

The inability of PMSF to abrogate low dose enhancing activity or high dose inhibitor activity (Figure 5) also indicated that the active sites of the positive and negative immunoregulatory molecules contained no serine (the amino acid altered in some proteins by the activity of PMSF).

Polyacrylamide gel electrophoresis of supernatants from Mϕ treated or untreated, with indomethacin, or proteolytic enzyme inhibitors (PMSF and benzidine) showed that PMSF and benzidine treatment yielded a deep staining protein band not evident on the normal or indomethacin-treated Mϕ supernatants (Figure 6). Though no activity assay has yet been done on this band, its location in the gel identified it as a strongly charged anion. It is noteworthy that anion exchange chromatography indicated the presence of a negatively charged (anionic), inhibitory protein (Figure 8).

In talking of enhancing and inhibitory factors, note that they influence only the ability of T cell to proliferate in response to an allogeneic stimulus (Table XI). Little or no immunoregulatory activity occurred when Mϕ supernatants were added to wells containing only syngeneic cells. Apparently Mϕ immunoregulation can occur only after initial contact has been made between a responder T cell and an allogeneic target (the foreign lymphocyte). Since syngeneic cells (by definition) make no such antigenic contact, they are affected by neither the enhancing nor inhibitory properties of Mϕ supernatants. If inhibitor factor was either thymidine or prostaglandin, the nonspecific nature of these suppressors would have lowered activity in the control [R + R_m] population. Conversely, if enhancing factor was

merely a reducing agent which increased in vitro T cell viability (96, 110), such nonspecific enhancement would have raised activity of the control [R + R_m] population. Lack of such fluctuation in the activity of variously treated control cultures, argues for the presence of genuine Mϕ immunoregulatory factors.

Anion exchange column chromatography allowed us to separate some of these factors by charge (Figures 7 to 11). When the heterogenous Mϕ supernatants were fractionated over a DEAE Sephadex column, numerous peaks of enhancing and inhibitory activity appeared. This was in keeping with other work reporting multiple peaks of both positive (167) and negative (222) immunoregulatory activity. There is, however, the possibility that the great number of hydrolytic enzymes (225) released by Mϕ, may partially degrade the regulatory factors, significantly altering their charge but not totally abrogating their activity. Removal of a charged amino acid from a regulatory factor would affect the chromatographic pattern on a DEAE column, but would not significantly affect its molecular weight. Our efforts to establish the molecular weight of our factors (thereby verifying the validity of our multiple peak phenomenon) were originally hampered by the method of supernatant concentration (lyophilization), which created a molecular aggregate not separated by passage through a Sephadex G-200 column. Since saturation with ammonium sulfate precipitated enhancing and inhibitor factors (Table X), this "salting out" procedure will be employed to obtain concentrated supernates to be used in molecular weight determinations.

Titration and heat treatment experiments strengthen conclusions derived from results of anion exchange experiments -- within the Mϕ supernatant, inhibitor and enhancing factors existed as separate entities. Whether these positive and negative immunoregulators can be subdivided still further into many, biochemically distinguishable enhancing or inhibitor factors is a subject still open to question.

At first, the low dose MLR enhancement and high dose inhibition by Mϕ (Figure 12), or their supernatants (Tables XII to XIV) may appear to be explainable by postulating that Mϕ supernatants contain but one enhancing factor, which at high doses, represents "too much of a good thing." It is however important to note that inhibitor factor is not merely an artifact created by an excess of enhancing factor. If this were true, heat treatment of Mϕ supernatants would destroy the excess labile enhancing factor and abrogate high dose inhibitory activity in high dose Mϕ supernatants. However, even after heat treatment, high dose Mϕ supernatants still suppressed the R + S_m MLR response -- causing alloreactivity-induced ^{3}H-TdR incorporation to fall below background syngeneic R + R_m counts (Table V). Thus heat treatment of Mϕ supernatants revealed the presence of two distinct factors, a heat stable MLR inhibitor factor and a heat labile enhancing factor (Figures 13 to 16). The titration profile of non-heat treated supernatants revealed a double peak of enhancing activity (Figure 13 and 15). Perhaps this biphasic curve represents the affect of distinct enhancing factors operating at different optimum concentrations. It further reinforced the concept of multiple enhancing factors discussed earlier in connection with the biochemical data. The titration profile

also showed that Mϕ supernatant regulation was a concentration dependent phenomena -- high dose inhibition and low dose enhancement of MLR reactivity.

The appearance of these Mϕ inhibitor factors is not necessarily a pathological aberration. The Mϕ could function as a homeostatic regulator; a monitor, eliminating the possibility of a "runaway" immune response to antigen. One could theorize that, in response to antigen presence, inhibitor as well as enhancing factors are released simultaneously by Mϕ (or subpopulations of Mϕ). However, each factor has a threshold level at which T cell activation or deactivation occurs. Antigen presence, results in a gradually increasing population of Mϕ as well as lymphocytes. At the initial stages of antigen response, Mϕ are present in a low concentration and the signal delivered by the enhancer overrides the inhibitor signal. Therefore, lymphocytes are free to launch a full scale immune attack upon the antigen. Though antigen is gradually cleared from the system, concentrations of Mϕ and lymphocytes continue to rise. At some point, the concentration of inhibitor factor will increase to a level high enough to reverse the activation signal and shut down DNA synthesis. This hypothesis presents a self regulating immune response; Mϕ factors monitoring and limiting the possible juggernaut of lymphocyte blastogenesis. This theory correlates well with cellular assays showing T cell suppression of DNA synthesis in the presence of high concentrations of whole Mϕ (Figure 12).

The above studies showed that Mϕ do possess distinct inhibitor factors. Therefore, in examining the mechanism behind TBM T cell hyporeactivity, I wished to see how <u>inhibitor factor from TBM Mϕ</u> contributed

to the immunologically debilitated status of the tumor-bearing host. To examine in vitro positive and negative Mϕ regulation of lymphocyte alloreactivity; normal and TBM Mϕ supernatants were added to MLR assays containing T cells from normal and TBM. In agreement with Calderon et al. (29, 30) our experiments revealed the presence of a heat sensitive enhancing factor and a non-cytotoxic heat stable inhibitor factor. These factors were released not only by normal peritoneal Mϕ but also by those from TBM. In fact, compared to their normal counterparts, heat-treated TBM Mϕ supernatants, as well as untreated low and high dose TBM Mϕ supernatants, caused significantly less inhibition of T cell ^{3}H-TdR incorporation (Tables XV to XVII). Based on their ability to augment T cell blastogenesis, TBM Mϕ supernatants possess a greater degree of enhancing activity than do normal Mϕ supernatants. These supernatants may be the result of the increased reticuloendothelial activity, reported in Mϕ from host animals with progressively growing tumors (22, 54, 67, 118, 249). At this point I can only speculate as to whether TBM Mϕ supernatants contain i) a greater quantity of enhancing factor, ii) a lesser quantity of inhibitor factor, iii) more active (greater number of?) enhancing factors, iv) or a less potent inhibitor factor. However the gel electrophoresis experiments (Figure 17 and 18) indicated that TBM Mϕ supernatants may possess additional enhancing factors, not present in normal Mϕ supernatants.

Though TBM Mϕ supernatants possessed the ability to enhance normal T cell alloreactivity (Tables XV to XVII), T cells from TBM demonstrated little MLR reactivity upon exposure to any concentration

of Mϕ supernatant from any host animal (Table XVIII). This lack of activity in T cells from animals in late stages of tumor growth is well documented (21, 121, 185). Kirchner et al. (134, 135), working with Maloney Sarcomas, attributed T cell hyporeactivity to high suppressive concentrations of Mϕ present in the tumor-bearing host. This theory is attractive since our laboratory (58, 59) and others (73, 203) show an increase in Mϕ proliferation during tumor growth. Acting as a foreign antigen, the tumor may cause Mϕ proliferation and an initial T cell activation due to high concentrations of activating factor. As tumor growth accelerates, in vivo concentrations of Mϕ continue to rise, releasing threshold levels of inhibitor factor. In accordance with this theory, inhibitor factor is not only an enforcer of the normal CMI homeostatic mechanism but, in addition, an antiproliferative factor designed to halt tumor proliferation (76, 114). However it is important to note that the mechanism of tumor-induced T cell hyporeactivity can not be attributed to the presence of a unique species of suppressor Mϕ, since TBM Mϕ supernatants enhanced (rather than inhibited) MLR reactivity.

It has been shown by us (58, 59) and others (245) that heavy tumor burden is often accompanied by in vitro abrogation of Mϕ-depleted T cell blastogenesis. Since this suppression was not due to the presence of a TBM suppressor Mϕ, experiments were undertaken to determine what caused TBM T cell hyporeactivity in Mϕ depleted populations. Data (presented in part D of the Results section) indicated that a mildly adherent (to nylon wool) tumor-induced splenic T cell was responsible for abrogation of MLR reactivity in Mϕ-depleted TBM T cell

populations. It is doubtful that the low Δ cpm value for TBM T cells was due to a population of Mϕ since TBM Mϕ-depleted spleen cells contain <1% Mϕ. In addition, other work in our laboratory (Tables XV to XVII) has established that TBM Mϕ in low concentrations augment rather than inhibit T cell MLR reactivity.

My suppressor T cell work fully agrees with the rat T_s cells research of Folch and Waksman (78). In addition to depletion experiments (Table XX and XXI), the T_s cell population was characterized using admixture experiments (Tables XXII to XXV). This is important, for it is in this way that I could most clearly see T_s cell reversal of Mϕ enhancement and an apparent "regulatory hierarchy" dominated by T_s cell inhibition. Kirchner et al. (136, 138) as well as Veit and Feldman (236), working with the Moloney sarcoma model, have ascribed suppressor cell activity, not to T cells, but splenic Mϕ (72). This does not contradict results of our investigation of TBM hyporeactivity, since we (42, 43) and others (105, 159) have demonstrated high dose, concentration dependent inhibitor activity in Mϕ (Figure 12) and Mϕ supernatants (Figure 13). It is becoming clear that immune regulation is a complex series of checks and balances; a system resulting in regulation by both Mϕ and T cells.

In our system, TBM lymphocytes were passed through a single nylon wool column and doubly plated to obtain a T cell population >99% Mϕ-free as judged by esterase staining. Although this technique depleted T cells of Mϕ,it did not reverse MLR hyporeactivity in the TBM T cell (Table XX and XXI). Not until a second nylon wool column passaging (which removed the mildly adherent T_s cell) could the now "purified"

TBM T cells give a full MLR response to allo-antigens upon addition of enhancing concentrations of Mϕ or Mϕ supernatants (Table XX and XXI). In cell mixing experiments, anti-Thy 1 serum sensitive T_s cells (Table XXII), extensively plated to remove Mϕ, abrogated normal i) whole spleen cell MLR reactivity (Figure 19), ii) splenic T cell MLR reactivity (Table XXIII and XXIV), iii) splenic T cell PHA reactivity (Figure 20).

While addition of low concentrations of Mϕ or Mϕ supernatants never inhibited normal or purified TBM T cell blastogenesis, (Table XXV) T_s cell supernatants could induce inhibition, and, in fact reverse enhancing effects of Mϕ supernatants (Table XXVI). This inhibition was not due to thymidine release from cultured T_s lymphocytes (127) and was unaffected by dialysis (31). It was also not an <u>in vitro</u> artifact of "cell crowding" since addition of equal numbers of normal cells to MLR and PHA cultures did not generate the suppression seen when using T_s cells (Table XXVII).

Inhibition of T cell blastogenesis, whether the result of exposure to T_s cells or high concentrations of Mϕ, has often been equated to the pathological breakdown of the host immune system (77, 94). High dose Mϕ inhibition may be the result of a normal non-pathological homeostatic mechanism, functioning to eliminate "immunological overkill" in response to an antigen (170). It is possible that, T_s cells, as described in Jerne's network theory (119), also function normally to counterbalance blastogenic hyperactivity in antigen stimulated amplifier T cells and proliferative T cells. It has been pointed out (111) that tumors possess specific stimulatory antigens which are

recognized as foreign and can induce T cell blastogenesis -- hence the splenomegaly seen in TBM spleens (142, 212, 213). Since proliferative T cells trigger activation of cytotoxic effector cells (5, 109, 216), an abundance of proliferative T cells is immunologically desirable. An overabundance of these cells is however, undesirable, since it siphons off energy needed to produce cytotoxic killer T cells. It is possible that, in a tumor system, or other antigenic system (141, 165), abrogation of *in vitro* T cell blastogenesis is not an indicator of specific *in vivo* immunoincompetence. The T_s cell may restrict the activities of responder cells, shifting T cell populations from a proliferative to a cytotoxic function. Kirchner *et al*. (138) have observed that the cytotoxic response of T cells to specific tumor antigens was enhanced not depressed in TBM spleen cells, though TBM T cell DNA synthesis remained low. The T_s cell may direct the energies of cytolytic T cells into killing tumor cells, sharply limiting, the activity of any cells normally reactive to other stimuli (i.e., antigen, mitogen and allogeneic cells). However, when T_s cells are removed, proliferative cells (as opposed to cytotoxicity) are once again able to respond fully to *in vitro* antigen, mitogen or allogeneic lymphocyte stimulation (as shown in our results). Because T_s cells may be linked with production of cytotoxic cells, *in vitro* suppression of T cell cytotoxicity is not observed in TBM (138). However, regarding *in vivo* T cell cytotoxicity toward a primary tumor, it is a question of "too little, too late." The rate of *in vivo* tumor growth, far exceeds the destructive capabilities of the newly generated cytotoxic cells. The immune system is unable to contain a well established tumor. A second inoculation of

tumor cells will, however, be immediately destroyed by an immune system already directed by the T_s cell to focus activity on the tumor, to the exclusion of other stimuli. This retention of *in vivo* competency towards a secondary tumor graft in the face of primary tumor growth has been called "concomitant immunity" by Kearney and Nelson (129). Others have shown *in vivo* immunocompetence by inducing resistance to a tumor graft after excision of the original tumor (3). It is important to note that, from our results we can only firmly attest to the presence of a splenic T_s cell abrogating blastogenesis; analysis of its "function" in the immune response is still a matter of conjecture. Whether T_s cell presence and its accompanying blastogenic inhibition does (82), or does not (111) inhibit T cell tumoricidal activity, is a question not yet definitely settled. The question itself, while interesting to the researcher, is largely an academic one in the eyes of the cancer bearing patient. Whether T_s cell-induced blastogenic hyporeactivity, aids or inhibits tumoricidal activity, the fact remains that tumor presence is a tremendous drain on the immunological system. The energy expended in slowing down neoplastic growth, leaves the body in no position to fight off any of the numerous secondary infections awaiting an opportunity to attack the vulnerable host. Thus, as stated earlier in the Introduction, neoplastic pathogenicity is often a function of systemic debilitation of the CMI response. I feel that this dissertation, with its coverage of Mϕ and T cell regulation of normal and TBM T cell blastogenesis (MLR reactivity) lays the groundwork for clinically oriented experiments correlating tumor growth not only with *in vitro* T cell cytotoxicity and MLR reactivity,

but also with *in vivo* manifestations of the CMI response (i.e. delayed hypersensitivity, allograft rejection, concomitant immunity).

Though *in vivo* demonstration of Mϕ and T cell immunoregulation has been limited to Farrar's adoptive transfer experiments (69) and Dr. Elgert's ^{125}I-human serum albumin localization assay (paper accepted for publication), our laboratory lacks for no number of interlocking *in vitro* models of immunoregulation. Part E of the Results section, is the immunological equivalent of "Free Parking" for the reader. It is a recap of Mϕ and T_s cell negative immunoregulation in the MLR response. In addition, it also brings out the regulatory similarities (and dissimilarities) between by allo-antigen work (MLR) and Farrar's mitogen (PHA) (69) and soluble antigen (DNP-BSA) system (70). I will not dwell upon this section extensively since my work has been covered in the preceding four result sections, and Farrar's (previous Ph.D graduate student in Dr. Elgert's laboratory) work, if given adequate coverage here, would result in a lengthy tome, (Ph.D dissertation), some 180 pages long.

Briefly, T cell proliferation is an important phase in the measurement of host animal immunocompetence (95). T cell blastogenic regulation, either positive (97) or negative (82), has most often been ascribed to Mϕ (214) or a subpopulation of T cells (78). In addition, there has been much discussion as to whether this regulation is contact dependent (149) or mediated by soluble factors (97). I report here that tumor-induced T_s cell-mediated suppression did not require contact since inhibition was also accomplished using a soluble factor(s) (Table XXIX); whereas Mϕ, in mitogen and antigen experiments, governed

primarily via direct contact (Table XXX). However, Mϕ supernatants yielded a heat stable inhibitor as well as a heat labile activator factor, effective only in regulating mixed lymphocyte reactivity (Table XXXI). The inability of Mϕ supernatants to alter T cell reactivity to mitogen or soluble antigen may be accounted for by postulating the following differences in the mode of stimulation. Assume in the MLR, that cell to cell contact between responder and stimulator T cell can be made in the absence of Mϕ (156). This initial antigenic signal does not induce blastogenesis until triggered by a second signal provided by Mϕ supernatants. In the case of soluble antigen, the Mϕ initiates the first signal. Without Mϕ presentation, there is no contact between responder cell and antigen (154, 155, 202, 210). Mϕ supernatants can not provide this responder-antigen contact -- they are therefore unable to modulate T cell-antigen stimulation. In the case of mitogen reactivity, one sees the opposite side of the immunological coin. As a powerful agglutinin, PHA, at optimum concentrations (89, 155), aggregates T cells, producing nonspecific total T cell blastogenesis. It bypasses the second signal altogether. Allogeneic reactivity lies somewhere between the two extremes; not dependent upon Mϕ to bring about antigen contact but dependent upon Mϕ to provide a second signal once contact has been established.

Getting back to T_s cell regulation, comparison of spleen cell proliferation in normal and TBM revealed dualistic suppression of lymphocyte blastogenesis by T_s cells as well as Mϕ. Mϕ regulation was a concentration dependent phenomenon. However neoplastic cell presence

did not reflect the presence of a suppressor Mϕ, whereas, T_s cell presence correlated directly with tumor burden.

From the immunoregulatory summary presented above, Mϕ and tumor-induced T_s cells were indicted as inhibitors of lymphocyte proliferation.

Thus far, in this Discussion, any dealings with enhancing activity has been of a decided Mϕ oriented nature. If Jerne's network theory is to be believed (119) it is only a matter of time before discussion must logically turn to the flip side of TBM T cell inhibition -- positive T cell immunoregulation. This brand of T cell "logical positivism" is demonstrated in part F of the Results. Though we (43, 59) and others (81-83) have most often examined the suppressor activity of lymphocytes derived from tumor-bearing hosts, it is probable that their function is not so much inhibitory as regulatory. When discussing positive and negative Mϕ regulation, Nelson postulated a homeostatic mechanism (170). Jerne's network theory is a T cell extension of this concept of immunological homeostasis and hypothesizes the existence of counterbalancing amplifier and suppressor cells (119). In previous articles we (68, 69) and others (52, 85, 86) have postulated that the positive or negative regulatory capacity of certain T cell subpopulations may depend on the activity of the cell population being regulated. Thus a low level of responder cell blastogenesis, due to inadequate quantities of Mϕ (Figure 21 and 22) or Mϕ supernatant (Table XXXII), could be enhanced by addition of a small percentage (10%) of TBM T cells treated or untreated with mitomycin C. Enhancement following mitomycin C treatment (Figure 21) negated the

possibility that TBM T cell augmentation of Mϕ-depleted T cell MLR activity could be due to blastogenesis within the added TBM T cell population. It also indicated that positive TBM T cell regulation was not dependent upon DNA synthesis. The specific intracellular mechanisms of TBM T cell enhancement and inhibition are probably different since TBM T cell suppressor activity is lowered by treatment of T_s cells with mitomycin C (Table XXVIII). However, untreated tumor-induced regulator T cells inhibited high level MLR reactivity, though the same population of TBM T cells enhanced low level lymphocyte blastogenesis (Table XXXIII). Whether this dual regulatory effect is due to one suppressor-helper T cell "hermaphrocyte" or subpopulations of TBM T cells is a subject under current investigation. In any case it appears that the manifestation of immunosuppression is governed by the blastogenic status of the proliferating T cell. It may be that suppression is an inducible state; suppressor T cells turned on by the feedback inhibitory action of the highly proliferative T cells. The nature of this "feedback inhibitory action" is a point of controversy. Thus far, no one has isolated a unique soluble factor released by all highly proliferating lymphocytes. However, Fox et al. (80) and Burger (28) isolated a unique membrane antigen, visible in a variety of cells, but only during active DNA synthesis. Perhaps this is the immunological red flag attracting the attention of potential suppressor cells.

It is possible however that the mitotic surface antigen is no more than a non-functional marker. Inhibition may be due, not to the specific T_s cell recognition of unfortunate blastogenic cells, but

rather to the increased vulnerability of proliferating T cells brought on by physiological differences, setting them apart from normal non-proliferating cells (84). This vulnerability of proliferative cells may stem from changes in membrane viscosity (117) and membrane cholesterol content (116). When acted upon by Mϕ or T cell regulators, their altered membranes may transmit an intracellular second messenger to the nucleus which results in abrogation of DNA synthesis. Since cyclic AMP production is governed by membrane bound enzymes (6), it has been postulated that cyclic AMP is the intracellular mediator of the inhibitor signal sent by regulator T cells (241).

Returning from the realm of conjecture, my results, while not entirely plugging the loop-hole ridden area of immunoregulatory theorys, throw light upon a few of its key components: i) Mϕ as well as tumor-induced T cells, possessed both positive and negative blastogenic immunoregulatory activity, ii) Mϕ regulation was a concentration dependent phenomenon -- high concentrations inhibitory and low dose enhancing, iii) tumor-induced T cell regulation was dependent upon the state of target cell proliferation. Taken together, these facts suggest a model of immunoregulation, outlined in the following section.

VI. MODEL

A. Concentration Dependent Mϕ Immunoregulation of MLR Reactivity (Figure 23)

1. Responder T cells have receptors for foreign mixed lymphocyte cultural (∿) as well as Mϕ enhancing (⌐) and inhibitor factors (⌐).
2. Responder T cells make contact with viable foreign stimulator lymphocytes (first signal) but show limited proliferation in the absence of a positive Mϕ signal (second signal).
3. Mϕ migrate to the site of lymphocyte interaction; initiating T cell blastogenesis by releasing optimum quantities of enhancing factor, but suboptimum concentration of inhibitor factor. (For the sake of simplicity, regulatory factors will be considered in the singular case.)
4. As T cell proliferation continues, Mϕ continue to migrate to the site of activity. This results in the accumulation of inhibitor factor at a concentration high enough to reverse the effects of enhancing factor, and halt blastogenesis.

Brevity may be the soul of wit, but a few explanatory notes have been added to flesh out the bare bones of the schematic diagram in Figure 23. In stage #1 of the model, the MLC antigen on the stimulator lymphocyte should not be mistaken for the antigens governing transplantation rejection. The gene governing MLC antigen expression is adjacent to the gene governing these transplantation antigens (143, 220, 233, 246). Though the MLR is often conveniently used for histocompatibility testing, its most closely related in vivo

CONCENTRATION DEPENDENT Mϕ IMMUNOREGULATION OF MLR REACTIVITY

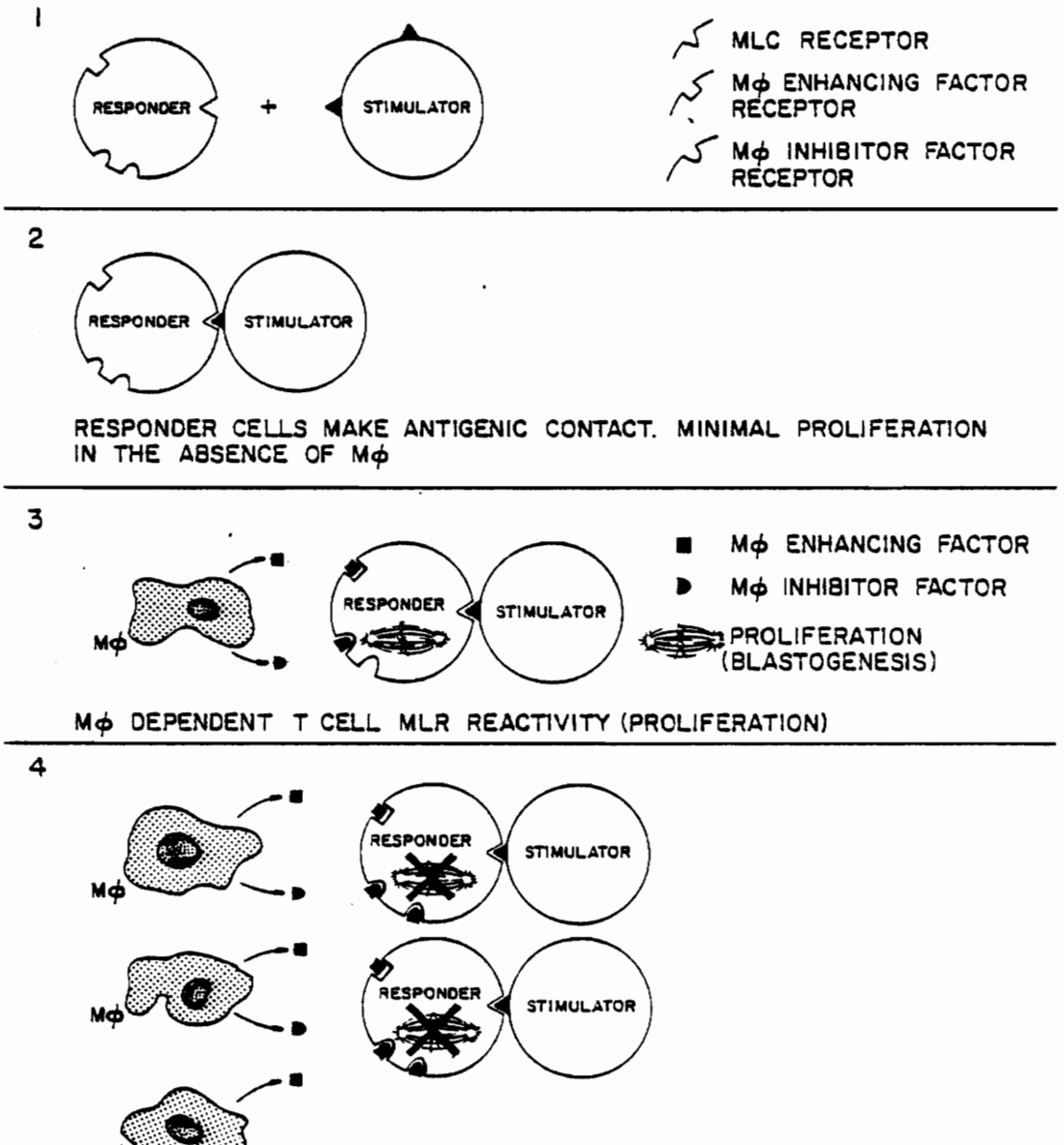


Figure 23

immunological reaction is the graft-vs-host response (18, 32, 33). Also in stage #1, evidence of T receptor sites for Mϕ regulatory factors has been borne out by results showing that our enhancing factor may be partially absorbed out by splenic T cells (47).

In stage #2, it is important that MLC antigens be presented by intact viable, mature foreign leucocyte (or epidermal cell) (115). Since non-viable cells, fail to stimulate T cells, presumably, another, as yet undefined, metabolic process must occur between responder and stimulator cell, prior to blastogenesis.

In stage #3, it is not known whether enhancing and inhibitor factors are released by a single type of Mϕ or by subpopulations of Mϕ. We are also uncertain of the exact nature of the intracellular second messenger transmitting the regulatory message to the T cell nucleus -- though cAMP and cGMP have been implicated (6, 241).

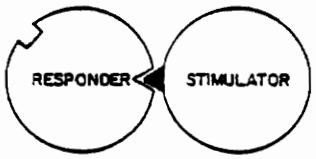
In stage #4 Mϕ migration creates a large extracellular pool of inhibitor factor which is great enough to inactivate the, now numerous, proliferating responder T cells.

B. TBM T Cell Immunoregulation by Blastogenic Feedback from Responder Cells (Figure 24)

1. Contact is made between responder T cell and foreign stimulator cell. In the absence of a second signal, no proliferation occurs.
2. MLR reactivity occurs following addition of regulatory T cells (or their factors). Blastogenesis also results in display of a membrane antigen, induced only during responder cell mitosis. Responder cells, in addition, undergo a membrane change, making them vulnerable to the activity of T cell inhibitor factor.

TBM T CELL IMMUNOREGULATION BY
BLASTOGENIC FEEDBACK FROM
RESPONDER CELLS

1

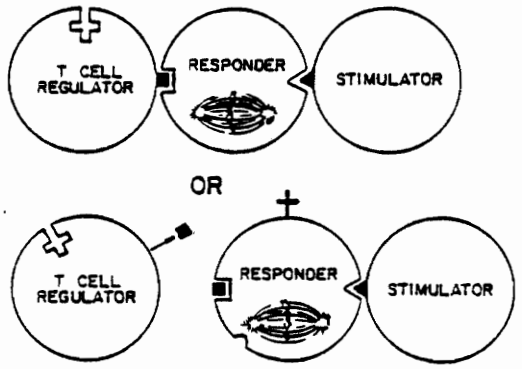


T CELL ENHANCING FACTOR RECEPTOR

MLC RECEPTOR

CONTACT BUT NO PROLIFERATION IN THE ABSENCE OF A SECOND SIGNAL

2



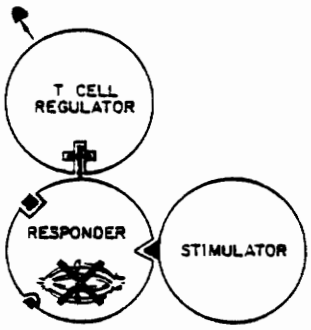
T CELL POSITIVE REGULATORY FACTOR

MITOTIC ANTIGEN ON BLASTOGENIC RESPONDER CELL

RESPONDER MEMBRANE, VULNERABLE TO INHIBITOR FACTOR

PROLIFERATION

3



T CELL NEGATIVE REGULATORY FACTOR

ABROGATION OF RESPONDER CELL PROLIFERATION BY T CELL INHIBITOR FACTOR

Figure 24

3. Vulnerable membrane of proliferating responder cell receives inhibitor factor. DNA synthesis is suppressed perhaps via inhibition of DNA polymerase activity. It is not completely established that mitotic antigen on responder cell is the trigger for release of T_s inhibitor factor.

T cell regulation may be an immunological failsafe mechanism. Mϕ, after antigenic overload, may be tied up and unable to migrate to a second site of antigenic invasion. T cells can fill the gap left by an inadequate number of Mϕ, not present in great enough quantities to turn off an overblown response, or, in severe Mϕ depletion, to even turn it on. The two models presented here give only a general concept of what might be occurring in vivo. The following section entitled "Future Directions" outlines experimental strategy and tactics to be used in tackling the many remaining immunoregulatory questions.

VII. FUTURE DIRECTIONS

A. Immunological Strategy

Heretofore, my examination of the host's immunoregulatory system has been based almost entirely on results from *in vitro* lymphocyte proliferative assays. Information from these experiments has been valuable in determining the *in vitro* mechanism of blastogenic regulation. However, *in vitro* proliferative assays, like all tools (even good ones), have certain limitations. The following key questions in immunoregulation can not be satisfactorily answered by sole use of *in vitro* proliferative assays:

i) What are the biochemical characteristics of isolated regulatory factors?

ii) What is the relationship between T cell blastogenesis and its ability to destroy foreign tissue?

iii) What is the *in vivo* biological relevance of *in vitro* immunoregulatory mechanisms?

We have no intention of abandoning *in vitro* proliferative assays. Rather, we wish to couple them to other experimental systems which will enable us to answer questions dealing, not only with T cell *proliferation* but also with *immunochemistry*, *cytotoxicity* and *in vivo* applicability. The following experimental tactics section presents a detailed analysis of the immunological issues and answers confronting future researchers.

B. Experimental Tactics

It would be foolish to discard such a fruitful source of information as the in vitro proliferative assay. A great deal of information remains to be obtained from the MLR assay system. Below are listed but a few of the planned future experiments:

i) Incubate normal T cells with Mϕ regulatory factors. Add only those treated T cells to MLR cultures to determine if Mϕ factors act directly on responder populations, or act indirectly, by triggering a population of regulatory T cells.

ii) Determine if inhibitor factor exists in intracellular Mϕ pools by sonicating Mϕ in the presence of the proteolytic enzyme inhibitor PMSF.

iii) Determine the kinetics of Mϕ immunoregulation by adding supernatants at varying time intervals after MLR culture incubation.

iv) Determine if receptor sites for Mϕ regulatory factors can be removed from responder cells by pre-incubation with various proteolytic enzymes.

v) To determine if Mϕ inhibitor factor suppressed T cell blastogenesis by triggering responder cell prostaglandin production, add inhibitor factor to responder ($R + S_m$) populations cultured in indomethacin.

In addressing the biochemical question, the stand taken by members attending the 12th International Leucocyte Culture Conference was summed up admirably by one of the self proclaimed wags from the National Institute of Health who drolly asserted that "the days of lymphocyte dregs and chicken-soupology are over." The emphasis on

biochemical purification is justified by the need to examine immunoregulation on a molecular level. Only after purification of the factors involved can one begin to investigate intracellular mechanisms. Though in vivo activity is hardly the result of neatly compartmentalized homogeneous secretions, it is still important to purify and identify the individual molecules exhibiting inhibitor or enhancing qualities. One can scarcely imagine Jacob and Monod describing E. coli metabolism using a heterogenous mixture of sugars; neither can immunoregulatory mechanisms be outlined using an amorphous mixture of dissimilar molecules. In scratching the biochemical surface of immunoregulation I have left behind a number of interesting projects in lymphokinology:

i) Separate the positively charged DEAE fractions by running them over a cation exchange column.

ii) Look for differences in the DEAE biochemical and activity profile of supernatants from untreated and PMSF incubated Mϕ.

iii) Use column chromatography to determine molecular weight, and assay fractions for the multiple peaks of inhibitor and enhancing activity seen in Mϕ supernatants fractionated on DEAE columns.

iv) Run column chromatography assays on heat treated supernatants to see if this treatment abrogates positive immunoregulation of the characteristic enhancing fractions.

v) Slice and assay polyacrylamide gels for activity, to determine the relationship (if any) between activity and the staining pattern of the gel.

On a cellular level, to understand how the T cell is rendered incapable of halting tumor growth, it is important to see that T cells are a heterogeneous population comprised of: i) proliferative T cells which initiate rapid cell division after recognizing an antigen as foreign, and ii) cytotoxic T cells which are thought to lyse foreign cells in response to the triggering action of proliferative T cells. Examination of both T cell proliferation and cytotoxicity (measured by ^{51}Cr release from lysed foreign target cells) will hopefully aid in answering the following questions:

i) How does the degree of T cell cytotoxicity compare with the level of T cell proliferation? Though many researchers have shown that T cell cytotoxicity is dependent upon the presence of a proliferative T cell, does it necessarily follow that T cell proliferation indicates the presence of cytotoxic T cells? This is a key question, since a high rate of T cell proliferation is a meaningless measurement of immunocompetence if it does not in some way reflect an increased ability to destroy foreign cells.

ii) Do the same factors which govern T cell proliferation also regulate T cell cytotoxicity? Do Mϕ regulate the cytotoxic as well as the proliferative T cell response? Is there a suppressor T cell which directly blocks T cell cytotoxicity as it does T cell proliferation?

Investigation of in vitro immunoregulation, whether on a cellular or a biochemical level, is little more than an academic exercise if it can not be linked to an in vivo system of activity. We have begun to establish the in vivo relevance of Mϕ and T cell immunoregulation with adoptive transfer experiments demonstrating T cell

blastogenic suppression following Mϕ or T_s cell i.v. injection. To further demonstrate biologic relevance of in vitro immunoregulation in PHA, MLR and cytotoxicity experiments, mice will be inoculated with regulatory factors or implanted with chambers containing Mϕ or regulatory T cells. The porous implant chambers will confine cells but allow soluble factors to circulate freely. By running the appropriate controls, in vivo immunoregulation will be measured using, as models of cell mediated immunity, the following assays:

i) Allograft rejection.

ii) Delayed hypersensitivity.

iii) Suppression of secondary tumor growth in the presence of continued primary tumor growth (concomitant immunity).

iv) Suppression of a secondary tumor growth following excision of the primary tumor.

In the research world of mice and men, the latter creature sometimes gets the short end of the stick. As any diet soft drink manufacturer can tell you, it is important to establish the connection between murine tumorigenesis and human neoplastic growth. Realistically, it is a quantum leap from the CO_2 incubator in 5014 Derring Hall, to the pharmaceutical shelf at Squibb, Inc. However, it takes little imagination to see that purified immunoregulatory lymphokines may prove to be an important clinical tool of the physicians. For example; where enhancement of the immune system is desirable, as in tumor growth, bacterial attack or virus infection, a timely injection of LAF (lymphocyte activating factor), MAF (Mϕ activating factor), or MIF

(Mϕ migration inhibitory factor), may be literally "just what the doctor ordered." Already, doctors are experimenting with intralesional injections of BCG in hopes of activating Mϕ into a tumoricidal state.

On the other hand, there are times when it is best to cool down an overzealous proliferative cell. To inhibit transplantation rejection, abrogate hypersensitivity, and reverse the debilitating effects of auto-immune disease, it may some day be possible to purchase, from your friendly druggist, a packet of IDS (inhibitor of DNA synthesis) pills or a bottle of concentrated PIF (proliferation inhibitory factor). At this time interferon is immunoregulatory inhibitor of choice.

Obviously, these applications are somewhere far in the future, but, without the "draft horse" (Farrar's term) research of today, those applications would stay forever in that future.

..So the patient biologist plods along,
piling up empirical observations
as honestly as he can --
getting what satisfaction he may,
from the fact that he is helping,
by infinite increments, to reduce the
scope of biological vagueness.

Hans Zinsser

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VITA

Kevin Michael Connolly

I was born January 10th 1952 in Baltimore, Maryland (affectionately pronounced "Baldamore" by the natives). To my relief, and the chagrin of my genetics professor, I received a B.A. in Natural Science from Johns Hopkins University in 1974. After some little debate, I abandoned Hopkins' advanced program in creative writing, in favor of scientific graduate research at VPI&SU. Having worked as an undergraduate for two years in a leukemia ward at the Baltimore Cancer Research Center, I was attracted to Dr. Elgert's program in tumor immunology. Though I consider the receipt of my Master of Science degree (1977) a high point in my career, the happiest moment in my life occurred July 9, 1978 when Cynthia Saul became my wife. Upon receiving my Ph.D degree, I will assume a post-doctoral position at the Medical College of Virginia in the tumor immunology laboratory of my good friend Dr. Alan M. Kaplan.

Portions of this research have been presented by Dr. Elgert at the 12th International Leucocyte Culture Conference, and by me at the 1978 meeting of the American Association of Immunologist and the 77th Annual Meeting of American Society for Microbiology (plus numerous regional presentations).

Kevin Michael Connolly

POSITIVE AND NEGATIVE IMMUNOREGULATION OF NORMAL AND TUMOR-BEARING MOUSE T CELL BLASTOGENESIS

by

Kevin Michael Connolly

(ABSTRACT)

Using the mixed lymphocyte reaction (MLR) as a correlate of cell-mediated immunity, an examination was made of positive and negative blastogenic immunoregulation by syngeneic peritoneal macrophages (Mϕ) and splenic T cells from tumor-bearing mice (TBM). As analysis of T cell proliferative response progressed an intricate pattern of immunoregulatory checks and balances unfolded involving both Mϕ and TBM cells.

In their capacity as regulators, Mϕ acted, not only to enhance MLR reactivity, but to inhibit it. Results indicated that: i) Mϕ regulation was a concentration dependent phenomena -- high concentrations of Mϕ (or their supernatants) inhibited MLR reactivity, while low doses enhanced MLR reactivity; ii) inhibition occurred via a non-toxic, heat stable, nondialyzable (and therefore non-thymidine) factor; iii) enhancement occurred via a heat labile nondialyzable factor.

Since normal Mϕ possessed a factor which, in high concentrations, inhibited T cell blastogenesis, tests were run to determine if the MLR hyporeactivity of T cells from TBM could be attributed to a unique tumor-induced inhibitory Mϕ. Contrary to expectations, TBM Mϕ

supernatants, when compared to their normal counterparts on a volume-to-volume basis, showed an increased (not decreased) ability to enhance MLR reactivity.

In light of results showing TBM Mϕ enhancement of MLR reactivity, T cell hyporeactivity in TBM was explained after observation of the following dual regulatory mechanism of suppression: i) on a purely quantitative basis, the high in vivo concentration of Mϕ in spleens of TBM inhibits spleen cell response to alloantigen; ii) there also exists a population of mildly nylon wool adherent tumor-induced splenic T cells which elaborate a soluble factor capable of overriding any Mϕ enhancing effect.

In their capacity as regulators, tumor-induced splenic T-cells act, both to enhance and inhibit MLR reactivity. Whereas Mϕ regulation is concentration dependent, in the case of the T cell regulator, regulation is based, not upon the relative concentration of the regulator cell, but upon the level of responder cell activity, i.e. Mϕ-depleted MLR cultures (showing minimal proliferation) were enhanced by regulator TBM T cell addition, while Mϕ augmented MLR cultures or PHA stimulated cultures (with a high rate of blastogenesis) were inhibited by the same concentration of TBM regulator cells.

Centering around more stringent biophysical and biochemical characterization of Mϕ supernatants, the latest work has resulted in the biochemical separation of Mϕ supernatants into inhibitor and enhancing components. Using anion exchange chromotography and slab gel electrophoresis, inhibitor and enhancing factors have been separated by charge. Treatment of Mϕ with indomethacin did not abrogate release

of inhibitor factor, suggesting that it was not prostaglandin. Enhancing factor, obtained from Mϕ sonicates as well as supernatants, could be distinguished from lymphocyte activating factor by its inability to induce a thymocyte PHA response.

Thus, analysis of regulation in the cell-mediated immune system has resulted in the elucidation of a most elaborate scheme of immunoregulation involving both Mϕ and T cells.