Tumor-Induced Immunosuppression: Contribution of a High Molecular Weight Inhibitor and Prostaglandin E$_2$

by

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A heat-stable soluble inhibitor of T cell proliferation was demonstrated in splenic and peritoneal macrophage (Mφ) culture supernatants. Concentrated supernatants were prostaglandin E2 (PGE$_2$)-free and yet inhibited proliferation in the mixed lymphocyte reaction (MLR) and mitogen assays. The high mw inhibitory factor was apparently > 67 kd, as shown by S-200 Sepacryl chromatography and gel electrophoresis. DEAE-Cellulose chromatography suggested that the pI of the inhibitory factor was < 7.7. Isoelectric focusing revealed that the Mφ-mediated inhibitory activity differed in charge, with a pI of 6.5-7.6 for normal hosts and 4.0-6.0 for tumor-bearing hosts (TBH). Normal and TBH Mφ supernatants showed different hydroxylapatite fractionation, with the latter being resistant to proteolytic enzymes but sensitive to neuraminidase. Lectins such as wheat germ agglutinin, concanavalin A, Ricin communis and Bandeirea simplicifolia were not useful in affinity purification of the high mw inhibitory monokine. Sugar-BSA conjugates suggested that inhibitory activity was vested in a terminal β1,4 linked galactose. The inhibitory activity was apparently hydrophobic and heat-stable, but heat-stability was lost if supernatants were boiled at an acidic pH. The high mw monokine inhibited the proliferation of P388D$_1$ and A4A cells, but enhanced the proliferation of BW 5147.3 cells. Time course addition to the MLR revealed that PGE$_2$ may be required for inhibitory activity to be manifested early (0 and 24 hr) but not if the high molecular weight (mw) inhibitor was added late (> 48 hr). Indomethacin blocked activity of the inhibitory factor early in the MLR using normal host T cells and augmented the proliferation of TBH T cells in the MLR. Both normal and TBH Mφ supernatants suppressed the generation of interleukin 2 but with a dose- and time-dependent difference. Cell cycle analysis of mitogen-
stimulated cells revealed that TBH Mφ supernatants enhanced cell cycle progression at 42 hr, but that both normal and TBH Mφ supernatants suppressed the number of cells in S phase at 66 hr.

To determine the phenotype of Mφ that produced the high mw inhibitor, Mφ were modulated with anti-Mac-1, -2, or -3 monoclonal antibodies (mAb) or depleted with mAb plus complement and cultured with or without indomethacin. P388D₁ cells, used as a control, revealed that the anti-Mac mAb were reacting specifically and not via Fc receptors. Culture supernatants added to the MLR showed that the Mφ suppressor phenotype shifted from Mac-3⁺ in the normal host to Mac-2⁺ in the TBH. Production of PGE₂ was susceptible to indomethacin, but in general the relative production of PGE₂ by the Mφ subpopulations remained the same. Depletion of Mac-1⁺ Mφ caused a higher increase in PGE₂ production than did activation, suggesting that Mac-1⁺ Mφ down-regulated PGE₂ production. In contrast, no Mac-1⁺ Mφ-mediated regulatory function occurred in the TBH. Mac-2⁺ Mφ were the primary producers of PGE₂ in the TBH, but not in the normal host. Thus, immunosuppression in the TBH was at least partly due to the inability of Mac-1⁺ and/or Mac-3⁺ Mφ to control production of PGE₂ by Mac-2⁺ Mφ, which then acts in conjunction with the high mw inhibitor to stop T cell proliferation.
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I set out to do.
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## Introduction

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- **Medium**
- **Monoclonal Antibody Generation and Purification**
- **P388D₁ Treatments**
- **Macrophage Treatments**
- **Mixed Lymphocyte Reaction**
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- **Suppressive Activity and Protein Content of Supernatants Derived from Anti-Mac-Treated Mφ**
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# Chapter III

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<tr>
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<td>bovine serum albumin</td>
</tr>
<tr>
<td>Con A</td>
<td>concanavalin A</td>
</tr>
<tr>
<td>CONC</td>
<td>concentrated</td>
</tr>
<tr>
<td>cpm</td>
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<tr>
<td>CstF</td>
<td>cytostatic factor</td>
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<tr>
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<td>³H-TdR</td>
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<td>IDS</td>
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<td>i.p.</td>
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<td>kd</td>
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<td>MAF</td>
<td>macrophage-activating factor</td>
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<td>mAb</td>
<td>monoclonal antibodies</td>
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<td>MASH</td>
<td>multiple automated sample harvester</td>
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<td>min</td>
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<td>MLR</td>
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<td>mw</td>
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<td>-------------</td>
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<tr>
<td>NAG</td>
<td>N-acetyl-glucosamine</td>
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<tr>
<td>NeuAc</td>
<td>N-acetyl-neuraminic acid (sialic acid)</td>
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<td>nor</td>
<td>normal</td>
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<tr>
<td>NRIGG</td>
<td>normal rat IgG</td>
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<td>PGE₂</td>
<td>prostaglandin E₂</td>
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<td>polyethylene glycol</td>
</tr>
<tr>
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<td>phytohemagglutinin</td>
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<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
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<tr>
<td>RM</td>
<td>regular monocytes</td>
</tr>
<tr>
<td>SAS</td>
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</tr>
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<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<td>TBS</td>
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<tr>
<td>TNF</td>
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<td>wheat germ agglutinin</td>
</tr>
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Introduction

The theme of this dissertation is to determine the mechanism(s) of how macrophage (Mφ) factors suppress the proliferation of T lymphocytes. In particular, I will focus on the effects of tumor development on Mφ-derived soluble factors and the tumor-induced changes in both the factors and their immunoregulatory effects. This involves many intertwined ideas and concepts.

Proliferation is a hallmark of the immune response. T and B lymphocytes are both uniquely capable of clonal proliferation, whereby one cell becomes many. It should be realized that resting "naive" lymphocytes, although they can proliferate and produce daughter cells which represent individual clones of the original, produce daughter cells which are different from the original. A more appropriate term is a cascade, which implies differentiation is a result of proliferation. This is driven by antigens and or soluble factors (lymphokines), and the daughter cells may be of helper, suppressor, cytotoxic, inducer, transducer, etc., phenotypes.

The paradigm with which this proliferation is understood is based on Neils Jerne's 1980 Nobel Prize winning network theory. While originally formulated to explain B cell antibody production, it has proved useful in dissecting T cell relationships and the immune response in general. Basically, Jerne felt that the immune response is in a dynamic state of constant amplification and suppression and the responses we measure are because of temporary perturbations in immune homeostasis. Foreign antigens, then, would temporarily disrupt the equilibrium between two or more counter-
acting cells, causing an immune response against the antigen. This response would then be controlled, or suppressed, and the homeostatic equilibrium reestablished. This interaction and control among immune cells is mediated via both cell-cell contact and soluble factors. If proliferation/differentiation results in phenotypically or functionally different cells, then the cells or factors which counteract them must also be developed. Simply stated, for every up there is a down in the immune system. It comes as no surprise, then, that there are T lymphocytes which help as well as suppress the immune response, Mφ which are required for both the initiation and suppression of T cell responses, and soluble factors which augment or inhibit functional responses.

For immune system homeostasis to occur, there must be a mechanism for cell-cell interaction and control. Studies directed at these questions have revealed that cells of the immune system interact via both cell-cell contact and by the release and reception of soluble chemical signals. These signals are now variously referred to as cytokines, lymphokines, monokines, interleukins, chalones, etc. While the vast majority of these substances are not well characterized biochemically, and are instead understood in terms of their biological activity, a few have been isolated, cloned, and generated in large quantities. These include interleukin 1 (IL 1), interleukin 2 (IL 2), interferon-γ (IFN-γ) and tumor necrosis factor (TNF). The availability of large quantities has circumvented the major hurdle in research on these soluble mediators, that is, they are present and operative at extremely low (nano-molar to pico-molar) quantities.

T cell proliferation is understood as the culmination of a cascade of events operating on two levels: the individual cell and the cell population. On the one hand, activation of the individual cell is accompanied by a calcium influx, and may or may not be accompanied by internalization of the soluble signal. In the broader sense, recruitment and activation of the population of responding lymphocytes must also occur for the immune response to be effective.

T cell proliferation is dependent on Mφ acting as accessory cells. Lipsky and Rosenthal (158-160, 237) showed that Mφ and T cells interact via cell-cell contact and in fact formed clusters of cells. The first step of T cell activation usually involves the presentation of antigen by Mφ. Mφ present the antigen in conjunction with their Ia molecules, for which the T cells have receptors. It is possible that soluble factors are released in the microenvironment of the Mφ-T cell contact.
One such Mφ-derived factor is IL 1 which has the notable property of augmenting the production of IL 2 and thereby initiating T cell proliferation (72, 73, 184, 208). The release of IL 2 by T cells engenders the elaboration of IL 2 receptors (167) on those T cells that produce IL 2 (autocrine pathway) and on other resting T cells (exocrine pathway). This serves as an amplification step in the immune response, as does the concomitant release of Interleukin 3 (IL 3) and IFN-γ. IL 3 is a hemopoietic growth factor (245) while IFN-γ, also known as Mφ activating factor (MAF) (247, 269, 297), can cause augmented production by Mφ of IL 1 (24, 71, 183), which then serves to increase the number of activated T cells. IFN-γ also reduces the production of prostaglandin E2 (PGE₂) by Mφ (24). While there is some evidence that PGE₂ is required for T cell proliferation to occur (165, 172, 313), PGE₂ appears to down modulate T cell proliferation in the vast majority of systems (41, 42, 76, 101, 264, 303).

T cell proliferation is a cascade with the eventual development of cytotoxic T lymphocytes (CTL), the final effector cell of the pathway. CTL and suppressor T cells share the same Lyt-2,3+ phenotype (CD8 in humans) and are both produced as the result of the same cascade. This lends support to the concept of immune homeostasis and to the idea that T cells which result from proliferation/differentiation are not the equivalent of the T cell which received the initial activation signal.

The system outlined above has many inherent checks and balances, with the Mφ in the role of initiator, regulator, and suppressor of T cell proliferation. This dualistic role of positive and negative effector cell has been delineated using Mφ (116, 315) and Mφ-derived culture supernatants (48, 49, 315). One thing that has become apparent is that Mφ display a concentration-dependent immunoregulatory activity, in that while Mφ are required for T cell proliferation, high doses of Mφ are suppressive (47, 48, 315). Mφ have also been shown to stimulate or inhibit the same response, depending on the level of the response, i.e., the T cell response is homeostatically controlled (50).

We have studied this system of Mφ-T cell interactions to determine how tumor growth tips the balance towards immunosuppression, i.e., how Mφ and T cell suppressor activities go uncontrolled. Tumor-bearing hosts (TBH) are severely immunosuppressed, as evidenced by a lack of T
cell proliferation in response to specific or non-specific stimuli. I have attempted to determine the 
Mφ role in this immunosuppression, and the influence of tumor growth on Mφ-T cell interactions. 
I have concentrated on the soluble factors that Mφ produce, in particular a high molecular weight 
(mw) factor which suppressed T cell proliferation. Previous work in this laboratory demonstrated 
that Mφ from normal and TBH produce two soluble compounds which inhibit T cell proliferation. 
One is PGE₂, a 352 mw arachidonic acid metabolite whose production is shut down by the addition 
of indomethacin (244), and the other is a large, non-dialyzable molecule which is still produced in 
the presence of indomethacin (51). It was known that this high mw (> 12,000 daltons) inhibitory 
molecule was heat-stable, and was produced concomitantly with a heat-labile molecule that en-
hanced T cell proliferation. The consensus from those studies was that there was no difference in 
the high mw inhibitory molecules produced by normal and TBH Mφ. The goal of my research 
was to determine if there were in fact any detectable differences. While rigorous purification and 
characterization was impossible due to insufficient quantities of starting material, I present evidence 
in this dissertation that there was a qualitative change in the high mw inhibitory molecule as a 
consequence of tumor development.

Based on previous work, my objectives were to determine the mechanism of tumor-induced 
immunosuppression by answering the following questions:

1. Is there a tumor-induced alteration in the phenotype of suppressor Mφ, i.e., the producers of 
PGE₂ or the high mw inhibitory monokine?

2. In particular, is there a change in the phenotype of Mφ which are responsible for factor-
mediated immunosuppression?

3. Is there a tumor-induced qualitative change in the immunosuppressive factors produced by 
Mφ, i.e., their kinetics of activity or interaction with each other and with other soluble factors 
such as IL 2?
My hypothesis was that since Mφ-T cell interactions are largely factor-mediated, alterations in factors could be the cause or result of tumor-induced immunosuppression. The possibility existed that tumor growth caused a change in Mφ-T cell populations or even their relative proportions, and that this change would be reflected in altered levels of immunoregulatory factors or even in new factors.

Some of the evidence for this hypothesis was that Mφ from TBH produced increased amounts of PGE₂, a known immunosuppressive compound due to its activation of T suppressor cells, etc. (55, 146, 121, 211). Plescia et al. (214) showed this increased PGE₂ was instigated by the production of PGE₂ by immunosuppressive tumor cells. Mφ, with their ability to migrate through tissues, could be activated at the site of the tumor, and then migrate to the spleen or peritoneal cavity where they would induce a systemic immunosuppression. Ting and Rodrigues (277) showed tumor cells evade the immune response by interacting (in vitro) with splenic Mφ which then activated the suppressive activities of peritoneal Mφ. The sequence was very important because pre-exposure of tumor cells to splenic Mφ was required before the splenic Mφ could then induce the peritoneal Mφ to suppress T cell proliferation. This ability of the tumor to switch on the host's own suppressor mechanisms was an important observation, and also implied that splenic and peritoneal Mφ were not one and the same. The direct contribution of PGE₂ or other monokines to this system was not determined (277).

Taking a cue from the above findings, and previous work in our laboratory showing that Mφ produce PGE₂ and a high mw immunosuppressive factor, I set out to determine the biochemical characteristics of the high mw inhibitory molecule and start the laboratory down the road to determining its mechanism of action. I succeeded in separating the two factors and showed, using PGE₂-free preparations, that the high mw inhibitory factor was >67,000 mw by gel filtration chromatography and a charged molecule, possibly a glycoprotein. Hydroxylapatite chromatography revealed that the high mw inhibitor from normal Mφ supernatants eluted later than its TBH counterpart. Experiments also showed the neuraminidase sensitivity of the inhibitory activity in supernatants derived from Mφ from TBH but not normal hosts. This agreed with the lowered pI of the factor seen in TBH Mφ supernatants. The factor was clearly delineated from
PGE$_2$ by its size, time course of action, and effect on cell cycle position of the target cells. Addition of the high mw inhibitor, indomethacin, and PGE$_2$ to the mixed lymphocyte reaction (MLR) at various times after initiation revealed that PGE$_2$ was required to manifest the inhibitory effect of the high mw inhibitory monokine on T cells in the early stages of activation. At later stages of activation, T cells were not as susceptible to PGE$_2$ but were susceptible to the high mw inhibitor. Interestingly, the TBH Mφ factor caused an early enhancement of cell cycle progression of mitogen-stimulated splenic T cells that was not seen using normal host Mφ supernatants. Both the normal and TBH factors suppressed the generation of IL 2 by alloantigen-stimulated T cells measured at 48 and 90 hr after initiation of the assay. We also demonstrated a shift in phenotype of suppressor Mφ as the tumor developed, with the suppressor Mφ being Mac-3$^+$ in normal hosts and Mac-2$^+$ in TBH. This shift was accompanied by a drop in Ia antigen expression and Ia antigen-mediated activities by TBH Mφ (91). Experiments directed at delineating the Mφ subpopulation which produced the high mw factor resulted instead in elucidating that the Mac-2$^+$ Mφ was responsible for the production of PGE$_2$, probably because down-regulation of PGE$_2$ production by Mac-1$^+$ Mφ was missing in the TBH. This partly explains the finding that the Mac-2$^+$ Mφ was the suppressor phenotype in the TBH. Mac-3$^+$ Mφ were not found to play an important role in the production of PGE$_2$. The high mw factor, acting in concert with increased PGE$_2$ production seen in TBH, undoubtedly played a role in the immunosuppression seen in TBH.

The literature review which follows is a discussion of the concepts needed for an understanding of the role of suppressor Mφ and their inhibitory monokines in the immune system. The road map I will follow takes the reader on a tour of Mφ, their role in augmenting and suppressing T cell proliferation, and the factors involved in performing these functions. I have generally restricted myself to a discussion of antigen-nonspecific factors, with an emphasis on Mφ and their factors. The literature review closes on the mechanisms of how tumor growth upsets the careful balance that exists in the immune system between Mφ and T cells, resulting in immunosuppression. The meat of the dissertation will present my data on the presence of PGE$_2$ and a high mw inhibitory monokine in Mφ culture supernatants, the biochemical characteristics of the high mw inhibitor, and the tumor-induced changes in these factors. I hope the reader will come away with an under-
standing of immunoregulatory pathways and interactions and the complications introduced by tumor growth.
Literature Review

Mφ were discovered by Metchnikoff in 1882 when he noticed that transparent starfish larvae possessed motile cells which surrounded and attempted to engulf foreign material (a rose thorn) injected into the host (106). While this is not an immune response, it is useful to note that this phagocytic response is necessary for survival of the host. In this context, immunity, with its complex mechanisms and interactions, may be thought of as a luxury. The Mφ, then, has been elevated from its sentinel role of cellular garbage man to its present luxurious role as the initiator and regulator of both humoral and cell-mediated immunity (43, 285).

Mφ originate from bone marrow pluripotent precursor cells and differentiate along the myeloid lineage from promonocytes to monocytes in the blood stream to the end cells that take up residence in various anatomical locations and are called Mφ. Mφ either do not proliferate in vitro or do so very slowly (292). Mφ are operationally defined (287, 293) based on the following characteristics: (i) they are mononuclear cells; (ii) they possess peroxidase and esterase activity; (iii) they bear surface receptors for antibody and complement; (iv) they exhibit phagocytic and pinocytotic abilities; and (v) they may be activated. Activation is a loose term for increased phagocytosis, increased quantity of acid hydrolases, more active metabolism, and increased bactericidal activity. A sixth criteria of varied and prolific secretory ability is also central to the definition of a Mφ (107, 284, 285, 293). These characteristics can be grouped under the Mφ roles as stimulator cells, accessory cells,
Mφ Heterogeneity

There is no consensus as to whether Mφ are a heterogeneous group of cells due to variable activation states (1) or due to the presence of subpopulations (14, 15, 283). Mφ-activation of different T cell populations in various in vitro assays is indicative of Mφ subpopulations and will be discussed later. Conveniently, Mφ have been characterized by their expression of Ia antigens (164, 260, 261, 283), receptors for complement components and Fc fragments of antibodies (58, 293, 294), and Mac-1, -2, and -3 antigens (258). Ia antigens demonstrate Mφ activation as their expression can be induced by LPS (113) and Listeria bacteria (16). Ia antigens are required for Mφ-T cell interactions and presentation of antigens to T cells, but their expression is not correlated with phagocytosis (283). Other Mφ surface antigens, such as Mac-1, Fc receptors, and C3 receptors, are constitutively produced and continuously present (294) on essentially all Mφ. On the other hand, the Mac-2 antigen is found predominantly on thioglycollate-elicited peritoneal Mφ (119), while the Mac-3 antigen is expressed on resident peritoneal Mφ (263, 304). Both the Mac-2 and Mac-3 antigens develop late in the progression from bone marrow stem cells to differentiated tissue Mφ (304). Mac-2 and Mac-3, then, show that Mφ heterogeneity is the result of either differentiation or subpopulations. Mφ subpopulations have been demonstrated using antibodies (240,
242, 267), and it has further been shown that antibody-defined Mφ subpopulations can be activated for tumor cell cytotoxicity (86, 127, 272). Another demonstration of Mφ subpopulations comes from experiments using buoyant density centrifugation (274). These separated Mφ produce IL 1 and/or PGE₂ (3, 99, 133, 200).

Up to this point, I have discussed Mφ in isolation, but in fact, they are but one member of an immune system which is composed of T lymphocytes, B lymphocytes, dendritic cells, natural killer cells, natural cytotoxic cells, and others, all of which can be separated into subpopulations, by anatomical location, and by susceptibility to Mφ-mediated control. T lymphocytes are responsible for the cell-mediated arm of the immune system, and are of importance to this dissertation because of their role in tumor cell recognition and destruction, as first demonstrated by Winn (312). In fact, Winn showed that this activity was due to cooperation between Mφ and T cells.

**T Cell Proliferation**

T lymphocytes are unusual in that they can undergo a clonal proliferation burst, which is actually the basis of the protective response of the immune system. A variety of *in vitro* methods exist to stimulate T cells to proliferate, including the addition of mitogens (18, 96, 205) and foreign cells bearing alloantigens (11, 61). It was Bain *et al.* who discovered that an *in vitro* indicator of lymphocyte stimulation was blast transformation upon exposure to foreign lymphocytes (11). Blast transformation, a morphologically detectable change from small to large lymphocytes, was most easily quantified by measuring increased ³H-thymidine (³H-TdR) uptake and incorporation due to enhanced DNA synthesis (22, 105). Thus, Dutton (61) was able to measure the degree of immunocompetence by assaying ³H-TdR incorporation in the presence of foreign lymphocytes (the mixed lymphocyte reaction, or MLR) (114, 275). This proliferation in response to foreign cells implies a recognition event, and correlates with the *in vivo* ability to reject foreign tissue (such as grafts or tumors which are histoincompatible) (215).
T lymphocytes are the cells which proliferate in response to stimuli, and this proliferation results in a variety of cell-mediated immune activities such as T helper cell induction of antibody production (62) and cytotoxic T cell destruction of foreign tissue (243). T cell activation, a prelude to proliferation, is associated with an early increase in the cytosolic calcium concentration (122, 198, 281, 307). There is evidence that the increase in cytosolic calcium, which follows surface receptor triggering, is preceded by the breakdown of phosphatidylinositol biphosphate into 1,2-diacylglycerol and inositol triphosphate, the latter causing the release of calcium from intracellular stores (191, 192, 225, 226). Calcium is also involved in the activation of protein kinase C (34). These physiological events may be mirrored by using calcium ionophore and phorbol esters, which together will induce lymphokine secretion and cell proliferation (182).

Initiation of the cascade of T cell proliferation and differentiation by Mφ begins with Mφ stimulation by antigen and subsequent production of IL 1 (150, 166). IL 1 and antigen induce T helper cells (L3T4+ in mice and CD4+ in humans) to generate IL 2 (97, 149). These antigen-specific T cells can only be activated by cells which are adherent and positive for Fc-γ receptors and Ia antigens (69), i.e., Mφ. IL 2 then feeds back on the cells which produce it, in both an autocrine and exocrine fashion, triggering its receptor, and causing the T cells to produce IFN-γ (72, 78, 129, 138, 279). IL 2 and IFN-γ will then act on the precursors of CTL to generate mature, antigen-specific CTL (45, 145, 181), which represent the effector cell and the culmination of the cascade resulting from the initial introduction of antigen. An important distinction that must be made is that T helper cells respond to Class II antigens (Ia molecules) as their first signal but are not activated until they receive a second signal such as IL 1 which is produced by Ia+ Mφ. CTL, on the other hand, respond to Class I antigens (histocompatibility or transplantation antigens) as their first signal and are activated by a second signal such as IL 2 produced by activated T helper cells. Once CTL are activated they can react with target cells that express the inducing Class I antigen in the absence of IL 2.
Mφ-T Cell Interactions

The prevalent understanding at this point is that Mφ play a role in the induction of T cell proliferation. T cells from guinea pigs (236), mice (96), and humans (154, 205) when exposed to the T cell-specific mitogen phytohemagglutinin (PHA) show an enhanced \(^{3}\)H-TdR uptake and incorporation in the presence of Mφ. In an antigen system, Mycobacterium-induced lymphocyte proliferation was also augmented by the \textit{in vitro} presence of Mφ (248, 302). Alloantigen-induced T cell proliferation in the MLR has also been shown to be enhanced by the presence of Mφ (155, 233, 232). It is interesting to note that lymphocyte reactivity may be augmented by allogeneic or xenogeneic Mφ (46, 161, 178, 282), as well as by syngeneic Mφ. It is possible that this is due to allogeneic or xenogeneic Mφ possessing foreign histocompatibility antigens, putting the Mφ in the role of allogeneic target or stimulator cells. In the physiologically relevant case of syngeneic Mφ, it appears more likely that Mφ enhancement of T cell proliferation is due to the active synthesis of soluble factors (monokines), but a case can be made for the importance of Mφ Ia antigens in the contact-dependent enhancement of T cell proliferation.

T cell proliferation assays, besides being dependent on Mφ for activation and enhancement, also show a hierarchy in their requirement for Mφ. The relative order of Mφ requirement or dependency (from greatest to least) among \textit{in vitro} reactivities in man is (i) pokeweeds mitogen activation of helper T cells required for immunoglobulin synthesis, (ii) antigen-induced T cell proliferation, (iii) antigen-induced lymphokine production, (iv) Con A and pokeweeds mitogen-induced proliferation and, (v) PHA-induced T cell proliferation (221). It is interesting that Novogrodsky \textit{et al}. (196) reported that down-modulation of the PHA response by PGE\(_2\), a Mφ product, is minimal, thereby indicating that if Mφ are not required for activation, they are also not required for suppression. The differential requirements for Mφ and the fact that distinct T cell populations are required for various \textit{in vitro} activities lends credence to the idea of Mφ subpopulations (221).
Contact-dependent enhancement by Mφ of T cell proliferation has been ascribed to an Ia* cell (280). Ia* Mφ are able to present antigen to T cells and thereby prime them, in other words, render them susceptible to IL 1, the second signal necessary for T cell proliferation. This is a temporal sequence because IL 1 obviates the need for Ia* Mφ once the T cells are primed (59, 60). The presence of Ia antigens appears important in an earlier phase of Mφ-T cell interaction. Ia antigens (or DR antigens on human cells) can be down modulated by the addition of PGE₂ (254) and up modulated by the addition of IFN-γ (108), two potent immunomodulators. Ia antigen expression is reduced on Mφ from individuals with Hodgkin’s lymphoma (188) and Mφ from tumor-bearing mice (91). Note however that some studies have implicated Ia* Mφ as suppressor Mφ (156, 266).

Monokine-mediated Mφ enhancement of T cell proliferation is most generally ascribed to production of IL 1, or lymphocyte activating factor, by Mφ (56). IL 1 is a 12,000-16,000 mw protein which allows PHA stimulation of murine thymocytes. IL 1 is known to exist in two forms (207). IL 1 production may be induced in P388D₁ cells by the addition of bacterial LPS, factors from PHA-stimulated T cells, or phorbol myristic acetate (185-187). The P388D₁ Mφ-like cell line has been utilized as a convenient source of this monokine. At present, IL 1 is available in recombinant form (162). At least two, probably non-exclusive, mechanisms of action of IL 1 have been suggested. Oppenheim et al. observed elevated cyclic AMP levels in IL 1-stimulated thymocytes, which could put the cells in an immunoreactive state (206). The IL 1 used in these experiments was derived from adherent mononuclear cells, and may have been contaminated with other lymphokines, which may account for this unusual result. Conlon et al. and others suggested that IL 1 induces IL 2, a T cell product which then allows mitogen-induced proliferation (44, 252). The present consensus is that G₀ phase T cells respond to antigen (the first signal) presented by Ia* Mφ and proceed to G₁. These G₁ cells are then responsive to IL 1 (the second signal), resulting in the production of IL 2 and IL 2 receptors, and subsequent progression to S phase and proliferation (201).

Mφ also produce a mitogenic protein (288) and plasminogen activator which is thought to play a role in cell proliferation (290, 295). Note that Mφ also produce an inhibitor of plasminogen ac-
Mφ-Mediated Immunosuppression

As mentioned in the Introduction, Mφ can also play a role in the negative regulation of T cell proliferation (5, 315). T cell proliferation is the culmination of a series of events in a multi-step pathway. This scenario results in many possible sites for Mφ-mediated inhibition to occur. Mφ suppression of T cell proliferation can be either contact-dependent or mediated by soluble factors. It is interesting to note that Mφ suppression of T cell proliferation can be passive (loss of Ia molecules necessary for Mφ-T cell interaction) or active (actual production of immunosuppressive factors). Rinehart et al. showed human monocytes enhanced while Mφ inhibited lymphocyte proliferation to PHA and allogeneic lymphocytes, and suggested, by exclusion, that Mφ suppression was contact dependent (229). Fernandez and Macsween (83) showed that high concentrations of human monocytes inhibited T cell proliferation in the autologous MLR, but not in PHA-induced blastogenesis. The use of Marbrook chambers demonstrated the inhibitory activity of soluble products from Mφ (83). Wing and Remington found that high concentrations of Toxoplasma gondii-activated, but not normal, unactivated, mouse peritoneal Mφ inhibited mitogen and alloantigen-induced murine lymphocyte proliferation, and that Mφ culture supernatants from activated Mφ are also effective (311). Travniczek et al. show that suppression of mitogen-induced T
cell proliferation was due to an Ia-Mφ which strongly bound ganglioside treated sheep erythrocytes, possibly indicating that the ganglioside receptor may be a useful marker of suppressor Mφ (280). We show that there is a loss of Ia antigen expression on TBH Mφ which is reflected in a loss of Ia antigen-mediated activities (91).

The majority of the literature on Mφ immunoregulation suggests that suppression of T cell proliferation is not contact-dependent, but rather is mediated by the release of soluble inhibitory factors (197, 284, 287, 294, 300). Mφ inhibition of T cell proliferation has been variably attributed to Mφ production and release of low molecular weight compounds such as arginase (144) which removes the essential amino acid arginine from the medium, and thymidine (203, 204) resulting from Mφ ingestion and degradation of foreign cells and their DNA, releasing the cold thymidine which would then compete with the ³H-TdR added to assess proliferation. The non-thymidine nature of Mφ supernatant-mediated suppression was demonstrated by competition experiments (49) and by showing reductions in ³²P (311), ³H-uridine (128), and ³H-leucine (301) incorporation. In addition, Fernbach et al. correlated ³H-TdR incorporation with lymphoblast counts and showed a real abrogation of T cell proliferation upon exposure to Mφ supernatants (84). Activated adherent peritoneal Mφ also synthesize and release polyamine oxidase. In the presence of spermine formed by actively dividing lymphocytes, the oxidase generates products that inhibit lymphocyte proliferation. This inhibition is reversible, and is more dramatic in the presence of fetal calf serum (5).

Mφ inhibition of T cell proliferation has also been attributed to prostaglandins, which is more cogent to my research. PGE₂, in particular, is present in Mφ culture supernatants and exerts effects ranging from inhibition of Ia antigen expression on Mφ (254) to down modulation of IL 2 production (40-42) and transferrin receptor expression on T cells (42) and the G_{1a}-G_{1b} cell cycle transition of mitogen stimulated murine T cells. This cell cycle transition is IL 2-dependent (303). Prostaglandins inhibit the IL 2-induced proliferative response of cloned antigen-specific CTL (12) as well as the proliferative response of human lymphocytes (100) and plaque-forming antibody responses of rat spleen cells (163, 177). Moreover, PGE₂ inhibits the development of antigen-specific CTL (177) as well as natural killer cell activity (104). PGE₂ is known to elevate cyclic AMP levels.
in the IL 2-dependent CT6 T cell line. This accumulation can be blocked by the addition of IL 2, which also decreases the basal level of adenylate cyclase activity and stimulates protein kinase C activation and translocation across the membrane (13). This is manifested as a restoration of proliferation and generation of lymphokine-activated killer cell activity (76). This is one of the few instances where an enhancing mediator overcomes the effects of a suppressive mediator.

A host of other Mφ-derived inhibitors that are less rigorously defined than PGE₂ have been described. Calderon et al. (31) described a murine Mφ factor which inhibited DNA and protein synthesis that is heat-stable (boiling for 10 min), but dialyzable (1400 mw) and pronase sensitive. This factor is active on tumor cells and mitogen-stimulated spleen cells and is detected in Mφ culture supernatants within 24 hr in the absence of stimulation. Calderon et al. (32) noted that once this factor is removed by dialysis, a stimulatory molecule which is heat-labile and trypsin-resistant was found in fractions corresponding to 15,000-20,000 mw. Production of the stimulatory molecule was enhanced by phagocytosis, lending support to the idea of Mφ playing an early regulatory role in immune induction. The existence of enhancing and inhibitory factors in the same Mφ culture supernatant has also been reported by us (47, 49).

Larger mw Mφ-derived inhibitory factors have been demonstrated by Chen et al. (35), who found a heat-stable 110,000 mw factor that is pronase-sensitive but trypsin-resistant in murine Mφ culture supernatants. This factor could be cytotoxic as exposure for 48 hr resulted in cell death. The inhibitory factor shown by Toh (278) from normal and activated rat Mφ is 20,000-30,000 mw and sensitive to trypsin and pronase. Werb and Chin (308) showed resident and thioglycollate-elicited Mφ produced apolipoprotein E, a 33,000 mw glycosylated factor which inhibits lymphocyte proliferation. Kilbourn et al. showed that BCG-activated peritoneal Mφ produce an inhibitor of DNA synthesis and also complex I, II, and III of the mitochondrial electron transport system. These activities were associated with factors of 55,000, 80,000, and 150,000 mw (134).

Mφ-like cell lines are also used as sources of inhibitory monokines. Wilkins et al. (310) stimulated the human histiocytic lymphoma, U937, with Con A or PHA and produced an inhibitor of human peripheral blood lymphocyte proliferation. This inhibitor of DNA and protein synthesis is 65,000 mw by ACA 54 gel filtration, heat (56°C) and acid (pH 2) labile, noncytotoxic, and
reversible within 24 hr of removing the cells from the inhibitor. This inhibitor appears to be specific for lymphoid cells. This factor was subsequently shown to be neither IFN-α nor IFN-γ (309). Fujiwara et al. showed that the U937 cell line spontaneously produces an acid and base labile factor which inhibits IL 1 activity and IL 2 receptor expression, and is 67,000-130,000 mw (88, 89). Interference with the production and action of IL 1 and IL 2 has also been shown by Krakauer (140) in a factor from silica-treated THP-1 cells, a monocytic leukemia cell line. This 60,000-70,000 mw factor does not act on IL 2-independent cell lines, and is sensitive to heat, trypsin, chymotrypsin, and protease (140).

Mφ Cytotoxic Factors

The possibility exists that these Mφ-derived inhibitory factors are cytotoxic factors under the disguise of low doses. Kramer and Granger (141) reported that murine peritoneal Mφ from tumor-sensitized mice release a nonspecific cytotoxic factor upon exposure to allogeneic target cells in vitro. This toxin elutes from Sephadex G-100 in fractions corresponding to 47,000 and 150,000 mw and is pronase-sensitive and heat-stable (100°C, 15 min) (141). Reed and Lucas (227) showed rat peritoneal Mφ release a cytotoxic factor within 2 hr of adherence to tissue culture-treated plastic. Release of this 45,000 mw factor was spontaneous and dropped off after 48 hr, at which time its production could be stimulated by the addition of endotoxin. This is apparently the first study to correlate a soluble cytotoxic activity with in vitro Mφ-mediated killing.

Pincus showed production of a toxic factor from guinea pig Mφ in the absence of an inducing agent within 2 hr after adherence to plastic. This factor is stable to boiling for 1 hr, has an estimated mw of 1000, and is inactivated by acid phosphatase and phospholipase D, while pronase, lipase, and amylase had no effect. The possibility remains that this factor is a glycolipid or phospholipid (212, 213, 251).

The cytotoxins reported by Matthews (175, 176) appear to be identical to the Mφ-derived tumor necrosis factor (TNF) activity reported by Carswell et al. (33) and later by Mannel et al. (173).
TNF has since been found to be identical to cachectin, an endotoxin-induced monokine capable of suppressing lipoprotein lipase activity in cultured adipocytes (21), and the possibility has been raised that cachectin/TNF is lytic for tumor cells by selective suppression of protein synthesis (19). Cachectin/TNF purified from RAW 264.7 mouse Mφ-like cells is an 87,000 mw protein with a pI of 4.7 which dissociates into five subunits of approximately 17,000 mw each in the presence of SDS or 6 M urea (19). Other Mφ-derived cytotoxic factors have also been described. Drysdale et al. isolated a 45,000 mw cytotoxic factor from monocytes (57). Takeda et al. isolated a 55,000 mw cytotoxic factor from the N/P-7-1 mouse Mφ hybridoma clone which shows up at 17,000 mw on SDS-PAGE, has a pI of 5 and is trypsin-sensitive and labile at pH < 6.0 (270). Currie (52) showed that arginase released from activated Mφ is cytotoxic for tumor cells. IL 1 is even known to be cytotoxic for certain tumor cell lines, mainly the A375 human melanoma cell line (202), and interestingly, IL 1 suppresses lipoprotein lipase activity in cultured 3T3-L1 adipocytes, although at much higher doses and potentially via a different mechanism than TNF (20).

TNF augments the "hemorrhagic necrosis" of tumors by endotoxin (33) and is very similar to the cytostatic factors demonstrated by Nissen-Meyer and Hammerstrom (193), who reported that cultured human monocytes release a cytostatic activity upon in vitro activation with lymphokines and LPS. This activity is due to two factors (cytostatic factor I and II, or CstF I and CstF II) which can be separated on the basis of charge and size and inactivated with proteinase K. CstF I is a 55,000 mw factor with a pI of 5.3 which eluted in the flowthrough from Con A-Sepharose. CstF II is a 40,000 mw factor with a lower, but undetermined, pI, which is partially retained on a Con A-Sepharose column (193). TNF and CstF I and II all behave similarly on ion-exchange chromatography, bind poorly to Con A-Sepharose, have similar mw, and appear to have isoelectric points between 5.0 and 5.4 (175, 176, 193). The difference is that CstF does not have cytotoxic activity, although it may be cytolytic at high doses or in the microenvironment of the monocyte-target cell.
Immunosuppressive T Cell Factors

T suppressor cells develop as a natural consequence of T cell proliferation (228, 250) which could explain the generation of T suppressor cells _in vivo_ in our tumor model system (75, 77, 79). If mitogen is added to T cells, proliferation occurs. The interesting observation is that if these cells are then admixed with fresh T cells and fresh mitogen is added, the suppressor T cells which have developed will now prevent proliferation from occurring. While Mφ are required for the development of suppressor T cells (222), the activity of the suppressor T cells is attributed, at least partially, to an inhibitor of DNA synthesis (IDS) produced by the mitogen-stimulated T cells, which is a 20,000 mw glycoprotein with an acidic pI (123, 189). This factor raises the intracellular level of cyclic AMP (124) in target cells, as does PGE₂ (13). We have also described an inhibitor of DNA synthesis and DNA polymerase produced by tumor-induced T cells (75, 77).

Another contributor to this phenomena is PGE₂ which induces suppressor T cells (41) and stimulates T cells to produce a prostaglandin-induced T suppressor factor (PITS), which inhibits their proliferation (305). Malkovsky _et al._ (171) showed Lyt-2⁺ T cells from mice exposed to contact sensitizers release a nonspecific inhibitor of contact sensitivity and DNA synthesis when armed with T suppressor factor and triggered with antigen. This suppression is eliminated by adding exogenous IL 2 and absorbed out with either activated Lyt-1⁺ or Lyt-2⁺ lymphocytes. The T suppressor factor mentioned above is apparently many things to many people. The pattern that has emerged, however, is that T suppressor factors either bind antigen or idiotype, contain determinants encoded by the major histocompatibility complex, and are restricted in their activity to genes mapping to the MHC or Igh variable locus (discussed in Ferguson _et al._ 82). Ferguson _et al._ have developed a monoclonal antibody that recognizes the 68,000 mw T suppressor factor that is specific for T cells reactive against sheep red blood cells (81, 82). It is interesting that Lau _et al._ documented the presence of a suppressor activating factor, produced by a human T cell line, which induces the production of T suppressor factors (151). This induced production is itself inhibited by PGE₂, implying a possible feedback circuit (151).
Chiba et al. (36) showed that mitogen-stimulated rat spleen cells produce a 45,000 mw inhibitor of DNA synthesis that is separable from IFN-γ by Con A-Sepharose chromatography and is a trypsin-sensitive sugar-free lymphokine (36). Production of this factor is inhibited by indomethacin which indicates a regulatory role for prostaglandins, and the cell responsible is a suppressor T cell subset distinct from the producers of IL 2 and IFN-γ (37). This factor inhibits DNA synthesis in a species-unrestricted manner and thus inhibits the proliferation of a variety of normal and neoplastic cells. The mechanism of action was at least partly via inhibition of IL 2 production and action (38). T cells can also mediate their suppressive activities via Mφ. Aune et al. (8, 9) described a soluble immune response suppressor (SIRS) of 14,000 and 21,000 mw produced by mitogen-stimulated T cells but processed into an active form as a result of the peroxidase enzymes of Mφ.

Other Inhibitory Factors

There is another group of molecules which may or may not be produced by Mφ which are capable of suppressing T cell proliferation. This group includes the lipoproteins, glycoproteins, glycolipids, glycosphingolipids (GSL), and gangliosides, and is mentioned here because the high mw inhibitor found in our system bears some striking similarities to these factors and their activities. Numerous studies show the ability of GSL, some of which contain sialic acid (gangliosides), to inhibit lymphocyte proliferation during in vitro assays (174). The inhibitory molecules include (in increasing order of potency and carbohydrate complexity) GM₃, GM₂, GM₁, and GD₁a, all of which bind to IL 2 in vitro and thereby prevent IL 2-dependent proliferation (209). Lengle et al. (153) showed that gangliosides, in particular trisialoganglioside, are capable of complete inhibition of Con A-induced uridine, leucine, and thymidine incorporation by T cells, but not carbohydrate metabolism, cell viability, or Con A binding, even if added as late as 24-28 hr in a 48-hr assay. While maximum binding of gangliosides occurs within 30 min, no inhibition of proliferation occurs if unbound gangliosides are washed out after 4 hr, i.e., the gangliosides are either not internalized.
or have a reversible effect (153). Ladisch et al. (147) demonstrated gangliosides purified from YAC-1 lymphoma ascites fluid inhibit the proliferative response of murine splenocytes to Con A and to soluble and cellular antigens at doses similar to the levels required for purified gangliosides. The mechanism of inhibition by gangliosides appears to be by binding IL 2 (209) or by suppressing the influx of calcium after stimulation with Con A or the calcium ionophore A 23187 (143). It is useful to note that treatment of ganglioside preparations with trypsin or protease for 24 hr or boiling for 15 min does not reduce their inhibition of IL 2 activity (209), which corroborates our data discussed here on heat stability and lack of sensitivity to proteolytic enzymes. Robb (230) showed that ganglioside inhibition of proliferation can be divided into IL 2-reversible and IL 2-irreversible mechanisms, the latter of which are predominant during the initial stage of cellular activation, i.e., proliferation of mitogen-activated human peripheral blood lymphocytes. This is analogous to the data presented in this study. Offner and Vandenbark (199) noted that gangliosides (but not the parent galactocerebroside, ceramide, or sialic acid) can inhibit stimulation by guinea pig myelin basic protein of an encephalitogenic T helper cell line. Further work showed that GSL modulation is occurring via the CD4 molecule on human, rat, and mouse T lymphocytes in a rapid, dose-dependent and reversible manner.

The use of free gangliosides and GSL in in vitro assays has provided useful information on the biochemical basis of their suppression of proliferation, but raises questions as to their in vivo relevance (174). For instance, most GSL are found to be membrane-bound, and there appears to be very little sugar specificity of their action (30, 153, 209). This is important because tumor cells in general show more glycosylation (110), and their immune suppression may be due to shed membrane vesicles which are glyclosylated (174). Using sugar-protein conjugates and β-galactosidase, we have shown that there is some sugar specificity to the inhibitory activity. This link to a protein carrier may explain the large size associated with the inhibitory activity found in the Mφ supernatants. Wolf and Merler (314) showed a T cell-derived inhibitor of lymphocyte proliferation is actually a biologically active lipid bound to inactive proteins. Dialysis against hypotonic, but not isotonic, solutions causes separation of the low molecular weight lipid from its protein carrier. The reverse of this, that the inhibitory activity is vested in the protein portion of the lipoprotein, was
found by Curtiss and Edgington (53) who isolated a low density lipoprotein from human serum which inhibits the \textit{in vitro} proliferation of human T cells. The inhibitory molecule is $2-3 \times 10^6$ mw and 25% protein, 75% lipid. This factor does not inhibit lymphocytes at periods $> 19$ hr after stimulation and has no effect on the erythrocyte rosette function of lymphocytes. This factor is thus differentiated from an immunoregulatory serum lipoprotein which does not modulate the erythrocyte rosette function and is found in serum of patients with hepatitis B virus (39).

\textbf{Contribution of Tumors to Mφ-Mediated Immunoregulation}

So far, I have discussed Mφ-T cell interactions mainly from the perspective of the role of the Mφ in immunoregulation, cytostasis, and cytotoxicity. The contribution of tumor growth to Mφ-mediated immunoregulation is a complex issue. The central theme is that tumors somehow evade the host response directed against them, and TBH are often found to be severely immunosuppressed. Determining the mechanism of this immunosuppression is an ongoing endeavor that has centered on elucidating the tumor-induced changes in both the subpopulations of Mφ and T cells and the factors they use for communication and regulation.

Impairment of host immune responsiveness often accompanies the appearance of tumors. This impairment is not necessarily due to direct contact between the tumor and the immune system cells, as the tumor may remain isolated and yet the immune system becomes suppressed. One of the causes of this "long reach" of the tumor cells could be shed membrane vesicles (110, 174, 218, 219) or antigens which act to induce suppression or a form of tolerance (antigen-specific suppression). A second mechanism is the development of antibodies against tumor cells which block an effective response against the tumor (115). Another possible mechanism of action is for the tumor to elaborate soluble factors which could mediate a systemic immunosuppression. Katzmann showed plasmacytoma tumor cells release a high mw factor that causes the appearance of host Mφ which in turn release a suppressive factor with an apparent mw of 10,000 to 20,000 (131). Further work on the plasmacytoma model by Kennard and Zolla-Pazner showed that the hosts
bearing plasma cell tumors have an inability to mount a primary antibody response but their T cell proliferative responses were normal. They showed that the plasmacytoma cells produce a > 30,000 mw noncytotoxic factor which induces normal MΦ to produce a noncytotoxic factor of < 8,000 mw. This low mw factor suppresses primary antibody responses in vitro but has no effect on mitogen-induced T cell proliferation (132). This two-step mechanism is somewhat analogous to the mechanism delineated by Ting and Rodrigues (277) whereby tumor cells turn on splenic MΦ to render peritoneal MΦ suppressive. Ting and Rodrigues used two Friend virus-induced leukemias, one syngeneic and the other allogeneic to their hosts, and two chemically induced tumors, one induced with benzopyrene and the other with methylcholanthrene. The source or type of tumor has no effect on their interpretations (277). Plescia et al. (214) showed that four different immunosuppressive tumors (two spontaneous, two methylcholanthrene-induced) release PGE₂ which initiates PGE₂ production by MΦ, thereby rendering them immunosuppressive. Pope showed tumor cells release a low mw factor capable of activating suppressor T cells (217). Putnam and Roth (220) identified an immunosuppressive glycoprotein from the metastatic murine melanoma line K-1735. This glycoprotein is 10,000-12,000 mw, bound lentil lectin, is heat-stable, and acid and base labile. This factor suppresses splenocyte proliferation, protein synthesis, IL 2-mediated blastogenesis, and MLR-induced responses, but has no effect on cell viability (220). Roth et al. (239) reported that a potassium chloride extract of 18 different fresh human tumors yields a supernatant that is inhibitory to human peripheral blood lymphocytes in any number of proliferation assays and even the induction of cytotoxicity. Extracts of normal or fetal tissue have no effect. This inhibitory factor is heat-stable and resistant to papain, chymotrypsin, protease, and lipase, but is sensitive to trypsin and is bound by a lentil lectin column. Further investigation of an extract from a liposarcoma revealed a 70,000 mw glycoprotein with determinants that cross-reacts with antisera against HLA-DR and IgM μ chains (238). This factor causes reversible suppression of T cell proliferation and inhibits IL 2-induced proliferation (238). Hersey et al. showed that a culture supernatant from melanoma cells suppresses the induction of CTL by inhibiting the production of IL 2. This activity is found in fractions corresponding to 44,000 and 7,000 mw, and has no effect on IL 1 production or on the action of preformed IL 2 (117). Ebert et al. (63) dem-
onstrated that the colon cancer line HT29 produces a factor that blocks mitogen-induced T cell proliferation and the production of IL 2. The effect is reversible and is seen even if the factor is added late in the assay, indicating that it affects cell division after activation of T cells has occurred. Since IL 2 does not reverse the inhibition, and the factor does not affect IL 2-induced proliferation, the mechanism of action appears to be IL 2-independent. The factor has an apparent mw of 56,000 and is sensitive to heat and endopeptidases (63).

The above studies implicate tumor cells as producing factors which are directly responsible for the immunosuppression seen in TBH. There is also a large body of literature which deals with the indirect effects of tumors. Goodwin et al. described Mφ-dependent, PGE₂-mediated immunosuppression in some patients with sarcoidosis (102). Patients cells synthesize almost twice as much PGE₂ as do cells from normal individuals, however addition of indomethacin to patient's cells increases the mitogen response to only 40% of normal, while removal of glass adherent cells results in a response which is 76% of normal. Goodwin et al. have also demonstrated that peripheral blood mononuclear cells from patients with Hodgkin's lymphoma synthesize four times as much PGE₂ when compared to cells from normal individuals (103). They went on to show that when Hodgkin's lymphoma patient’s cells were tested for PHA reactivity in the presence of indomethacin, their reactivity increases 180% and could no longer be distinguished from controls. This is analogous to data presented here. The analogy between our tumor model and Hodgkin's lymphoma is also supported by the work of Nagai et al. (188) who showed, as we did (91), a loss of Ia antigen expression on cells from individuals bearing tumors.

Analysis of the immune cells of animals with tumors has shown the existence of non-neoplastic suppressor cells which are usually resolved as Mφ (137). The induction of these suppressor cells by tumor cells and their products has already been discussed (131, 132, 214, 277). While tumor growth can induce or manifest the activity of suppressor cells, for immune homeostasis to be a relevant idea the suppressor cells must pre-exist the tumor, but be under control. This falls into place nicely with the idea of immune surveillance, which holds that cells (Mφ) patrol the body and rid it of unwanted or aberrant cells (tumor cells). Unfortunately, in the presence of tumors, immune surveillance has failed and the immune system proliferative responses are
suppressed. Suppressor cells are simply more identifiable, then, in the presence of tumors or immunosuppression in general.

Dr. Elgert’s laboratory has approached this problem from the perspective of the response of the host cells that results in tumor-induced immunosuppression. An early paper by Kirchner et al. showed that suppressor cells (Mφ) exist in the spleens of mice bearing primary Moloney sarcoma virus induced tumors (136). One of the salient contributions of our laboratory was the demonstration that immunosuppression in our model system was due to the presence of both inhibitory Mφ and suppressor T cells acting in concert (50, 66). This observation was corroborated by Pope et al. (216). The model system used in our laboratory is a methylcholanthrene-induced transplantable non-metastatic fibrosarcoma. The tumor cells are routinely passaged by intramuscular injection resulting in a palpable tumor, which is then followed by splenomegaly (4-5 fold increase in spleen size) and an increase in both the number of splenic Mφ and their relative percentage (increase from 5% to 22%) (66). While others have reported that this increase in Mφ numbers is in itself immunosuppressive, and that the immunosuppression was due to a quantitative change in the Mφ from TBH (306), we have shown that there is a qualitative change in the suppressor Mφ population, with the suppressor Mφ shifting from Mac-3+ in the normal host to Mac-2+ in the TBH (93). We have also shown a decrease in the number of Ia+ Mφ as the tumor develops (91), which corresponds with an increase in PGE₂ production by TBH Mφ (55, 121, 146, 211) and a lessened ability of TBH responder T cell preparations to respond to the anti-proliferative effects of PGE₂ (55). Other alterations in T cells that occur with tumor growth are a drop in IL 2 production (28) and IL 3 production (26, 27), both of which can be prevented by injection of Corynebacterium parvum concomitant with the tumor (231). This drop in immune-amplifying interleukins is either the cause or effect of the development of a clone of tumor-induced suppressor T cells which have been shown to produce an inhibitor of DNA synthesis and DNA polymerase (75, 77). This T suppressor cell-derived inhibitor also inhibits the response of B cells to LPS, a T-independent antigen (79), indicating that, in this system, the factor could work directly on B cells without requiring an intermediate T cell. The proliferative response to LPS was also inhibited by Mφ but not by Mφ factors (79). TBH T cells have been shown in our hands to be anergic to stimulation
by alloantigens (64, 67), mitogens (50, 66, 74), and in delayed-type hypersensitivity assays (65, 92). To this end, we have shown, using antibody plus complement depletion of T cell populations, that there is a suppressor T cell phenotype in TBH spleens whose proliferation is enhanced by the addition of an inhibitory Mφ supernatant derived from normal host Mφ (170). This lends credence to the idea of different T cell populations in normal vs. TBH spleens and to differences in normal vs. TBH Mφ factors. This is in agreement with and in fact extends the work of Goldyne (98) who showed that Mφ could be separated on bovine serum albumin buoyant density gradients into populations that secrete various levels of PGE₂. Human T cells, separated the same way, were shown to exhibit varying levels of responses to PGE₂ in the presence of PHA. I have extended this by showing that tumor growth altered the normal balance between these populations and factors, tipping the balance in favor of immunosuppression.
Chapter I

TUMOR-INDUCED VARIABLE GLYCOSYLATION OF A HIGH MOLECULAR WEIGHT INHIBITORY MONOKINE(S)

Introduction

The immune system is a finely tuned homeostatic mechanism whose cells constantly amplify and suppress each other in a dynamic equilibrium. In this context, antigens or tumors disrupt the equilibrium, resulting in a measurable response. Tumor growth suppresses the immune response (4, 66, 87, 216). This immunosuppression is manifested as a loss in T cell proliferative ability (66, 265, 315). The suppression is due to suppressor T cells (47, 109), or their factors (77, 262), low levels of immune-amplifying interleukins such as interleukin 2 (IL 2) (224, 276) or their receptors (253), inhibitory macrophages (Mφ) (4, 50, 136, 216), and/or an increase in immunosuppressive molecules such as prostaglandin E₂ (PGE₂) (55, 94, 211) or the suppressive factors they induce (214, 234).
Mφ play a pivotal role in the immune system as both effector and regulatory cells (197, 286). Mφ regulatory functions are often mediated by soluble factors, or monokines, which serve as immunoregulatory signals between Mφ and between Mφ and other immune cells. Evidence for the maintenance of homeostasis by Mφ comes from the natural presence of suppressor Mφ (306, 311) and spontaneous production of inhibitory monokines (88, 89), PGE$_2$ (94, 98), and immune-amplifying signals such as interleukin 1 (IL 1) (56). Using a methylcholanthrene-induced fibrosarcoma as a model system, we reported that peritoneal Mφ from normal and tumor-bearing hosts (TBH) and their supernatants suppress mitogen- (66) and alloantigen-induced (64) proliferation. Although PGE$_2$ is a contributing factor to this inhibitory activity, it is not the sole factor. Adding exogenous PGE$_2$ to the mixed lymphocyte reaction (MLR) showed that physiological levels of PGE$_2$ could only account for 38% of the inhibitory activity seen with whole Mφ supernatants (55). The remainder of the inhibitory activity is due to a nondialyzable, ammonium sulfate precipitable, heat-stable factor (48), which can be generated in the presence of indomethacin (51). Since both PGE$_2$ and the inhibitory monokine are heat-stable Mφ products which inhibit lymphocyte proliferation, separation of the two factors was critical to determining their contribution to Mφ-mediated immunosuppression.

We have reported a tumor-induced increase in the number of Mφ (66, 50) and a tumor-induced qualitative change in Mφ phenotypes and their production of immunoregulatory factors (93, 169). We report here that there also was a qualitative difference between normal and TBH Mφ-derived soluble factors. We demonstrated a high molecular weight (mw) molecule(s) that was concentrated on a 30 kilodalton (kd) exclusion ultrafiltration membrane, and that this preparation had heat-stable inhibitory activity and yet was PGE$_2$-free as determined by radioimmunoassay. Inhibitory activity was found in fractions from S-200 Sephacryl chromatography corresponding to > 67 kd. Concentrated normal and TBH Mφ supernatants were fractionated on DEAE-Cellulose, and the inhibitory activity was found to have a pI of < 7.7. Isoelectric focusing (IEF) of normal and TBH Mφ supernatants localized inhibitory activities at pI 6.5-7.6 and 4.0-6.0, respectively. Hydroxylapatite was also useful in fractionating inhibitory activity and showed a difference between normal and TBH supernatants. Proteolytic enzymes were used to determine the biochemical nature
of the inhibitory activity, and while the molecule may be a protein, its active portion was not necessarily protein in nature. There was no reduction in inhibitory activity of TBH Mφ supernatants following proteolytic enzyme treatments. The TBH, but not the normal host, Mφ supernatant inhibitory activity was sensitive to neuraminidase. This suggested either the presence of an additional inhibitory molecule in the TBH Mφ supernatants that was absent in the normal host Mφ supernatants, or the factor underwent variable glycosylation (addition of sialic acid) as the tumor developed. Concentrated normal and TBH Mφ factors demonstrated a similar time course of activity in the MLR, and possibly required the presence of PGE₂ for their inhibitory activity to be manifested, at least in the first 48 hr of the MLR. Analysis of the effect of Mφ supernatants on the generation of IL 2 in the MLR revealed a differential dose- and time-dependent inhibitory activity for normal and TBH Mφ supernatants. Cell cycle analysis of mitogen-stimulated cells that were treated with Mφ supernatants at time of initiation revealed that TBH Mφ supernatants caused an early enhancement of cell cycle progression, but both normal and TBH Mφ supernatants caused a decrease in the percentage of cells in S phase when measured at 66 hr. Taken together, normal and TBH Mφ produce a high mw inhibitory monokine which is distinct from PGE₂ and from each other. The data is discussed in terms of the influence of tumor growth on Mφ-derived immunoregulatory factors.
Materials and Methods

Mice

Male BALB/c mice were obtained at 4-6 wk of age from either Dominion Laboratories, Dublin, VA, or the Virginia-Maryland Regional College of Veterinary Medicine Vivarium, Blacksburg, VA. Male C3H/He mice were obtained at 4-6 wk of age from Dominion Laboratories. Mice were injected intramuscularly in the left hind leg with a single-cell suspension of a methylcholanthrene-induced transplantable nonmetastatic fibrosarcoma (66). Normal and TBH mice were tested for LDH virus by Microbiological Assoc., Bethesda, MD, and were found to be negative.

Medium

RPMI 1640 (Flow Laboratories, McLean, VA, or Hazleton Dutchland, Denver, PA) supplemented with 50 µg/ml gentamicin (GIBCO, Grand Island, NY), 2 gm/L sodium bicarbonate (Fisher Scientific), and 25 mM HEPES (Sigma, St. Louis, MO) was filter sterilized and used in all cell culture and dialysis experiments. Fetal Bovine Sera (FBS; Flow Laboratories or Hazleton) was used in biological assays; RPMI medium supplemented with FBS and $4 \times 10^{-5} M$ 2-mercaptoethanol is called complete medium.
MΦ and MΦ Supernatants

MΦ were obtained from single cell suspensions of spleens or from mice intraperitoneally injected with 2.0 ml of sterile thioglycollate at Day-0 (normal) or at Day-17 of tumor development (TBH), and peritoneally lavaged 4 days later with cold RPMI medium. Cells were plated on 150 mm tissue culture-treated dishes for at least 2 hr at 37°C in a 5% CO₂ incubator, nonadherent cells rinsed off, and adherent cells scraped and resuspended to 4 x 10⁶/ml. Cells were then replated on plastic dishes, and incubated at 37°C. Four days after peritoneal lavage, the serum-free supernatants were harvested, centrifuged at 800 x g for 5 min to remove cells, and the remaining supernatant centrifuged at 10,000 x g for 20 min, and frozen for later concentration on a stirred cell ultrafiltration device (Model 52, Amicon Corp., Lexington, MA) under Nitrogen pressure (40 psi) using a YM30 30,000 mw exclusion, low protein binding membrane. Concentrated samples were washed with five 40 ml volumes of RPMI medium in the stirred cell to completely remove low mw compounds. Supernatants were concentrated 10- to 100-fold and sterile filtered on Millex GV 0.22 μm syringe filters (Millipore Corp., Bedford, Ma.) before use in biological activity assays.

PGE₂ Analysis

Briefly, PGE₂ levels in MΦ supernatants and in MLR supernatants were determined using a competitive radioimmunoassay using dextran-coated charcoal, tritiated-PGE₂ standards and specific rabbit anti-PGE₂, performed as previously described (2). This assay was sensitive to 5 pg/0.1 ml.
Molecular Weight Determination

Concentrated supernatants were applied to a 1 x 85 cm Sephacryl S-200 column (Pharmacia) equilibrated in 0.005 M sodium phosphate, pH 7.5, with 0.15 M NaCl. The column was calibrated with blue dextran, rabbit muscle aldolase (161 kd), bovine serum albumin (67 kd), ovalbumin (42 kd), chymotrypsinogen A (25 kd), lysozyme (14 kd) and myosin (16 kd). Individual (normal host) or pooled (TBH) Mφ supernatant fractions (0.8 ml) were dialyzed against RPMI medium in BSA-coated 12,000-14,000 mw cutoff dialysis bags (Spectrapor 4) and filter sterilized before use in the MLR.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sample heterogeneity was assessed by SDS-PAGE on 10% polyacrylamide resolving gels using a 3% stacking gel under reducing and denaturing conditions in Laemmli buffers (148). Samples and mw markers were boiled in sample buffer containing SDS and 2-mercaptoethanol for 2 min before application on the gel. Electrophoresis was performed at a constant current of 30 milliamps. Staining was accomplished with the silver stain method of Merril (180).

Enzymatic Sensitivity

A panel of enzymes was used to determine the biochemical classification of the inhibitory activity. Concentrated TBH splenic Mφ culture supernatants or RPMI medium were treated with enzymes at 1 mg/ml for 3 hr at 37°C. The enzymes used were lactate dehydrogenase, protease from Streptomyces griseus, trypsin, lysozyme, elastase, peptidase, protease from Bacillus subtilis, pronase, papain, and α-chymotrypsin, all purchased from Sigma Chemicals. All treatment groups were
boiled for 10 min before addition to the MLR, and added at 50 µl/well at time of initiation of the MLR. In a separate experiment, phospholipase A₂, ribonuclease, trypsin, and neuraminidase linked to agarose (Sigma) were incubated with TBH Mφ supernatants for 3 hr at 37°C, centrifuged, and the treated supernatants added to the MLR.

DEAE-Cellulose Chromatography

DEAE Cellulose was used in a batch method to separate Mφ supernatant factors on the basis of charge. DEAE was equilibrated in 0.005 M sodium phosphate buffer at either pH 7.0 or 7.7. Concentrated Mφ supernatants were dialyzed in 12-14,000 mw cutoff BSA-coated dialysis bags against the same buffer and applied to the DEAE. Elution was accomplished with a step gradient of 0.005 (equilibration buffer) to 0.5 M sodium phosphate of the same pH. Fractions were dialyzed against RPMI medium in BSA-coated dialysis bags and sterile filtered for use in biological assays.

Flatbed Isoelectric Focusing

IEF was performed as reported earlier by us (55). Briefly, concentrated normal and TBH Mφ supernatants were applied to Sephadex IEF (Sigma) containing ampholytes (Serva Fine Chemicals, Garden City Park, NY) in the pH range of 3-10. Focusing was performed on a glass plate (115 x 230 mm) with the gel thickness being 1 mm. Ethylenediamine (1.0 M) and sulfuric acid (0.1 M) were the anode and cathode solutions, respectively. The gel was electrofocused at 30 W for 2 hr across the shorter dimension, then cut into 15 equal fractions, each of which was placed into 1.0 ml RPMI medium. After standing for 2 hr at 4°C, fractions were filtered to remove the gel, dialyzed, sterile filtered, and tested for activity in the MLR.
Hydroxylapatite Fractionation

Hydroxylapatite (DNA grade, Biogel HP, Bio-Rad Laboratories, Rockville Centre, NY) was equilibrated in 0.05 M sodium phosphate buffer, pH 7.3, and used in a batch method in a Bio-Rad econo-column with a fritted disc. One inch height of matrix was packed tightly by aspiration with a syringe. One ml of concentrated sample or RPMI medium was applied to the column, aspirated through and eluted with a step gradient of 2 ml of 0.05, and one ml of 0.3, 0.6, and 0.9 M sodium phosphate buffer, pH 7.3. All fractions were subsequently dialyzed against RPMI medium in BSA-coated bags before use in biological activity assays.

Mixed Lymphocyte Reaction

Biological activity of Mrp supernatants was assessed in a Mrp-depleted BALB/c anti-C3H one-way MLR. A single cell suspension of BALB/c (H-2d) splenic responder cells were fractionated by nylon wool columns (126). Nonadherent T cells were eluted with warm RPMI medium and placed on a Sephadex G-10 (Sigma) and glass bead column and also incubated for 30 min. Cells were eluted and plated on 150 mm tissue culture dishes (Hazleton Dutchland) for 1 hr at 37°C. Nonadherent cells were washed off and resuspended to 2 x 10^5 viable cells/50 μl and dispensed in 96-well microculture plates (Flow Laboratories). C3H/He (H-2b) splenic stimulator cells were treated in a similar fashion, but were treated with 25 μg mitomycin-C (Sigma) per 10^7 cells for 20 min at 37°C, and washed 4 times. Alternatively, stimulator cells were X-irradiated for 4 min resulting in a dose of 2400 rads. Stimulator cells were dispensed at 4 x 10^5 viable cells/50 μl/well. Responder and stimulator cells were found to contain <0.5% esterase positive cells. Supernatants or column fractions were added to the MLR at time of initiation. In one experiment cited, responder cells from normal and TBH mice were used, and indomethacin (Sigma, first solubilized at 1 x 10^-2 M in ethanol) was titrated from 1 x 10^-4 M to 1 x 10^-9 M (final concentration, 100
μl/well added at time of initiation). In the time-course addition experiment, supernatants, PGE₂ (Sigma), indomethacin, or RPMI medium were added to the MLR at various times after initiation of the reaction. Constant well volume of 200 μl was maintained, and indomethacin was used at a final concentration of 1 x 10⁻⁷ M. Assays were incubated for 90 hr at 37°C, then pulsed for 6 hr with 1 μCi/well of tritiated thymidine (³H-TdR) (Amersham Corp., Arlington Heights, IL), harvested onto glass fiber filters (Whatman 934-AH), and counted in a Beckman LS230 liquid scintillation counter. Counts are expressed as percentage of control (responder plus stimulator cells with no supernatant addition).

Cell Lines

P388D₁ macrophage-like cells, BW 5147.3 lymphoma cells, and A4A T helper cells were cultured in vitro in complete medium in 75 cm² tissue culture flasks. Cells were routinely split to maintain viability and subcultured at approximately 1 x 10⁵/ml each time. Cell lines were used to assess the inhibitory properties of Mφ culture supernatants. For proliferation assays, cells were cultured in 96-well flat bottom tissue culture dishes at 1 x 10⁵/ml. Supernatants to be tested were added at time of initiation of the cultures, and the cultures incubated at 37°C for 42 hr. Cultures were pulsed, harvested, and counted as described above for the MLR.

Determination of IL 2 Levels

The effect of concentrated Mφ supernatants on the generation of IL 2 in the MLR was determined by aspirating the contents of the well at specified times, and removing the cells by centrifugation. Supernatants derived from Mφ or from MLR-stimulated cells were then tested for IL 2 activity using IL 2-dependent CT-6 cells. A programmable HP-97 calculator was used to calculate the IL 2 units according to the following formula as previously described (29):
\[ IL_2 \text{ units/ml} = \frac{\text{Sample dilution } 50\% \text{ maximum cpm}}{\text{Standard dilution } 50\% \text{ maximum cpm}} \times 100 \]

Mφ supernatants contained no IL 2 activity. Supernatants from cultures of responder plus stimulator cells (maximum proliferation, no Mφ supernatant addition) were assigned a value of 100 units of IL 2 activity.

**Mitogen-Induced Proliferation and Cell Cycle Analysis**

Biological activity of Mφ supernatants was also assessed using concanavalin A (Con A)-induced proliferation. BALB/c spleen cells were Mφ-depleted as described above for the MLR. All admixtures were performed at time of initiation of the assay. Con A was used at a final concentration of 0.8 µg/well in flat-bottom microculture dishes. Proliferation was assessed with a 6-hr pulse of \(^3\)H-TdR starting 66 hr after initiation, and the assay was harvested at 72 hr. Quadruplicate wells were also harvested for cell cycle analysis by aspirating the well contents at 42 and 66 hr, centrifuging and aspirating the supernatants, resuspending the cell pellets in 100 µl of RPMI medium, and then adding 100 µl of ice-cold methanol. Cell suspensions were then treated with 20 µl (15 units) of ribonuclease A (Sigma, Type II A, bovine pancreas, previously boiled to remove any contaminating DNase) for 1 hr at room temperature. Samples were subsequently stained with propidium iodide in 0.1 M sodium citrate and analyzed on an EPICS V flow cytometer (142). Appropriate gating was performed for one parameter analysis of cell cycle position (so G₂ peak position corresponds to twice the ploidy of the G₀ + G₁ peak position).
Results

Levels of PGE₂ in Mφ Supernatants

PGE₂ levels were determined by radioimmunoassay and are shown in Table 1. TBH Mφ produced more PGE₂ than their normal counterparts. After concentration using an Amicon ultrafiltration stirred cell with a YM30 30,000 mw exclusion membrane, PGE₂ was present only at background levels of detection (detection limit of the assay was 5 pg/0.1 ml). These PGE₂-free concentrated supernatants were used in all subsequent experiments. While PGE₂ levels were consistently reduced to background levels, MLR inhibitory activity was still found in the concentrated samples.

Gel Filtration Fractionation of the Inhibitory Factor

Figure 1 illustrates the elution profile of a 100-fold concentrated peritoneal Mφ culture supernatant applied to a Sephacryl S-200 column. In Fig.1, fractionation of normal host Mφ supernatant, even-numbered fractions were scanned for protein content (absorbance at 280 nm) in a spectrophotometer while odd-numbered fractions were dialyzed to RPMI medium before use in the MLR. In Figure 2, fractionation of TBH Mφ supernatants, all fractions were scanned at 280 nm, and adjacent fractions pooled before dialysis. In both cases, the peak of inhibitory activity is found in fractions corresponding to > 67 kd. This agrees with previous data on the nondialyzability and non-prostaglandin nature of the inhibitory factor (48, 51). Figure 3 is a silver-stained SDS polyacrylamide gel of the odd numbered fractions corresponding to the peak of inhibitory activity from the S-200 column fractionation of concentrated normal peritoneal Mφ supernatants. The inhibitory fractions have several high mw bands corresponding to > 67 kd.
Table 1. Levels of PGE$_2$ in Mφ Supernatants

<table>
<thead>
<tr>
<th>Supernatant Source</th>
<th>Original Pool$^2$</th>
<th>20 X Concentrate$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Splenic</td>
<td>20.2</td>
<td>0</td>
</tr>
<tr>
<td>Normal Peritoneal</td>
<td>37.6</td>
<td>5.2</td>
</tr>
<tr>
<td>TBH Splenic</td>
<td>33.4</td>
<td>3.5</td>
</tr>
<tr>
<td>TBH Peritoneal</td>
<td>41.3</td>
<td>0</td>
</tr>
</tbody>
</table>

$^1$PGE$_2$ was determined by radioimmunoassay. Levels are reported in pg/0.1 ml of supernatant. Background detection limit for the assay is 5 pg/0.1 ml.

$^2$Pools were frozen pooled samples, approximately 200 ml, from which the concentrates were made.

$^3$20 X Concentrates were generated by Amicon stirred cell ultrafiltration using a YM30 30,000 mw exclusion membrane.
Figure 1. Gel filtration chromatography of normal host Mφ supernatants: One ml of 100-fold concentrated normal host peritoneal Mφ supernatant was applied to a Sephacryl S-200 column (1 x 85 cm) and 0.8 ml fractions were collected. Absorbance at 280 nm for even numbered fractions (•-•) and MLR inhibitory activity of odd numbered fractions (○-○) were determined after chromatography of normal host Mφ supernatant. The column was calibrated using blue dextran (Vo), rabbit muscle aldolase (R, 161 kd), bovine serum albumin (B, 67 kd), chick ovalbumin (O, 42 kd), chymotrypsinogen A (C, 25 kd), and lysozyme (L, 14 kd). All fractions were dialyzed to RPMI medium and sterile filtered before use in the MLR.
Figure 2. Gel filtration chromatography of TBH Mφ supernatants: One ml of 100-fold concentrated TBH peritoneal Mφ supernatant was applied to a Sephacryl S-200 column (1 x 85 cm) and 0.8 ml fractions were collected. Absorbance at 280 nm was determined for all fractions (•--•) and MLR inhibitory activity of pools of adjacent fractions (o--o) were determined after chromatography of TBH Mφ supernatant. The column was calibrated using blue dextran (Vo), bovine serum albumin (B, 67 kd), and myosin (M, 16kd). All fractions were dialyzed to RPMI medium and sterile filtered before use in the MLR.
Figure 3. SDS-PAGE of inhibitory fractions from gel filtration chromatography of normal host Mφ supernatants: Proteins in odd-numbered fractions corresponding to the peak of MLR inhibitory activity from the Sephacryl S-200 column were resolved on 10% polyacrylamide gels. Lanes 1 and 8 contain mw standards (BSA, ovalbumin, chymotrypsinogen A, and lysozyme). Lanes 2 to 7 contain column fractions 41, 43, 45, 47, 49, and 51, respectively. Arrows indicate high mw proteins whose concentrations rise and fall in parallel with inhibitor activity.


Enzymatic Sensitivity

A panel of enzymes was used to determine the biochemical nature of the inhibitory activity. Figure 4 shows that the inhibitory activity of concentrated TBH splenic Mφ culture supernatants was not sensitive to boiling nor was it sensitive to any of the enzymes used. Control experiments showed that the enzymes, boiled before addition to the MLR, had no direct inhibitory activity, and in fact were enhancing in most cases. Since boiling does not affect the activity of some enzymes, we also performed an experiment using agarose-linked enzymes which could be centrifuged out of the preparation after the incubation period. When agarose-linked enzymes were used, the boiling step was omitted. Figure 5 demonstrates that neuraminidase was the only enzyme which removed the inhibitory activity. This effect was not seen using normal Mφ supernatants.

DEAE-Cellulose Fractionation of Concentrated Supernatants

DEAE-Cellulose was used in an effort to purify the inhibitory activity from the concentrated supernatants. Using DEAE-Cellulose at pH 7.7, all inhibitory activity in normal or TBH concentrated Mφ supernatants was firmly bound, and was not eluted even with the use of 0.5 M phosphate, as shown in Figure 6. Figure 7 shows that at pH 7.0, DEAE-Cellulose fractionation of normal host Mφ supernatants resulted in MLR inhibitory activity both in the flowthrough and fractions eluted with higher molarity phosphate buffers.

Determination of pI

DEAE-Cellulose chromatography revealed that the inhibitory activity was vested in a charged molecule which irreversibly bound to the DEAE at pH 7.7 and eluted at high salt concentrations.
**Figure 4.** Enzymatic sensitivity of TBH supernatants inhibitory activity: Concentrated TBH splenic Mφ supernatant was added to the MLR directly (TBH) or after boiling for 10 min (BOIL). Enzymes were used at 1 mg/ml, and all treated samples were incubated for 3 hr at 37°C. All samples were boiled for 10 min before addition to the MLR at time of initiation. Enzymes used were lactate dehydrogenase (LD), protease from *Streptomyces griseus* (P-S), trypsin (TR), lysozyme (LY), elastase (EL), pepsin (PE), protease from *Bacillus subtilis* (P-B), pronase (PR), papain (PA), and α-chymotrypsin (CH).
Figure 5. Enzymatic sensitivity using agarose-linked enzymes: TBH splenic Mφ supernatants were treated with phospholipase A$_2$ (PA2), ribonuclease (RIBO), neuraminidase (NEURA), and trypsin (TRYP), all of which were linked to agarose. Supernatants were treated for 3 hr at 37°C, centrifuged to remove the agarose-linked enzymes, and added to the MLR at time of initiation.
Figure 6. DEAE-Cellulose fractionation at pH 7.7: DEAE-Cellulose fractionation of normal and TBH peritoneal Mφ concentrated (20-fold and 10-fold, respectively) supernatants was done at pH 7.7. Whole supernatants (A) were concentrated (B) and dialyzed to 0.005 M phosphate buffer, pH 7.7 (C) and applied to the DEAE-Cellulose equilibrated in the same buffer. D represents the column flowthrough and E through I represent a step gradient from 0.005 to 0.5 M sodium phosphate buffer. Separation was performed via a batch method to maintain constant volumes. Samples C-I were dialyzed to RPMI medium and sterile filtered before addition to the MLR at time of initiation.
Figure 7. DEAE-Cellulose fractionation at pH 7.0: DEAE-Cellulose fractionation of normal host peritoneal Mφ concentrated supernatants was done at pH 7.0. Supernatants were concentrated 20-fold and equilibrated to 0.005 M phosphate buffer, pH 7.0, and applied to the DEAE-Cellulose. Separation was performed via a batch method to maintain constant volumes. Fractions were eluted with increasing ionic strengths of sodium phosphate buffer. The 0.1 M fraction was found to be lacking bands of 43, 55, 76, 82, and 87 kd by 10% SDS-PAGE. A = normal host Mφ supernatant, concentrated 20-fold. B = 20-fold concentrated supernatant dialyzed to 0.005 M buffer. C = DEAE flowthrough. D-H = elution with 0.005, 0.01, 0.05, 0.1, and 0.5 M sodium phosphate buffer, respectively. B-H were dialyzed back to RPMI before use in the MLR. Control is the allogeneic response of normal splenic T cells in the MLR.
at pH 7.0. Isoelectric focusing experiments were then done to determine the pI of the factor. Table 2 illustrates the MLR reactivity of culture supernatants from normal and TBH Mφ. Total protein was determined using a Biorad protein assay with BSA as the standard and normalized so that each supernatant had equal protein levels; 40 μl (100 μg) was applied to the IEF gel. Inhibitory activity was seen in fractions from IEF corresponding to pH 6.5-7.6 with normal host Mφ supernatants, and pH 4.0-6.0 for TBH Mφ supernatants. A less dramatic area of inhibitory activity is found in TBH Mφ supernatants around neutrality, and an area of MLR enhancing activity is seen in fractions corresponding to a pI of approximately 6. The presence of enhancing and inhibitory fractions in the same supernatant agrees with previous data and was not pursued (51).

**Hydroxylapatite Fractionation of Inhibitory Activity**

Hydroxylapatite was used to further characterize and purify the inhibitory factor. Figure 8 shows that inhibitory activity was found in the normal Mφ supernatant fraction eluted with 0.6 and 0.9 M phosphate. Figure 9 shows that inhibitory activity of TBH Mφ supernatants eluted predominantly in the 0.3 M phosphate fraction, and that inhibitory activity was also found in the 0.6 and 0.9 M phosphate fractions. Total protein content, as measured by a Biorad protein assay with BSA as a standard, was approximately 1 mg/ml in the concentrated samples but decreased to < 100 μg/ml in the inhibitory fractions. All fractions were dialyzed to RPMI medium before testing in the MLR.

**Effect of the High mw Inhibitory Monokine on Proliferation of Cell Lines**

Concentrated Mφ supernatants (PGE₂-free) were added to cell line proliferation assays at time of initiation. Figure 10 shows that P388D₁ Mφ-like cells and A4A T helper cells were sensitive to the inhibitory effects of the high mw monokine. The A4A cells showed a differential response
Table 2. Regulation of Normal Host T Cell MLR Reactivity by IEF Fractions from Mφ Supernatants

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Normal</th>
<th>pI</th>
<th>TBH</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLR Control</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Whole Mφ Supernatant^2</td>
<td>17</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Concentrated Mφ Supernatant^2</td>
<td>0.3</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>Mφ Supernatant Filtrate^2</td>
<td>16</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>IEF Fraction #</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>77</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>74</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>70</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>65</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>60</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>50</td>
<td>6.5 - 7.6</td>
<td>73</td>
</tr>
<tr>
<td>7</td>
<td>53</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>68</td>
<td>75</td>
<td></td>
</tr>
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<td>9</td>
<td>67</td>
<td>103</td>
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<td>10</td>
<td>133</td>
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<td>11</td>
<td>126</td>
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<td>12</td>
<td>159</td>
<td>56</td>
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<tr>
<td>13</td>
<td>86</td>
<td>4.0 - 6.0</td>
<td>56</td>
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<td>14</td>
<td>94</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>83</td>
<td>57</td>
<td></td>
</tr>
</tbody>
</table>

^1In the absence of Mφ supernatant, MLR reactivity is represented as 100% activity. Results are expressed as a percentage of control cpm when supernatant fractions were added to the MLR.

^2Peritoneal Mφ from normal and TBH were cultured in serum-free medium for 4 days at 4 x 10^6 cells/ml. Total protein was determined and normalized to 2.5 μg/μl. An aliquot of unconcentrated supernatant was tested in the MLR and the remainder concentrated 100-fold by ultrafiltration; 40 μl (100 μg) was used in the IEF. Concentrated supernatants, filtrates, and fractions were tested for MLR reactivity.

^3Brackets indicate the major inhibitory fractions in the MLR.
Figure 8. Hydroxylapatite fractionation of normal host peritoneal Mφ supernatants: Hydroxylapatite fractionation of normal host peritoneal Mφ supernatant inhibitory activity. One ml of concentrated supernatant was applied to a hydroxylapatite column equilibrated in 0.05 M phosphate buffer, pH 7.3. Fractions were dialyzed to RPMI, sterile filtered, and added to the MLR at time of initiation. Data presented are the column flowthrough (FT) and the 0.05, 0.3, 0.6, and 0.9 M phosphate elution fractions.
Figure 9. Hydroxylapatite fractionation of TBH peritoneal Mφ supernatants: Hydroxylapatite fractionation of TBH peritoneal Mφ supernatant inhibitory activity. One ml of concentrated supernatant was applied to a hydroxylapatite column equilibrated in 0.05 M phosphate buffer, pH 7.3. Fractions were dialyzed to RPMI, sterile filtered, and added to the MLR at time of initiation. Data presented are the column flowthrough (FT) and the 0.05, 0.3, 0.6, and 0.9 M phosphate elution fractions.
to normal and TBH Mφ supernatants. Proliferation of BW 5147.3 lymphoma cells was enhanced. Neither the A4A or the BW 5147.3 cells were inhibited by 50 nM of PGE₂, a relatively high dose. PGE₂ was not added to P388D₁ cells as these cells produce PGE₂ constitutively.

**Titration of Indomethacin in the MLR**

MLR responder cell populations were consistently depleted of Mφ before use. Indomethacin was used to (i) assess the contribution of PGE₂ production in the MLR and thereby ascertain the effect of low levels of contaminating Mφ and (ii) determine if normal and TBH responder cells exhibited a different proliferative response in the presence of indomethacin. Figure 11 shows a titration of indomethacin from $1 \times 10^{-4} \text{M}$ to $1 \times 10^{-9} \text{M}$. Data is presented as cpm to avoid correcting for two different controls. At high doses, an apparent toxicity occurred, as proliferation was dramatically reduced. Proliferation of TBH responder cells was restored to normal levels by a dose of indomethacin of 10 μM ($1 \times 10^{-5} \text{M}$).

**Time Course of Activity in the MLR**

To begin to determine the mechanism of action of the high mw inhibitory activity, determine its effect on both cell activation and on activated cells, and further distinguish it from PGE₂, various preparations were added to the MLR at 0, 24, 48, 72, and 90 hr after initiation (Fig. 12). Data is presented as a percentage of the cpm obtained by adding RPMI medium to the MLR at each time point. Well volumes were maintained at a constant 200 μl. PGE₂ suppressed the MLR, but only if added during the early phases (0-48 hr). This PGE₂-mediated suppression was mirrored by enhancement of the MLR by indomethacin up to 24 hr. The concentrated PGE₂-free normal or TBH Mφ supernatants maintained a relatively constant degree of inhibitory activity through the first 72 hr of the time course. If the high mw inhibitor and indomethacin were simultaneously...
Figure 10. Effect of the inhibitor on the proliferation of cell lines: P388D₁, BW 5147.3, and A4A cells were cultured in vitro and used in proliferation assays. Cells were taken from the 37°C 5% CO₂ incubator, checked by trypan blue dye exclusion to ensure that their viability was >90%, and added to flat-bottom 96-well microculture plates at 1 x 10⁵ viable cells/well. 100 µl/well of concentrated normal (///) and TBH(\_) peritoneal Mφ supernatants were added at time of initiation. PGE₂ was added at a final concentration of 50 nM (///). Cultures were pulsed at 42 hr and harvested at 48 hr.
Figure 11. Titration of indomethacin in the MLR using normal and TBH responder cells: Mφ-depleted splenic responder cells from normal and TBH BALB/c mice were prepared and the MLR performed as described in the Materials and Methods. 100 µl of indomethacin was added to a final concentration in the well of 1 x 10^{-6} M (100 µM) to 1 x 10^{-9} M (0.001 µM). Cultures were pulsed and harvested as described for the MLR. The data is presented as ^3^H-TdR incorporation, with proliferation of the normal and TBH responder cells in the absence of indomethacin given as (none).
added to the same wells, an intermediate effect was seen during the first 24 hr, after which the effect of the indomethacin was masked. This could also be interpreted as no inhibition when added at 0 or 24 hr, but inhibitory activity is seen at the 48 and 72 hr time points of addition. Concentrated normal and TBH peritoneal Mφ supernatants showed the same trends on a time course addition.

**Effect of the Inhibitory Monokine on the Generation of IL 2 in the MLR**

Table 3 illustrates the levels of IL 2 generated in the MLR by control and Mφ supernatant-treated allogenically-stimulated cell populations. The data is expressed as units of IL 2 activity relative to controls, which were assigned a value of 100. Controls were the responder plus X-ray irradiated stimulator cells with no Mφ supernatant added, and were labeled NONE in Table 3. Both normal and TBH concentrated Mφ supernatants (PGE₂-free and IL 2-free) were found to inhibit the generation of IL 2 in the MLR when added at 50 μl/well at the time of initiation. If the supernatants were first diluted 1:5, their inhibitory activity decreased, but the normal and TBH Mφ supernatants exhibited a dose- and time-dependent difference. Diluted normal host Mφ supernatants completely lost their ability to inhibit IL 2 generation when measured at 90 hr, whereas TBH Mφ supernatants completely lost their inhibitory activity, in fact enhanced the production of IL 2, when IL 2 levels were measured at 48 hr.

**Effect of the Inhibitory Monokine on Cell Cycle Progression**

To determine the effect of the inhibitory supernatants and PGE₂ on cell activation, a mitogen stimulation assay was chosen to provide cells for cell cycle analysis by flow cytometry. Table 4 shows normal and TBH concentrated Mφ supernatants, previously determined to be PGE₂-free, were capable of inhibiting proliferation in both the alloantigen-induced MLR and Con A-induced mitogenesis. PGE₂, at a final concentration of 25 μg/ml, significantly inhibited ³H-TdR incorpo-
Figure 12. Time course of activity in the MLR: To determine the interrelationships between the high mw inhibitory molecule, PGE$_2$, and indomethacin, various preparations were added to the MLR at 0, 24, 48, 72, and 90 hr after initiation. Well volumes were maintained at a constant 200 µl. Data is presented as a percentage of the cpm obtained by adding RPMI medium to the MLR at each time point. Indomethacin (□) was added at a final concentration of 1 x 10$^{-7}$ M. Concentrated supernatant from normal host peritoneal Mφ was added by itself (+) or concomitantly with indomethacin (◇). Concentrated TBH peritoneal Mφ supernatant was also added by itself (△) or concomitantly with indomethacin (x). PGE$_2$ was also added to individual wells at a final concentration of 50 nM (○).
Table 3. Effect of Supernatant Treatment on the Generation of IL 2 in the MLR

<table>
<thead>
<tr>
<th>Mφ supernatant addition to the MLR</th>
<th>48 hr</th>
<th>90 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>NONE</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>NOR Mφ</td>
<td>40</td>
<td>5</td>
</tr>
<tr>
<td>NOR Mφ 1:5</td>
<td>61</td>
<td>100</td>
</tr>
<tr>
<td>TBH Mφ</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TBH Mφ 1:5</td>
<td>167</td>
<td>67</td>
</tr>
</tbody>
</table>

MLR cultures were initiated as described in Materials and Methods. Mφ supernatants were added at time of initiation. MLR culture wells were aspirated at 48 and 90 hr and analyzed for IL 2 activity.

IL 2 activity was determined using IL 2-dependent CT-6 cells and units of IL 2 activity calculated using a programmable HP-97 calculator by the following formula:

\[
IL 2 \text{ units/ml} = \frac{Sample \text{ dilution}_{50\% \text{ maximum cpm}}}{Standard \text{ dilution}_{50\% \text{ maximum cpm}}} \times 100
\]
ration in both the MLR and mitogen assay. Without any mitogen, cells showed a marginal increase in the percentage of cells in S phase (8.8 vs 11.5%, defined as background levels) at 42 and 66 hr. With mitogen, very little difference was detected in the percentage of cells in S phase at the two time points. Addition of both normal and TBH MΦ supernatants resulted in increased proportions of cells in S, relative to the 42 hr + Con A control. However, the addition of both normal and TBH MΦ supernatants resulted in low (background) levels of cells in S phase at 66 hr, in agreement with the low $^3$H-TdR incorporation seen after treatment with these supernatants. Addition of PGE$_2$ caused no change in the percentage of cells in S phase relative to the mitogen control, even though PGE$_2$ had a dramatic effect on $^3$H-TdR uptake and incorporation.
<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>100%</th>
<th>+ TBH</th>
<th>+ NOR</th>
<th>+ PGE₂</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MLR cpm¹</strong></td>
<td>4643 ± 126</td>
<td>150326 ± 10870</td>
<td>58897 ± 5553</td>
<td>36795 ± 3157</td>
<td>281 ± 82</td>
</tr>
<tr>
<td><strong>Con A cpm²</strong></td>
<td>1149 ± 276</td>
<td>223898 ± 18414</td>
<td>113870 ± 1416</td>
<td>126860 ± 4712</td>
<td>288 ± 94</td>
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</table>

42 hr Con A³

<table>
<thead>
<tr>
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<th>+ TBH</th>
<th>+ NOR</th>
<th>+ PGE₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>%G₀ + G₁</td>
<td>90.9</td>
<td>78.9</td>
<td>57.2</td>
<td>72.2</td>
</tr>
<tr>
<td>%S</td>
<td>8.8</td>
<td>20.0</td>
<td>41.8</td>
<td>26.6</td>
</tr>
<tr>
<td>%G₂ + M</td>
<td>0.3</td>
<td>1.1</td>
<td>1.0</td>
<td>1.2</td>
</tr>
</tbody>
</table>

66 hr Con A³

<table>
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<tr>
<th>Stage</th>
<th>CONTROL</th>
<th>+ TBH</th>
<th>+ NOR</th>
<th>+ PGE₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>%G₀ + G₁</td>
<td>88.5</td>
<td>82.3</td>
<td>88.1</td>
<td>86.7</td>
</tr>
<tr>
<td>%S</td>
<td>11.5</td>
<td>17.2</td>
<td>11.0</td>
<td>12.0</td>
</tr>
<tr>
<td>%G₂ + M</td>
<td>0</td>
<td>0.5</td>
<td>0.9</td>
<td>1.3</td>
</tr>
</tbody>
</table>

¹MLR was pulsed at 90 hr and harvested at 96 hr. Control is responder cells plus X-irradiated syngeneic responder cells. 100% represents responder cells plus X-irradiated allogeneic stimulator cells in the absence of Mφ supernatants. + TBH, + NOR, and + PGE₂ indicate the cells were treated with 50 µl of concentrated Mφ supernatants or a final concentration of 25 µM PGE₂.

²Con A-induced proliferation assay was performed concomitantly with the MLR but was pulsed at 66 hr and harvested at 72 hr. Control is cells in the absence of mitogen. 100% is the proliferation of cells in the presence of Con A. Additions were equivalent to the MLR.

³Well contents of a replicate 96-well plate were aspirated at 42 and 66 hr, centrifuged, cells resuspended in 100 µl of RPMI medium, fixed with 100 µl ice cold methanol, and incubated with ribonuclease for 1 hr at room temperature. Cells were stained with propidium iodide immediately before flow cytometry evaluation. Percentage of cells in cell cycle stages was determined by one parameter analysis based on G₀ + G₁ cells having a ploidy of 1, G₂ + M cells having a ploidy of 2, and S cells having an intermediate value.
Discussion

This chapter describes an inhibitor(s) of alloantigen- and mitogen-induced T cell proliferation found in splenic and thioglycollate-elicited normal and TBH Mφ culture supernatants. We showed that the inhibitory activity was not PGE₂-mediated, but was due to a large (> 67 kd) charged factor which was glycosylated. Tumor growth resulted in a change in glycosylation, decrease in pI, and early enhancement of mitogen-stimulated cells (as shown by cell cycle analysis). The lack of effect of proteolytic enzymes on inhibitory activity did not rule out the possibility of a glycoprotein, but indicated that the protein component was not involved in the active site or that the inhibitory activity was insensitive to proteases. Presence of neuraminidase sensitivity of TBH and not normal Mφ supernatants suggested a tumor-induced variable glycosylation or production of an additional factor. The presence of a sialic acid residue agreed with the data obtained from IEF showing a lower pI for the inhibitory activity from TBH Mφ culture supernatants. A second, less dramatic band of activity was seen in TBH Mφ supernatants near neutrality, lending support to the presence of more than one inhibitory factor in the TBH. Elution of a major inhibitory fraction at 0.3 M phosphate in TBH but not normal host Mφ supernatants using hydroxylapatite supported the ideas of both normal and TBH differences and the presence of an additional factor in TBH Mφ supernatants.

Since the inhibitory activity was found in fractions corresponding to > 67 kd, and generated in the presence of indomethacin (48) we could rule out the possibility that our factor was prostaglandin, arginase (144), or thymidine (203). Since radioimmunoassays revealed that there was only background levels of PGE₂ in the concentrated Mφ supernatants, we can also rule out the contribution of bound PGE₂ to the inhibitory activity (130). Thymidine is also ruled out by the time course addition experiment showing no inhibition if the high mw inhibitor is added at the time of pulse, and interferon is ruled out on the basis of size (23) and previous data showing very low levels of interferon in our Mφ supernatants (51). Various investigators have isolated Mφ-derived inhibitory factors. Toh (278) describes an inhibitor of DNA synthesis derived from normal and
activated rat Mφ that is 20-30 kd and sensitive to both trypsin and protease. This factor eluted from hydroxylapatite at pH 6.8 with 0.4 M phosphate buffer, and several peaks of inhibitory activity were also recovered from a DEAE-Cellulose column. Aune et al. (10) describe a soluble immune response suppressor (SIRS) of 14 and 21 kd. Chen et al. (35) describe a factor that is 110 kd, trypsin-resistant and pronase-sensitive in Mφ culture supernatants. Krakauer (140) describes a monocytic leukemia cell line (THP-1) which, when grown in serum-free medium in the presence of silica, produces an inhibitor of IL 1- and mitogen-induced proliferation. This factor interferes with both the production and action of IL 2 and is sensitive to heat, trypsin, chymotrypsin, and protease. Kilbourn et al. (134) show BCG-activated peritoneal Mφ produce a conditioned supernatant that inhibits DNA synthesis and also complex I, II, and III of the mitochondrial electron transport system of target cells. Activities are associated with factors of 55, 80, and 150 kd. Werb and Chin (308) show variable glycosylation of apoprotein E, a 33 kd immunosuppressive factor produced by resident and thioglycollate-elicited Mφ, but not by hydrogen peroxide-producing Mφ elicited by BCG, pyran copolymer, Corynebacterium parvum, or endotoxin. The presence of an inhibitory activity in normal host as well as TBH Mφ culture supernatants points to the presence of suppressor Mφ and possibly a homeostatic mechanism. Suppressor Mφ have been implicated as the cause and/or effect of tumor-induced immunosuppression (306). Spontaneous production of inhibitory factors by non-stimulated Mφ has recently been noted by Fujiwara et al. (88, 89) using a U937 human Mφ-like cell line. This factor inhibits IL 1 activity and IL 2 receptor expression, and is acid- and base-labile, ammonium sulfate precipitable, 67-130 kd, and partially heat-stable. The biochemical nature of the compound(s) is presently unknown.

The lack of proteolytic enzyme sensitivity, heat stability, sensitivity to neuraminidase, and apparent hydrophobicity, as demonstrated by irreversible binding to phenyl-Sepharose (Chapter 3), suggests that our inhibitory activity may be vested in a glycosylated, non-protein, compound. Numerous studies have demonstrated the ability of glycosphingolipids (GSL), some of which contain sialic acid (gangliosides), to inhibit lymphocyte proliferation during in vitro assays (174). The inhibitory molecules include (in increasing order of potency and carbohydrate complexity) GM₃, GM₂, GM₁, and GD₁₄, all of which were found to bind IL 2 in vitro and thereby prevent IL
2-dependent proliferation (209). While the ability of our inhibitory monokine to bind IL 2 is not currently known, it was interesting that the normal and TBH high mw inhibitory factors suppressed the generation of IL 2 in the MLR.

Lengle et al. (153) showed that gangliosides, in particular trisialoganglioside, are capable of complete inhibition of Con A-induced uridine, leucine, and thymidine incorporation by T cells, but not carbohydrate metabolism, cell viability, or Con A binding, even if added as late as 24-28 hr in a 48-hr assay. While maximum binding of gangliosides occurred within 30 min, no inhibition of proliferation occurred if unbound gangliosides are washed out after 4 hr, i.e., the gangliosides are either not internalized or had a reversible effect (153). This time course of activity is similar to the data presented in this report, that the inhibitory monokine could be added up to 72 hr after initiation of the MLR and still demonstrate inhibitory activity. In unpublished experiments, we have not been able to demonstrate absorption or removal of the inhibitory activity using P388D1 cells, which is also similar to the results reported by Lengle et al. (153). Ladisch et al. (147) demonstrated gangliosides purified from YAC-1 lymphoma ascites fluid inhibit the proliferative response of murine splenocytes to Con A and to soluble and cellular antigens at doses similar to the levels required for purified gangliosides. The mechanism of inhibition by gangliosides appears to be by binding IL 2 (209) or by suppressing the influx of calcium after stimulation with Con A or the calcium ionophore A 23187 (143). It is useful to note that treatment of ganglioside preparations with trypsin or protease for 24 hr or boiling for 15 min did not reduce their inhibition of IL 2 activity (209), which corroborates our data on heat stability and lack of sensitivity to proteolytic enzymes. Robb (230) showed that ganglioside inhibition of proliferation is divided into IL 2-reversible and IL 2-irreversible mechanisms, the latter of which are predominant during the initial stage of cellular activation, i.e., proliferation of mitogen-activated human peripheral blood lymphocytes. This is analogous to the data presented in this study, and is of interest because IL 2 is known to override the inhibitory effects of PGE2 (76), and our high mw inhibitory monokine appears to require PGE2 for its inhibitory activity to be manifested on T cells in the early stages of activation.
While our inhibitory monokine shares some biochemical and physical properties with the above listed molecules, it is substantially larger in size. This can be explained as association of a protein with the active moiety, with the protein conferring size and possibly stability and specificity of action. Interestingly, Wolf and Merler showed a T cell-derived inhibitor of lymphocyte proliferation is a biologically active lipid bound to inactive proteins (314). Curtiss and Edgington showed the reverse, that the inhibitory activity of a low density lipoprotein from human serum is vested in the protein portion (53).

T cell proliferation in the MLR is the culmination of a series of antigen-driven and factor-driven events which initiate a cascade of intracellular events. Inhibition can occur at several steps along this pathway. For instance, PGE₂, a Mφ product, can inhibit la antigen expression (254), and IL 2 production (42), and can activate T suppressor cells (41), and induce the production of suppressor factors by T cells (PITS) (234) and inhibit the IL 2-dependent G₁₄-G₁₈ cell cycle transition (303). In our system, PGE₂ acted early in the MLR. The suppressive effects of PGE₂ were mirrored by the enhancing effects of indomethacin during the early phases of the reaction. Both the normal and TBH high mw inhibitor, in contrast, appeared to maintain a certain level of activity throughout the MLR. This could imply a nonspecific activity on T cells at different stages of activation. Note that the time course addition experiment (Fig. 12) involves adding supernatants to cells which are already committed to proliferate, and then measuring their proliferation with a 6 hr pulse at 90 hr. Alternatively, one can address the question of cell cycle progression after concomitant exposure to both mitogen and inhibitor (Table 4). To do this, inhibitor and mitogen were added at time of initiation of the assay, and cells analyzed at 42 and 66 hr. Flow cytometry data on Con A-stimulated splenocytes showed that TBH Mφ supernatants had a significant enhancing effect early (41.8% S at 42 hr) but suppressive effect late (11% S at 66 hr). Normal host Mφ supernatants showed less early enhancement, but equivalent suppression at 66 hr. Both the normal and TBH Mφ supernatants showed dramatically different effects from PGE₂, suggesting that they operate via different mechanisms.

A lack of correlation between ³H-TdR incorporation and cell cycle progression has been noted by others and may explain the discrepancy seen using PGE₂. In analyzing strain variation in
murine DNA and RNA synthetic responses, Buckley and Wedner (25) found that incorporation of isotopes into nucleic acids is not an accurate reflection of cell activation. Differences in isotope incorporation are not reflected in the number of activated cells, extent of S phase, time of peak response, etc. Betel et al. noted that a 20-fold increase in $^3$H-TdR incorporation results in only a 2-fold increase in the proportion of proliferating cells as measured by acridine orange staining in a flow cytometer (17). This lack of strict corroboration between $^3$H-TdR incorporation and cell cycle analysis may contribute to the discrepancy of a lack of $^3$H-TdR incorporation in the presence of PGE$_2$, and yet a cell cycle profile similar to Con A-stimulated cells. When analyzed microscopically, PGE$_2$-treated Con A-stimulated cells showed a complete lack of clustering, both relative to controls and to Mφ supernatant-treated cells, which showed essentially the same degree of clustering as Con A-treated cells (unpublished data). Interestingly, the A4A T helper cells exhibited different sensitivity to normal and TBH Mφ supernatants, and was not sensitive to the inhibitory effects of PGE$_2$. Lyt subsets of T cells show differential susceptibility to normal and TBH Mφ supernatants, with Lyt-2,3$^+$ TBH T suppressor cells showing increased proliferation on exposure to normal host Mφ supernatants (170). All these phenomena support the conclusion that normal and TBH Mφ supernatants had a mechanism of action different both from each other and from PGE$_2$. The possibility exists, however, that PGE$_2$ was required for the inhibitory activity to be manifested, at least on T cells in the early stages of activation. This was evidenced by the lack of inhibitory effect when cells were treated with both the inhibitor and indomethacin at early time points in the MLR time course addition experiment (Fig. 12). Interestingly, the high mw inhibitor suppressed proliferation of syngeneic P388D$_1$ Mφ-like mastocytoma cells, which produce PGE$_2$, but not proliferation of BW 5147.3, an allogeneic thymoma T cell line (Fig. 10). While PGE$_2$ may be required for inhibitory activity of the high mw factor to be manifested, the high mw factor did not appear to cause the elaboration of PGE$_2$. If the PGE$_2$-free Mφ supernatants were added to the MLR and levels of PGE$_2$ determined at 48 and 90 hr, they were found to range from 4 to 10 pg/0.1 ml, with no significant difference between controls and supernatant-treated cells.

The combination of heat stability (48) and generation in the presence of indomethacin (51), time course of activity, size (>$67$ kd), lack of proteolytic enzyme sensitivity, and sensitivity in TBH
to neuraminidase distinguished the activity reported here from other Mφ-derived inhibitory factors. We have demonstrated a progressive loss of Mφ Ia antigen expression as the tumor develops (91), and that inhibitory Mφ are Mac-3+ and Mac-2+ in normal and TBH, respectively (93). We also show that the Mac-2+ peritoneal Mφ is the producer of PGE₂ in the TBH, and that Mac-1+ Mφ control of PGE₂ production is missing in the TBH (169). We have now extended these findings on tumor-induced qualitative differences in Mφ subpopulations and their immunoregulatory activities. Data presented in this report pointed to a qualitative difference in the soluble mediators produced as a result of tumor development. This was shown by hydroxylapatite chromatography elution profile, pI, and neuraminidase sensitivity. While we have demonstrated variable glycosylation (sialic acid residue involved in inhibitory activity of TBH but not normal supernatants), we cannot yet say if this was the same or a different molecule. The presence of more than one molecule is suggested by data showing a requirement for PGE₂ to inhibit T cells in the early but not the late stages of proliferation. Previous work using whole Mφ supernatants showed a generally inhibitory normal but not TBH Mφ supernatant causes increased proliferation of TBH Lyt-2,3+ splenic T cells (170). Data presented in this report extends this observation on the existence of a difference between normal and TBH Mφ supernatants.

The possibility of induction or activation of a suppressor T cell by the high mw inhibitor remains to be elucidated. It is interesting that suppressed proliferation of Mφ-depleted TBH T cells in the MLR can be restored to normal levels by the addition of indomethacin. Suppressed T cell proliferation in TBH is attributed to in vivo exposure to high levels of PGE₂ (55) low levels of Ia antigens on Mφ (91), lack of IL 2 (26) and IL 3 (28) and induction of T suppressor cells which produce an inhibitor of DNA polymerase (77). Since the high mw inhibitory monokine required PGE₂ for its inhibitory effect to be manifested on T cells early in activation, and indomethacin can “cure” the lack of proliferation of TBH T cells, it is possible that TBH T cells are suppressed, or produce immunosuppressive factors, due to in vivo exposure to the high mw monokine in conjunction with PGE₂.

The fact that the inhibitory monokine was glycosylated could have implications for its biological half-life (190) and/or its affinity for the receptor on the target cell. Recognition and pro-
liferation in the MLR is dependent on Ia antigens which are glycosylated (179). Transformation of normal cells into tumor cells is accompanied by different glycosylation of surface antigens which could lead to differences in immune recognition and avoidance (110, 111). It was possible that variable glycosylation of soluble mediators caused different recognition phenomena. Increased biological half-life of the high mw inhibitor, coupled with increased PGE$_2$ production in the TBH (55, 211), could help account for the immunological anergy seen in the TBH.
PROSTAGLANDIN E₂ PRODUCTION BY Mac-2⁺ MACROPHAGES:
TUMOR-INDUCED POPULATION SHIFT

Introduction

Immunoregulation by macrophages (Mφ) is mediated by soluble factors. For instance, Mφ
produce prostaglandin E₂ (PGE₂), an arachidonic acid metabolite (146) which contributes to in-
flammatory responses (121) and suppression of T lymphocyte proliferation (55). PGE₂ is known
to down-modulate Ia antigen expression on Mφ (254), induce suppressor T cells (41), inhibit
interleukin 2 (IL 2) production, transferrin receptor expression (42) and the IL 2-dependent
G₁₅₋G₁₆ cell cycle transition of mitogen-stimulated T cells (303).

While the effects of PGE₂ on surface antigen-defined cells and on surface antigen modulation
are known, the correlation between cell surface antigen expression (markers of activation, differen-
tiation, and/or subpopulations) and PGE₂ production is not known. Others have reported Mφ
phenotypes vary in their ability to produce PGE2 based on countercurrent centrifugal elutriation (3) or buoyant density gradients (133, 200), and have also correlated this with interleukin 1 production (133, 200) and activation states (99). To determine the correlation between MΦ phenotype and PGE2 production, we used a series of rat-anti-mouse monoclonal antibodies (mAb) developed by Springer (257) which recognize the Mac-1, -2, and -3 surface antigens. The Mac-1 heterodimer is constitutively expressed by MΦ and monocytes (118, 256, 304), and is involved in early MΦ differentiation (223) and in adherence, aggregation, directed migration, and phagocytosis of C3bi-coated particles by MΦ (6, 7). Phorbol esters and chemoattractants induce a 5-10 fold increase in Mac-1 expression (259), which is lacking in cells from patients with inherited Mac-1 deficiency (54). The Mac-2 molecule is preferentially found on thioglycollate-elicited peritoneal MΦ (119), while the Mac-3 antigen is expressed on resident peritoneal MΦ (263, 304). The Mac-2 and Mac-3 antigens are expressed late in the development of nonadherent bone-marrow progenitor cells as they mature into MΦ (304). Although the Mac-1, -2, and -3 antigens may be shared by individual MΦ or by MΦ populations, we correlated mAb-defined MΦ phenotypes with alterations in both the ability to suppress an allogeneic mixed lymphocyte reaction (MLR) and PGE2 production by MΦ from normal and TBH mice.

Tumor growth induces a population of suppressor T cells and inhibitory MΦ (66), both of which produce soluble factors which inhibit T cell proliferation. In our system, MΦ produce two immunosuppressive monokines, PGE2 (55) and a high molecular weight factor which is glycosylated (168) and produced in the presence of indomethacin (51). We have also found a progressive loss in Ia antigen expression and Ia antigen-dependent activities of MΦ (91), a drop in IL-2 (28) and interleukin 3 levels (27), and a shift in the MΦ subpopulations as the tumor develops, with the inhibitory MΦ from normal and tumor-bearing hosts (TBH) being Mac-3+ and Mac-2+, respectively (90). This suggested a correlation between the Mac antigen-defined phenotype of peritoneal MΦ and the ability to suppress the MLR by producing PGE2, and that this phenotype changes with tumor growth. Thioglycollate-elicited peritoneal MΦ were treated with mAb either to modulate cells by membrane activation (receptor-ligand interaction) or to deplete antigen-defined phenotypes in the presence of complement. Conditioned-medium was generated from these treated
Mrp populations in the presence or absence of indomethacin to determine the contribution of PGE₂ and non-PGE₂ inhibitors of T cell proliferation. The use of supernatants from mAb plus complement-treated Mrp in an allogeneic MLR revealed not only that the suppressor phenotype was Mac-3⁺ and Mac-2⁺ in normal and TBH, respectively, but that this suppression was inversely correlated with protein content of the supernatants. Since Mrp produce PGE₂, which is immunosuppressive and is not detected by protein assays, we analyzed supernatants for PGE₂ by radioimmunoassay. While the producer of PGE₂ in the normal host could not be directly delineated, PGE₂ assays of Mrp supernatants suggested that: (i) both Mac-1⁺ and Mac-3⁺ Mrp were involved in the regulation of PGE₂ production in normal hosts, (ii) depletion of normal host Mac-2⁺ Mrp had no effect on PGE₂ production, (iii) Mac-1⁺ Mrp regulation of PGE₂ production was absent in the TBH, (iv) Mac-3⁺ Mrp were involved in the regulation of PGE₂ production in the TBH, and (v) Mac-2⁺ Mrp were the primary producers of PGE₂ in the TBH.
Materials and Methods

Mice

Male BALB/c mice were obtained at 4-6 wk of age from either Dominion Laboratories, Dublin, VA, or the Virginia-Maryland Regional College of Veterinary Medicine Vivarium, Blacksburg, VA. Mice were injected intramuscularly in the left hind leg with a single cell suspen-
sion of $4 \times 10^5$ methylcholanthrene-induced transplantable nonmetastatic fibrosarcoma cells (55). Mice were injected intraperitoneally with 2.0 ml of sterile thioglycollate (Difco Laboratories, Detroit, MI) at 0 days (normal host) or 17 days of tumor development (TBH), and sacrificed for peritoneal lavage 4 days later. Normal and TBH mice were tested for LDH virus (an immunosuppressive murine virus) by Microbiological Assoc., Bethesda, MD, and were found to be negative.

Medium

RPMI 1640 (Hazleton Dutchland, Denver, PA) supplemented with 50 μg/ml gentamicin (Gibco, Grand Island, NY), 2 gm/L NaHCO₃ (Fisher Scientific, Raleigh, NC) and 25 mM HEPES (Sigma, St. Louis, MO) was filter sterilized and used in all cell culture procedures.

Monoclonal Antibody Generation and Purification

Hybridoma cell lines M1/70, M3/38, and M3/84 (producing rat anti-mouse Mac-1, -2, and -3 mAb, respectively), originally cloned by Springer et al. (256, 257), were obtained from the American
Type Culture Collection, Rockville, MD. Hybridoma cells were cultured in 2 L roller bottles containing 250 ml RPMI medium supplemented with 10% heat-inactivated fetal calf serum (Hazleton) at 37°C. Hybridoma cultures were maintained between 2 and 4 x 10^5 cells/ml by periodically examining viability by trypan blue dye exclusion and reculturing when viability fell below 85%. Supernatants from hybridoma cultures were harvested every 3 days and remaining cells replenished with fresh complete medium. Cell-free supernatants were either stored at -70°C for later use or immediately purified by ammonium sulfate precipitation and DEAE-Cellulose chromatography (90). Briefly, a pH 7.4 ammonium sulfate solution was added to whole Mφ culture supernatants to yield a 45% saturated solution. After standing at 4°C overnight, precipitates were collected by centrifugation at 10,000 x g for 30 min, resuspended in 0.05 M Tris buffer pH 8.0, dialyzed against the same buffer for 24 hr, and applied to a DEAE-Cellulose column. Protein was eluted from the column in 3 steps with 0.05 M Tris buffer pH 8.0 containing 0, 0.05 or 0.1 M NaCl and collected in 2 ml fractions. Fractions containing rat mAb were detected by Ouchterlony immunodiffusion with goat-anti-rat antibody (Fc-specific) (Cappel Laboratories, Malvern, PA). Positive fractions were pooled and concentrated 20-fold by ultrafiltration using a YM10 membrane filter. Dialysis of the concentrated mAb fractions was performed in RPMI medium at 4°C for 24 hr. The final mAb preparations were assayed for antibody concentration and purity by comparison with normal rat IgG (NRIGG; Jackson Laboratories, Bar Harbor, ME) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and radial immunodiffusion. All protein determinations were performed using a Biorad protein assay kit (Biorad Laboratories, Richmond, CA) using a bovine serum albumin standard (Sigma).

**P388D1 Treatments**

P388D1 cells were cultured in RPMI medium supplemented with 10% heat-inactivated fetal calf serum (Hazleton). Cells were maintained as confluent cultures of adherent cells by splitting the cultures every 2-3 days using 75 cm² tissue culture flasks (Fisher). P388D1 cells were washed and
resuspended to $4 \times 10^6$ viable cells/ml (trypan blue dye exclusion) in serum-free RPMI medium. Cells were then treated with 10, 25, 50, and 100 µl of antibodies (1 mg/ml) added to 1.0 ml of cells. Antibody-treated P388D1 cells were then cultured for 4 days at 37°C in a 5% CO₂ atmosphere, supernatants harvested, sterile filtered, and stored at -70°C.

**Macrophage Treatments**

Mφ were obtained from 6-18 mice intraperitoneally injected with 2.0 ml of sterile thioglycollate, and peritoneally lavaged 4 days later with cold RPMI medium. Equivalent amounts of cells were plated on 150 mm tissue culture-treated dishes (Miles Laboratories, Elkhart, IN) for at least 2 hr at 37°C in a 5% CO₂ incubator, at which time nonadherent cells were washed off with warm RPMI medium. For convenience, Mφ were left on the plate in 9.0 ml of RPMI medium and treated with 1.0 ml of antibodies at 1 mg/ml concentration. There was no difference in sensitivity to mAb treatment of adherent or scraped Mφ. At times, complement (Low-Tox rabbit complement, Accurate, Westbury, NY) was added at a 1/20 final dilution. Plates were then incubated for 90 min at 37°C in a 5% CO₂ incubator, nonadherent cells again washed off, and the remaining adherent cells scraped with a rubber policeman, centrifuged, and resuspended to $4 \times 10^6$ viable cells/ml as determined by trypan blue dye exclusion. Mφ were then incubated at 37°C for 4 days at $4 \times 10^6$ cells/ml. Four-day cultures contain maximum inhibitory activity (51) because under these conditions, Mφ produce PGE₂ and a high molecular weight factor that inhibits MLR reactivity, is noncytotoxic, is not a "thymidine artifact" and is generated in the presence of indomethacin (51). In fact, PGE₂ only accounts for 38% of the inhibition of MLR reactivity using Mφ-derived supernatants (55). Thus, indomethacin (Sigma) was added to some Mφ cultures at the start of the 4-day incubation at a final concentration of $1 \times 10^{-7}$ M. This dose was chosen to interrupt PGE₂ biosynthesis (244) and at the same time have negligible effects on other cellular functions (85) or cause shunting among the arachidonic acid metabolism pathways (291). After 4 days, supernatants were harvested, sterile filtered, and stored at -70°C.

Chapter II
Mrp were resuspended to equivalent cell numbers (4 x 10^6 viable cells/ml) after mAb or mAb plus complement treatments. This was done to allow for the fact that while the anti-Mac-1, -2, and -3 mAb all have the same degree of antibody plus complement-mediated lysis (50-55%) (93), the expression of these surface antigens is not equivalent. We find normal host peritoneal Mφ are 80% Mac-1^+, 53% Mac-2^+, and 66% Mac-3^+, while TBH peritoneal Mφ are 77% Mac-1^+, 37% Mac-2, and 100% Mac-3^+ by immunofluorescence. Background fluorescence levels were 22% and 7% for normal and TBH thioglycollate-elicited peritoneal Mφ, respectively, treated with NR IgG (93), which suggests Fc receptor differences between normal and TBH Mφ. Treatment groups were referred to as modulated (mAb-treated) or depleted (mAb plus complement-treated), although we feel that some of the “depleted” Mφ may well be modulated by the presence of a mAb plus complement conjugate, and not lysed. Note that resuspension to equivalent numbers after treatment selects for the remaining cells.

**Mixed Lymphocyte Reaction**

MLR reactivity was initiated between BALB/c (H-2^b) Mφ-depleted responder T cell populations and C3H (H-2^a) stimulator cells (51, 55). Spleens from at least 4 mice were excised and passed through a wire sieve, yielding a single cell suspension. Whole spleen cell preparations were washed and resuspended in RPMI medium with 5% fetal calf serum (FCS). Responder cells were placed on nylon wool columns and incubated for 45 min at 37°C. The nonadherent cells were eluted with FCS-containing RPMI medium, centrifuged, and placed on Sephadex G-10 columns for 30 min at 37°C. The nonadherent cells were eluted as before, centrifuged, and placed on plastic tissue culture dishes for 2 hr. Nonadherent BALB/c cells were counted with a Coulter counter and dispensed in complete RPMI medium at 2 x 10^5 T cells per well in a 96-well "U" bottom microculture dish (Flow Laboratories). Plated C3H splenocytes were counted, resuspended to 2 x 10^7 cells/ml, and treated with Mitomycin-C (Sigma) (final concentration of 25 µg/ml) for 20 min at 37°C. The C3H stimulator cells were used at a concentration of 4 x 10^5 cells per well. Fifty µl of
Mφ supernatant were added to quadruplicate wells. Test groups varied with respect to Mφ source and dilution of supernatant. While Mφ supernatant dilutions of 1:1, 1:5, 1:10, and 1:20 were used, only the 1:5 dilution is reported in Table 1 as it represents the linear portion of the titration curve. Control groups received RPMI medium in place of Mφ supernatants. The reaction proceeded for 96 hr at 37°C and was pulsed with 1 μCi of tritiated thymidine (³H-TdR; spec. act. 6 Ci/m mol, Amersham Corp., Arlington Heights, IL) per well 6 hr before termination. Cells were harvested onto glass fiber filters (Whatman 934-AH). After drying, the samples were counted in a Beckman LS230 liquid scintillation counter.

**PGE₂ Analysis**

Mφ supernatants were analyzed for PGE₂ content using a competitive radioimmunoassay (RIA) using dextran-coated charcoal, a tritiated-PGE₂ ligand and specific rabbit anti-PGE₂ (Seragen, Boston, MA) as described elsewhere (2). This assay system is sensitive to 5 pg/0.1 ml. Statistical analysis and p value determinations were made using Students t test, with all values compared to their appropriate NRIgG controls.
**Results**

Suppressive Activity and Protein Content of Supernatants Derived from Anti-Mac-Treated Mφ

To determine the role of Mφ subpopulations in monokine production, we examined culture supernatants of mAb plus complement-treated Mφ subpopulations. After treatment with mAb plus complement, normal and TBH Mφ cultures were established by resuspension to 4 x 10⁶/ml, incubated for 4 days in serum-free RPMI medium, and Mφ factor-mediated modulation of MLR proliferation was assessed. Table 5 illustrates the effect of treatment on production of suppressor factors and the protein content (μg/ml) of the respective supernatants. Supernatant dilutions of 1:5 were used to compare normal and TBH Mφ-derived regulatory factors, because the 1:5 dilution reflected the most linear portion of the 1:1 to 1:20 titration curve. As the proliferation of allogenically-stimulated responder cells was always greater in the absence of Mφ supernatants, 0% MLR reactivity was defined with supernatants derived from NRlG plus complement-treated normal and TBH Mφ. A loss of MLR suppression (increased MLR reactivity) occurred when using supernatants from normal host Mφ depleted of Mac-1⁺ and Mac-3⁺ Mφ (36% and 83% increase in MLR reactivity, respectively), or from TBH Mφ depleted of Mac-2⁺ Mφ (64% increase in MLR reactivity). Loss of suppression was associated with an increase in protein content (relative to NRlG plus complement-treated controls) of the supernatants.

**PGE₂ Levels After Antibody Treatment of P388D₁ Cells**

P388D₁ mastocytoma cells were chosen as a control population for nonspecific activation (they express Fc and complement receptors) and specific activation (they express Mac-1, -2, and
Table 5. Percentage of MLR Reactivity and Protein Content of Supernatants Derived from Anti-Mac-Treated Mφ

<table>
<thead>
<tr>
<th>Mφ supernatant source</th>
<th>Normal</th>
<th>TBH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% MLR reactivity</td>
<td>protein (µg/ml)</td>
</tr>
<tr>
<td>NRIgG</td>
<td>0</td>
<td>42</td>
</tr>
<tr>
<td>Anti-Mac-1</td>
<td>36</td>
<td>70</td>
</tr>
<tr>
<td>Anti-Mac-2</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>Anti-Mac-3</td>
<td>83</td>
<td>60</td>
</tr>
</tbody>
</table>

1 Mφ were treated with NRIgG or mAb plus complement, with the remaining cells resuspended to 4 x 10⁶ cells/ml and cultured in serum-free RPMI medium for 4 days. Both normal and TBH were used as Mφ sources.

2 mAb treatment was carried out as described in Materials and Methods with NRIgG and three anti-Mac mAb.

3 MLR reactivity was observed following the addition of Mφ supernatants to BALB/c T cells stimulated by allogeneic C3H splenocytes. 50 µl of a 1:5 supernatant dilution were added per well. Percentages were calculated by the following formula:

\[
\text{Percentages} = \frac{\text{anti-Mac} + C \text{ cpm} - \text{NRIgG} + C \text{ cpm}}{\text{NRIgG} + C \text{ cpm}} \times 100
\]

4 Protein concentrations were determined using a Biorad protein assay using BSA as a standard and are expressed in µg/ml.
-3 antigens) (294). Varying amounts of antibodies were added to $4 \times 10^6$ P388D₁ cells. Cells were cultured for 4 days, the supernatants were harvested, centrifuged, and assayed for PGE₂ by radioimmunoassay. In the absence of antibody treatment, the P388D₁ cells produced 151 pg of PGE₂ per 0.1 ml of culture supernatant. The dose response curve shown in Fig. 13 reveals minor differences between the antibody treatments, but these were not titratable phenomena. Note that antibody concentrations were kept at 1 mg/ml, and the 100 μl treatment group was the corollary to all the other experiments cited where 1.0 ml of antibody was added to 10.0 ml of Mφ.

**PGE₂ Production by Modulated and Depleted Mφ Subsets**

To determine if PGE₂ was responsible for the suppression of MLR reactivity and if there was a tumor-induced change in the production of PGE₂ by peritoneal Mφ, we used mAb to modulate or deplete Mφ subpopulations and analyzed their subsequent PGE₂ production. Figure 14 represents PGE₂ levels produced by thioglycollate-elicited peritoneal Mφ from normal and TBH cultured in the absence of indomethacin. The data is expressed as a percentage of the appropriate control treatment group (NRIgG with or without complement). Modulation of Mac-1⁺ normal host Mφ resulted in a 51% increase ($p < 0.05$) in levels of PGE₂. Depletion of Mac-1⁺ normal host Mφ (with complement) resulted in a 208% increase ($p < 0.005$) in PGE₂ production by the remaining cells. In contrast, neither modulation nor depletion of Mac-1⁺ Mφ in the TBH had an effect on PGE₂ production. Depletion or modulation of Mac-2⁺ cells had no effect on PGE₂ production by normal host Mφ while only depletion of Mac-2⁺ cells in the TBH resulted in a 69% decrease ($p < 0.001$) of PGE₂ production. The modulation of normal and TBH Mac-3⁺ cells resulted in a 51% ($p < 0.05$) and 105% ($p < 0.01$) increase in PGE₂ production, respectively. Depletion of normal and TBH Mac-3⁺ Mφ also resulted in an increase in the production of PGE₂ (64% [$p < 0.05$] and 85% [$p < 0.01$], respectively).
Figure 13. PGE₂ production by antibody-treated P388D₁ cells: 4 x 10⁶ P388D₁ cells were treated with 10, 25, 50, or 100 µl of antibody (1 mg/ml) and cultured for 4 days as described. No antibody treatment resulted in the production of 151 pg per 0.1 ml of culture supernatant, as determined by radioimmunoassay. Values are given in pg per 0.1 ml for P388D₁ treated with normal rat IgG (□), anti-Mac-1 (+), anti-Mac-2 (◇), and anti-Mac-3 (△).
Figure 14. PGE$_2$ production by modulated and depleted Mφ subpopulations: Thioglycollate-elicited peritoneal Mφ from normal and 21-Day TBH were treated with NRlgG or anti-Mac-1 (Mac-1), anti-Mac-2 (Mac-2), or anti-Mac-3 (Mac-3) in the presence or absence of complement. Treated Mφ were washed and resuspended to 4 x 10$^6$/ml and cultured for 4 days in serum-free RPMI medium in the absence of indomethacin as described in Materials and Methods. PGE$_2$ production by normal (/ /) and TBH (\\) peritoneal Mφ after modulation by antibodies and by normal (////) and TBH (\\\\) peritoneal Mφ after depletion by antibodies plus complement is presented as the percentage of the PGE$_2$ production of the appropriate NRlgG control treatment group, represented by the 0% change line.
PGE2 Production by Modulated and Depleted Mφ Subsets in the Presence of Indomethacin

Indomethacin was added to mAb-treated Mφ subpopulations to determine the effect of an arachidonic acid metabolite inhibitor on PGE2 production by Mφ subpopulations, provide a corollary to the indomethacin-free Mφ cultures, and determine the contribution of non-PGE2 inhibitors of T cell proliferation. A dose was selected which had an effect on PGE2 biosynthesis (PGE2 levels decreased by approximately 50%) but had negligible effects on other cellular functions or pathways (85, 244, 291). Figure 15 illustrates PGE2 levels, expressed as a percentage of the appropriate NR IgG control, produced by Mφ cultured in the presence of indomethacin. Indomethacin was added once at the initiation of the culture period (after antibody treatment and resuspension to 4 x 10^6 cells/ml). All treatment groups showed approximately 50% less PGE2 production in the presence of indomethacin except for normal host Mφ treated with anti-Mac-3 (370 pg/0.1 ml in the presence versus 260 pg/0.1 ml in the absence of indomethacin). Depletion of Mac-1+ cells in normal hosts increased PGE2 production by 160% (p < 0.001). Depletion of Mac-2+ cells in the normal host caused a 36% increase (p < 0.025) in PGE2 production. Conversely, depletion of Mac-2+ cells in the TBH caused a 56% drop (p < 0.001) in PGE2 production. Modulation and depletion of normal, but not TBH, Mac-3+ cells in the presence of indomethacin caused a 150% (p < 0.01) and 145% (p < 0.005) increase in PGE2, respectively.
Figure 15. PGE$_2$ production by modulated and depleted Mφ subpopulations in the presence of indomethacin: Thioglycollate-elicited peritoneal Mφ from normal and 21-Day TBH were treated with NRlgG or anti-Mac-1 (Mac-1), anti-Mac-2 (Mac-2), or anti-Mac-3 (Mac-3) in the presence or absence of complement. Treated Mφ were washed and resuspended to $4 \times 10^9$/ml and cultured for 4 days in serum-free RPMI medium in the presence of $1 \times 10^{-7} M$ indomethacin as described in Materials and Methods. Production of PGE$_2$ by normal (//) and TBH (\) peritoneal Mφ after modulation by antibodies and by normal (////) and TBH (\\) peritoneal Mφ after depletion by antibodies plus complement is presented as the percentage of the PGE$_2$ production of the appropriate NRlgG control treatment group, represented by the 0% change line.
**Discussion**

Mφ produce a variety of compounds in response to environmental stimuli (107), which suggests variable activation and/or differentiation states and possibly the presence of Mφ subpopulations. Mφ can be divided by their ability to produce monokines (3, 99, 133, 200). Boraschi et al. have shown that IFN-γ, a Mφ activating agent, can inhibit PGE₂ release and induce IL 1 production by mouse peritoneal Mφ (24). Goldyne and Stobo (99) separated human peripheral blood monocytes into five fractions on discontinuous density gradients and show variable PGE₂ production by the fractions, with no simple correlation with activation states. Akiyama et al. (3) found two populations of human peripheral blood monocytes from healthy donors, called intermediate (IM) and regular (RM) monocytes, following countercurrent centrifugal elutriation. These monocytes differ in phenotypic and functional activities; in particular their baseline PGE levels are $1,582 \pm 970$ pg/ml for IM and $801 \pm 417$ pg/ml for RM cultured at $1 \times 10^6$ cells/ml for 48 hr in medium containing 10% serum. These monocytes also differ in their ability to release PGE after stimulation with poly I:C, with RM showing a higher stimulation index than IM. Ohmann and Babiuk (200) using bovine alveolar Mφ showed percoll density gradients can be used to separate populations of Mφ that differ in their spontaneous release of IL 1 and PGE with one fraction being a high spontaneous producer of both IL 1 and PGE. In contrast, Khansari et al., using discontinuous bovine serum albumin gradients, separated the PGE₂-producing from the IL 1-producing human monocyte subpopulation (133). We have extended this information by examining the production of PGE₂ by mAb-defined subpopulations of thioglycollate-elicited peritoneal Mφ from normal and TBH, and showed that there was a tumor-induced shift in (i) the phenotype of suppressor Mφ, (ii) their ability to produce PGE₂, and (iii) their ability to control the production of PGE₂.

Monoclonal antibodies plus complement were used to deplete subpopulations of Mφ which were then used to generate culture supernatants. These were tested for their ability to suppress proliferation in the MLR. All supernatants tested were inhibitory, but a partial loss of inhibition
was seen after depletion of Mac-1⁺ and Mac-3⁺ Mφ in the normal host, and depletion of Mac-2⁺ Mφ in the TBH. Supernatants that were less inhibitory than their NRIgG plus complement-treated controls showed increased protein concentrations. This inverse relationship was investigated further by assaying supernatants of treated Mφ for PGE₂, which showed a loss of PGE₂ production after depletion of Mac-2⁺ Mφ in the TBH, which could account for the 64% loss of MLR suppression. No loss of PGE₂ production was associated with anti-Mac-1 or anti-Mac-3 depleted Mφ in the normal host, suggesting that their suppression of the MLR is not necessarily mediated by PGE₂. Note that the Biorad protein assay does not detect PGE₂ (data not shown), which could account for the inverse relationship between lack of suppression and increased protein content. More importantly, increased levels of PGE₂ did not necessarily correlate with increased immunosuppression in the MLR. For example, depletion of normal host Mac-3⁺ Mφ resulted in a 64% increase in PGE₂ production, yet an 83% recovery of MLR reactivity. This lends support to the existence of other, non-PGE₂, inhibitory molecules, which can be generated in the presence of indomethacin (51, 55).

There is ample evidence that IgG2b and IgG2a subclasses of murine IgG can activate, via their Fc region, PGE₂ production by P388D₁ cells (194, 195, 210, 241, 268). Mφ Fc receptors are known to be activated by particles with subsequent release of prostaglandins (210, 241). Activation of the P388D₁ Fc receptor for IgG2b initiates the cyclooxygenase pathway, probably by activating phospholipase A₂ (268), which could result in release of PGE₂ and activation of membrane adenylate cyclase. Binding of the Fc receptor for IgG2a by antigen-complexed IgG2a also activates membrane adenylate cyclase, although by a different mechanism (195). However, the rat immunoglobulin subclass effect on PGE₂ synthesis through Fc receptor binding is unknown. Since P388D₁ cells are H-2⁺ and express the antigens of interest (293), and the subclasses of the rat-anti-mouse mAb are IgG2b (256), IgG2a (119), and IgG1 (257) for anti-Mac-1, -2, and -3, respectively, we initially used P388D₁ cells to aid in determining the contribution of Fc binding to PGE₂ production in our system. While P388D₁ cells are not equivalent to Mφ, and rat IgG subclasses are not equivalent to murine IgG subclasses, PGE₂ production by P388D₁ cells after treatment with mAb alone was always less than its untreated counterpart and exhibited no change over a titration
from 10-100 μg mAb per 4 x 10^6 cells per ml. This suggested that the rat mAb reacted with P388D1 surface antigens specifically and not through their Fc receptors.

Receptor-ligand binding by complement fragments reportedly results in increased PGE_2 production (112), which is relevant here because the Mac-1 receptor is the C3bi receptor (7). This was not necessarily found in our system, as modulation or depletion of TBH Mφ by anti-Mac-1 had no effect on PGE_2 production. While increased PGE_2 production by normal host Mφ after anti-Mac-1 modulation could be explained as an interaction with the C3bi receptor (120) or the Fcγ2b receptor/phospholipase A_2 system (268), this would not explain the increase in PGE_2 after depletion of normal host Mac-1^+ Mφ.

Indomethacin, at 1 x 10^-7 M, is a specific inhibitor of the cyclooxygenase pathway (244) which generally reduced the levels of PGE_2 by approximately 50%. Indomethacin treatment corroborated data on the production of PGE_2 by Mφ subsets without arachidonic acid inhibitors, except for Mac-3^+ Mφ. This was manifested both by lack of indomethacin sensitivity of the anti-Mac-3-treated normal host Mφ and the differential sensitivity to indomethacin seen in anti-Mac-3-treated normal versus TBH Mφ. This could reflect a tumor-induced alteration in Mφ functions, i.e., TBH Mac-3^+ Mφ are not the functional equivalent of normal host Mac-3^+ Mφ. The reason for the apparent lack of indomethacin sensitivity of normal host Mac-3^+ Mφ PGE_2 production is unclear.

In the normal host, depletion of Mac-1^+ Mφ caused an increase in protein content of the culture supernatants and a loss of monokine-mediated suppression of the MLR. Since PGE_2 production increased from cells remaining after depletion of Mac-1^+ Mφ, it was inferred that the Mac-1^+ peritoneal Mφ was not the producer of PGE_2, but was instead a regulatory cell, and leaves open the possibility of production of other inhibitory monokines. This regulation of PGE_2 production by normal host Mac-1^+ Mφ appeared to be a dampening effect, since depletion had a more dramatic effect on PGE_2 production than modulation (receptor-ligand interaction). Absence of this effect in TBH suggested that Mac-1^+ Mφ did not play this role in the TBH, or that there was a shift in Mφ phenotypes and associated functions (on an individual or population basis) as the tumor developed. An alternative explanation is that the mAb plus complement-mediated depletion only
removed 50-55% of the Mφ, thus subsequent renormalization of cell numbers might increase the percentage of PGE₂-producing Mφ. In other words, a 50% depletion of nonsecreting cells, with subsequent resuspension (enrichment) back to original cell numbers, could yield a 100% increase in PGE₂ levels in cultures of the surviving cells. This explanation, however, does not account for the differences seen between normal and TBH Mφ, other than to suggest again a tumor-induced population shift.

The use of culture supernatants from anti-Mac-3 depleted normal host Mφ in the MLR implicated Mac-3⁺ Mφ as producers of immunosuppressive monokines. Since the Mac-3⁺ Mφ were not necessarily producers of PGE₂, it leaves open the possibility that normal host Mac-3⁺ Mφ produce other immunoregulatory compounds in our system. Resident peritoneal Mφ appear to be Mac-1,3⁺ (263, 304), which is interesting because normal host Mac-1⁺ Mφ were also implicated as contributing to the suppression seen in the MLR. The contribution of shared antigens and production of other monokines by Mφ subsets in this system remains to be determined.

Depletion of Mac-2⁺ Mφ in the TBH resulted in a significant loss of both MLR suppression and PGE₂ production in the presence or absence of indomethacin. Thus, the Mac-2⁺ Mφ must be the producer of PGE₂ in the TBH, which accounts, at least in part, for the immunosuppression seen in the MLR. The possibility of a population shift between normal and TBH was supported by loss of Mac-1⁺ and decrease of Mac-3⁺ Mφ-mediated regulation of PGE₂ production as the tumor developed and lack of evidence for substantial PGE₂ production by Mac-2⁺ Mφ in normal hosts. Note also that depletion of Mac-2⁺ Mφ in the normal host and TBH did not result in increased PGE₂ production (or restoration to control levels) by the remaining cells, confirming that both normal host and TBH Mac-1⁺ and Mac-3⁺ Mφ were not the producers of PGE₂.

The production of PGE₂ by TBH Mac-2⁺ Mφ, and the control by Mac-3⁺ Mφ was supported by the data of Shibata and Volkman (249) using Corynebacterium parvum activation of Mφ from ⁸⁹Sr-treated bone marrow-depleted mice. One of their conclusions is that the PGE₂-producing suppressor Mφ in the spleen were bone marrow-derived and not resident. This agrees with our data that Mac-2⁺ Mφ (thioglycollate-elicited [119], not Mac-3⁺ resident [263, 304]) were the PGE₂ producers, at least in the TBH.
The immediate significance of this study rests on the fact that PGE\(_2\) mediates its immunosuppressive effects by decreasing IL 2 production (42), Ia antigen expression (254), and also activating suppressor T cells (41). We have documented (28, 91) all but PGE\(_2\)-induced suppressor T cell activation in our system. A tumor-induced suppressor T cell mediates immunosuppression by producing soluble inhibitors of DNA synthesis (66, 75). In addition, MLR-induced proliferation of TBH splenic responder cells can be restored to normal host levels by the addition of indomethacin (Fig. 11). Taken together, these data point to a central role for PGE\(_2\) in tumor-induced immunosuppression. This study implicated the Mac-3\(^*\) and Mac-2\(^*\) Mφ from normal and TBH, respectively, as being responsible for inhibition of MLR-induced proliferation. The TBH Mac-2\(^*\) Mφ was the producer of PGE\(_2\), which at least partly explained the loss of MLR-inhibitory activity after depletion of TBH Mac-2\(^*\) Mφ. These results are especially interesting in light of Goldyne's report on buoyant density fractionated Mφ and T cells, which showed fractionated Mφ produce varying amounts of PGE\(_2\), and the fractionated T cells respond differently to the PGE\(_2\) (98). Delineating the complete interaction between tumor-altered Mφ populations and their factors with tumor-altered T cell populations in our system is presently under experimentation.

A Mφ population shift resulting from tumor growth could potentially cause a change in shared antigens on both the individual and population level. This tumor-induced shift in phenotype, coupled with a functional alteration, i.e., the tumor-induced loss of the dampening function of Mac-1\(^*\) Mφ on PGE\(_2\) production by Mac-2\(^*\) Mφ, may explain both the increased PGE\(_2\) production generally associated with TBH Mφ and why this study did not delineate the PGE\(_2\) producer in the normal host Mφ. Flow cytometry, positive selection, and a more extensive panel of mAb are currently being used to determine the combination of shared antigens which delineate the progression of Mφ from normal to TBH phenotype.
Chapter III

GLYCOSYLATION AND HYDROPHOBICITY OF THE HIGH MOLECULAR WEIGHT INHIBITORY MONOKINE

Introduction

Cells of the immune system communicate with each other, and regulate each other's activities, via soluble mediators (201). Dissection of immunoregulatory pathways and cellular interactions has progressed rapidly with the cloning and subsequent commercial availability of interleukin 1 (IL 1) (162), interleukin 2 (IL 2), interferon-γ, colony stimulating factors, and now tumor necrosis factor. Biochemical characteristics of these factors are now being determined with much greater accuracy. IL 1, for example, has been found to exist in two forms, α and β (207). Tumor necrosis factor has been found to be the equivalent of cachectin, a macrophage (Mφ)-derived modulator of metabolism (19). Interferon-γ has been found to be responsible for a variety of disparate activities, notably Mφ activating factor, induction of Ia antigens on Mφ, and regulation of Fc receptor ex-
expression. Interferon-$\gamma$ exhibits size and charge heterogeneity due to variable glycosylation, which appear to have no effect on its biological activity (297).

Previous work in this laboratory showed the high mw inhibitor was a heat-stable molecule which was generated in the presence of indomethacin (51). Interestingly, the same supernatants were found to have a heat-stable inhibitor and a heat-labile enhancer of T cell proliferation (48). These supernatants were found to contain little, if any, interferon or IL 1 (51). The heat stability of the high mw inhibitory monokine was of greatest interest to us, and provided the basis for the series of experiments presented here. We have already provided data (168) showing that the high mw inhibitor was not prostaglandin E$_2$ (PGE$_2$), which is important because both of these molecules are heat-stable (boiling for 10 min does not abrogate their inhibitory activity). It is easy to conceptualize a small molecule like PGE$_2$ (352 mw) being heat-stable. The high mw molecule is $> 67$ kd, however, and its heat stability is harder to accept, especially in light of the fact that most proteins, enzymes especially, have an activity which is dependent on their conformation, and boiling is a convenient way to denature enzymes. An explanation must be found, then, that accommodates the size ($> 67$ kd), lack of effect of proteolytic enzyme sensitivity, and charge of the high mw monokine. There are examples of lipids, lipoproteins, glycoproteins, and glycosphingolipids which are inhibitory to T cell proliferation (153, 174, 230, 314). This group of molecules is not susceptible to boiling or proteolytic enzymes (209), and the high mw inhibitory monokine may belong in this broad classification. Experiments described in this chapter on the hydrophobicity and potential glycosylation of the high mw inhibitory monokine provide some evidence for this assertion.
Materials and Methods

Mice

Male BALB/c mice were obtained at 4-6 wk of age from either Dominion Laboratories, Dublin, VA, or the Virginia-Maryland Regional College of Veterinary Medicine Vivarium, Blacksburg, VA. Male C3H/He mice were obtained at 4-6 wk of age from Dominion Laboratories. Mice were injected intramuscularly in the left hind leg with a single-cell suspension of a methylcholanthrene-induced transplantable nonmetastatic fibrosarcoma (66). Normal and TBH mice were tested for LDH virus by Microbiological Assoc., Bethesda, MD, and were found to be negative.

Medium

RPMI 1640 (Flow Laboratories, McLean, VA, or Hazleton Dutchland, Denver, PA) supplemented with 50 μg/ml gentamicin (GIBCO, Grand Island, NY), 2 gm/L sodium bicarbonate (Fisher Scientific), and 25 mM HEPES (Sigma, St. Louis, MO) was filter sterilized and used in all cell culture and dialysis experiments. Fetal Bovine Sera (FBS; Flow Laboratories or Hazleton) was used in biological assays; RPMI medium supplemented with FBS and 4 x 10^-5 M 2-mercaptoethanol is called complete medium.
Mrp and Mφ Supernatants

Splenic and thioglycollate-elicited peritoneal exudate cells were plated on 150 mm tissue culture-treated dishes for at least 2 hr at 37°C in a 5% CO₂ incubator, nonadherent cells rinsed off, and adherent cells (Mφ) scraped and resuspended to 4 x 10⁶/ml. Cells were then replated on plastic dishes, and incubated at 37°C. After four days of culture, the serum-free supernatants were harvested, centrifuged at 800 x g for 5 min to remove cells, and the remaining supernatant centrifuged at 10,000 x g for 20 min, and frozen for later concentration on a stirred cell ultrafiltration device (Model 52, Amicon Corp., Lexington, MA) under nitrogen pressure (40 psi) using a YM30 (30,000 mw exclusion, low protein binding) membrane. Concentrated samples were washed with five 40 ml volumes of RPMI medium in the stirred cell to completely remove low mw compounds. Supernatants were concentrated 10- to 100-fold and sterile filtered on Milllex GV 0.22 μm syringe filters (Millipore Corp., Bedford, Ma.) before use in biological activity assays.

β-Galactosidase Purification

β-galactosidase provided by Dr. David Smith (Department of Biochemistry and Nutrition, VPI&SU) was purified from jack bean meal using the procedure of Li and Li (157). Briefly, 250 g of jack bean meal was stirred in 1.5 L of water, centrifuged, and the supernatant precipitated overnight with ammonium sulfate (16.4 g/100 ml supernatant) in the cold. This was then centrifuged (10,000 x g, 20 min) and the precipitate discarded. The pH of the supernatant was adjusted to 5.5 with 1.5 M sodium citrate pH 2.7. Ammonium sulfate was again added (18.1 g/100 ml solute) and stirred overnight in the cold. This solution was centrifuged as above, the supernatant discarded and the precipitate resuspended in 0.02 M Na₃HPO₄ pH 8.0 and dialyzed in the same buffer in the cold. The dialysate was then centrifuged, applied to a DE53 column and eluted with 0.02 M PO₄⁻ at pH 8.0. To determine activity, 100 μl of β-galactosidase was added to 1.0 ml of
p-nitrophenyl-β-galactoside (2 mM in RPMI medium) and incubated at 37°C. At appropriate times, the reaction was stopped by addition of 3.0 ml of 0.2 M sodium borate, pH 9.8, and the absorbance determined in a spectrophotometer at 400 nm after blanking with RPMI medium. Activity assays were performed by Dr. Howard Krivan of the VPI&SU Anaerobic Microbiology Department.

Lectin Fractionation with Wheat Germ Lectin

Wheat germ lectin (from *Triticum vulgari*) linked to agarose (Sigma), which selectively binds N-acetyl-glucosamine and neuraminic acid residues, was used to fractionate the inhibitory activity. One ml of Amicon concentrated normal and TBH peritoneal Mφ supernatants were incubated with the lectin overnight at 4°C in 1.5 ml centrifuge tubes. These were then spun at 10,000 x g for 5 min and the supernatants aspirated. The lectin was then washed 5 times with 1.0 ml volumes of RPMI medium (1 hr incubation at 4°C, spun, and aspirated). One volume of 1 M N-acetyl-D-glucosamine (Sigma) was then added to the lectin and incubated at 4°C for at least 3 hr, then spun and aspirated. The lectin was then washed with 1 ml of RPMI medium. All fractions were dialyzed to RPMI medium in BSA-coated Spectrapor 4 dialysis bags (12,000-14,000 mw cutoff) and filter sterilized before testing in the MLR.

Lectin Fractionation with Bandeirea simplicifolia

A lectin derived from *Bandeirea simplicifolia* was bound to cyanogen-bromide activated Sepharose in Dr. Smith's laboratory. This column was provided to me equilibrated in Tris buffered saline (TBS), pH 7.5 with 0.02% NaN₃. The column was washed extensively and a 1 ml sample of normal or TBH peritoneal Mφ concentrated supernatant was applied and followed with one ml volumes of buffer or buffer containing 1 M α-methyl galactoside (before collection of the fifth
fraction). Fractions were dialyzed to RPMI medium in BSA-coated Spectrapor 4 dialysis bags and filter sterilized before testing in the MLR.

**Fractionation using Hydrophobic Interaction Chromatography**

Phenyl-Sepharose was used to fractionate concentrated Mφ supernatants. The phenyl-Sepharose column was used in a batch technique and 1 ml of swollen gel was equilibrated with saline and 1 ml of concentrated TBH peritoneal Mφ supernatant or RPMI medium was added. Incubations of samples and elution buffers were carried out at 4°C for 30 min. Elution buffers were 10% saturated ammonium sulfate (SAS), 10% SAS made up in 10% polyethylene glycol (PEG), 5% SAS in 15% PEG, or 0% SAS in 20% PEG. Note that the SAS decreases while the PEG increases. All volumes were 1.0 ml, and all samples were dialyzed to RPMI medium and sterile filtered before testing in the MLR.

**Mixed Lymphocyte Reaction**

Biological activity of Mφ supernatants, column fractions, sugars, and sugar-protein conjugates were assessed in a Mφ-depleted BALB/c anti-C3H one-way MLR. A single cell suspension of BALB/c (H-2d) splenic responder cells were fractionated by nylon wool columns (51, 55, 126). Nonadherent T cells were eluted with warm RPMI medium and placed on a Sephadex G-10 (Sigma) and glass bead column and also incubated for 30 min. Cells were eluted and plated on 150 mm tissue culture dishes (Hazleton) for 1 hr at 37°C. Nonadherent cells were washed off and re-suspended to 2 x 10^5 viable cells/50 μl and dispensed in 96-well microculture plates (Flow Laboratories). C3H/He (H-2k) splenic stimulator cells were treated in a similar fashion, but were treated with 25 μg mitomycin-C (Sigma) per 10^7 cells for 20 min at 37°C, and washed 4 times. Alternatively, stimulator cells were X-irradiated for 4 min resulting in a dose of 2400 rads. Stimulator cells
were dispensed at $4 \times 10^5$ viable cells/50 µl/well. Responder and stimulator cells were found to contain < 0.5% esterase positive cells. Test samples were added to the MLR at time of initiation. Constant well volume of 200 µl was maintained. Assays were incubated for 90 hr at 37°C, then pulsed for 6 hr with 1 µCi/well of tritiated thymidine ($^3$H-TdR) (Amersham Corp., Arlington Heights, IL), harvested onto glass fiber filters (Whatman 934-AH), and counted in a Beckman LS230 liquid scintillation counter. Counts are expressed as percentage of control (responder plus stimulator cells with no supernatant addition).
Results and Discussion

Hydrophobicity of the High mw Inhibitory Monokine

Previous work showed that the inhibitory monokine was non-dialyzable (>12,000 mw). Dialysis proved to be a vexing problem, however, with yield losses which sometimes resulted in total loss of inhibitory activity upon dialysis. Coating of the dialysis bags with BSA in the cold to prevent non-specific adsorption resolved this problem, and gave us a clue that the inhibitory activity may be somewhat hydrophobic. An attempt was then made to use high pressure liquid chromatography (HPLC). This was done with a gel filtration column (TSK 3000 SW) with a useful range from 4,000 to 400,000 mw and a Waters HPLC system (laboratory of Dr. James Palmer, Department of Food Science, VPI&SU). The column was run with 0.9% saline that had been previously filtered, and was first calibrated with mw standards, and their elution profile recorded on a strip chart connected to a UV detector set at 280 nm. Samples placed on the HPLC column were TBH Mφ supernatants that had been concentrated and run on hydroxylapatite. The 0.3 M fraction was known to be inhibitory and was used on the column. Concentrated Mφ samples were not used directly so as to avoid placing a growth medium (RPMI medium) on the column, which would simply invite bacterial contamination, and to avoid overloading the column with too many proteins or too high a concentration. The sample volume applied to the column was 50 µl. This resulted in an increase in the apparent baseline of the effluent, so another sample was applied, with the same result of no resolution whatsoever. Molecular weight standards were injected into the column, and these also no longer gave resolvable peaks. Apparently, the sample (but not necessarily the high mw inhibitor) had precipitated on the bed of the column, as it was subsequently flushed out using a solution of acetic acid and methanol, which resulted in a large broad peak on a refractive index recorder. Calls to technical service representatives of several companies resulted in the understanding that our factor(s) must be hydrophobic to have precipitated under the condi-
tions we used. Further experimentation using a column of phenyl-Sepharose and eluting the column with decreasing percentages of saturated ammonium sulfate and increasing percentages of polyethylene glycol (all fractions were subsequently dialyzed to RPMI) resulted in no recovery of inhibitory activity from either normal or TBH Mφ supernatants (Fig. 16). This confirmed our suspicions of hydrophobicity but did not aid in our purification of the high mw inhibitor.

Evidence in Favor of a Glycoprotein

Heat stability is not usually a characteristic of proteins, but it is characteristic of some glycoproteins. Glycoproteins are not stable to boiling at acidic pH, however. In fact, this acid hydrolysis is one of the hallmarks of glycoproteins. In Fig. 17, samples of normal and TBH peritoneal Mφ concentrated supernatants were boiled for 10 min after first adjusting their pH down to 5 or 4 with 0.1 N HCl. Samples were dialyzed and filtered before addition to the MLR. The inhibitory activity of both supernatants was abrogated by this treatment, lending support to the glycoprotein nature of the inhibitory activity.

Lectin Column Fractionation

A series of lectins were used in an effort to find a useful technique for affinity purification of the high mw inhibitory monokine. Lectins employed included (i) Wheat germ agglutinin (*Triticum vulgaris*), specific for N-acetyl-glucosamine and sialic acid, bound to agarose (Sigma); (ii) *Bandeirea simplicifolia*, specific for α-1,3-galactose and α-1,3-galactose N-acetyl-glucosamine, bound to cyanogen-bromide-activated Sepharose (Dr. David Smith); (iii) *Ricin communis* A, specific for β-D-galactose, bound to cyanogen-bromide-activated Sepharose (Dr. David Smith) and (iv) Con A (*Canavalia ensiformis*) specific for α-D-glucose and α-D-mannose, bound to Sepharose CL 4B (Sigma).
Figure 16. Fractionation on phenyl-Sepharose: Figure 16 shows the results of fractionation of TBH peritoneal concentrated Mφ supernatants, with the fractionation of RPMI medium given as a control treatment. Data show the relative effects of adding TBH Mφ supernatant (TBH) or RPMI medium (RPMI) to the MLR. One ml of each sample was placed on the column and eluted with 10% saturated ammonium sulfate (10S), 10% S together with 10% polyethylene glycol (10S/10P), 5% S with 15% P (5S/15P), and 0% S with 20% P (0S/20P). All samples were dialyzed to RPMI medium and filter sterilized before addition to the MLR.
Figure 17. Heat treatment of concentrated Mφ supernatants: Normal and TBH concentrated peritoneal Mφ dialyzed supernatants were inhibitory in the MLR. Supernatants were adjusted to pH 5 or 4 with 0.1 M HCl, and some were boiled for 10 min. All samples were dialyzed to RPMI medium (pH 7.4) before addition to the MLR.
Data presented in Fig. 18 using a wheat germ agglutinin column shows that inhibitory activity from normal host peritoneal \( \text{M}\varphi \) supernatants did not bind to the column at all. It appeared that some activity from TBH peritoneal \( \text{M}\varphi \) supernatants was bound, because there was an increase of cpm from 6,533 \( \pm \) 290 to 10,417 \( \pm \) 228. No activity from either normal or TBH \( \text{M}\varphi \) supernatant was washed from the column by RPMI medium or 1 \( M \) N-acetyl-glucosamine, the competitive sugar. This indicated that N-acetyl-glucosamine was not involved in the active moiety of the inhibitory monokine or even the monokine itself. The possible weak binding to the column by TBH \( \text{M}\varphi \) supernatants was a possible indication that N-acetyl-glucosamine or sialic acid was involved in the TBH inhibitory activity. Since I had previously seen neuraminidase sensitivity in TBH \( \text{M}\varphi \) supernatants, and wheat germ agglutinin requires a strong sialic acid signal to bind to it (high degree of sialylation), I felt that this was some supportive evidence for the presence of a sialic acid residue on the TBH high mw inhibitor.

Data presented in Fig. 19 on fractionation of normal and TBH peritoneal \( \text{M}\varphi \) concentrated supernatants using the lectin \textit{Bandeirea simplicifolia} reveals that inhibitory activity from normal \( \text{M}\varphi \) supernatants eluted immediately and in fractions 3, 4, and 7. The bed volume of the column was approximately 3 ml, and all fractions were 1 ml to match the sample volume. The competitive sugar \( \alpha \)-methyl galactoside was added before fraction 5 was collected. For inhibitory activity to come off before addition of the competitive sugar implied a retarding effect, assuming it was after the void volume. Fraction 7 may be of interest. Inhibitory activity did not appear to be selected in any of the fractions from TBH \( \text{M}\varphi \) supernatants, except fraction 3.

Data on fractionation using Con A-Sepharose or \textit{Ricin communis} A-Sepharose are not presented. \textit{Ricin communis} is a large (32,000 mw) molecule which apparently leached off the columns in amounts which were non-detectable by Dr. Smith but which obliterated proliferation in biological assays (dialyzed wash buffer was 100% inhibitory in the MLR). Con A-Sepharose fractionation was inconsistent, with the inhibitory activity being found either in different fractions, no fractions, or many fractions. It would be interesting to run these columns in the absence of RPMI medium, which contains 2 mg of glucose per liter of medium. Unfortunately, this would involve dialysis to a glucose-free salt solution, running the column, and dialysis back to RPMI.
Figure 18. Fractionation on wheat germ agglutinin: Figure 18 shows the addition of normal (\textit{\textw{\(\)) and \textit{\textw{\(\)} peritoneal Mφ supernatants to the MLR (CONC) and their fractionation on wheat germ agglutinin (WGA). Samples were incubated with the lectin overnight at 4°C, spun and aspirated, and added to the MLR at 100 μl/well at time of initiation (WGA). The lectin was washed 5 times with 1 ml volumes of RPMI (W1 - W5) and then treated with 1 ml of 1 M N-acetyl-glucosamine (NAG). All samples were dialyzed to RPMI medium and filtered before use in the MLR.
Figure 19. Fractionation on Bandeirea simplicifolia lectin: Figure 19 shows fractionation of 1.0 ml of normal and TBH peritoneal Mφ supernatant. Samples were added to the MLR either as direct concentrates (CONC) or after dialysis (DIAL) to RPMI medium. The column was eluted with TBS buffer (FR 1-4) or TBS buffer containing 1 M α-methyl galactoside (FR 5-7). The column was run in the cold, and all fractions dialyzed to RPMI medium and filtered before use in the MLR.
medium. My unfortunate prediction is that this double dialysis will result in too great of a loss of the inhibitory activity. A western blot technique whereby the blots are detected with Con A, anti-Con A (Sigma), and protein A peroxidase may prove more useful (80).

**β-Galactosidase and Sugar-BSA Conjugates**

Initial experiments suggested that the inhibitory activity was sensitive to β-galactosidase, but further experimentation did not confirm this. While no direct correlation can be made with the inhibitory monokine, the sugar-BSA conjugates may well provide a useful model system for delineating the mechanism of action, target cell, etc. of inhibitory monokines in general. We first determined the extent of contamination of the β-galactosidase preparation with other enzymes. The β-galactosidase preparation provided by Dr. David Smith had no detectable α-galactosidase activity, while β-hexosaminidase and α-mannosidase activities were detected at 1/250th and 1/50th of the levels of β-galactosidase activity, respectively. We then determined that β-galactosidase exhibits time-dependent utilization of its substrate in RPMI medium at pH 7.4 and 37°C (0.1 units of β-galactosidase plus 1 ml of 2 mM p-nitrophenyl β-galactoside in RPMI medium, incubated at 37°C, stopped with 3 ml of 0.2 M sodium borate, pH 9.8, and read at 400 nm in a spectrophotometer gave the following results at 5, 8, and 23 hr of 0.67, 0.83, and 1.10 OD). This activity was obliterated by prior boiling of the enzyme.

The addition of β-galactosidase directly to the MLR at the same concentration used to treat Mφ supernatants or sugar-BSA conjugates had no effect on 3H-TdR incorporation (control = 99,524 ± 11,185; + β-galactosidase = 10,1936 ± 3,411). Addition of sugar-BSA conjugates enhanced proliferation over control levels except in the case of lactose-BSA or acid-hydrolyzed 6′sialyl-lactose-BSA, both of which inhibited 3H-TdR incorporation. This was in contrast to the data obtained using free sugars, all of which were inhibitory to 3H-TdR incorporation.

Sugar-BSA conjugates (gift of Dr. David Smith) revealed that there is some sugar specificity to the suppression of lymphocyte proliferation in the MLR. This was interesting because virtually
any free sugar I had used was inhibitory to proliferation. These included α-methyl-mannoside, lactose, galactose, 6’sialyl-lactose, and 3’sialyl-lactose. Others have noted that free sugars do not support cell proliferation (30). Coupling of a sugar to a protein must change the recognition or internalization pattern of the compound. It is possible that free sugars simply overwhelm cell surface receptors, whereas sugar-protein conjugates act in a more specific fashion. Fig. 20 shows the effects of adding 6’sialyl-lactose-BSA, a high mw fraction from Lewis B negative individuals linked to BSA, lactose-BSA, lacto-N-tetrose-BSA, and BSA itself to the MLR. With the exception of lactose-BSA, the sugar-BSA conjugates and BSA itself had no effect on MLR-induced proliferation at any of the doses used. Out of the group, lactose-BSA is the only one with a β-1,4-galactose as its terminal sugar. Lacto-N-tetrose is galactose linked β-1,3 to N-acetyl-glucosamine linked β-1,3 to galactose linked β-1,4 to glucose (as glucitol) linked to BSA. Boiling of the 6’sialyl-lactose-BSA in 0.01 M HCl, pH 2, for 20 min effectively removes the sialic acid from the 6 position, leaving lactose-BSA. This procedure had no effect on BSA, but caused 6’sialyl-lactose-BSA to become suppressive (95,457 ± 4523 cpm before acid hydrolysis, 25,709 ± 2060 cpm after). The next experiment involved using lactose-BSA conjugates which had different rates of substitution, ranging from 11 to 23.6 to 37 to 49 moles lactose per mole of BSA. All of these were suppressive in the MLR when added at 1 mg/ml, as shown in Fig 21. When these samples were treated with β-galactosidase, however, they lost their inhibitory activity, again suggesting that the terminal lactose was important in the suppression mediated by the sugar-BSA conjugates. This link to a protein carrier may explain the large size associated with the inhibitory activity found in the Mφ supernatants. It is useful to note that Wolf and Merler (314) reported a T cell-derived inhibitor of lymphocyte proliferation is actually a biologically active lipid bound to inactive proteins. Interestingly, dialysis against hypotonic solutions caused separation of the low mw lipid from its protein carrier and subsequent loss of inhibitory activity. This may have bearing on our dialysis problems.
Figure 20. Activity of sugar-BSA conjugates in the MLR: Sugar-BSA conjugates were prepared by Dr. David Smith and added to the MLR after filter sterilization or X-ray irradiation. Samples were titrated from 100 µg/well to 1 µg/well (100 µl/well added at time of initiation). Figure 20 shows the addition of 6′sialyl-lactose-BSA (6SL-B), high mw sugars from Lewis B negative individuals (LE-B), lactose-BSA (LAC-B), lacto-n-tetrose-BSA (LNT-B), or BSA itself to the MLR.
Figure 21. β-galactosidase abrogation of LAC-BSA inhibition: Figure 21 shows that the addition of lactose-BSA at mole:mole substitution rates of 11.6, 23.6, 37, and 49, were all inhibitory to the MLR (1 mg/ml, 100 μl/well added at time of initiation). β-galactosidase was added directly to the MLR at a 1/10 dilution of stock (stock enzyme preparations were all approximately 1 unit/ml in PBS) to correspond to the dilution used in treatment of sugar-BSA conjugates. The "0" bar graph represents the proliferation control with no lactose-BSA added ( / ) or with the addition of β-galactosidase enzyme ( / ). Lactose-BSA conjugates were treated with β-galactosidase for 5 hr at 37°C, then added to the MLR at 100 μl/well at time of initiation (BGAL TRT).
Summary

The main objective of the studies presented in this dissertation was to characterize a high mw inhibitory monokine found in Mφ culture supernatants. Two objectives intimately related with this were to determine the population of Mφ which produced PGE₂ and/or the high mw inhibitory monokine and determine the effects of tumor growth on the factors or their producers.

Previous work in this laboratory shows that if Mφ are cultured at a density of 4 x 10⁶ cells/ml for four days they produce a supernatant that is inhibitory to T cell proliferation (46). If the culture conditions are changed to include indomethacin (48) (an inhibitor of PGE₂ production [85, 244, 291]), the supernatant is still inhibitory, indicating that PGE₂ is not the sole contributor to the inhibitory activity. If one of two proteolytic enzyme inhibitors, phenyl-methyl-sulfonyl-fluoride or benzidine, were added at the initiation of the Mφ culture, a culture supernatant is produced that is even more inhibitory (48). This indicated that the inhibitory activity could be vested in a proteinaceous, non-PGE₂ material. These Mφ supernatants are found to have enhancing as well as inhibitory activities, and to have an inhibitory activity which could be fractionated on DEAE-Cellulose (51), but at that time no qualitative differences between normal and TBH Mφ supernatants were indicated.

A difference was known to exist between normal and TBH Mφ. Early work showed that there is a quantitative increase in both the percentage and actual number of Mφ found in the spleen of
TBH mice, which contributes to the splenomegaly found in TBH (66). Recently published experiments have demonstrated that there is an increase in the number of peroxidase-positive peritoneal Mφ in the peritoneal exudates of TBH (93), indicating that the bone marrow is undergoing a rapid turnover and production of cells in response to the tumor challenge. This increase in the number of immature (peroxidase-positive) Mφ in the peritoneal cavity of TBH is undoubtedly associated with a tumor-induced decrease in Mac-2* Mφ and increase in Mac-3* Mφ in the peritoneal cavity (90). This phenotypic shift in the Mφ population has been extended to a functional alteration of both the activities and the soluble mediators those Mφ produce. We have demonstrated that the suppressor phenotype shifts from Mac-3* to Mac-2* as the tumor develops (93). Data presented in this dissertation extends this finding by showing that the Mac-2* Mφ is the producer of PGE₂ in the TBH, and that this is probably due to the loss of Mac-1* Mφ-mediated down regulation of PGE₂ production.

Experiments done to dissect the role of T cells in the immunological anergy seen in TBH animals revealed that TBH splenic T cells contain a mildly nylon wool-adherent population of T cells which produces an inhibitor of DNA synthesis and DNA polymerase (66, 75, 77). Further work revealed that TBH splenocytes produce very low levels of IL 2 (28) and IL 3 (26, 27), and that the drop in IL 2 and IL 3 occurs at approximately the same time the tumor becomes palpable, the splenomegaly occurs, and the drop in proliferation assays is seen. In other words, immunosuppression sets in approximately 10 days after injection of the tumor, and its causes are multifactorial.

Further work on the Mφ-T cell interaction facet of the immunosuppression revealed that TBH Mφ culture supernatants contain more PGE₂ than their normal counterparts (55). This is in agreement with the literature on a variety of tumor model systems in mice and humans (102, 121, 146, 211). Data presented here could explain the mechanism of this phenomena. I have shown that PGE₂ production by TBH Mac-2* Mφ was probably the result of a loss of Mac-1* Mφ down-regulation of PGE₂ production. In other words, tumor growth induced a change in Mφ phenotype, which was apparently manifested as an altered balance between the subpopulations, and subsequent uncontrolled production of PGE₂. It is interesting that PGE₂ is able to down-modulate the pro-
duction of IL 2 (42), and we have already documented the decline in IL 2 production as tumor growth progresses (28).

Previous work has also shown that concomitant with an increase in PGE$_2$ production there is a decrease in the susceptibility of TBH T cells to PGE$_2$-mediated down-regulation in the MLR (55). This was originally published as an anomaly but can be better interpreted in light of recent findings by us that there is a tumor-induced loss of Ia antigen expression on TBH Mφ, probably as a consequence of high in vivo levels of PGE$_2$. The lack of Ia antigen-mediated stimulation of allogeneic C3H T cells could not be corrected by treating the Mφ with indomethacin. Loss of Ia antigens was shown by immunofluorescence and the use of Mφ as stimulators of an allogeneic MLR (91), and has since been confirmed using flow cytometry.

All MLRs were done using Mφ-depleted T cells, and the Mφ contamination by esterase staining and latex particle uptake has typically been found to be <5%, usually <2%. Mφ are the primary producers of PGE$_2$ (121), and are required both for initiation and suppression of T cell proliferation. Data presented in this dissertation using indomethacin in a time course addition into the MLR revealed that the small contamination of Mφ, while necessary for the reaction to proceed, probably are producing PGE$_2$ in vitro, as indomethacin added at the initiation of the MLR caused an increase in proliferation of both normal and TBH responder cells. While the presence of Ia antigens and production of PGE$_2$ has not been correlated, it now appears that the lack of sensitivity of TBH T cells to PGE$_2$ down-modulation (55) is because Ia$^+$ Mφ are necessary for proliferation to be initiated (283). These Ia$^+$ Mφ are missing in the TBH, so PGE$_2$ was not able to act via down-modulation of Ia antigen expression, thereby mitigating its effect.

In my discussion of past work that has come out of this laboratory I have alluded to data presented in this dissertation. There is at least one more piece of old information, however, that is necessary for an interpretation of the new data. In early experiments directed at delineating the change in T cell subpopulations and their responsiveness to Mφ factors, we found the first indications for a tumor-induced qualitative alteration in the soluble factors that Mφ produce. Using anti-Lyt plus complement, we were able to deplete T cell populations, and use either the whole, helper, suppressor, or an add-back population of normal and TBH splenic T cells as responders in

Summary
an MLR. These cell cultures were then treated with supernatants from normal or TBH Mφ. Doing this, we determined that TBH suppressor T cells (Lyt 2,3+) proliferate in response to a generally inhibitory normal Mφ supernatant (170). This was the only combination to exhibit this effect, and while it is not an in vivo situation, it does show that normal and TBH T cells and Mφ supernatants were not functional equivalents. The reader should be aware that those Mφ supernatants probably contained low levels of PGE₂ which was not removed in the concentration procedure.

The data presented in this dissertation show that there was a high mw inhibitory monokine, in addition to and distinct from PGE₂, found in the culture supernatants of splenic and thioglycollate-elicited peritoneal Mφ from normal and TBH. This statement alone tells the reader that the high mw monokine was probably produced as part of the steady state condition of Mφ, and possibly explains the natural existence of suppressor Mφ in the immune system of the normal host. Note that suppression can be passive (lacking the antigens required to initiate T cell proliferation) or active (production of a suppressive mediator). Data presented here showed that in normal hosts, Mφ-mediated suppression was active (production of PGE₂ and the high mw inhibitor). In the TBH, suppression was both active and passive. High levels (or prolonged exposure to high levels in vivo) of PGE₂ produced by Mφ will result in the activation of suppressor T cells and the loss of Ia antigens on Mφ. These secondary effects, in conjunction with production of the high mw inhibitor, will lead to immunosuppression. It is possible that glycosylation of the high mw inhibitor endows the molecule with a longer biological half-life, which would mimic the prolonged exposure to high levels seen with PGE₂. We have shown that immunosuppression can also be mediated by loss of production of IL 2. This was seen both when examining TBH T cells and by examining the effects of the high mw inhibitor on alloantigen-activated normal host T cells in the MLR. Inhibition of production of IL 2 by the high mw inhibitor, in conjunction with PGE₂ production and loss of Ia antigens in the TBH, would virtually prevent T cell activation and proliferation, probably to the extent that the immune system cannot correct itself and achieve homeostasis.

The high mw inhibitor required the presence of PGE₂ for its immunosuppressive effect to be manifested on T cells in the early stages of activation in the MLR. Once the T cells were activated,
there did not appear to be as stringent a requirement for the presence of PGE\textsubscript{2}. Besides showing activity on different T cell subpopulations (or activation stages), I also showed different effects on cell cycle progression if measured early or late in a mitogen-induced proliferation assay. Unfortunately, one cannot directly compare a mitogen-induced proliferation assay and an alloantigen-induced MLR. These assays differ in the responding T cell populations, the percentage of T cells that respond, the dependency on M\textsubscript{φ}, and the time required for an optimal response. It is useful to compare normal and TBH M\textsubscript{φ} supernatants and note that while the time course activity profile (with and without indomethacin) were similar, their effects on cell cycle progression were not, even though the net result of inhibition was the same. It is interesting that the TBH high mw inhibitor exhibited early enhancement of cell cycle progression, as proliferation of T cells is a requirement for suppressor T cells to develop. The presence of enhancing and inhibitory factors in the same M\textsubscript{φ} supernatant has already been noted (49), but is different from the phenomena reported here, as proliferation and cell cycle progression were both inhibited when measured late in the assay. This experiment supports the idea of more than one inhibitor in TBH M\textsubscript{φ} supernatants, as opposed to the presence of enhancing molecules, or that inhibition overrides enhancement. This is similar to the finding that normal M\textsubscript{φ} supernatants enhanced the proliferation of TBH suppressor T cells (170), which would result in immunosuppression.

Now that I have shown that indomethacin blocked the activity of PGE\textsubscript{2}-free M\textsubscript{φ} supernatants when added to normal host T cells at the initial stages of activation, and that TBH T cell proliferation can be "rescued" by the addition of indomethacin, it would be interesting to determine the susceptibility of TBH T cells to PGE\textsubscript{2} and the high mw inhibitor in an MLR time course experiment by measuring both proliferation and IL 2 levels. This would possibly shed light on the active/passive argument proposed earlier, and allow one to draw an analogy between naive vs. activated T cells and T cells from normal vs. TBH. My speculation, however, is that high \textit{in vivo} levels of PGE\textsubscript{2}, coupled with the presence of the high mw inhibitory monokine, prevent the activation of T cells necessary for an effective response against the tumor. Another experiment needed to prove this speculation is to examine the development of cytotoxic T lymphocytes as a result of MLR-induced proliferation after treatment with the high mw inhibitor and/or PGE\textsubscript{2}.
Experiments directed at elucidating the subpopulation of Mφ responsible for production of the high mw inhibitor instead revealed the producer of PGE₂ in TBH were Mac-2⁺ Mφ. In addition, we also showed that the suppressor phenotype in normal host was Mac-3⁺, with Mac-1⁺ Mφ playing a smaller role. Obviously, the determination of shared antigen expression is important to solving this puzzle, and is being actively pursued. Neither the Mac-1⁺ or Mac-3⁺ Mφ, however, is a producer of PGE₂, which leaves them available as producers of the high mw inhibitor. Interestingly, the resident peritoneal Mφ is apparently Mac-1,3⁺ (personal communication, Dr. Carleton Stewart). If we assume constitutive, but low level, production of the high mw inhibitor in normal hosts as part of a homeostatic mechanism, it may come as no surprise that the resident population (whose numbers are relatively low) is the producer of the high mw inhibitor. It is also possible then, and equally speculative, that there is a hierarchy of communication amongst the Mφ populations in their respective anatomical residences. This idea is analogous to the mechanism proposed by Ting and Rodrigues, who showed that splenic Mφ, after exposure to tumor cells, induce peritoneal Mφ to become suppressive (277).

If one can assume a communication link between peritoneal and splenic Mφ, and assume their ability to thereby educate each other, then it is easy to understand the necessity of a different factor, or alteration of an old factor, to somehow change the signal that the Mφ send and receive. Data presented here, and summarized in the following table, showed that there was a tumor-induced alteration in the high mw inhibitory monokine produced by Mφ. Admittedly, I cannot yet say whether a "new" factor was produced or an "old" factor was altered.

The high mw inhibitory monokine described in this dissertation appeared to be different from the inhibitory factors presently described in the scientific literature. The most intriguing similarities appear to be with factor(s) produced by the U937 human histiocytic lymphoma line. Fujiwara et al. (88, 89) have shown U937 cells spontaneously produce a factor that is 67,000 to 130,000 mw, partially heat-stable, acid-labile, ammonium sulfate-precipitable, and capable of inhibiting IL 2 receptor expression. Personal communication with Dr. Jerold Ellner, in whose laboratory the U937 work was done, unfortunately revealed that the biochemical classification or interaction with PGE₂ of the inhibitory compound(s) is unknown and not being pursued. However, spontaneous
Table 6. Characteristics of Inhibitory Mφ and Mφ Supernatants

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Normal</th>
<th>TBH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>High mw inhibitor</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Size</td>
<td>&gt; 67 kd</td>
<td>&gt; 67 kd</td>
</tr>
<tr>
<td>Hydroxylapatite elution</td>
<td>0.6, 0.9</td>
<td>0.3, 0.6, 0.9</td>
</tr>
<tr>
<td>pH</td>
<td>6.5 - 7.6</td>
<td>4.0 - 6.0</td>
</tr>
<tr>
<td>Neuraminidase sensitivity</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>Requires PGE₂ early</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Indomethacin blocks early activity</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Heat-stable</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Generated with indomethacin</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td><strong>Mφ supernatants</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGE₂ level</td>
<td>less</td>
<td>more</td>
</tr>
<tr>
<td><strong>Mφ</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Produce PGE₂</td>
<td>?</td>
<td>Mac-2⁺</td>
</tr>
<tr>
<td>Control PGE₂</td>
<td>Mac-1⁺</td>
<td>--</td>
</tr>
<tr>
<td>Ia antigen expression</td>
<td>high</td>
<td>low</td>
</tr>
<tr>
<td>Suppressor phenotype</td>
<td>Mac-3⁺</td>
<td>Mac-2⁺</td>
</tr>
</tbody>
</table>

Summary
production of a high mw, heat-stable inhibitor would fit well with my assumption of an inhibitory molecule necessary for immune homeostasis. While the precise biochemical nature of the high mw inhibitory monokine described here remains to be determined, the evidence leaned in favor of a glycosylated factor that was not necessarily a protein, as shown by resistance to proteolytic enzymes, and heat-stability which was lost when boiling at acidic pH. This would fit with descriptions of inhibitory lipoproteins, glycoproteins, and glycosphingolipids. Unfortunately, there is very little in the literature directly implicating Mϕ as the source of these compounds. A point in favor of Mϕ is that they synthesize and/or secrete virtually everything except albumin (107, 271), and the future may well show that they are the source of the compounds in question. This will become more important as more is learned about how sugar moieties give specificity to biological reactions and cellular interactions, and could give insight to the ramifications of a tumor-induced variable glycosylation. The use of sugar-BSA conjugates may also shed light on this, with the finding that while no specificity was vested in monomer sugars, they did exhibit specificity when linked to a protein carrier, with a terminal galactose in a β1,4 linkage appearing to be important. These conjugates may prove useful in finding the target cell of the high mw inhibitory monokine.

The possibility exists that the high mw inhibitor was actually directed at tumor cells themselves. This was supported by previous work on tumor cells (66, 75, 77) and by the observation that the high mw factor was active on cell lines in addition to syngeneic T cells, and fits the data on Mϕ involvement in immune surveillance and tumor rejection. Another possibility is that tumor cells in the TBH may produce PGE₂ (214, 277), or other low mw compounds (255), as a means of dampening T cell proliferation, depressing chemotactic responses, inhibiting phagocytosis, decreasing Fc receptor expression, and decreasing spreading and attachment to substrates (246). Substances which inhibit the accumulation of Mϕ in vitro may also depress the localization of macrophages at neoplastic sites (255). The interaction between the high mw inhibitory monokine and tumor cells or their factors remains to be determined, but it raises the interesting possibility that the tumor cells, in conjunction with altered Mϕ populations and their factors, interfere with T cell activation and proliferation.
Figure 22. Model of Immunoregulation in the Normal Host: This schematic depicts the interaction of PGE₂ and the high mw inhibitory monokine (HMWI) with helper and suppressor T cells. See text for description.
Figure 23. Model of Immunoregulation in the TBH: This schematic illustrates the tumor-induced loss of Ia antigens on Mφ, loss of IL 2, increased suppressor T cell activity, and possible production of two high mw inhibitory monokines (HMWI). See text for description.
At this point I will propose a model of how the inhibitory monokine works, which should provide a paradigm for future experiments. Figure 22 illustrates that normal T cell proliferation and development starts with an unactivated T cell. In the presence of an appropriate stimulus (tumor cells), the T cell should proliferate with the help of Ia⁺ Mφ and IL 1. This proliferation is normally controlled, or dampened, by subsequent production by Mφ of PGE₂ and the high mw inhibitor (depicted as HMWI). These two factors prevent the elaboration of IL 2, and PGE₂ is known to down-regulate the expression of Ia antigens on Mφ. In the case of the TBH T cell (Fig. 23), concomitant production and action of the high mw inhibitor and PGE₂ (does one induce production of the other in an autocrine feedback loop?) prevents the elaboration of Ia antigens on Mφ and of IL 2 by T cells, resulting in no proliferation. Proliferation has to occur, however, for the development of tumor-induced suppressor T cells. The data supports both high mw monokine-induced proliferation of TBH suppressor T cells and enhancement of T cell proliferation if measured early. It is possible that tumor-induced suppressor T cells develop in vivo at an early time point, before proliferation is shut down by the excess presence of the high mw monokine and PGE₂. It would then be possible for these tumor-induced suppressor T cells to cause increased production of PGE₂ and possibly induce variable glycosylation of the high mw inhibitory monokine. This cause and effect relationship has not been determined, and my present interpretation is to assume two high mw inhibitory monokines in the TBH, one of which acts early in the T cell activation process and requires PGE₂ and the other acting farther down the cascade but causing the generation of suppressor T cells, which could be aided by PGE₂. PGE₂ is produced by Mac-2⁺ Mφ in the TBH, but Mac-2⁺ Mφ are not the producers of PGE₂ in the normal host. The evidence suggests that Mac-3⁺ Mφ are the suppressor phenotype in the normal host, with some contribution from Mac-1⁺ Mφ. In the TBH, it could be said that the Mac-3⁺ Mφ contribution was lowered, and that the Mac-1⁺ Mφ contribution was absent, except for loss of control of PGE₂ production. While we do not know the shared antigen expression at this time, it is possible that Mac-3⁺ Mφ are the producers of the high mw inhibitor(s) in both the normal and TBH.

Future work on purification and characterization of the high mw inhibitor, besides the experiments mentioned above, can proceed in several directions:

Summary
1. A specific technique that may prove to be useful in purification is hydroxylapatite chromatography using HPLC methods. Note that hydroxylapatite chromatography was one of the few techniques that selected for the inhibitory activity. Unfortunately, it did not provide enough material to make a subsequent chromatography step feasible.

2. A longer range goal, and the subject of recent grant submissions from this laboratory, would be to construct Mϕ hybridomas to effectively freeze the Mϕ in its progression from the normal to the TBH phenotype and functional capabilities. These hybridomas would provide a constant source of cells which could be tested for production of the high mw inhibitory factor, and thereby provide sufficient starting material for significant progress.

3. It would then be useful to use T cell lines, or antibody-selected T cell populations from normal and TBH, and determine their ability to proliferate, produce interleukins, and develop into cytotoxic T lymphocytes, in the presence of PGE$_2$-free preparations of the high mw inhibitor. Specific comparisons could then be made on the ability of PGE$_2$ and the high mw inhibitory monokine to alter T cell surface antigen expression (markers of development/differentiation), cyclic AMP levels, and cell cycle progression (with the aid of known metabolic inhibitors).

In order to make further progress in our understanding of immunoregulation and its complex pathways and networks, factors such as the high mw inhibitory monokine must be generated in sufficient quantities for biochemical analysis and biological testing. This has already happened with IL 2, TNF, IFN-γ, and others, which are now cloned and commercially available. The interrelationships between these factors and the cells which produce and respond to them is now being determined with greater accuracy. Once the factors are understood, it will then be possible to delineate the alterations in their relationships that tumor growth, aging, parasitic infections, etc. impose on the immune network. Factors such as the high mw inhibitory monokine may then find application as immunotherapeutic agents to dampen uncontrolled immune responses in graft rejection or autoimmune diseases or even be used to down-regulate the proliferation of tumor cells or suppressor T cells. The advantage of this approach is that it would make use of the patient's
own storehouse of biological response modifiers, obviating the need for therapeutic regimens with more toxic and unpredictable side effects.


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Appendix 1

Generation of Rat Antibodies to $M_0$ Factors

1. RATS. Outbred Fischer rats were purchased and stored at the Virginia-Maryland Regional College of Veterinary Medicine Vivarium. Rats were immunized on a bi-weekly basis with subcutaneous injections divided over four sites on the back (one near each limb). The total injection was usually 1 mg (1 ml) of conditioned supernatant. The first injection was antigen in complete Freund’s adjuvant, and all subsequent injections used incomplete Freund’s adjuvant (1 part antigen to 3 parts adjuvant, kept on ice and sonicated with 10 second bursts until emulsified). The emulsification is tested by putting a small drop on water and seeing if it separates: if it separates, keep sonicating. Rats were bled before (prebleed) and 10 days following immunizations by first anesthetizing them with a mixture of halothane and nitrous oxide (courtesy of Brad Krueger), then making an incision with a scissors in the center of a triangle formed by the jaw, sternum, and distal end of the clavicle. The incision is then teased apart with forceps, and the anterior jugular vein exposed as it exits the chest cavity and enters the neck. A 3 ml blood sample is then obtained using a 5 cc syringe and a 22 gauge by 0.5 inch needle, and the sample placed in a 15 ml conical test tube for clotting and transport. The wound is closed using two or three surgical clips, and the rat returned to his cage. Blood samples are rendered cell free by first rimming the clots with a wooden stick, placing the stick in the center of the
clot in the tube, and allowing the clot to condense around the stick overnight at 4°C. Remaining cells are then removed by centrifugation at 1500 rpm for 10 min.

2. ANTIGENS. Antigens consisted of culture supernatants from P388D1 or RAW Mφ-like cell lines. Cells were grown serum-free on 75 cm² tissue culture flasks for four days at an original resuspension of 4 x 10⁶/ml. The conditioned medium from the first day was discarded due to high levels of fetal calf serum. The third antigen originally consisted of the 0.3 M phosphate hydroxylapatite fraction of TBH splenic Mφ supernatants, but was later replaced with concentrated splenic TBH Mφ culture supernatant. All the supernatants were originally concentrated 20-fold (using an Amicon 52 stirred cell ultrafiltration system with a YM30 membrane and 40 psi of Nitrogen gas) before hydroxylapatite fractionation.

3. OUCHTERLONY TESTING. Serum samples, both pre and post immunization, were tested for the presence of antibodies against the antigens. Ouchterlony tests were performed using low EEO agarose (Sigma) melted in the microwave or autoclave (1% in 0.9% saline) and poured into Petri plates (10-15 ml/plate). Wells were punched in a circular pattern and 20 μl of antiserum or antigen added to the well, allowed to dry, and the additions repeated up to three times (a total of 60 μl added to each well). A 1/10 dilution of fetal calf serum was used as a control. Plates were sealed with parafilm and incubated in the humid 37°C incubator.

4. SDS-PAGE. SDS gels are made immediately before use according to the protocols published by Laemmli (148). Glass plates are scrubbed with a brillo pad, rinsed exhaustively in distilled water, and placed into the gel stand using the grommets. Tighten the apparatus so the comb fits snugly at the top. The separating and stacking (top) gel are then prepared, exercising caution because acrylamide is a neurotoxin. The separating gels are either 7.5% or 10%, and the stacking gel is 3%, and are made according to the following table (numbers given in mls):
<table>
<thead>
<tr>
<th></th>
<th>3%</th>
<th>7.5%</th>
<th>10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% acrylamide 0.8% bis</td>
<td>1.0</td>
<td>7.5</td>
<td>10</td>
</tr>
<tr>
<td>1.4 M tris-Cl pH 8.9</td>
<td>0</td>
<td>7.5</td>
<td>7.5</td>
</tr>
<tr>
<td>with 0.4% SDS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(16.95 g Tris Base in 80 ml ddH₂O, pH 8.9 with HCl, add 4ml 10% SDS, bring to 100ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.25 M tris-Cl pH 6.8</td>
<td>0.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(15.14 g Tris Base in 80 ml ddH₂O, pH 6.8 with HCl, ddH₂O to 100ml)</td>
<td></td>
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<tr>
<td>Temed</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>H₂O</td>
<td>8.0</td>
<td>14.7</td>
<td>12.2</td>
</tr>
<tr>
<td>10%SDS</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5% Ammonium Persulfate</td>
<td>0.5</td>
<td>0.3</td>
<td>0.3</td>
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</tbody>
</table>

Ammonium Persulfate is added immediately before pipetting the gel into the plates, as it catalyzes the polymerization reaction. It is convenient to make the 3% gel in a 30 ml beaker, and the separating gel in a 50 ml erlenmayer flask. Add the ammonium persulfate to the separating gel, and pipette it into the gel apparatus; allow it to polymerize for about 2 hr. Add separating gel so that approximately 1 inch is left free under the bottom of the gel comb. The top of the separating gel is covered with water (added very slowly across the gel) so the gel hardens in the absence of an air interface. After polymerization, remove the water from the gel surface, add ammonium persulfate to the 3% gel, pipette it into the apparatus, and add the comb.
After polymerization (about 1 hr), remove the comb and the grommet, and add the
electrode buffer (3.0 g Tris base, 14.4 g glycine, 10 ml 10% SDS, ddH₂O to 1 liter).
If the apparatus is tilted and electrode buffer added to the bottom chamber first, few
bubbles result under the gel plates. Any bubbles must be removed using a 9 inch
Pasteur pipette which is bent at the end. Samples can now be added to the lanes
(volume is determined by the comb). Samples are made up using 5x sample buffer
containing 62 mM Tris HCl, pH 6.8, 2% SDS, 5% 2-me, 7.5% glycerol, and a few
μl of bromophenol blue and pyronin Y. Samples should be standardized to equivalent protein concentrations. Generally, 80 μl of sample is added to 20 μl of 5x sample buffer, and the samples boiled in eppendorf tubes for 2 min. Samples are cooled, and loaded into the lanes using Hamilton syringes, which are rinsed 3-4 times with electrode buffer between samples. Load the samples quickly, attach the electrical leads (the black wire attaches to the negative pole of the power source and to the top of gel) and switch on the power supply. Electrophoresis is done at 15 mAmps until the proteins stack up at the stacking/separating gel interface, and then the current is increased to a constant current of 30 mAmps. The gel is run until the tracking gels reach the bottom of the gel. Pyronin Y will reach bottom before bromophenol blue.

5. WESTERN BLOT. At this point, remove the gel using a small spatula to separate
the plates. Scrape off the 3% stacking gel and discard it. The gel is now placed onto
a sheet of nitrocellulose cut to the approximate dimensions of the gel. For transfer
to be accomplished in the Hoeffer transfer apparatus, a sandwich is made using half
of the cassette holder, the mesh-like support piece, a piece of Whatman 3MM filter
paper, the nitrocellulose, the gel, filter paper, support, and the other half of the cas-
sette holder. It helps to first wet the nitrocellulose and filter paper in transfer buffer
before using, taking care not to touch the nitrocellulose with your fingers. Transfer
buffer (kept at 4°C) is 9.08 g Tris base, 43.25 g glycine, 600 ml methanol, and
ddH₂O to 3.0 liters. Make a notch in the gel and nitrocellulose (usually the bottom-left hand corner) and put the sandwich together so you know which side the nitrocellulose is on -- transfer must proceed towards the nitrocellulose. Once the cassette is all lined up, snap it together, and place it into the transfer apparatus with a stir bar in the cold. Make sure the gel is covered with buffer, and attach the power supply. Typically, transfer is done overnight at 20 Amps.

To determine if transfer is complete, a section of nitrocellulose corresponding to lanes may be cut off and placed, with its gel, into a solution of amido black (stock solution = 1% w/v amido black in 35% acetic acid, working solution is 50 ml stock in 200 ml ddH₂O) for 30 min. This is then destained with several changes of 7% acetic acid in 10% methanol. This will detect the presence of proteins and the locations of the molecular weight standards.

The blotted nitrocellulose is blocked by incubating with 100 ml of 5% carnation nonfat dry milk in PBS. This must be washed extensively to remove unbound milk, and this step may be deleted if all subsequent steps are performed in the presence of PBS with 0.05% tween (PBS-T). In this experiment, blots are reacted with rat-anti-Mφ supernatants. These antisera were diluted 1:100 in PBS-T, and incubated on a rotator for 1 hr, and washed extensively with PBS-T. Primary antisera is detected using goat-anti-rabbit conjugated with horseradish peroxidase (Sigma) diluted 1:500 in PBS-T. Incubation proceeds for 1 hr, and the blots washed extensively with PBS-T. The substrate, 4-chloro-1-naphthol (Sigma) is then added. Substrate is made up as a stock of 60 mg in 20 ml cold methanol, stored at 4°C protected from light. This stock is diluted 1:5 in PBS just before use (into 100 ml PBS) and 60 µl of 30% H₂O₂ added. The substrate reaction is stopped with water.

6. MLR TESTING. The rat-anti-Mφ supernatant antibodies were used to treat Mφ supernatants before addition to the MLR. Antiserum samples were heat inactivated (56°C, 30 min) before being used to treat TBH splenic Mφ supernatants or RPMI
medium. Treatments were done at 4°C either for 2 hr or overnight. To conserve reagents, 25 µl of antiserum was used to treat 25 µl of supernatants, and 100 µl of RPMI medium added at the end of the treatment period to provide enough sample for testing in the MLR at 25 µl per well and to provide a sample for SDS-PAGE.
Appendix 2

Sorting of Mφ Populations

1. Mφ. Mφ are obtained by peritoneal lavage, typically at 10:00 PM of the day preceding the planned sorting, plated for 2 hr at 37°C, nonadherent cells washed off with warm RPMI medium, and the Mφ scraped and counted by trypan blue dye exclusion. Mφ are resuspended to 4 x 10⁶/ml, and aliquoted to tubes depending on the number of treatment groups.

2. Mφ LABELING. Mφ are labeled with NR1gG, anti-Mac-1, -2, and -3 primary antibodies. This is done in the cold (cells on ice in the cold box) to avoid membrane internalization. NR1gG is obtained from Jackson Laboratories (#012-0003) or Accurate (#E4324) and is made up as a sterile 10 mg/ml solution. The anti-Mac antibodies are derived from hybridoma supernatants that have been cut with ammonium sulfate (final concentration 50%), dialyzed to 0.05 M pH 8.0 Tris buffer in the cold, and fractionated on a DEAE-Cellulose column. Presence of antibody was confirmed by Ouchterlony using goat-anti-rat IgG, directed against the heavy and
light chains (Cappel, #0113-0081). These antibodies are dialyzed to RPMI medium and their final volume adjusted to represent a 20-fold concentration from the original hybridoma supernatant. Typically, 25 μl of NRIgG and 100 μl of 20-fold concentrated anti-Mac antibodies were used to treat 1.0 ml of Mφ at 4 x 10⁶/ml. Labeling took place during a 60-90 min incubation on ice. Typically, this was done using 15 ml conical pyrex tubes, which make it easy to wash the cells four times after labeling with the primary antibodies. The cells were then resuspended to their original volumes and labeled with the secondary antibody, which was fluorescein isothiocyanate (FITC) conjugated to the affinity purified F(ab)₂ fragment of goat anti-rat IgG (heavy and light chain specific, Cappel #1713-3151) used at 1/500 in RPMI medium. After a minimum of 30 min on ice, the cells were washed 4 times with cold RPMI medium and checked for fluorescence on the Olympus IMT-2 microscope. This is done by placing 20 μl of the cell suspension on a slide, smearing with the side of the pipette tip, and observing for fluorescence.

3. FLOW CYTOMETRY. Cells were transported to the School of Veterinary Medicine and analyzed on the EPICS V flow cytometer (Model 752, Coulter Electronics, Hialeah, Florida) equipped with a 5 watt innova 90 argon laser (Coherent, Inc.) tuned to an output of 488 nm wavelength. This is close to the absorption maxima of FITC, which is 492 nm. Parameters such as flow rate are determined by the operator (Mr. Robert Haywood Pyle, School of Veterinary Medicine, VPI&SU), but should be consistent from run to run, which assumes that the sample concentration is also consistent (Mφ at 4 x 10⁶/ml). Labeled cells are filtered through a 50 μM nylon mesh screen to remove clumps and injected into the (sterile) saline flow stream where they intersect the laser. Three parameters per cell were collected: forward angle light scatter (FALS), an indication of cell size, 90 degree light scatter, and green fluorescence. Laser excitation was normally 300 milliwatts Scattered light is sequentially analyzed on a real-time basis as follows: scattered light first passes through a
488 nm dichroic filter and is diverted to photomultiplier tube 4, representing 90 degree light scatter, which is a measure of cell granularity and nuclear/cytoplasmic ratio. Remaining light goes through a 457-502 nm laser blocking filter and a 515 nm long pass filter which effectively blocks any remaining photons coming directly from the argon laser. The remaining light, which is all of wavelength > 515 nm, enters a 550 nm dichroic reflector, which diverts light between 515 and 550 nm to a 525 nm band pass filter, which sends a beam of light to photomultiplier tube 3. 525 nm is the peak fluorescence emission of FITC. All data collection and analysis were done with the multiparameter data acquisition and display system from Coulter Electronics. Forward angle light scatter was collected in a linear integral fashion with a gain setting of 2. The 90 degree light scatter was collected in a log integral fashion with a high voltage setting of 525 and no gain. Green fluorescence was collected on a linear integral scale with a high voltage of 1100 and a gain of 20.

4. CELL SORTING. For cell sorting to be effective, you must have a minimum of $10^7$ cells (at $4 \times 10^6$/ml), with a high percentage of those labeled. Higher concentrations are probably recommended as they will speed the sorting procedure. A plot was obtained of cell number on the y axis versus fluorescence intensity on the x axis. From this, the operator sets gates which determine the parameters upon which the cells will be sorted. Cells were then sorted on this parameter into two fractions, positive and negative by giving electrical charges to the droplets containing cells which pass through the flow stream. These charged droplets were then diverted into tissue culture dishes (35x10 mm, Falcon #3001) which contained RPMI medium to maintain viability. Sorted cells can then be checked for viability on the flow cytometer by propidium iodide dye exclusion (using a 590 nm dichroic and a 610 nm long pass filter). The plated Mφ were then cultured for four days at 37°C in a 5% CO₂ incubator.
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