Phenotypic and Functional Changes in Populations of Murine Macrophages During Tumor Growth

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(ABSTRACT)

Four macrophage (Mφ) surface antigens (Ia, Mac-1, -2, and -3) were examined for their association with Mφ regulatory functions. Observations of antigen expression on Mφ derived from normal or tumor-bearing hosts (TBH) showed that changes occurred in the antigen-defined phenotypes of Mφ which evolve during tumor growth. These changes in antigen expression were correlated with notable changes in Mφ immunoregulatory functions. Experiments using only normal host-derived Mφ showed that in the presence of complement (C), monoclonal antibodies (mAb) directed against Mφ could lyse targeted Mφ and that enrichment of the remaining cells provided populations of Mφ that were altered in their regulatory functions. Analysis of mAb-treated Mφ in the absence of C, suggested that the alterations observed in the presence of C were not due to ligand-receptor activation of peritoneal Mφ and that antibodies alone were not altering Mφ viability.

When anti-Mac-1, -2, and -3 antibodies, were used to modify accessory cell activity of whole spleen cell (WSC) or splenic adherent cell (SAC) preparations from normal or TBH, differential susceptibilities of the Mφ were noted. Ligand-receptor activation of WSC by anti-Mac-1 was observed in normal but not TBH WSC. With C, anti-Mac-1 and -3 each reduced normal and TBH WSC proliferation. To evaluate the possible role of different types of SAC in T cell lectin responsiveness, adherent cells were collected and depleted by antibody plus C treatment and added back to normal T cells. Removal of Mac-1^+ normal host SAC stimulated the supportive accessory function of the remaining SAC. Enhancing accessory cell function diminished after removal of normal host Mac-2^+ or TBH Mac-1^+ SAC. In summary, SAC from normal host demonstrated an accessory cell function corresponding to a Mac-1^- phenotype, which was either replaced or obscured by the predominance of a Mac-1^+ phenotype in TBH.

Variable Ia antigen expression by Mφ was examined during tumor growth. Tumor growth induced progressive loss of Ia antigen expression on Mφ. TBH splenic Mφ supported Concanavalin A-induced proliferation of syngeneic T cells (Ia antigen-independent) but did not support syngeneic T cell proliferation in the mixed lymphocyte reaction (MLR) (Ia antigen-dependent). Irrespective of tissue source, normal and TBH Mφ differed in their MLR stimulatory capabilities. In general, splenic Mφ preparations were better stimulators of allogeneic T cell blastogenesis in the MLR than thioglycollate-elicited peritoneal Mφ. Expression of Ia antigens by normal but not TBH Mφ were diminished by 24-hr *in vitro* plating of the peritoneal Mφ. Indomethacin treatment showed Prostaglandin E_2 was not a direct *in vitro* factor in Ia antigen-mediated reduction of splenic Mφ MLR stimulatory activity. Taken together, this data suggested a loss of Mφ Ia antigen expression, resulting in a decrease in Ia antigen-mediated functional activities during tumor growth.

To continue the assessment of Mφ phenotypes and to determine if alterations in Mφ function during tumor growth included changes in the secretion of soluble regulators of T cell activities, anti-Mac-1, -2, and -3 mAb were used to modulate monokine-mediated regulation of T lymphocyte proliferation. The mAb anti-Mac-1, -2, and -3 (plus C) exhibited differential depletion of normal and TBH Mφ. There was a distinct increase in the number of peroxidase-positive Mφ during tumor growth. Peroxidase-positive TBH Mφ were susceptible to C-mediated lysis by anti-Mac-1 and -3 but not anti-Mac-2, whereas no direct relationship was observed among normal host-derived Mφ. Immunofluorescence of mAb-binding showed a decrease in Mac-2^+ cells in TBH Mφ populations that was accompanied by an increase in Mac-3 expression. Anti-Mac-2 treatment significantly reduced the ability of TBH Mφ to produce a soluble suppressor(s) but did not alter normal host Mφ-derived suppressor production. In contrast, anti-Mac-1 and -3 treatment of normal host Mφ significantly reduced suppressor production but had diminished effects on TBH Mφ. Anti-Ia plus C treatment of splenic or peritoneal Mφ derived from normal or TBH showed that selection of Ia^- Mφ increased the secretion of PGE and also increased the T cell suppressor activity in Mφ culture supernatants. Collectively, these data suggest that tumor-induced aberrations

in immunoregulation can be attributed to differences in Mφ subpopulations which were discriminated by their surface membrane components.

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Table of Contents

List of Illustrations

List of Tables

Introduction

The accessory role of macrophages (Mφ) in both humoral and cell-mediated immunity was established in the early 1970's (157, 158, 212, 215). Since then numerous investigators have demonstrated the pivotal role of Mφ as both negative and positive modulators of immune responses. In other words, Mφ are capable of enhancing or suppressing the immunoreactivity of other immunocytes. This control is of obvious importance since it is the Mφ that primarily encounters foreign antigens. Thus, the processes of phagocytosis and subsequent antigen presentation initiate the immune response and by virtue or inherent Mφ control mechanisms, it is this action that also initiates the immune control circuit. This event is central to the immune response and involves a highly sophisticated cellular communication process. Signals within this process can be derived by either cell contact or by soluble immune modulators (lymphokines) that are released by the interacting cells. Cell-cell interactions proceed at the membranes of the interacting cells and therefore require the interaction of cell-surface antigens (62). The production (27) and reception (100, 268) of monokines are also dependent on membrane receptors, since monokines must be transported out of Mφ (through the membrane) and incoming lymphokines contact and interact with Mφ surfaces where a signal must be transduced across the membranes of the Mφ. Therefore, the membranes of Mφ are critical to the process of immune control.

Recently, the production of monoclonal antibodies (mAb) to immunocyte cell-surface antigens has facilitated the detailed study of leukocyte functions (113). From these studies, numerous models have evolved that describe the interaction of various specialized cell types which perform specific functions. Furthermore, the same studies have provided an outline of maturation processes that lead to the differentiation of specialized cell types (82, 152, 175, 190). However, with respect to Mφ, information correlating structure and function and maturation processes has been slow in accumulating. Most Mφ studies have focused primarily on Ia antigens because this antigen is directly involved in the cell-cell recognition process critical in antigen presentation and self recognition (21). Other Mφ cell-surface antigens have recently been identified and sometimes correlated with Mφ effector functions (82, 126, 152).

The classical definition of Mφ refers to them as a ubiquitous large mononuclear cell type that is capable of phagocytizing particulate matter and storing vital dyes. Numerous studies describing the morphology and functions of Mφ have far exceeded this early definition that labeled Mφ as immunological scavengers. Mφ are now associated with a variety of immunological activities ranging from the original effector role as a scavenger to an elaborate post as an immunoregulatory cell. The diversity of Mφ function has prompted the inception of new criteria for Mφ classification.

The variety of immunological functions attributable to Mφ suggests that these cells either represent a single population of pluripotent cells or a heterogeneous mixture of definable subpopulations with distinct immunological activities. Support of the subpopulations hypothesis is garnered by numerous studies which have correlated many diverse Mφ functions with enriched or selected subpopulations of cells. Stages in Mφ development are paralleled by distinct changes in phenotype, morphology, function, secretory activity, enzymatic activity, and membrane antigen expression (62, 64, 254). The correlation of these variable characteristics is the key in understanding the development and specialized functions of Mφ

This study examines changes that occurred in the body's defense system during the onset and development of cancer or, more specifically, those changes which involve Mφ. This defense system otherwise known as the immune system, identifies and destroys countless aberrant cells that

could develop into tumors. Occasionally, this intervention fails and a cancerous cell survives, subsequently forming a tumor.

A central unanswered question in the study of cancer is, "How do the initial cancerous cells and subsequent tumor avoid the host's immune surveillance system?" A current hypothesis proposes that a normal functioning immune system may become suppressed by environmental- or host-derived factors. Usually, the correlation between immunosuppression and tumor development has been established by both *in vitro* and *in vivo* assessments of host responsiveness (79, 277, 300). Evaluation of tumor-bearing host (TBH) immune responses demonstrates that the host is typically hyporesponsive to cellular antigens. Typically these studies describe reductions in T cell proliferative responses (83), increased T-suppressor cell numbers (83), and altered lymphokine production (34). From these studies one can see that much is known about the role of T cells in cancer-associated immune dysfunction. However, the exact role of the Mφ, an equally important cell in cellular immune responses, has remained elusive. The implications for Mφ involvement are on the basis of its pivotal role as an immune sentinel that not only monitors and destroys aberrant cells but also actively regulates the activities of other immune cells. Several studies (74, 109, 261, 262) including this one, hypothesize that the induction of immunosuppression could begin with the Mφ at some point during tumor growth.

Studies during the past decade show that intercellular communication is an essential part of the immune response (111, 269, 270). Molecular and cellular evaluations, using *in vitro* and *in vivo* models, have revealed changes in normal cellular interactions during tumor burden. A point of interest in tumor immunology is the Mφ, which serves the dualistic role of positive and negative effector (15, 109, 298). Although some of its regulatory activities require direct cell-cell contact, Mφ can regulate immune responses via the secretion of monokines (111). Evaluations of these soluble effector molecules reveals that their production can be controlled by stimuli originating either from within the immune system (i.e., from other leukocytes) or externally (i.e., foreign antigens or tumor products). In a "normal" animal these molecules and cells exist in a dynamic state of equilibrium. When the system is induced, a cascade-like proliferation of immune cells and their factors occurs. However, in the TBH immune homeostasis is lost and a state of equilibrium is

never regained (34). The result of this impairment is the inability of TBH to identify and/or extinguish neoplastic growth. Since homeostasis is a normal function of the immune system, it would appear that an aberration may occur in the mechanisms which control the differentiation and proliferation of cells responsible for tumor immunity.

Using approaches that have been successful in the delineation of altered T cell functions, several phenotypic and functional characteristics were used to compare normal and TBH-derived Mφ. The recent availability of mAb specific for Mφ has made functional analysis of Mφ populations possible. Antibody-mediated depletion of immunocyte populations, a technique which has been successful in the delineation of various leukocyte functions, was used to dissect Mφ populations. On the basis of previous evidence that suggested a potential for Mφ involvement in TBH hyporesponsiveness, we addressed the following questions:

1. What variations in Mφ regulatory function occurred during tumor development?

2. What changes occurred in Mφ antigen expression during tumor growth?

3. What correlation(s) existed between antigen expression and regulatory functions?

We have attempted to answer these questions by using Mφ-specific mAb to remove or block antigen-defined Mφ phenotypes and select for Mφ or Mφ-like cells that remain. With this approach, we were able to delineate Mφ subsets that were involved in tumor immune dysfunction. The antigens examined in this study were Ia, Mac-1, -2, and -3. The functions we attempted to correlate with these antigens were: (i) Mφ stimulatory function, (ii) accessory cell function, and (iii) Mφ-mediated suppression. The functional assays used to examine these parameters were lectin stimulated T cell cultures and mixed lymphocyte reactions (MLR). The six chapters contained within this dissertation show provocative and novel correlations between Mφ control of T cell responses and Mφ membrane antigen expression. Furthermore, they illustrate the alterations that occur in Mφ phenotypes and function as a result of tumor burden. The following literature review examines the scientific basis for these studies and provides corollaries with other studies in this area.

The presentation of the results that follows is arranged into six separate chapters that incorporate many of the ideas promoted in the literature. Collectively, the chapters are arranged in somewhat of a chronological order and represent the sequence of events during this research. Finally, in the Summary the individual studies are discussed cumulatively and a model is proposed that illustrates the author's view of Mφ phenotypes and regulatory functions.

Literature Review

Mφ-Mediated Immunoregulation

Before the identification of lymphokines (leukocyte-derived molecules that dictate the differentiation of immune cells) the mechanisms of immunoregulation remained an enigma. However in the past two decades much information has been gained through molecular and cellular evaluation of *in vivo* and *in vitro* models. One parameter of immune control that has been delineated is the role of the Mφ as an immunoregulatory cell. Mφ immunoregulation includes both humoral (160) and cellular responses (213, 214, 215). Proliferation assays using T cells stimulated by lectins demonstrate an absolute dependency of T cell proliferation on Mφ accessory cells. These and other studies (103, 227, 245) show that the dependency is particularly notable at low Concanavalin A (Con A) doses and was correlated with the number of Mφ added. Usually reconstitution of Mφ to Mφ-depleted spleen cell cultures is optimum at Mφ concentrations of approximately 5-10% (245), which is equivalent to the 8% percentage values recorded for *in vivo* splenic Mφ populations. These results explain the notable cell-cell binding observed between Mφ and T cells. Physical interactions between T cells and Mφ at the cellular level had been previously described by Lipsky and Rosenthal (157, 158, 159, 216, 217). These earlier studies showed that Mφ and T cells interact via cell-cell contact in what were termed lymphocyte-Mφ clusters. The process of Mφ-T cell ad-

herence was not dependent on the tissue origin of Mφ and was trypsin sensitive. Furthermore, adherence did not occur in the absence of cations nor did it occur if nonviable Mφ were used. The sensitivity of the adherence process to proteolytic treatment implied that there was a requirement for membrane proteins. Although physical contact of Mφ and T cells augments the accessory function of Mφ and enhances T cell proliferation, it is not an absolute requirement, since T-lymphocyte proliferation occurs when these cells are separated by semipermeable membranes (212, 214). These observations prompted studies that delineated mechanisms of Mφ immunoregulation which involved soluble products initially called mitogenic proteins (75). During the ensuing years, researchers demonstrated that the relationship between T cells and Mφ can occur via both cell-cell interaction and the release and/or reception of soluble regulatory molecules (lymphokines). Eventually, several Mφ-derived soluble immunoregulators which enhance T cell proliferation were identified, such as T cell activation factor (TAF) (213), interleukin 1 (IL-1) (177, 178), and lymphocyte activating factor (LAF) (176, 179, 180). Although IL-1 and LAF were initially identified as separate entities current feelings are that they are similar or even identical substances (264). As a Mφ product, IL-1 is viewed as an initial signal in cellular immune cascades and is recognized for its ability to augment the production of IL-2 and thereby initiate T cell proliferation (76, 77, 178, 196). Besides the functional characterizations of IL-1 activities, the molecule has been characterized biochemically (178). Purified concentrates of IL-1, obtained from either the cell line $P388D_1$ or lipopolysaccharide (LPS) stimulated splenic Mφ show that the molecule has a molecular weight of 12-16 Kd. Isoelectric heterogeneity has been demonstrated in what appears to be homogenic preparations of IL-1 that were purified by molecular sizing techniques (295). These variations are perceived by some as an indication of both functional as well as morphological heterogenic expression of a group of IL-1 molecules (195). Nevertheless, biochemical characterizations have enabled researchers to identify and purify IL-1, which has in turn allowed for kinetic studies of IL-1 activities. Characterization of IL-1 function shows that the molecules bind to lymphocytes 12-24 hr after lymphocyte stimulation (130, 181) and thus the binding may be dependent on expression of a receptor during a critical point in the cell cycle. The solitary role of IL-1 as the only Mφ-derived enhancing monokine is questionable, since studies have suggested the ex-

istence of other stimulatory factors of considerably higher molecular weight in Mφ culture supernatants (41, 294, 295). For example, B cell activation factor (BAF) (294) and genetically related factor (GRF) (70) appear biochemically distinct from IL-1 but are capable of enhancing lymphocyte proliferation.

Besides their role as positive or enhancing immunoregulators, Mφ are also capable of suppressing the immune response. The mechanisms of Mφ immunosuppression are not clearly understood, as correlations between suppression and characterized or purified soluble inhibitors are incomplete. However, one unique feature is apparent, that whenever Mφ can augment a particular immune response they can also inhibit it (298). Keller (134) demonstrated this phenomenon using antigen-reactive T cells. In these experiments, splenic Mφ induced cytotoxic activities in T cells via the release of lymphokines whereas the addition of peritoneal Mφ to the T cells reversed the process. The suppressive activity of Mφ also proceeds via either cell-cell contact mechanisms (40) or via the production of soluble factors (10). If regulation occurs by cell contact then mechanisms of cell-cell suppression may be major histocompatability complex (MHC) restricted events. This hypothesis is consistent with studies that show a definite relationship between Mφ-mediated immunosuppression with Ia^- Mφ (153, 206, 291, 292) and a dependency on Mφ to responder T cell ratios (291). Youdim (298) describes this as a collective Ia-dependent phenomenon, that is derived from the displacement of enhancing Ia^+ accessory cells by Ia^- Mφ.

Mφ secrete immunosuppressive substances which down regulate immune response by inhibiting T cell activation (275) or by restricting T cell proliferation (10, 193). Soluble suppressor molecules such as arginase (143), complement components (222), prostaglandins (97, 286), cyclic AMP (32), interferon (186), and apolipoprotein (54, 255, 290) have been associated with Mφ regulatory functions. Examining Mφ production of regulatory factors, shows that the mode and degree of T cell suppression is often related to the activation state of the Mφ (10). For example, Mφ activated by pyran co-polymer (170) or by *Toxoplasma gonidii* (293) are more suppressive than normal Mφ. Also, Erb *et al.* (70) demonstrated that inhibition and enhancement can be either antigen-specific or nonspecific. This hypothesis is of particular importance *in vivo*, as noted by Allison (40), where host conditions such as tumor burden can be major factors in Mφ-mediated

immune control systems (95). Another contributing factor to secretion of monokines is the anatomical origin of the Mφ. For example, Youdim (298) demonstrated that splenic adherent cells perpetuate and support the cytotoxic response of cytotoxic T cells whereas the same T cell activity is suppressed by peritoneal Mφ. Similar dissociations between splenic and peritoneal Mφ regulatory functions were observed in Con A-stimulated T cells.

For the most part, Mφ immunosuppression by soluble factors is described but the mechanisms are not well characterized. One mechanism that has been studied in detail is the immunosuppressive effects of Mφ-derived Prostaglandin E_2 (PGE_2). The best characterized member of this molecular family of membrane lipid by-products is PGE_2 (30, 118). Mφ synthesize large quantities of PGE_2 from endogenous stores of arachidonic acid. Perturbation of the plasma membrane (i.e., ligand receptor binding) is directly associated with the induction of PGE_2 synthesis (107). For example, Scott *et al.* (226) showed that PGE_2 synthesis by murine Mφ is tightly coupled to phagocytosis. Other stimulators of Mφ-derived PGE_2 synthesis include Fc-receptor binding (251), Con A binding (199) and C-receptor binding (108). PGE_2 suppression of lymphocyte functions can proceed either directly via the inhibition of T helper cells (94, 95) or indirectly through the induction of T suppressor cells (85, 285). Direct suppression of lymphocyte metabolism by PGE_2 appears to be linked to cAMP (282) and has been associated with down regulation of interleukin-2 (IL-2) production (282) and also down regulation of IL-2 receptor expression (259). Regardless of the mechanism, sufficient evidence exists which identifies the Mφ as both an inhibitory and enhancing cell. In response to this characteristic one raises the question of functionally distinct Mφ subpopulations.

Phenotypic Heterogeneity of Mφ

The existence of Mφ subpopulations requires that differentiation precede the development and expression of individual Mφ activities. In other words, specialized Mφ subpopulations might be derived either from a central line of differentiation which branches to yield specialized Mφ or

from several independent lines of Mφ differentiation. Presently, the theory of a central line of differentiation is more prominently accepted. However, the availability of reagents for examining Mφ differentiation have only recently been available. This accepted theory of Mφ differentiation describes the migratory maturation of progenitor bone marrow cells to peripheral blood monocytes which eventually migrate into a particular tissue where maturation into resident Mφ occurs (86, 87). Other highly controversial theories suggest either differentiation from endothelial precursors or transformation of lymphocytes. Direct evidence which demonstrates bone marrow derivation of Mφ precursors is found in studies which have successfully induced the maturation of bone marrow-derived stem cells into Mφ (152, 283). Advancement of this research may one day further delineate the differentiation processes of Mφ. One parameter of Mφ maturation that may eventually be explained by studies such as these is the terminal differentiation step. Currently, mature Mφ are described as nondividing terminal cells. An exception to this belief was noted by van der Zeijst *et al.* (301), who observed continual division by thioglycollate-elicited Mφ in the presence of Mφ growth factor. Analysis of the proliferating clones showed that these cells were in an activation state intermediate to resident and stimulated Mφ. In summary, van der Zeijst *et al.* suggest that resident Mφ can not be induced to divide but the newly immigrated Mφ will synthesize DNA and divide. Other controversy arises from the fact that tissue-dependent morphologies and functions have been shown (219). Collectively, these inconsistencies in observations might reflect the heterogeneity of Mφ populations and the possible existence of discrete Mφ subpopulations.

Phenotypic heterogeneity among Mφ was initially determined in the context of immunological and/or biochemical activities. These approaches are useful but their implications are not yet fully understood. One such type of approach involves the analysis of ectoenzymes. A variety of these enzymes are located on the outer surface of Mφ as membrane proteins. One of the classical characterizations of Mφ as a single population was demonstrated by Li *et al.* (155), who showed that these cells contain significant amounts of esterase. Since that finding, this characteristic is used as a phenotypic trait for identification of Mφ within mixed cell suspensions (44). Wachsmuth in 1975 (281) performed one of the first detailed studies of an ectoenzyme aminopeptidase which showed that thioglycollate-elicited Mφ have higher activity levels of this

enzyme than normal resident Mφ. In addition, time course studies performed at different points after initial stimulation suggest that enzyme activity increases slowly, which was interpreted by the author as an intratissue process of differentiation. A second ectoenzyme, 5'nucleotidase was also examined in thioglycollate-elicited and resident Mφ populations (281). Here, the enzyme activities were higher in resident than in elicited Mφ. More recently (65), mAb were used as probes to assay for the presence of 5'nucleotidase. These studies showed that the lower enzymatic activity noted in elicited Mφ was not because of modification of the enzyme but was because of a physical absence of the protein. A third enzyme marker and perhaps the most widely used for analysis of Mφ population is peroxidase. Several studies using *in vivo, in vitro,* and neoplastic Mφ have shown that this enzyme is an excellent marker determing the degree of Mφ maturity (88, 128, 220). The proposed scheme for enzyme production and localization derived by these studies is as follows: promonocytes produce the enzyme and store it in cytoplasmic granules (lysosomes), enzyme production ceases at the end of the promonocyte stage, enzyme levels progressively decrease during the monocyte phase but granules persist, and as the cells mature into phagocytic Mφ the enzyme content is negligible.

Another useful criteria used by some to examine Mφ heterogeneity and delineate Mφ subpopulations are differences in the buoyant density of these cells. Several studies have shown that peritoneal Mφ can be separated into four subgroups based on their differential buoyant density when centrifuged through a percoll gradient (44, 192, 252). Others (153, 224) demonstrated similar phenomenon when Mφ are centrifuged through a discontinuous gradient of bovine serum albumin (BSA). In at least one case, a study by Lee *et al.* (153), shows differential Ia antigen expression and endogenous peroxidase activity are localized in different Mφ subgroups collected by percoll gradient centrifugation. In attempting to correlate Mφ size with function, Lee *et al.* (153) show that peritoneal exudate Mφ are composed of a population of small (peroxidase positive) immunostimulatory cells and a population of large (peroxidase negative) immunosuppressive Mφ. More recent investigations show differential secretion of IL-1 and PGE_2 by density gradient purified human Mφ populations (136). High density Mφ appear to be the major source of IL-1 while lower density Mφ are the major producers of PGE_2.

With respect to the collective approaches to cell separations perhaps the most powerful and useful techniques are those separations based on differential cell-surface antigen expression. One of the first descriptions of Mφ populations in the context of cell-surface antigens was based on variable expression of surface Ia antigens (21). Using this as a simple criteria for classification, Mφ can be grouped into either Ia^+ or Ia^- populations. The identification of Ia on the surface of Mφ was performed with mAb which were either florescently or radioactively tagged. Using this technique Beller and Unanue (22) find that Ia^- cells not only lack Ia antigen but also fail to produce the substance intracellularly. This would imply that a stimulation-dependent control mechanism may regulate both the membranous expression of Ia and other dependent Mφ functions.

Both Ia^+ and Ia^- Mφ phenotypes bear Fc and C3 receptors, phagocytize particles and respond to lymphokines, however only Ia^+ cells are capable of cell-cell interactions with T cells (21, 215, 218). Sampling of various resident populations with anti-Ia suggests that there is a constant basal number of cells expressing Ia antigens (i.e., 10% Ia^+ cells in the peritoneal cavity) and that this level remains unchanged even after administration of eliciting agents (endotoxin, thioglycollate, mineral oil, and others). Recruitment of Mφ by *Listeria monocytogenes* or BCG results in a significant increase in Ia^+ cell numbers (50-100%) (25).

Further analysis on the variable expression of Ia antigens indicates that changes in the numbers of Ia^+ cells within a resident population of Mφ is dependent on a T cell product, Mφ Ia recruiting factor (MIRF) (23, 268). *In vitro* and *in vivo* studies with MIRF show that it does not act directly on mature Ia^- Mφ but rather on immature precursor cells. X-irradiation prevents MIRF-mediated induction of new Ia^+ cells in the peritoneum but adoptive transfer of bone marrow cells to mice so treated will result in increased expression of Ia^+ cells.

Additional studies with Mφ confirm that Ia is not expressed constitutively on Ia^+ cells (22). Using *in vitro* cultures of Mφ, the authors show that Ia expression occurs for approximately 24-48 hours of culture, after which it is no longer detectable on the cell surface. They also found that Ia expression can be maintained for longer periods of time if antigen is added to the cultures. Similar fluctuations in Ia expression are reported from *in vivo* studies (190). Thus, Ia antigen may represent

a useful marker in the delineation of independently differentiated Mφ subpopulations or as an indicator of specialized Mφ functions that require the expression of the antigen.

Other Mφ surface antigens have been identified which represent putative markers for Mφ subpopulations. The development of xenogeneic hybridization techniques has facilitated the production of monospecific antisera to unique antigens on the surface of Mφ. Springer *et al.* have tentatively identified four different antigens which are present on murine Mφ but absent on lymphocytes (208, 234, 236). The qualitative and quantitative expression of these antigens on different Mφ phenotypes was assayed for by adherence of labelled (^{125}I and fluorescent dyes) mAb. The authors suggest that the occurrence of all four of these antigens on thioglycollate-elicted peritoneal Mφ shows that they do not define qualitatively distinct subpopulations under "normal" conditions. However, they admit that quantitative expression of Mac-1, -2, and -3 on Mφ surfaces does appear variable and may provide an index for the identification of Mφ subpopulations (114, 115, 208).

Mac-1 has been identified in all Mφ populations thus far examined (236). Phenotypic studies with the anti-Mac series of mAb (114) have demonstrated that the Mac-2 (32,000 dalton protein) antigen is differentially induced by several eliciting agents. Furthermore, Mac-2 is expressed in a variable fashion on different resident Mφ populations, suggesting a possible tissue specific distribution of Mφ subpopulations. Ho and Springer (114) find that the expression of Mac-2 is increased on Mφ elicited by thioglycollate, whereas unelicited peritoneal Mφ and those elicited by protease peptone, Con A, LPS and *Listeria monocytogenes* are negative or contain much less Mac-2 antigen. Other studies (150, 297) also show distinct membrane protein differences in extracts of Mφ derived by thioglycollate as compared to protease peptone and further illustrate variability in Mφ phenotype in these conditions. Another Mφ antigen identified by the Springer group, Mac-3, is also a potential source of Mφ heterogeneity (117). It was initially thought that Mac-3 is found at very low frequency or is absent on cells other than Mφ. Within different groups of Mφ molecular characterization of this antigen (117) has revealed molecular weight heterogeneity that correlates somewhat with origin of the Mφ. In recent anatomical studies by Flotte *et al* (80, 81), Mac-3 was identified on dendritic cells as was the Mac-2 antigen.

More recent studies with mAb vs. Mac antigens (207) have been attempted with Mφ-like cell lines to determine if additional cellular and functional activities may be coordinated with antigen-defined subpopulations. Among the various observations reported, the presence of Mac-1 appears to be directly associated with the presence of C3 receptors among these cell lines. Furthermore, M-1 cells (myeloblasts or progenitor Mφ) are normally both Mac-1$^-$ and C3$^-$, but become positive for both traits after induction of the maturation process. Generally, among Mφ-like cell lines the expression of Mac-2 parallels Mac-1 expression except in the M-1 myeloblast line. In contrast, significant coordination is noted between Mac-1 and -3 expression in the M-1 model after stimulation with several different agents (LPS, lymphokine and WEHI-3 conditioned media). The connection between Mac-1 and -3 expression was further illustrated by the inhibition of Mac-3 induction by cells treated with anti-Mac-1. Evidence of inducible expression of the Mac-2 and -3 antigens on the surface of M-1 cells offers support to the idea that Mφ differentiation occurs in a series of antigen-definable stages.

Presently, more attention is being given the Mac-1 antigen at least in terms of functional significance. Biochemical (237) and genetic (239) studies with Mac-1 show a relationship between it and the T cell antigen, lymphocyte functional antigen (LFA-1). Both of these molecules show amino acid sequence homology (237) and mediate similar adhesive functions (238, 240). Immunological and biochemical peptide mapping show that the β-subunits are almost identical (237), yet the α-subunits are different. Most interesting of all, however, is the homology noted between the α-subunits and interferon (IFN-α) (237).

Other attempts to correlate specific immunological and biochemical activities of putative Mφ subpopulations with the presence of specific cellular antigens have been partially successful. Akagawa *et al.* (6) indicate that biochemical changes in the cell membrane occur concomitant with Mφ activation. The expression of a glucolipid, ganglio-tetraosyl-ceramide (GM1) is induced during activation of the Mφ cell line M-1 or peritoneal Mφ. Cytotoxicity functions are directly linked to GM1 expression. Other investigators have implicated glycosylation events on the Mφ membrane with expression of adherence characteristics and sensitivity to Mac-1 induction (265).

Sun *et al.* were successful at associating Mφ functions with antigen expression (249). They used Mφ generated by a variety of methods to stimulate the production of mAb against Mφ antigens. In this way, they were able to produce three mAb that correlate with three separate Mφ functions. They reference these isolates as being indicative of Mφ that have natural killer activity, lymphokine-induced Mφ-mediated cytotoxicity or neither. The reactivity of mAb-induced Mφ with natural killer (NK) cells (removal of NK activity) prepared from bone marrow suggests that the NK cells may actually represent a subpopulation of the monocyte differentiation lineage. Other studies indicating a similarity between Mφ and NK cells are reported (13).

Early on, a polyclonal antisera prepared in rabbits by Kaplan *et al.* (126, 127) was used to identify a cell surface antigen temporally expressed on C. parvum-induced Mφ. Peritoneal Mφ activated in this fashion have increased biochemical activity and are cytotoxic for tumor cells. With this antisera a correlation was detected between tumorocidal activity and the presence of the antisera-identified antigen. Since this initial study, other antigens are associated with Mφ functions. The identification of these antigens is described later in this review under the heading "Membrane antigen involvement with Mφ functions."

Despite the growing list of correlations between Mφ structure and function, the delineation of Mφ subpopulations is not yet possible. One reason for this is the great diversity of model systems and parameters (e.g., cell types and activation criteria) used to define Mφ subpopulations. There is a general concern that some degree of standardization is required, if we are to fully understand Mφ heterogeneity. Partial fulfillment of this requirement has been proposed by the Reticuloendothelial Society, they are: (i) Mouse peritoneal Mφ and human peripheral blood monocytes (PBM) are proposed as the standard model systems for Mφ investigations. (ii) Resident Mφ are accepted as Mφ collected without experimental elicitation. (iii) Avoidance of unclear terms such as, stimulated or primed, is suggested. By remaining within these prescribed boundaries, it is hoped that the information gained by independent researchers will be applicable to a central model of Mφ ontogeny.

Cumulative assessment of studies on Mφ phenotypes implies that these phenotypes may represent distinct Mφ subpopulations. However, since the new technology of mAb applications

has only recently been introduced into this field, the information correlating Mφ function with distinct cell surface markers has not yet been completed. If the past 10 years are indicative of future achievements, it appears that our understanding of Mφ phenotypes, activities and differentiation are at the threshold of clarification.

Role of Mφ In Tumor Immunology

So as to appreciate the role of Mφ in tumor immunology, one must first identify the Mφ as an anti-tumor mechanism in the immune defense arsenal against neoplastic growths. A clear participation of Mφ in tumor immunity has been shown by Hibbs and others (18, 28, 112). These studies demonstrate that activated Mφ have an inherent trait of tumor cell recognition and destruction. This sentinel-like activity is acquired from interactions with immune lymphocytes (131). However, this phenomenon of acquired immunity departs from the central dogma of Mφ nonspecificity and has been difficult to delineate. To further complicate the scenario, others (162, 207) show that Mφ cell lines are capable of destroying tumor target cells that they have presumably never encountered and more important without the cooperation of other leukocytes.

Aside from the dilemma of Mφ nonspecificity, the Mφ is undoubtedly a key player in anti-tumor immunity. The facilities of the Mφ for tumor identification and destruction are based on the diverse capabilities of the cell. Phagocytosis, motility, chemotaxis, inflammatory responses and cytotoxic mechanisms are Mφ traits which all lend themselves to anti-tumor activities (1). Although the traits individually are insufficient to control a neoplastic growth, collectively they can be used quite effectively in the abrogation of neoplasia.

It has been suggested that one of the events in tumor immunity is the uptake or phagocytosis of tumor-derived particles by the Mφ (16). In some instances this stage of immunity has been viewed as a precursor to the synthesis and release of Mφ-derived cytotoxic compounds and monokines (9, 112). The Mφ products are correlated with the activation state of Mφ in the TBH (167). Another trait demonstrated by Mφ useful in tumor immunity is their ability to move

directionally in response to a variety of chemotactic factors. The advantage of this trait is that Mφ can be elicited to an area and then directed to the source of the antigen by other leukocytes or tumor products (8, 9). Perhaps the most important Mφ characteristic in tumor immunity is the its ability to cooperate with and regulate other lymphocytes (73). Therefore immunoregulation of the tumor response places the Mφ in a central role as both effector and regulator. The most direct evidence for Mφ-mediated anti-tumor activity is demonstrated by cytotoxic mechanisms (127, 168). Here a regulatory relationship between Mφ and T cells is also displayed. T cell activation of Mφ plays an important role in Mφ cytotoxicity against syngeneic tumor cells (127, 171). Studies on Mφ cytotoxicity, specific and nonspecific (135, 169, 172), show that specific mechanisms require cell-cell contact between the Mφ and the target cell, while nonspecific mechanisms can occur via cell-cell interactions or the action of a soluble nonspecific cytotoxic mediator. The most intriguing of these is the mechanism of specific cytotoxicity because of its requirement of T cell-Mφ interaction prior to the cytotoxic event. Although the exact mechanism of the Mφ arming event is unknown a number of phenotypic traits change after this ability has been imparted to the Mφ (84, 249).

As mentioned, immunological evaluations of TBH indicate that cell-mediated immunity is significantly impaired. T cells (263, 273), Mφ (1, 273) and NK cells (93, 273) play integral roles in the rejection of tumors and may all be involved in this impairment to some extent. Since Mφ are known to actively participate in the host's response to neoplasia, it goes to reason that tumor survival may be accompanied by alterations in Mφ activities and because of their pivotal regulatory functions. Direct evidence supporting this hypothesis is found in studies that noted impaired Mφ activities after exposure to TBH-derived sera (1, 16). In fact, the Mφ is implicated as a specific target of tumor-derived products which induce immune dysfunction (121, 137, 261, 262). Furthermore, the degree of tumor-induced dysfunction is related to tumor-size, indicating that the dysfunction is a sequential event that occurs systematically with tumor growth. In these same studies the influence on Mφ by tumor growth is demonstrated in anatomical sites distant from the tumor such as the peritoneum and skin. One notable effect is the reduction in the number of Mφ that migrate into sites of inflammation other than the tumor (9). However, this effect is not typical

as others have demonstrated just the opposite effect (79) in the spleen. Disparities such as this are not uncommon in tumor immunology, since different types of tumors have different effects on different sites of the immune system. For example, although splenomegaly occurs in rats because of tumor burden, the activation state of Mφ isolated from the peritoneal cavity (260) may be quite different from those isolated from the spleen (261). The nature of observed variability in Mφ derived from TBH is open to debate and yet it is this parameter that may explain tumor-associated immune dysfunction. Alexander (9) notes that the cause of aberrations seen in Mφ populations may actually represent an "over taxed" stem cell system that can not keep up with the demands echoed by the immune system in response to tumor growth. Consequently, the maturation events of various immune cells such as Mφ would also be aimed at the tumor and not at secondary stimuli (i.e., thioglycollate, lectins, or alloantigens) and thus stimulation may yield variable Mφ populations, with respect to normal host versus TBH.

Based on studies which show differences in Mφ-mediated immune functions during tumor growth (79, 137, 138, 184, 203), the question arises "How does the presence of a tumor initiate alterations in the immune system?" Nowell *et al.* (188) describes tumor development as a series of alterations in heritable phenotypic properties by transformed normal cells which ultimately enhances the survival of the tumor. They also imply that subversive adaptation allows the tumor to avoid immune responses. Several studies which focus on tumor-derived immunomodulatory agents have shown that several soluble substances are produced by tumors which can directly effect the immune system (31, 104, 105, 211). One obvious Mφ function that tumors must overcome to survive is Mφ cytotoxicity. The *in vivo* cytotoxic function of Mφ is an important part of the host's anti-tumor arsenal (42). Urban and Schreiber (274) show that some tumor cells have a heritably reduced sensitivity to cytotoxic Mφ. They found that development of this survival trait occurred independent of the loss of tumor-specific antigen *in vivo* and suggested that it might involve resistances to Mφ-mediated cytotoxicity. Although the study did not show a direct correlation between tumor-derived products, within their model they noted them as a possible factor. It has long been known that tumor cells or their products are capable of depressing immune responses both *in vitro* and *in vivo* (16). Although the exact nature of these substances is still under investigation, re-

searchers are able to separate the substances into groups based on their molecular weight. Of particular interest are the smaller compounds between 1000 and 10,000 daltons. Continued investigations in this area have indicated that PGE_2 may be at least partially responsible for the immunomodulatory activities observed in tumor cell supernatants (12, 299). These molecules are produced by some tumor cells in large quantities as a consequence of rapid membrane metabolism. Prostaglandins are known to be potent immunomodulators (78, 84, 101, 201) and exert their effects either directly on responding lymphocyte populations or indirectly, by initiating altered Mφ regulation (i.e., down regulation of Ia expression or decreased IL-1 production). The role of the Mφ in tumor-induced immunosuppression has been demonstrated by experiments which use MLR (261). In this study, MLR were initiated in the presence of tumor cells and a normal response was observed unless Mφ which were previously exposed to tumor cells were added. The addition of splenic Mφ which were previously exposed to tumor resulted in suppression of the immune response. Other studies attribute disruption of immune functions in animals with tumors to circulating membrane vesicles that are released from tumor cells (204, 205). In these studies the vesicles caused a decrease in Mφ Ia antigen expression. Therefore, one more bit of evidence exists implicating the Mφ as a target of tumor factors.

Further evidence supporting the hypothesis of tumor-Mφ interactions during tumor growth and illustrating the complexity of the immunocyte interactions that follow this exposure is shown in studies by Herlyn and Koprowski (110). These studies clearly indicate that mAb (nonreactive with the tumor) can suppress tumor growth by interacting with Mφ. They show that Mφ reception of these IgG2a molecules via the Fc receptor result in the increased Mφ cytotoxicity, that was tumor cell specific. One explanation for this phenomenon offered by these and other researchers (2, 3, 4, 99) implicates Mφ activation as an important step in tumor rejection.

Membrane Antigen Involvement with Mφ Functions

Importance of the cell membrane in immunocyte function has been well characterized (19, 21, 62, 65, 66). The role of the Mφ membrane in immunoregulatory and effector functions has become an area of intense investigation since the advent of our understanding of this cell as a pivotal cell within the immune response. It has been indicated by certain studies that the Mφ (24, 43, 62, 178, 270) membrane may play an important role in the processing of either incoming or outgoing messages (124). Thus, one might expect the Mφ membrane to reflect any specialized activities by differential expression of membrane proteins involved in the regulatory process. Studies have shown that Mφ antigens are responsible for the reception of IgG (271), C3bi (26), and T-cells (Ia-mediated) (21). Thus, Mφ membrane antigens are integral components of many immunological functions. The role of membrane antigens as receptors can be three-fold: (i) binding of the ligand, (ii) transduction of the signal to the cytoplasm, (iii) the expression of new membrane proteins that may participate in specialized Mφ function. Perhaps an indication of the importance of membrane proteins is their involvement in various functions which is shown by studies that examine the insertion and turnover of membrane antigens. Mφ ingest up to 25% of their cell volume per hour and rapidly reconstruct their plasma membrane by utilization of phagosomes (67). The importance of the membrane is further exemplified by changes in antigen expression after Mφ exposure to modulators of Mφ activities. Exogenous or endogenous cGMP promotes expression of Mφ Fc-receptors (FcR), but the antigens are suppressed by cAMP (67). Thus, Mφ membrane proteins and their related ligand-receptor interactions are subject to control systems that involve second messages and self regulation (64). In these contexts, Mφ membrane antigens are cycled, processed, and/or modified in immunoregulatory cascades.

Herlyn and Koprowski (110) demonstrate the reception of IgGa mAb by Mφ FcR as a means of activating Mφ anti-tumor activity. Another receptor-ligand interaction that has a direct relationship to immunoregulation as well as effector function is the reception of maleylated proteins (124). In these studies enhanced secretion of neutral proteases, plasminogen activator, and

cytotoxic factor was observed from Mφ that were pulsed with and absorbed to their membrane maleylated-BSA. The same study also showed that the cytotoxic factor was found to be directly involved in anti-tumor cytotoxicity. A recent study by Koestler *et al.* (142) shows that reception of interferon (IFN) and/or Mφ activating factor (MAF) by Mφ can simultaneously induce antigen 158.2 anti-tumor cytotoxicity and microbicidal functions. An interesting feature of this study is the dissociation between LPS induction of the same antigen and microbicidal function while association between the antigen and anti-tumor cytotoxicity was maintained. From these findings, it appears that an antigen may be induced by a generalized activation step but the antigens function is limited to a particular process, such as tumor cytotoxicity.

Secondarily, membrane antigens may be directly involved in effector functions. In these terms the response of the Mφ to signals which trigger effector functions can be viewed like a cellular response to hormones. C3 receptors on Mφ are directly involved in adherence (197) and phagocytosis (255). Furthermore, Mφ membrane molecules Ia and FcR have been shown to be released into the surrounding tissue and are potent immunoregulators (154). In the mentioned example, membrane proteins have a direct role in immunoregulatory functions, but in other less defined examples the membrane proteins are only characterized in terms of their presence or absence on a functionally distinct type of Mφ and thus represent only an indirect relationship between the antigen and the function. For example, Gp 160, Ia, Fc, M43 and M57 are antigens used to characterize cytotoxic Mφ that are involved in tumor rejection. But of these only Ia and Fc have been shown to have a direct relationship with cytotoxic processes (110, 256, 258). Kuribayashi and Masuda (145) reported a study which showed a direct relationship between FcR and Ia antigens (145). They found that FcR^+ cells were better stimulators in the MLR than were FcR^- cells. In addition, FcR^+ cells separated by fluorescence activated cell sorting (FACS) also proved to be Ia^+. Further correlation between these antigens and regulatory functions are demonstrated in studies by Raff *et al.* (206). They show that Ia^+ Mφ are necessary for differentiation of suppressor T cells. Collective evaluation of these facts with other work which demonstrated the release of FcR by Mφ into the medium and its subsequent incorporation into T cells, makes FcR and Ia prime can-

didates as immunoregulatory Mφ membrane proteins that are involved in noncontact, soluble mediator interactions (154).

To date only a handful of reports are available which describe a direct relationship between Mφ effector functions and membrane antigens. Kaplan *et al.* (126, 127) generated a heterogeneous rabbit antisera against the $P388D_1$ cell line that, after preabsorption with the proper cells, detected an association surface antigen on cytotoxic Mφ. A correlation between the antigen, Mφ inhibition of lymphoproliferative, Mφ-mediated by cytotoxicity of tumor cells was demonstrated by experiments which showed a concomitant loss of the antigen and these functions after enzyme treatments of the Mφ. Taniyama and Watanabe (257) generated a hybridoma which secreted a mAb specific for tumorocidal Mφ (AcM.1) but failed to show direct involvement of the membrane antigen with the cytotoxic function. Fox and Petty (82) and Sun *et al.* (249) delineated a similar antigen, MAA-1 and M43 respectively, which are not directly involved in activated Mφ cytotoxic functions but were found on the subpopulation of Mφ which mediated this event. Aside from the correlations of Mφ antigens and function via mAb identification are the correlations that have been established between enzyme activity and effector function (290). For example, changes in Mφ ectoenzymes have been directly associated with Mφ-mediated anti-tumor activity (184). By virtue of the location of these proteins on the membrane, they represent surface antigens that have effector functions in tissue repair and cytotoxicity.

Direct involvement of Mφ membrane proteins with Mφ regulatory functions can be defined in terms of signal reception. For example, the interactions of various immunostimulants like, Con A, LPS, and BCG with Mφ most likely occur through membrane antigens and based on other cellular activation models, involve primary and secondary signals of activation (250). Not to be overlooked are the cytokines which function as intercellular messages during an immune response. Collectively, these molecules can evoke a wide range of alterations in Mφ functions. Migration inhibition factor (MIF) (141), MAF (36), interleukin-3 (IL-3) (37), and IFN (45) are examples of leukocyte derived molecules which alter Mφ activities. Indeed, identification of the receptors for these molecules and delineation of the mechanisms of their ligand-receptor regulatory functions are paramount steps in understanding Mφ functions.

Observations of immune regulatory systems in animals compromised by tumor burden reveals that aberrant cellular interactions are magnified during ordinary immune responses (191). These complications are viewed by some as the major factor in tumor-induced immune dysfunction. The result of this impairment is the inability of the TBH to effectively identify and/or extinguish neoplastic growth or to properly regulate the anti-tumor response of other leukocytes. The exact role of membrane antigens in this impairment is now unknown, but in view of the importance of the Mφ membrane in the immune response one might predict that aberrant changes in the immune response are paralleled by changes in Mφ membrane proteins. Reports are presented in the following chapters that may shed some light the structure-function relationship of Mφ phenotype and immune dysfunction during tumor growth.

Chapter I

USE OF mAb AS SELECTIVE TOOLS: DEPLETION VERSUS ENRICHMENT

Introduction

Mφ populations are known to be heterogeneous with respect to immunological functions (64, 260, 298). Of particular interest are those functions that involve or mediate regulation of T lymphocytes (260, 298). As a whole population, Mφ are capable of enhancing or inhibiting T cell proliferation via the secretion of soluble immunoregulatory molecules (monokines). Evidence exists which implicates Mφ phenotypic heterogeneity as a source of functional heterogeneity. Differences in cellular density (153) and antigen expression (249) have been correlated with distinctly different immune functions. As yet, no one has reported a correlation between three recently identified Mφ antigens, Mac-1, -2, and -3, with immunoregulatory functions.

Monoclonal antibodies (mAb) against the Mac antigens may be used as selective parameters, in the presence of C, to alter Mφ populations. In this study, we evaluated the effectiveness of anti- Mac-1, -2, and -3 as selective devises and correlated Mac antigen expression with the secretion of immunoregulatory substances from Mφ. Two basic approaches were used: (i) 4 x 10^6 peritoneal Mφ were depleted of Mac^+ cells and cultured or (ii) 4 x 10^6 peritoneal Mφ were depleted and subsequently Mφ not removed were enriched by resuspension to 4 x 10^6 cells/ml. The second procedure was notably better than the depletion procedure, probably because it magnified the loss of particular Mac^+ cell phenotypes.

Materials and Methods

Animals

Eight to 12 wk-old male BALB/c and C3H mice were obtained from Dominion Labs, Dublin, Virginia.

Medium and Cell Lines

Hybridoma cell lines M1/70, M3/38, and M3/84 originally cloned by Springer *et al.* (233), were obtained from the American Type Culture Collection, Rockville, Maryland. Cells were initially grown in 75 cm tissue culture flasks containing RPMI medium supplemented with 5 or 10% heat-inactivated fetal calf serum (FCS; Flow Laboratories, McLean, Virginia) and 4 x 10^{-5} *M* 2-mercaptoethanol. All media contained 50 mg/l Gentamicin (Grand Island Biologicals Co. [Gibco], Grand Island, New York) 2 g/l $NaHCO_3$, and 25 mM Hepes. Thus, complete medium consisted of 10% FCS plus mentioned reagents. For larger scale production of mAb, hybridoma cells were transferred to 2 L roller bottles and cultured in 250 ml RPMI medium supplemented with 10% FCS for 3 days. Hybridoma cultures were maintained between 2-4 x 10^5 cells/ml by periodically examining the viability (kept > 85%) with trypan blue staining and reculturing when viability declined.

Mφ Preparation

Peritoneal Mφ were obtained from BALB/c mice 4 days after intraperitoneal injection of sterile thioglycollate broth (Gibco). Mφ were selected for by plating lavaged cells on plastic dishes for 4-8 hr. Nonadherent cells were removed by rinsing with 37°C RPMI medium 4-5 times. Adherent cells were removed by scraping with a rubber policeman, eluted with 10°C RPMI medium, centrifuged at 500 x *G*, and resuspended in either RPMI medium plus normal rat IgG (NRIgG) or mAb plus C. Cells isolated in this manner are >95% esterase positive (68, 69). Mφ were resuspended to 4 x 10^6 cells/ml and incubated in mAb + C for 90 min at 37°C. After rinsing 3 times in cold RPMI medium viable cells were counted (trypan blue exclusion) and either resuspended to 4 x 10^6 cells/ml or left at existing cell numbers (enriched and depleted respectively). The Mφ cultures were incubated in serum-free RPMI medium for 4 days at 37°C. Culture supernatants were collected after incubation, sequentially centrifuged at 500 x *G* for 10 min and 10,000 x *G* for 30 min, and stored at -70°C.

Monoclonal Antibodies

Supernatants from hybridoma culture were harvested and replenished with fresh complete medium every 3 days and centrifuged as described for Mφ supernatants. Cell-free supernatants were either stored at -70°C for later use or immediately purified by ammonium sulfate precipitation and DEAE chromatography. Briefly, enough of a pH 7.4 saturated ammonium sulfate solution was added to Mφ culture supernatants to yield a 45% v/v solution and were stored at 4°C overnight. Precipitates were removed by centrifugation at 10,000 x *G* for 30 min. The resulting pellet was resuspended in 0.05 *M* Tris buffer pH 8.0, dialysed against the same for 24 hr, and applied to a DEAE-Cellulose column. Protein was eluted from the column 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 antibody

were detected by Ouchterlony immunodiffusion with goat-anti-rat antibody (Fc specific) from Cappel Laboratories, Malvern, Pennsylvania. Positive fractions were pooled and concentrated 20-fold by ultrafiltration using YM-10 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 (Cappel) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and radial immunodiffusion. All protein determinations were performed using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, California). Concentrations were determined by comparing sample values to a bovine albumin standard (Sigma Chemical Co., St. Louis, Missouri).

Mixed Lymphocyte Reaction

MLR reactivity was initiated between BALB/c (H-2d) responder T cell populations and Mφ-depleted C3H (H-2k) stimulator cells. Spleens from at least three animals were excised and passed through a wire sieve, yielding a single cell suspension. Whole spleen cell (WSC) preparations were washed and resuspended in RPMI medium supplemented with 5% FCS. Responder cells were placed on nylon wool columns and incubated for 45 min at 37°C. The nonadherent cell population was eluted from the columns with RPMI medium plus FCS, 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 RPMI medium, containing 10% FCS, 4×10^{-5} *M* 2-mercaptoethanol, and 50 mg/l Gentamicin (Gibco), at 2×10^5 T cells per well in a 96-well microculture dish (Flow Laboratories). Alternatively, plated C3H splenocytes were counted, re-suspended to 2×10^7 cells/ml, and treated with Mitomycin-C (final concentration of 25 μg/ml) for 20 min at 37°C. The C3H stimulator cells were used at a concentration of 4×10^5 cells per well. Fifty μl of Mφ supernatant was added to quadruplicate wells. Test groups varied with respect to Mφ source and dilution of supernatant. 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 tritiated thymidine (^{3}H-TdR; spec. act. 6 Ci/mM, Amersham Corp., Arlington Heights, Illinois) per well 6 hr before termination and harvest. Cells were harvested onto glass fiber filters (Whatman 934-AH). After drying, the adsorbed samples were counted in a Beckman LS230 liquid scintillation counter.

Cytotoxicity Assay

Thioglycollate-elicited peritoneal Mφ were pulsed with 100 μCi of ^{51}Cr (specific activity of 250-500 Ci/mmole; Amersham, Arlington Heights, IL) per 10^7 cells for 30 min at 37°C. The labeled cells were washed 4 times with RPMI medium. Mφ were dispensed in microtiter wells at a concentration of 1 x 10^5 cells per well, containing 100 μl of RPMI medium and various concentrations of mAb, which were added in 50 μl of RPMI medium. Immediately following the addition of mAb, 50 μl of low tox rabbit C (Accurate Biochemicals, Westbury, New York) diluted 1/6 in RPMI medium was added. After a 90 min incubation at 37°C the microculture plate was centrifuged at 800 x *G* for 10 min. The supernatant from the wells was harvested with a Titertek harvesting apparatus (Flow Laboratories). The supernatants were then counted with a gamma counter.

Results

Antibody-Induced Cytotoxicity

The effectiveness of anti-Mac mAb to induce C-mediated lysis of Mφ is depicted in Fig. 1. Dilutions were performed on mAb concentrates containing 1-2 mg/ml of anti-Mac antibodies. Normal host derived peritoneal murine Mφ were pulsed with ^{51}Cr and examined examined after 90 min of incubation, in the presence of the mAb and C. Above background ^{51}Cr release or lysis was observed for all three mAb at the higher concentrations. Since each of the mAb were concentrated approximately 20-fold, the lowest dilution represents the normal concentration of these mAb in spent hybridoma supernatants. In these assays maximum lysis (54-55%) was achieved at an mAb concentration of 1.0 mg/ml, which corresponded to an antibody to cell ratio of about 12.0 $\mu g/10^5$ cells (1.3×10^7 antibody molecules per cell). Close approximations of this ratio of antibody to cells was maintained in all subsequent antibody treatments.

Anti-Mac Treatment of Mφ

After treatment with mAb + C, Mφ cultures were established with or without resuspension to 4×10^6 cells/ml to determine the most appropriate approach for the analysis of Mφ-mediated modulation. Figure 2 illustrates the effect of depletion (DMAC) and enrichment (EMAC) on production of Mφ regulatory factors. The degree of suppression was observed in MLR as decreases in ^3H-TdR incorporation. Changes in suppression were determined by comparing each Mφ treatment group with its respective C-treated controls. With respect to each of the mAb the enrichment approach yielded supernatants that were consistently less suppressive in MLR. The depletion of Mφ expressing Mac-1 and Mac-2 resulted in supernatants with suppressive activities that approxi-

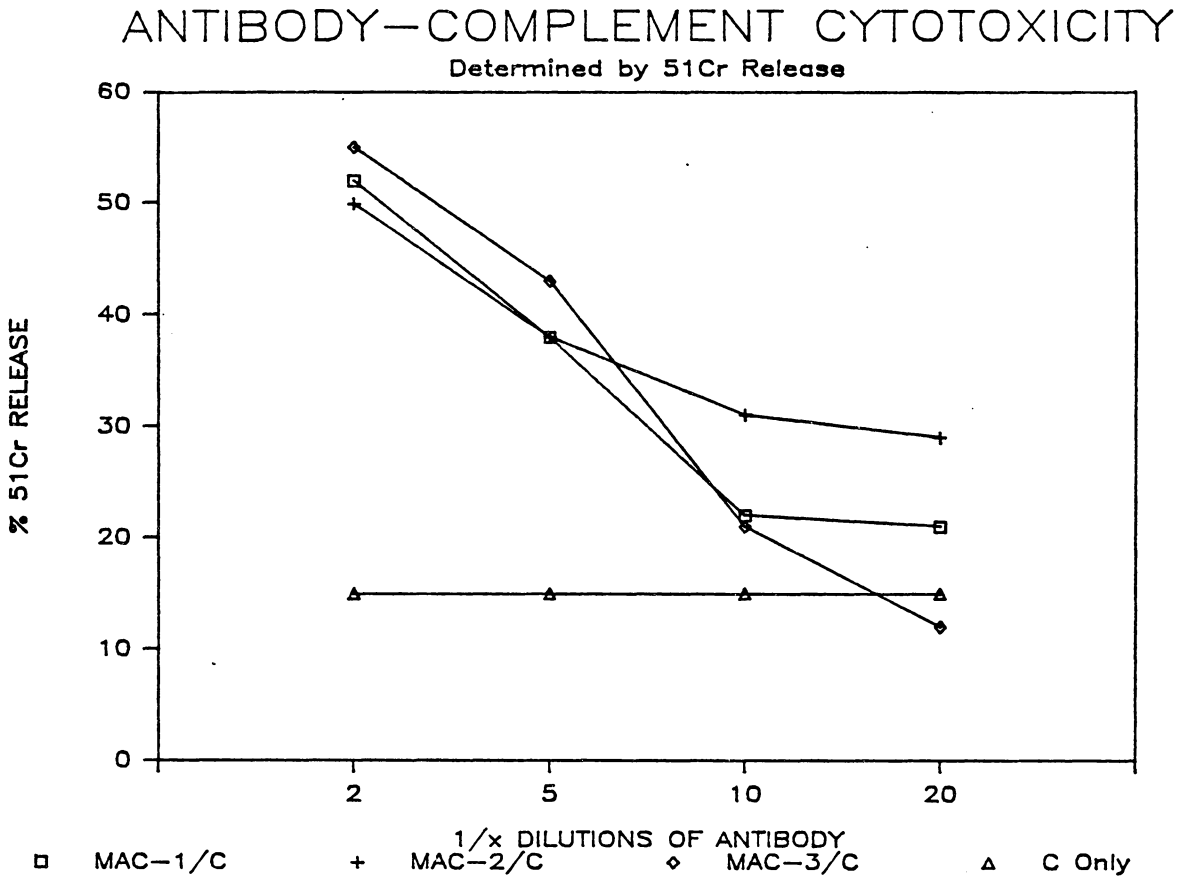

Figure 1. Antibody-complement cytotoxicity: The percentage lysis of normal peritoneal Mφ was determined from the amount of chromium released following treatment of the Mφ with anti-Mac mAb plus C. Complement controls (shown as control on the figure) represented the background or nonspecific lysis induced isotope released during the treatment period. Total release (data not) shown was determined from detergent lysed Mφ and represented 100% lysis. Shown on the graph are C only (△) as the control plus anti-Mac-1 (□), anti-Mac-2 (+), and anti-Mac-3 (◇) plus C treatments.

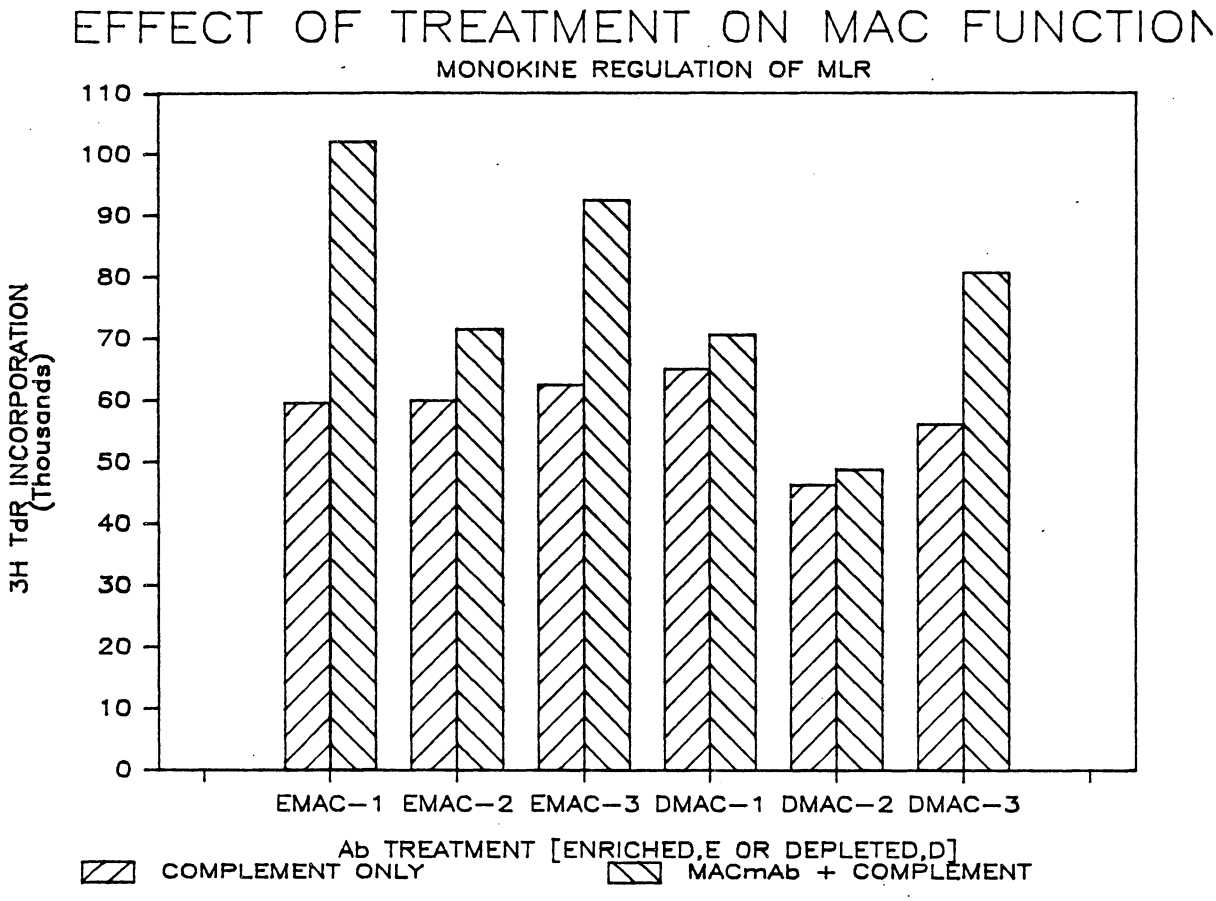

Figure 2. Effect of mAb + C treatment on monokine production: T cell proliferation was observed in MLR by measuring labeled thymidine incorporation into the DNA (see Materials and Methods). The addition of Mφ supernatants was performed at the onset of the MLR. The supernatants were derived from Mφ that were treated with antibody and C prior to *in vitro* culture. Shown in the graph are C control (///), anti-Mac-1 (\\\), anti-Mac-2 (\\\), and anti-Mac-3 (///) treatments. Suppression is observed as decreased label incorporation by the T cell cultures.

mated those demonstrated by their respective C controls. Alternatively, enriched anti-Mac-1$^+$ and anti-Mac-3-treated Mφ seemed to be more effective at reducing the inhibitory activity than anti-Mac-2-treated Mφ. In addition, anti-Mac-3 treatment demonstrated the least noticeable difference between enriched and depleted cultures, as both effectively altered the Mφ ability to produce suppressor factors.

Discussion

In past studies (10, 68), Mφ are shown to secrete molecules that inhibit T cell proliferation. In this study, we have shown that anti-Mac mAb plus C can be used to modify peritoneal Mφ populations. The selective depletion and/or enrichment of Mφ altered the secretory properties of peritoneal Mφ populations, rendering them in some instances less suppressive of T cell proliferation.

Anti-Mac-1 + C was effective at lowering the secretion of suppressive factors after enrichment. Following depletion, the Mφ remaining after anti-Mac-1 + C treatment generally produced culture supernatants that contained suppressor activities equivalent to those derived from control treatments. This showed that the remaining cells were less capable of producing the suppressive factors than those that were removed. A phenomenon such as this can not be explained based on cell numbers. It is known that the suppressive monokines are produced only at high cell densities (49). Thus, one might argue that depletion of the Mφ populations lowered cell numbers in the population and sufficient cells did not remain to produce the suppressive activity. However, when the Mφ were resuspended to 4 x 10^6 cells/ml differences in the supernatant inhibitory activities were noted.

In contrast, anti-Mac-2 plus C treatment was ineffective at lowering the suppressive activity secreted by Mφ. This characteristic was observed whether the cells were depleted or enriched. Since anti-Mac-2 + C treatment effectively lysed Mφ in the presence of C, this was not a result of inactive mAb. Also, one should note that cell number was not a factor since depletion with anti-Mac-2 was ineffective at reducing suppressive activities.

Anti-Mac-3 plus C treatment was the most effective, in the context of decreasing secretion of suppressor factors. The culture supernatants from either anti-Mac-3-depleted or enriched Mφ were less suppressive than their respective controls. This result showed that anti-Mac-3 plus C treatment was removing a segment of the Mφ population that produced suppressor factors and that could not be replaced by the remaining cells.

In light of these results, it appeared that the suppressive activity that was released during the four-day incubation period was produced by a Mφ that was Mac-1^+, -3^+ but Mac-2^-. A review of work by Stewart and Walker (244, 283) supports this hypothesis since they find that a distinction exists between Mac-2^+ Mφ and Mac 3^+ Mφ in the peritoneal population. They find that Mac-2^+ Mφ are primarily elicited by thioglycollate and are different from the Mac-3^+ cells that are resident Mφ. Collectively, this suggests that in the population of normal host peritoneal Mφ, the Mφ that produced the majority of the suppressor activity during 4 days of *in vitro* culture are the resident Mφ.

Chapter II

MODULATION OF Mφ FUNCTION BY LIGAND-RECEPTOR INTERACTION

Introduction

In recent years it has become apparent that ligand-receptor interactions mediate various types of intercellular communication activities within the immune system (27, 100, 268). These activities are ultimately responsible for homeostasis and the control of immune responses (23). The basic mechanism of these regulatory circuits includes the reception of a signal from the primary responder to a second cell. Soluble molecules or cytokines (41) are recognized as the communication signals. Mφ secrete various types of regulatory molecules in response to primary signals and antigen reception (41, 178, 179). Thus, their presently defined immunological function surpasses previous descriptions labeling them as merely a phagocytic scavenger.

Monoclonal antibodies against cell-surface antigens have been used successfully to either block or duplicate signals between cells (96, 132). In this study, we have employed mAb specific for the Mφ cell-surface antigens, Mac-1, -2, and -3, as probes to examine the role that these antigens may have in Mφ immunoregulatory functions. Our hypothesis was that antigens such as the Mac antigens may represent receptors for immunoregulatory compounds and thus mAb binding to these receptors may cause changes in the transmission of regulatory signals to other immune cells (i.e., T cells). Normal and TBH Mφ were examined since differences in the immunoregulatory activities of Mφ from these origins is known (48, 90). In this study, normal host Mφ produced more inhibitory supernatants after exposure to anti-Mac-1 and anti-Mac-2, as compared to controls which received no mAb. Alternatively, anti-Mac-2 and -3 treatments caused increased suppressive activity in TBH-derived Mφ culture supernatants. The mAb did not adversely affect $P388D_1$ cell proliferation. Since $P388_1$ cells are a neoplastic line of Mφ that express all three of the Mac antigens and have Fc-receptors, unaltered proliferation and noninduction of PGE secretion by these cells following mAb treatment suggested that the changes seen in peritoneal Mφ regulatory function were most likely independent of cytotoxicity or Fc-receptor activation. The resulting changes in Mφ regulatory activities following mAb treatment showed that the Mac antigens may be differentially involved in Mφ-regulatory functions. Furthermore, the data demonstrated differences in normal and TBH Mφ populations.

Materials and Methods

Animals

Eight to 12 week-old BALB/c mice were obtained from Dominion Labs, Dublin, Virginia. Tumor-bearing animals were generated by intramuscular injections of 3 x 10^5 cells/ml of a transplantable methylcholanthrene-induced fibrosarcoma. TBH were animals that had a palpable 21 day-old tumor.

Cell Culture

RPMI medium containing 20% FCS was used to grow the hybridoma cells. Peritoneal Mφ were collected 4 days after thioglycollate elicitation of the peritoneum (see Appendix 2). Production of Mφ supernatants was achieved by culture of the Mφ for 4 days in serum-free RPMI medium. Hybridoma cell lines M 1/70, M 3/38, and M 3/84 originally cloned by Springer *et al* (249), were obtained from the American Type Culture Collection, Rockville, Maryland. Hybridoma cells and mAb were prepared as described elsewhere (89).

Anti-Mac Treatment of Mφ

mAb treatments involved treating Mφ with 10-20 μg/10^5 cells (see Appendix 2). Mφ were incubated in the presence of mAb (without the addition of C) for 90 min at 37°C, washed 3 times with ice cold RPMI medium, resuspended to 4 x 10^6 viable cells/ml and culture *in vitro* for 4 days. In this study control Mφ cultures were treated with only RPMI medium. Because other studies

(246, 249) compare normal rat IgG to RPMI treatments show no cytotoxic effects and minimal or no membrane binding, RPMI controls were sufficient in this study.

Suppressive Culture Supernatants

Supernatants obtained from 4 day *in vitro* cultured Mφ (in the absence of serum) were spun at 500 x G for 10 min and 10,000 x G for 30 min. The cleared supernatant was filter sterilized by passage through a 0.22 μm filter. Collected supernatants were stored at -80 C until used.

Mixed Lymphocyte Reaction

MLR reactivity was initiated between BALB/c ($H\text{-}2^d$) Mφ-depleted responder T cell populations and Mitomycin-C (Sigma) inactivated C3H ($H\text{-}2^k$) stimulator cells. Enriched T cells were obtained by previously described procedures (89). Briefly, this involved nylon wool adherence and G-10 sepharose-glass bead filtration. Cells were suspended in RPMI medium that was supplemented with 5% FCS (FCS) and β-mercaptoethanol (10^{-5} *M*). Responder and stimulator cells were co-cultured for 96 hr at 2:1 cell-cell ratios (respectively). During the final 6 hr of culture responder T cell proliferation was measured by ^{3}H-TdR incorporation (initially added at 1 μCi per well). Proliferation was recorded as counts per minute (cpm) of harvested DNA on glass fiber filters.

Suppressive activity of Mφ-derived culture supernatants was examined by adding 50 μl of the supernatant to each well (yielding a total volume of 200 μl) at initiation of the MLR. MLR cultures which received only RPMI medium were used as controls. The degree of suppression mediated by Mφ supernatants was determined from decreases in the incorporation of ^{3}H-TdR.

Results

Suppressive Activity of Mφ Culture Supernatants After mAb Treatment

To assess the possible adverse effects of anti-Mac mAb binding to Mφ, the P388D_1 cell line was treated with the mAb and changes in viability were monitored as changes in proliferation. P388d_1 cells are proliferative Mφ cells that express all three of the Mac antigens. Since no changes occurred in the proliferation of these cells no adverse effects were attributed to mAb-receptor binding. Furthermore, other studies (165) show that Fc binding of these mAb to P388D_1 is minimal since they fail to activate PGE_2 production by these cells. In light of the results from P388 D_1 treatments, mAb were subsequently used to treat normal host peritoneal Mφ and the secretion of suppressor factors by the treated Mφ was monitored. Suppression was detected by the degree of decrease in T cell proliferation caused by addition of treated Mφ culture supernatants to MLR.

As noted in Fig. 3, Anti-Mac-1 treatment of Mφ caused increases in the suppressive activity of 4-day *in vitro* culture supernatants of normal Mφ but not TBH culture supernatants. This suppressive activity was observed as a decrease in T cell proliferation in MLR, as measured by ^{3}H-TdR incorporation. The difference in suppressive activity was seen at all dilutions of culture supernatants and was the greatest at a 1:10 dilution. Figure 4 showed that both normal and TBH anti-Mac-2 treated Mφ produced more suppressive culture supernatants than their respective controls. Alternatively, anti-Mac-3-treated normal host Mφ (Fig. 5) did not demonstrate any change in production of suppressor factor(s), but the same treatment caused a notable change in TBH-derived Mφ. Following anti-Mac-3 treatment TBH Mφ produced supernatants that were more suppressive than their RPMI-treated counterparts.

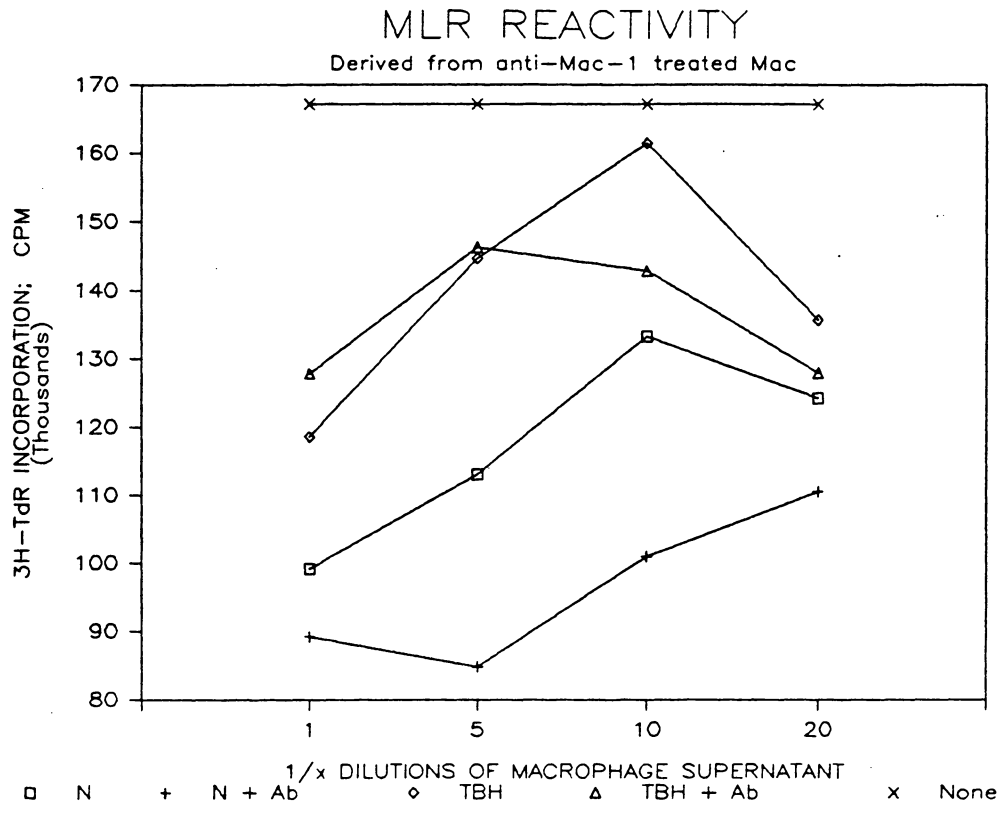

Figure 3. **Anti-Mac-1-mediated alterations in monokine secretion:** T cell proliferation after the addition of Mφ culture supernatants derived from mAb-treated Mφ T cell proliferation is shown as the amount of ^{3}H-TdR incorporation occurring in a MLR following the addition of the culture supernatants. The effects of the Mφ supernatants shown are from untreated normal (□) and TBH (◇) Mφ and anti-Mac-1-treated normal (+) and TBH (△) Mφ.

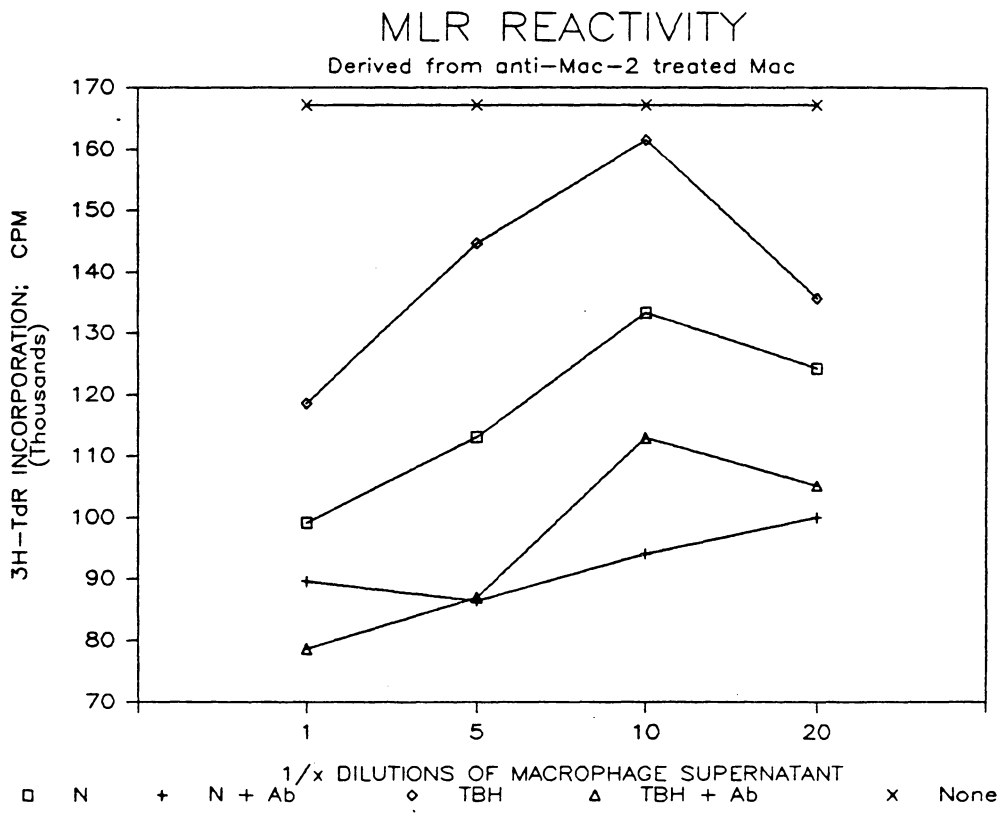

Figure 4. Anti-Mac-2-mediated alterations in monokine secretion: T cell proliferation after the addition of mAb-treated Mφ-derived culture supernatants. T cell proliferation is shown as the amount of ^{3}H-TdR incorporation occurring in a MLR. The effects of the Mφ supernatants shown are from untreated normal (□) and TBH (◇) Mφ and anti-Mac-2-treated normal (+) and TBH (△) Mφ.

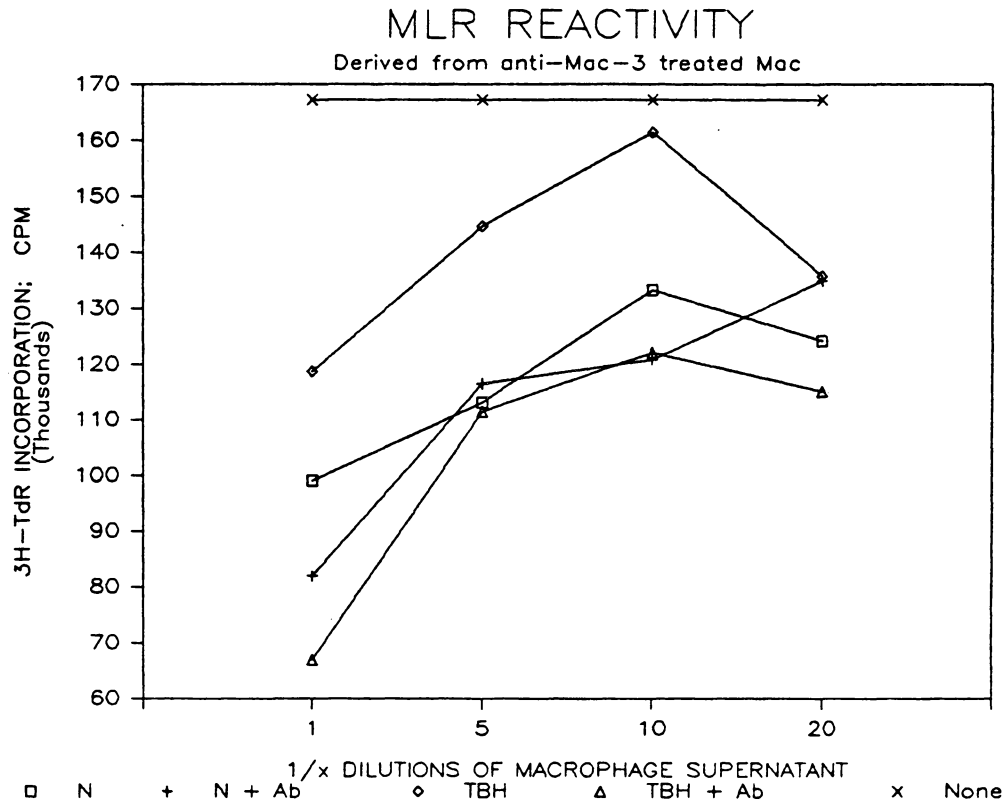

Figure 5. **Anti-Mac-3-mediated alterations in monokine secretion:** T cell proliferation after the addition of mAb-treated Mφ-derived culture supernatants. T cell proliferation is shown as the amount of ^{3}H-TdR incorporation occurring in a MLR. The effects of the Mφ supernatants shown are from untreated normal (□) and TBH (◇) Mφ and anti-Mac-3-treated normal (+) and TBH (△) Mφ.

Discussion

Modulation of surface receptors by mAb has been demonstrated (96), where ligand-receptor interaction results in the alteration or modification of cell functions. Here we have shown that anti-Mac mAb are capable of ligand-receptor interactions that cause changes in the production of Mφ suppressor factors. Furthermore, differential patterns of ligand-mediated alterations were observed between normal and TBH-derived Mφ.

In other studies (89, 90), we show that normal and TBH Mφ differs in their expression of Mac-1, -2, and -3 cell surface antigens. These studies also imply a correlation between Mφ immunoregulatory activities and the Mac antigens. Other researchers have already shown that Mac-1 is the C3b receptors (55) and by virtue of C3b functions may be associated with PGE_2 production (223). Ligand-receptor interaction between C3b and the C3b receptor causes increased PGE_2 production. Our results concur with these findings because anti-Mac-1 binding to normal Mφ caused an increase in the suppressive content of Mφ culture supernatants. The ineffectiveness of anti-Mac-1 treatment on TBH-derived Mφ reflects either a difference in their Mac-1 expression or differences in their regulatory activity. We have observed differences in Mac-1 expression among splenic Mφ, which also demonstrate differential activities between normal and TBH Mφ after anti-Mac-1 treatment.

The similarities observed between normal and TBH Mφ-mediated suppressor functions after anti-Mac-2 treatment are difficult to explain, since we have previously noted (89) a decrease in Mac-2 expression on peritoneal Mφ from TBH. However, quantitative expression of a receptor may or may not contribute to the observed alterations in Mφ function. For example, the activity of the cell expressing the antigen is probably more of a factor than the total number of cells (active and inactive) expressing the antigen.

The observation that anti-Mac-3 treatment caused an increase in suppressor secretion by TBH Mφ but not normal host Mφ, correlated with our previous observation that the suppressor Mφ in TBH populations was a Mac-2^+ cell type. It also implied that Mac-3 antigen expression

on the group of TBH Mφ responsible for soluble suppressor activities may be required and be directly associated with a regulatory function.

Chapter III

SPLENIC Mφ PHENOTYPES AND ACCESSORY CELL FUNCTION IN NORMAL AND TBH

Introduction

The correlation of function with specific accessory cell phenotypes is a prerequisite to our understanding of immune cell interaction. Delineation of Mφ immunoregulation is of particular interest in studies involving altered host systems such as a TBH. Several studies have implicated Mφ as contributors to tumor-induced immune dysfunction (34, 98, 262, 302). Of these, a limited number have defined functional subsets of Mφ on the basis of phenotypic variations (89, 91, 257, 258, 194). Tumor growth is thought to upset homeostasis at the level of the Mφ, resulting in the transmission of aberrant signals to T cells. We (68, 69) and others (73, 95, 106, 148, 276) show differences in the regulatory activities of normal and TBH Mφ.

One model system that allows for the study of immune cell interactions is the accessory cell-dependent T cell lectin-induced proliferative response (173, 214). Depletion of splenic adherent cells (SAC) from whole spleen cells (WSC) reduces T cell responsiveness to mitogens (214, 227, 245). Studies focusing on Mφ-mediated immunoregulation of T cell responses demonstrate that both cell-cell interaction (133) and soluble mediator production (111) are mechanisms used by Mφ to mediate homeostasis. The supportive accessory function of Mφ and Mφ-like cells is complemented by suppressive accessory functions which sometimes is attributable to a physically distinct Mφ-like cell. Thus, Mφ are pivotal cells in the regulation of T cell responses and their pluripotency focuses attention on the existence of functionally independent and phenotypically distinct subpopulations.

The recent availability of anti-Mφ mAb allows for characterizations of Mφ phenotypes. Springer and others have produced a series of mAb directed against Mφ surface antigens Mac-1, -2, and -3 (114, 115, 116, 208, 234, 240). Analysis of spleen cell populations indicates that these antigens are differentially expressed on Mφ (208, 234) and dendritic cells (81). Interpretation of these studies collectively suggests that splenic Mφ are Mac-1^+, -2^-, -3^+ and dendritic cells are Mac-1^-, -2^+, -3^+.

To assess the contribution of mAb defined SAC accessory cell in lectin-induced T cell proliferation in the normal host and to address alterations due to tumor growth, we selectively depleted different splenocyte populations by mAb-dependent C-mediated cytotoxicity. Mφ adherence to various surfaces and autofluoresence (244) restricted our choice of other selective separation techniques (296) to mAb-dependent C-mediated cytotoxicity methodology, which is not limited by these parameters. We and others have used similar depletion techniques in the functional characterization of Mφ (89, 249, 279) and lymphocytes (46, 166). In the present study, reductions in SAC populations by mAb + C reflected the expression of these antigens within the total population as similar percentage Mac^+ values were obtained by mAb-mediated indirect immunofluorescence. mAb + C-mediated depletion correlated Mac-1^+ and -3^+ but not Mac-2^+ splenocytes with accessory function in normal and TBH WSC. Lectin stimulation of normal T cells that were admixed with mAb + C treated SAC identified the Mac-2^+ phenotype as a func-

tionally dominant accessory cell in normal and not TBH SAC. In contrast, TBH SAC accessory activity was mediated by a Mac-1^+ phenotype. In summary, removal of Mφ or Mφ-like splenocytes with anti-Mac mAb from WSC and splenic adherent cell (SAC) preparations effectively altered subsequent regulation of lectin-induced T cell blastogenesis and thus identified functionally different accessory cells in normal and TBH.

Materials and Methods

Animals

Eight to 12 wk-old male BALB/c ($H2^d$) mice were used throughout (Dominion Labs, Dublin, VA). Tumor induction was achieved by intramuscular injection with a single-cell suspension of a methylcholanthrene-induced transplantable fibrosarcoma (3 x 10^5 BALB/c tumor cells) into the left hind leg (68, 69). Varying degrees of tumor burden were achieved by using animals inoculated at 7-day intervals. The tumor is palpable by Day-10. By Day-14, the TBH is maximally immunosuppressed and remains in that state until death. (The lectin-stimulated normal and Day-14 TBH WSC have a blastogenic response of 190,700 versus 53,476 cpm, respectively.) By examining TBH at three sequential intervals, kinetic parameters of accessory cell activity were assessed. Normal and TBH were tested by Microbiological Associates (Bethesda, MD) and were found to be free of immunologically pertinent viral contaminants (89).

Medium

All cells, unless stated otherwise, were grown in RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum (FCS; Flow Laboratories, McLean, VA) and 4 x 10^{-5} *M* 2-mercaptoethanol (2-ME). All media also contained 50 mg/l Gentamicin (Grand Island Biologicals Co., Grand Island, NY), 2 g/l $NaHC0_3$, and 25 mM HEPES.

Cell Lines

Hybridoma cell lines M1/70, M3/38, and M3/84 (which produce anti-Mac-1, -2, and -3, respectively), originally cloned by Springer *et al.* (234), were obtained from the American Type Culture Collection, Rockville, MD. These hybridomas produce rat mAb against mouse Mφ antigens Mac-1, -2, and -3 which are glycoproteins with M_r mass of 285 (dipeptide with 190 and 95 kDa subunits), 32, and 110 kDa, respectively. All three of the antibodies are effective at fixing C (89). Anti-Mac-1, -2, and -3 belong to the rat IgG subclass groups 2b, 2a, and 1, respectively. The cellular specificity of anti-Mac-1 is inclusive of all Mφ, NK cells, a proportion of bone marrow cells, and crossreacts with human Mφ (115, 116). Mac-2 is found on elicited peritoneal exudate cells (PEC) (114) and dendritic cells (81) but is present only in small quantities or absent from normal host splenic Mφ (114). Mac-3 is described as a constitutive Mφ antigen that is found on all mature Mφ (208, 234) and dendritic cells (81). Alternatively, Walker *et al.* (283) and Stewart *et al.* (244) have reported variable expression of Mac-3 on maturing bone marrow-derived pro-monocytes and a lack of expression on certain PEC.

Hybridomas were initially grown in 75 cm^2 tissue culture flasks containing RPMI medium supplemented with 5 or 10% FCS (Flow Laboratories). For larger scale production of mAb, hybridoma cells were transferred to 2-liter roller bottles and cultured in 250 ml RPMI medium with 10% FCS. Further details are published elsewhere (89).

Monoclonal Antibodies

mAb from hybridoma cultures were prepared as described previously (89). Hybridoma supernatants were either stored at -70°C or purified by ammonium sulfate precipitation and DEAE chromatography. Briefly, our purification procedures involved ammonium sulfate precipitation, DEAE-cellulose ion-exchange chromatography, Amicon concentration, and dialysis to RPMI me-

dium. The procedure yielded partially purified, 20-fold concentrated mAb. Rat mAb were detected with goat-anti-rat antibody (Fc-specific) (Cappel Laboratories, Malvern, PA) by immunodiffusion. The final antibody preparations were assayed for antibody purity and concentration by polyacrylamide gel electrophoresis and radial immunodiffusion, respectively, by comparison to normal rat IgG (NRIgG) (Cappel).

Cell preparations

Spleens from at least 4 animals were excised and passed through a wire sieve to yield a single cell suspension. These WSC were then handled in one of four different ways: (i) responder cells in lectin-induced proliferation assays, (ii) treated with mAb and assayed for lectin responsiveness, (iii) plated to obtain SAC which were mAb-treated and added to (iv) Mφ-depleted responder T cells. While WSC were derived from normal and TBH animals, only normal mice were used as a source of responder T cells in the SAC-T cell admixture experiments.

mAb Treatment of WSC

Single-cell suspensions of WSC from normal and TBH were counted and viability assessed by trypan blue exclusion, resuspended to 1 x 10^7 per ml, and treated in 4 ml of RPMI medium containing 50 μg/ml mAb or NRIgG in the presence or absence of C (Low-Tox Rabbit C; Accurate Chem. & Sci. Corp., Westbury, NY) for 90 min at 37°C (89). After washing 3 times the mAb-treated cells were resuspended in the same volume as the RPMI medium-treated WSC, which were resuspended at 2 x 10^5 per well in RPMI medium supplemented with 10% FCS and 4 x 10^{-5} *M* 2-ME for assessment of lectin-induced proliferation. Anti-Mac antibodies had no effect on lymphocyte viability.

SAC Preparation

Splenic Mφ were obtained from single-cell suspensions of WSC that had been treated with NH_4Cl to remove red blood cells. Normal and TBH spleen preparations contained 10-15% and 30-32% esterase positive SAC, respectively. Mφ were selected for by plating on plastic dishes for 2-4 hr in RPMI medium. Nonadherent cells were removed by rinsing with RPMI medium. SAC were harvested by scraping with a rubber policeman, washed with RPMI medium, centrifuged at 500 x *G*, and resuspended in RPMI medium. Harvested SAC (>98% esterase positive) demonstrated >90% viability. After isolation, the SAC were treated with NRIgG or mAb with or without C for 90 min at 37°C (89). Equivalent mAb + C-mediated lysis was observed with pre- and post-scraping cell preparations, suggesting that adherence and scraping did not contribute to regulatory differences between WSC and SAC. After rinsing 3 times with RPMI medium the treated cells were counted with trypan blue, resuspended to 4 x 10^6 cells/ml, and dispensed at 4 x 10^4/well as accessory cells into 96-well flat-bottom microculture plates (Flow Laboratories).

Indirect immunofluorescence with mAb was performed with SAC that were methanol fixed onto glass slides. Two hundred μl of the previously described SAC were spread over the surface of a glass slide and air dried for 15 min. The cell smears were fixed for 5 min in ice cold 100% methanol. The slide preparations were treated with 10% FCS in RPMI medium for 2 hr to block nonspecific binding of mAb. Smears were then immersed in cold whole hybridoma supernatants for 1 hr and subsequently rinsed with 3 changes of RPMI medium. Rinsed smears were immersed in 1:1000 dilution of rhodamine-labeled mouse $F(ab')_2$ anti-rat IgG (Jackson ImmunoResearch Laboratories, Avondale, PA) and incubated for 1 hr. Labeled cell smears were rinsed with two changes of RPMI medium, two changes of PBS (pH 7.4), and covered with 50% glycerol/PBS. Fluorescing cells were observed and enumerated using an Olympus IMT-2 inverted phase-fluorescence microscope.

Lectin-Induced T Cell Proliferation.

Non-mAb-treated WSC were placed on nylon wool columns and incubated for 45 min at 37°C. The nonadherent cells were eluted from the columns with RPMI medium plus FCS, centrifuged, and placed on Sephadex G10/glass bead columns for 45 min at 37°C. The nonadherent T cells were harvested (contaminated with $< 2\%$ esterase positive cells), counted with a Coulter counter, and dispensed at a cell density of 2×10^5 T cells per well in RPMI medium supplemented with 10% FCS. There was a $\log_{10}$ loss in the number of T cells following nylon wool separation (212). Concanavalin A (Con A) stimulation of purified T cells was minimal in the absence of Mφ or 2-ME, indicating that they were relatively free of contaminating accessory cells. BALB/c SAC were introduced into the microculture wells at 4×10^4 cells/well (108). Con A (Difco Laboratories, Detroit, MI) was added to the wells to yield a final concentration of 0.8 μg/well. This concentration of lectin is optimal for both normal and TBH splenocytes. TBH splenocytes are hyporesponsive to lectin which correlates directly to the decrease in the magnitude of splenocyte proliferation. WSC were compared to their respective normal or TBH counterparts to obtain percentage differences. Thus, differences in responses were directly associated with the mAb treatment. Sensitivity of the normal and TBH WSC to different mAb were compared on the basis of percentage change in lectin-induced blastogenesis of remaining cells. Further analysis of the cell type responsible for these changes were examined by treating SAC from normal and TBH with mAb plus C and examining altered accessory cell activities. All treatment groups of SAC were resuspended to the same cell density to account for quantitative differences. Therefore, any changes noted in proliferation of normal T cells to lectin was the result of qualitative differences from the added SAC population.

In both WSC and mixtures of T cells and SAC, Con A reactions proceeded for 72 hr at 37°C and were pulsed with 1 μCi tritiated thymidine (^{3}H-TdR; spec. act. 6 Ci/mM, Amersham Corp., Arlington Heights, IL) per well 6 hr before termination and harvest. Cells were harvested onto glass fiber filters (Whatman 934-AH). After drying, the adsorbed samples were counted in a

Beckman LS230 liquid scintillation counter. All determinations were done in quadruplicate and are shown as the mean cpm ± SEM.

Results

mAb-Induced Alterations in WSC Responses to Con A

To determine ligand activation by mAb binding on Mφ accessory cell activity, treatments were performed in the absence of C. Normal and TBH WSC responses to Con A after treatment with anti-Mac-1, -2, or -3, as compared to their equivalent NRIgG-treated counterpart, is illustrated in Fig. 6. Anti-Mac-1 treatment of normal host WSC caused a significant increase (70%) in T cell mitogenesis. Anti-Mac-1 had no detectable effect on TBH WSC responses. Ligand interaction with Mac-2 significantly reduced (28%) normal host WSC responsiveness; whereas, it was significantly increased (36%) in TBH WSC. TBH WSC were also significantly more responsive (34%) to lectin after anti-Mac-3 treatment. Binding of normal host WSC Mac-3 epitopes with anti-Mac-3 had no effect.

To evaluate the contribution of mAb-defined Mφ cell types in WSC responses to lectin, WSC were treated with mAb + C. Figure 7 compares the proliferative response of WSC after mAb + C to their corresponding NRIgG + C-treated control. Anti-Mac-1 + C treatment significantly reduced the Con-induced proliferation of normal (42%) and TBH (38%) WSC. Anti-Mac-2 and -3 + C treatments caused minor changes in normal host WSC mitogenesis, 14% and 18% respectively. Only a minor 18% change in TBH WSC response was observed after treatment with anti-Mac-2 + C; whereas, TBH WSC responsiveness was significantly reduced (46%) by anti-Mac-3 + C treatment.

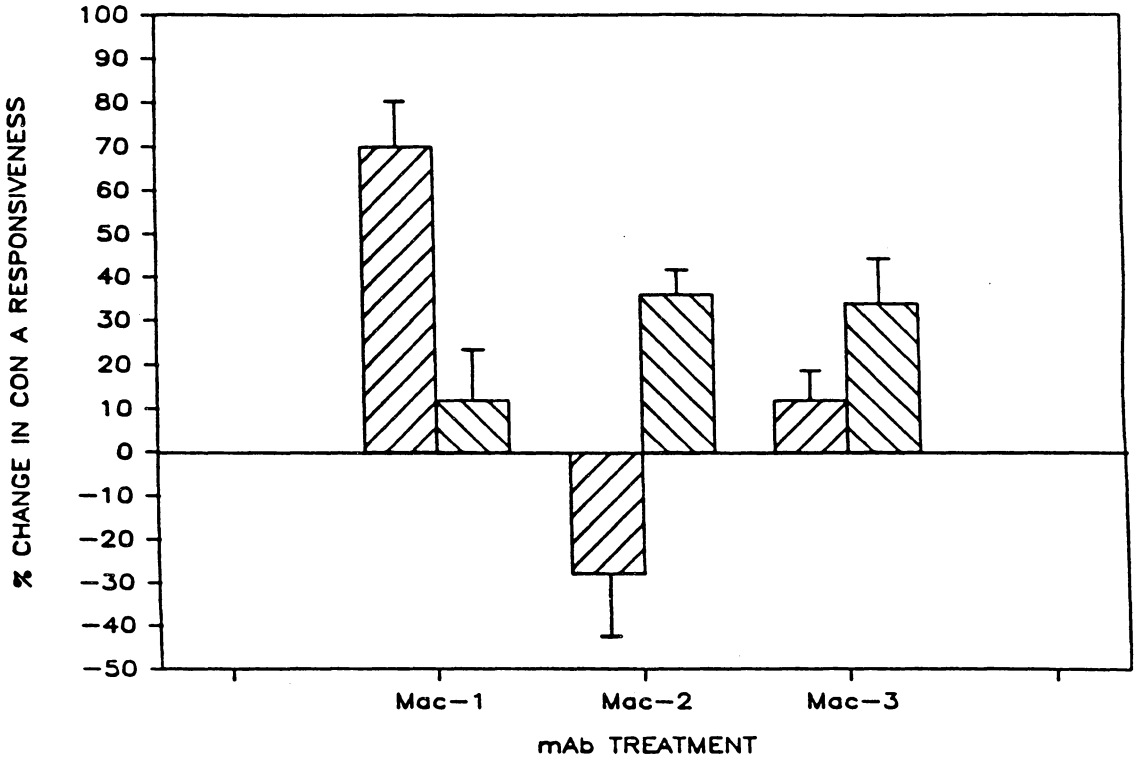

Figure 6. **Anti-Mac-mediated alterations of Mφ accessory function:** Ligand modulation of WSC response to Con A after anti-Mac mAb treatment without C. WSC from normal (///) and TBH (\\\) were treated with NRIgG (represented by zero percentage line), anti-Mac-1, -2, or -3, and activated with Con A. The degree of T cell proliferation was detected by ^{3}H-TdR incorporation. Percentage change in T cell proliferation was determined by comparing each treatment to NRIgG-treated WSC. The response of NRIgG-treated WSC used in the calculations of zero percentage change was 307,467 and 39,140 cpm for normal and TBH, respectively. Tumor-bearing mice were at least 14 days post tumor cell inoculation (PTI) and were immunosuppressed. WSC from animals at time points 14-21 days PTI were equivalent in their immunosuppression and accessory activity. Results are represented as the mean ± SEM for quadruplicate determinations.

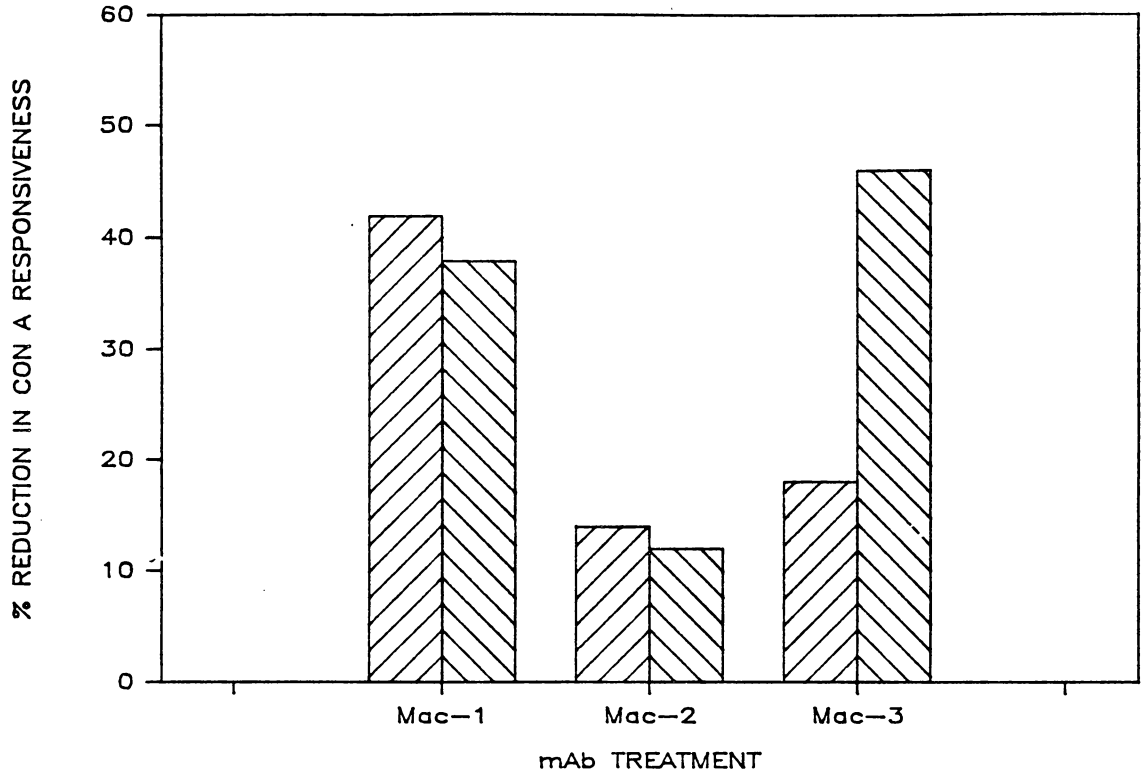

Figure 7. Modulation of WSC response to Con A after anti-Mac mAb treatment: Tests were performed with normal (///) and TBH (\\\) as described in Figure 6, except C was added to treatments. Percentage reduction in Con A responsiveness was obtained by comparing each treatment to NRIgG + C-treated WSC. The response of NRIgG + C-treated WSC used in the calculation of the zero percentage change were 307,892 and 40,989 cpm for normal and TBH, respectively. WSC from animals at time points 14-21 days PTI were equivalent in their immunosuppression and accessory activity.

Lectin Response of Anti-Mac + C-treated WSC Combinations

To determine functional overlap between mAb-defined SAC populations, different treatment groups were combined; that is, the resultant normal or TBH WSC of anti-Mac-1, -2, or -3 + C treatment were added together with similarly treated WSC from the same host (Fig. 8). Control WSC combinations received either two normal or TBH WSC populations treated with NRIgG + C. The combination of anti-Mac-1- plus -2- (Group A) or -3-treated (Group C) normal host WSC resulted in a proliferative response that was significantly reduced, 49% and 52%, respectively. Combinations of anti-Mac-1- with -2- (Group A) or -3-treated (Group C) TBH WSC were unchanged. Mixtures of anti-Mac-2- plus -3-treated (Group B) WSC showed opposing responses when comparing normal to TBH. That is, anti-Mac-2- plus -3-treated WSC (Group B) combinations were more responsive when derived from normal (+28%) than from TBH (-26%) (for each $P < 0.005$).

Indirect Immunofluorescence of mAb-treated SAC

To analyze the composition of SAC populations and to detect differences between normal and TBH SAC, mAb-labeled SAC were detected with rhodamine labeled mouse anti-rat IgG. The fluorescing cells from Mac-1, -2, -3, and NRIgG-treated normal and TBH SAC were examined. Percentage of cells expressing mAb-defined antigens was determined and is illustrated in Table 1. In normal host-derived SAC, Mac-1 was detected on 39% of the population whereas 63% of the TBH SAC expressed the antigen. Mac-2^+ cells were the least abundant, when compared to other mAb labeled cells, in both normal (27%) and TBH (40%) SAC. Mac-3 antigen expression was the highest in normal host SAC (80%) and decreased in the TBH (62%). In general, an inverse relationship was demonstrated between Mac-1 and -3, that is, Mac-1 expression increased with tumor development and Mac-3 expression decreased.

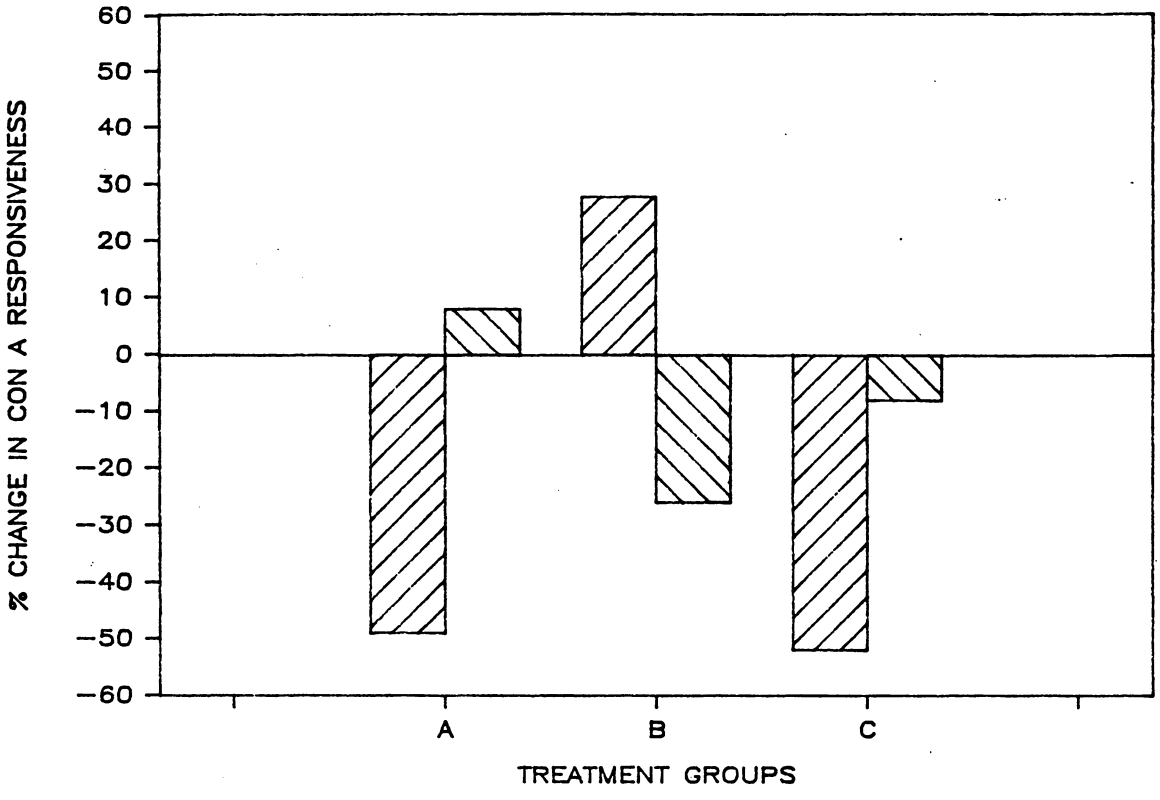

Figure 8. Modulation of admixed WSC treatment group responsiveness to Con A: WSC were treated as described for Figure 1, were mixed in pairs, and reacted with Con A. Mixtures were restricted to normal host plus normal host (///) or TBH plus TBH (\\\). Percentage reduction in Con A responsiveness was determined by comparing mAb + C-treated pairs to combinations of two NRIgG + C-treated WSC. The combinations include anti-Mac-1- plus -2-treated WSC (Group A), anti-Mac-2- plus -3-treated WSC (Group B), and anti-Mac-1- plus -3-treated WSC (Group C). Results are represented as the mean ± SEM for quadruplicate determinations. WSC from animals at time points 14-21 days PTI were equivalent in their immunosuppression and accessory activity.

Table 1. Indirect Immunofluorescence Analysis of mAb Binding to Normal and TBH SAC

Host cell source	Antibody used			
	NRIgG	Mac-1	Mac-2	Mac-3
Normal	33[a]	39	27	80
TBH	35	63	40	62

[a]Data are expressed as mean percentage positive cells from a pooled SAC population derived from 6 normal and 6 TBH mice. Cells were either treated with nonspecific normal rat IgG (NRIgG) or specific mAb (anti-Mac-1, -2, and -3).

Accessory Activities of mAb + C-modified SAC Population

To determine the accessory activity of enriched Mac$^-$ normal and TBH SAC, anti-Mac + C-treated SAC were added to lectin-stimulated normal host Mφ-depleted T cells. The data in Fig. 9 shows the response of T cells when combined with 4 x 10^4 treated SAC. SAC- and B cell-depleted normal host T cells that received no SAC responded poorly (< 10,000 versus > 40,000 cpm with the addition of NRIgG-treated SAC from any stage of TBH). SAC preparations which were not treated with NRIgG had similar accessory activities to NRIgG-treated SAC (data not shown). Thus, NRIgG + C-treated SAC represented control SAC accessory activities at each of the times post tumor cell inoculation (PTI) and were compared with mAb + C-treated SAC to determine the accessory function of anti-Mac defined Mφ populations. Anti-Mac-1 + C-treated normal host SAC were 87% more supportive of normal host T cell proliferation at 0 days PTI. Normal host SAC (0 days PTI) lost 66% of their supportive activity following anti-Mac-2 + C treatment. There was no change in accessory cell activity following anti-Mac-3 + C treatment of Day-0 PTI SAC. At 7 days PTI, anti-Mac-1 + C-treated SAC were equivalent to control SAC accessory cell activity. Anti-Mac-2 + C SAC-treated demonstrated a 50% reduction in accessory cell activity. Anti-Mac-3 + C-treated SAC had increased accessory cell activity (42%; $P < 0.025$). By 14 days PTI, anti-Mac-1-, -2-, and -3-treated SAC each were significantly ($P < 0.05$) less supportive of T cell proliferation with anti-Mac-1 + C treatment causing the most pronounced decrease in accessory cell function.

Accessory Cell Function of Combined mAb Plus C-treated SAC

Since Mac antigen-defined SAC subpopulations may overlap in accessory function, equal numbers of anti-Mac + C-treated SAC were combined and added to Mφ-depleted normal host T cells. The SAC combinations were only performed with cells from the same times PTI. A total

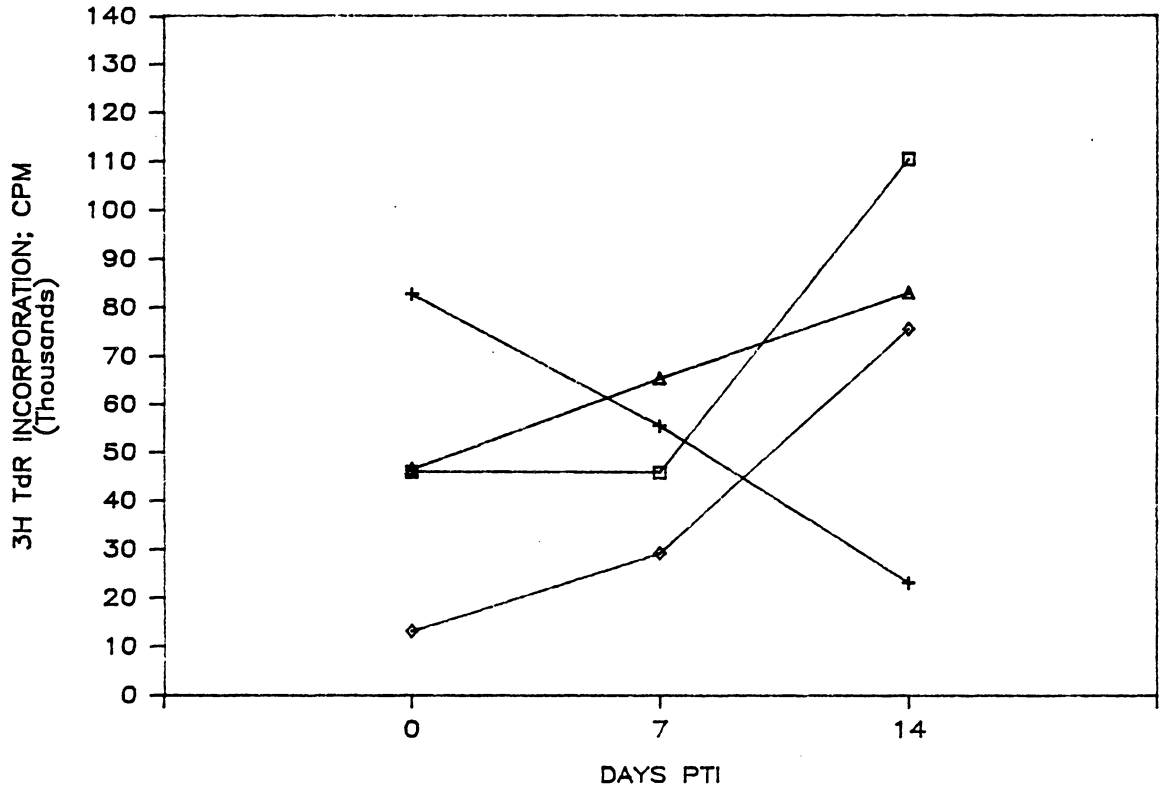

Figure 9. Accessory cell function of anti-Mac-treated SAC: SAC were treated with NRIgG (□), anti-Mac-1 (+), -2 (◇), and -3 (△) + C, washed, and mixed with normal host T cells at a 1:2 ratio. T cell response without SAC was < 10,000 cpm. The admixture (SAC plus T cells) populations was assessed for their ability to respond to Con A by ^{3}H-TdR incorporation. Results are represented as the mean ± SEM for quadruplicate determinations. 21-day PTI SAC were equivalent to 14-day PTI SAC and thus are not included in the figure.

cell number of 4 x 10^4 mAb + C-treated SAC were added to Mφ-depleted T cells. The T cell mitogenic responses following SAC admixtures are shown in Fig. 10. All combinations of SAC were supportive of T cell proliferation to some degree (greater than the response of T cells without SAC). All combinations were compared to T cells receiving NRIgG + C-treated SAC. At 0 days PTI, anti-Mac-1- plus -2- or -3-treated SAC were more supportive (90%) of T cell mitogenesis and became less supportive of T cell proliferation with tumor development. In contrast, combinations of anti-Mac-2- plus -3-treated SAC were significantly less supportive of T cell proliferation at 0 days PTI.

SAC Removal by mAb-induced Lysis

To determine the proportions of mAb-defined SAC subpopulations, lysis of mAb + C-treated SAC was assessed at different stages in tumor development and is illustrated in Figure 11 as percentage removal of SAC. The reduction in SAC number was determined by comparing NRIgG- and anti-Mac-treated SAC (all treatments were performed simultaneously). Anti-Mac-1 + C removed larger portions of TBH SAC populations than normal host SAC populations (35%, 83%, and 64% at 0, 7, and 14 days PTI, respectively). Anti-Mac-2 + C treatment demonstrated limited lytic activity (25% to 10%) during tumor growth. Anti-Mac-3 + C treatment removed a greater number of Mφ (65%) from "normal" host SAC (0 days PTI) than at any other time point. At 7 and 14 days PTI, anti-Mac-3 + C treatment removed 30% and 24% of the SAC population, respectively.

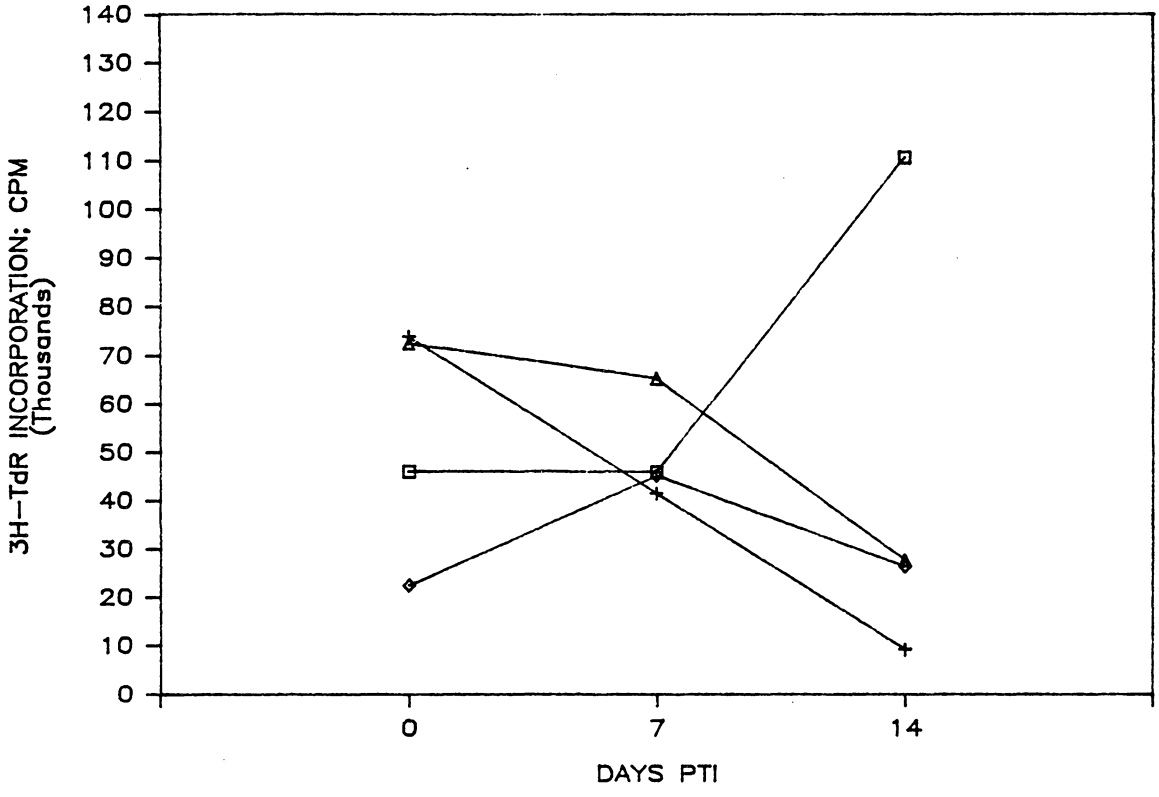

Figure 10. **Accessory cell function of combined anti-Mac-treated SAC:** Equal numbers of treated SAC from normal or TBH were added to normal host T cells. Combinations were restricted with respect to SAC origin (that is, only normal host SAC were mixed with normal host SAC). The degree of T cell reactivity was recorded as described in Fig. 9. Combinations included, anti-Mac-1- plus -2-treated SAC (+), anti-Mac-2- plus -3-treated SAC (◇), anti-Mac-1- plus -3-treated SAC (△) and NRIgG- plus NRIgG-treated SAC (□), from normal and TBH. 21-day PTI SAC were equivalent to 14-day PTI SAC and thus are not included in the figure.

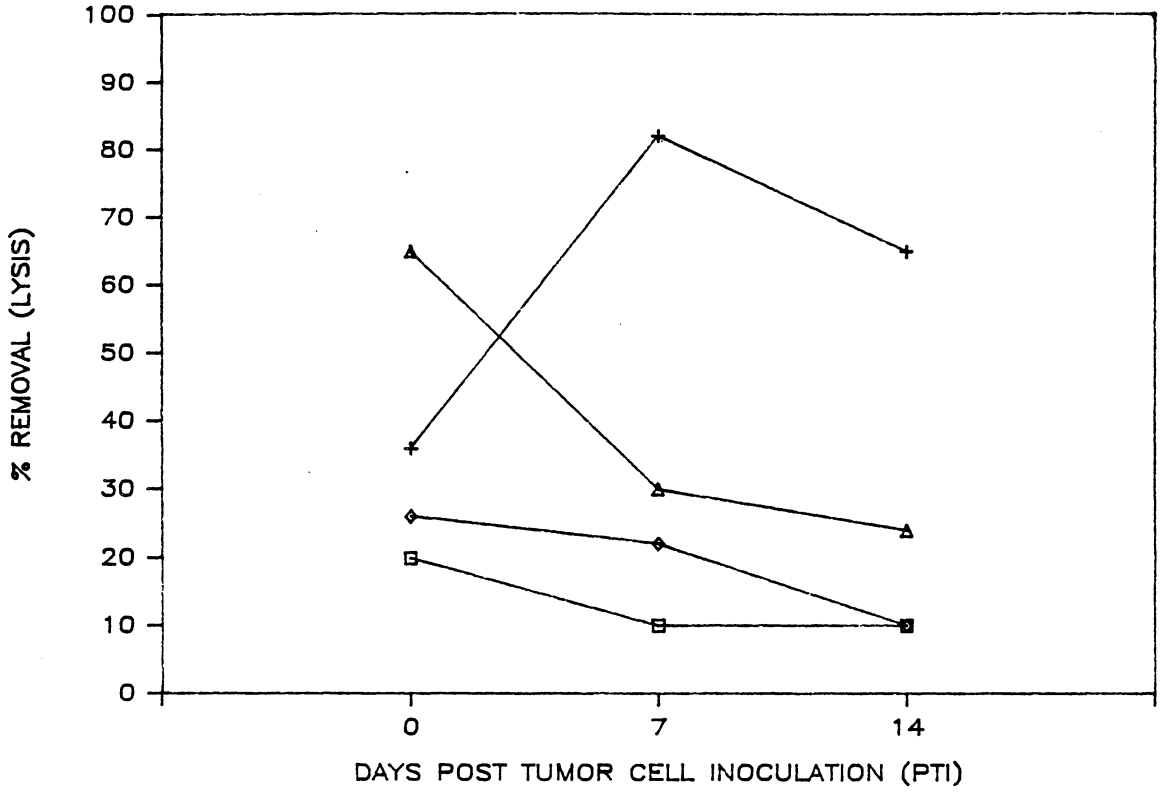

Figure 11. Percentage removal of SAC by anti-Mac + C treatment: Equal numbers of SAC were treated with NRIgG (□), anti-Mac-1 (+), -2 (◇), and -3 (△) + C for 90 min at 37°C, washed, and counted. The number of viable treated SAC remaining were compared to RPMI medium-treated cells to yield percentage of SAC removed.

Discussion

In this study, we demonstrated that the major accessory cell in both normal and TBH WSC is a Mac-1$^+$ cell. With respect to the specificity of the Mac-1 mAb, we can putatively identify this cell as either a Mφ, NK cell, or granulocytes and would tend to rule out significant dendritic cell involvement. Concomitant removal of accessory activity from TBH WSC by anti-Mac-3 + C treatment further identified the accessory cell as Mac-3$^+$ and thus ruled out the involvement of NK cells and granulocytes. Previously, investigators have primarily used depletion techniques that take advantage of physical traits of accessory cells to remove them from responding T cell cultures. Although these techniques are efficient in the removal of accessory function, as demonstrated by decreased T cell proliferation, they do not identify the accessory cell by phenotype. Physical depletions are not specific, thus mAb-mediated depletions because of their specific nature, were more representative of the functional cells phenotype.

Using admixtures of the mAb + C-treated WSC to examine overlap of antigen expression, we demonstrated that partial or complete reconstitution of accessory function was achievable with some but not all combinations of treatment groups. Note that Mac-1 is found on all Mφ, NK cells, and granulocytes; Mac-2 is found on elicited peritoneal Mφ and dendritic cells; and Mac-3 is found on resident peritoneal Mφ, splenic Mφ and dendritic cells (81, 114, 115, 116, 208, 234, 240). With normal host WSC, we observed reconstitution of lectin responsiveness only with the combination of anti-Mac-2- plus -3-treated WSC. Thus, based on the known expression of these antigens, this combination should be the only one that contains Mφ and lacks dendritic cells. We also found that anti-Mac-1 treatment removed a critical accessory cell that could not be completely restored by the addition of other cell types. mAb + C-treated TBH WSC demonstrated the opposite pattern of restoration (Fig. 8). Anti-Mac-1 plus -2-depleted and anti-Mac-1 plus -3-depleted TBH WSC both approximated control level responses to lectin-induced blastogenesis. Whereas, anti-Mac-2 plus -3-depleted were significantly less than their control counterparts. In short, a notable difference in the mAb sensitivity of normal and TBH resulted in altered reactivity of the remaining splenocytes.

This suggested that TBH WSC response to lectin was regulated by a different group or combination of accessory cell populations than normal host WSC.

Although we did not expect to find direct functional involvement of the Mac antigens, our mAb alone treatments of WSC indicated that at least one of the Mac antigens was somehow involved in regulation of the T cell-lectin response. Treatment of WSC with anti-Mac mAb without C demonstrated that Mac-1 ligand interaction with mAb could increase the response of WSC to mitogen. This modulation could reflect alterations in regulatory mechanisms associated with the C3bi receptor (Mac-1) (116). It is now thought that stimulation of the C3bi receptor with C3b fragments induces the release of PGE_2, inhibitors of T cell responses (198). Ligation of anti-Mac-1 to its receptor could alter this regulatory mechanism by (i) mimicking C3b binding and thus induce the same regulation as C3b or (ii), as with IL 2 or epidermal growth factor (59, 132), competitively displacing C3b or other related ligands without activating the system. The latter of these proposed mechanisms agrees with the observed increase in WSC response following anti-Mac-1 treatment. Since this mAb treatment of cells was performed without C or FCS, no exogenous source of C3b was introduced. Thus, one may speculate that anti-Mac-1 was blocking the production of an endogenous regulatory factor in normal but not TBH WSC.

Treatment with different anti-Mac mAb differentially modulated the accessory function of WSC. Since accessory activity is mediated by an adherent cell, we refined the testing procedure to examine SAC. First we determined that the degree of modulation was independent of each mAb ability to fix C, since percentage lysis or cell removal closely paralleled the percentage mAb detected by indirect immunofluorescence. Thus, for anti-Mac-1, -2, or -3, modulation was related to the specific removal of one population of SAC and the enrichment of the remaining cells. Mac-1 antigens are proposed by others (115) as being present on all Mφ. Fc receptors are also present on most Mφ, so the two values would be expected to be close if the two different reagents are binding to the same cell. Since there could be Fc binding by the Mφ, this only acts to strengthen the argument that the cells we looked were Mac-1^+. Moreover, Fc binding would not directly activate C fixation and therefore not be responsible for the observed lysis; in fact NRIgG + C was only responsible for minimal lysis. The take-home message is obscured by this argument, but is

of upmost importance. That is, the fact that increases and decreases in immunofluorescence (mAb binding) paralleled increases and decreases in mAb-induced cell lysis. Thus, one can interpret both the efficiency of lysis initiated by the mAb and the changes in SAC populations during tumor burden. Collectively, it was evident from both the percentage removal and immunofluorescence data that Mac-1^+ cell numbers increased and Mac-3^+ cell numbers decreased during the two weeks of tumor growth.

When treated normal and TBH SAC were added to enriched normal T cells, pretreatment of the SAC with anti-Mac-1 + C increased the accessory activity in normal host-derived SAC but caused an overwhelming loss of all positive accessory activity in TBH-derived SAC. In direct contrast, anti-Mac-2 + C treatment removed all accessory activity from normal-derived SAC, but only partially reduced accessory function in TBH-derived SAC. Anti-Mac-3 + C treatment of normal and TBH SAC, did not show any correlation between Mac-3^+ phenotype and accessory function.

Taken together, immunofluorescence, percentage lysis, and accessory activities all suggest that the recruitment of Mφ into the spleen during tumor development may have resulted in the accumulation of many accessory cells, which are predominantly Mac-1^+. Although the results ascribing accessory cell activity to Mac-1^- and 2^+ normal host SAC are contradictory to our observations with WSC, it is important to realize that the two experiments differ inasmuch as the SAC experiment is more refined and controls for variables not taken into account by the WSC experiments. For example, SAC numbers were standardized before and after mAb treatment and added to enriched normal host responder T cells. In this context, the results demonstrated that at an accessory cell to T cell ratio of 1:5, untreated or NRIgG + C-treated normal SAC were less supportive of normal T cell-lectin responses than were similarly treated TBH SAC. Other studies demonstrate similar changes in accessory cell activity during tumor growth (73) but do not identify the phenotypic nature of these changes. Within the parameters defined by the admixture of SAC to normal host T cells, we have demonstrated that either a very potent accessory cell was enriched in normal SAC by anti-Mac-1 treatment or a suppressive regulatory cell was removed. With respect to the treatment of normal and TBH WSC, accessory activity was reduced by anti-Mac-1 depletion.

In contrast, enrichment of Mac$^-$ normal host SAC selected for a potent accessory cell. With TBH SAC, Mac-1$^+$ cells were the predominant accessory cell. Selected and enriched Mac-1$^-$ TBH SAC were incapable of serving as accessory cells to normal T cells. Thus, a distinct difference in accessory cell function existed between normal and TBH SAC that was attributable to changes in mAb-defined SAC phenotypes. Differences in WSC and SAC data most likely demonstrate differences in the responding T cell populations. The possible causes include: (i) the nylon wool removal of a suppressor T cell that interacted with the SAC population (69), (ii) the nylon wool removal of a type-2 helper T cell (253), or (iii) the absence of a nonadherent regulatory Mac$^+$ cell in the SAC.

When independent mAb-depleted SAC were combined, accessory functions were restored. Differential patterns in the degree of restoration were observed with normal and TBH SAC. Combinations of normal host, anti-Mac-1 plus -3 and anti-Mac-1 plus -2 treated SAC, were more supportive of T cell lectin-induced proliferation than NRIgG + C-treated or anti-Mac-2 plus -3-treated SAC. All combinations of 14-day TBH SAC were less supportive of normal T cells than their NRIgG + C-treated counterparts. These observations demonstrated a change in accessory cell dependency that followed growth of the tumor. When compared with individual activities of the treatment groups (Fig. 9), anti-Mac-1-treated SAC appeared to be the dominant cells in the combined normal host SAC populations, as the lack of accessory activity was not reversed by the addition of other treated SAC. Anti-Mac-2 plus -3 combinations appeared to differ from their individual kinetics, while dominance of any one particular population was dependent on the stage of tumor development. At 14 days PTI, combined accessory cell activities were not all dominated by the anti-Mac-1 treatment group. For example, combinations of Mac-2$^-$ and Mac-3$^-$ SAC were less capable of accessory function than their individual groups. This may reflect a unique regulatory interaction between these two groups. Combinations of Mac-1$^-$ and Mac-3$^-$ SAC produced an accessory activity that was intermediate to their own individual activities. This type of interaction reflected a difference in the two populations, that is, the partial accessory activity of Mac-3$^+$ populations was further diluted by the addition of Mac-1$^-$ SAC. Collectively, the admixture of separate and combined SAC to normal T cells supported the hypothesis that Mac-1$^+$ cells accumulate in

the spleen during tumor growth and that these cells accounted for the increased accessory cell activity noted with TBH-derived SAC.

Since peroxidase activity is a measure of Mφ maturity (59), we have also analyzed SAC for endogenous peroxidase activity and found significantly higher activity in SAC from TBH than normal hosts (data not given). Walker *et al.* (283) draws a close correlation between small immature peroxidase positive Mφ and those expressing Mac-1 but no correlation between Mac-2 and Mac-3 with peroxidase activity. In light of their interpretation and our results, the hypothesis that maturing Mφ populations vary in phenotype and function appears plausible. Furthermore, tumor growth may alter *in vivo* Mφ maturation and subsequently cause changes in Mφ immunoregulation.

In summary, these data suggest that: (i) different regulatory functions may be ascribed to Mac-defined subtypes of Mφ and (ii) the presence of these subtypes varied during tumor burden. Mφ are implicated in tumor-induced immune dysfunction, yet studies show that the immunoregulatory dysfunction varies with respect to the *in vitro* assay, origin of the Mφ, and the isolation procedures used (117). In this context, the present study suggested that more than one Mφ subpopulation existed and the fluctuations in Mφ regulatory activity concomitant with tumor growth were the result of changes in a heterogeneous Mφ population. Our observations might be extended to incorporate the current hypothesis that during the recruitment of Mφ to the spleen and their residence, a series of maturation steps occur. The influences of tumor growth on splenic Mφ maturation may result in alterations in the phenotypic composition of Mφ populations, resulting in suppressed immune reactivity. Two possible explanations can be given to describe changes in SAC populations during tumor growth: (i) there was an active change in Mac expression on Mφ or Mφ-like cells initiated, either directly by tumor products or indirectly by other immune cells that have responded to the tumor or (ii) the change in splenocyte expression of Mac antigens was a result of migration (resulting in a loss of Mac expression) or immigration (resulting in an increase in Mac expression) of Mφ or Mφ-like cells.

Chapter IV

REDUCED Ia ANTIGEN EXPRESSION ON TBH Mφ AND CORRELATED Mφ REGULATORY FUNCTIONS

Introduction

Ia antigens play functional roles in Mφ-T cell interactions (20, 21, 52, 71). They can be continuously (122, 151, 284) or transiently (22) expressed. Correlation of Ia antigen expression with the ability of Mφ to present protein antigens to T cells is an integral part of major histocompatibility complex (MHC)-restricted antigen recognition (20, 21, 52, 71, 303). In one model of MHC-restricted antigen recognition, the MLR, proliferation will not proceed unless Ia antigen positive stimulator cells are present (225). The MLR demonstrates environmental control of Mφ Ia antigen expression as Mφ from different tissues and elicited by different stimuli have different stimulatory and accessory activities (23, 25, 51).

We (57, 68, 69, 89) and others (95, 262, 277) have demonstrated that TBH are severely immunosuppressed. Mφ play a role in this suppression as evidenced by their negative modulation of T cell responses (68, 69, 95, 125, 262, 277, 280). Tumor-induced immunosuppression by Mφ is associated with quantitative (68, 69, 287) and qualitative (206, 258) population differences. Alterations in Mφ Ia antigen expression could account for immune dysfunctions seen during tumor growth (123, 156, 185, 206).

Soluble immunoregulatory molecules are capable of modulating Ia antigen expression and thereby altering T cell responses. Prostaglandin E_2 (PGE_2), a known immunosuppressive agent, is a negative regulator of Ia antigen expression (57, 229, 230). When used with T cell-derived positive Ia antigen modulators, PGE_2 is capable of reversing the induction of Ia antigen expression (230). During tumor growth, we find elevated PGE_2 production by Mφ (57). PGE_2 can suppress cell-mediated immunity either directly via T cell interaction or indirectly by reducing Ia antigen expression on Mφ and subsequently altering Ia dependent accessory activity.

We hypothesized that reductions in Ia antigen expression by TBH Mφ correlate with TBH hyporesponsiveness. To determine the effect of tumor burden on Mφ Ia antigen expression we have used Ia-dependent functional assays and mAb directed against Mφ Ia antigens. We report here the progressive loss of Ia antigen expression by TBH Mφ relative to normal host Mφ. We have shown by immunofluorescence that TBH Mφ have low levels of Ia antigen expression. As accessory cells in an Ia antigen-dependent MLR, TBH Mφ did not support syngeneic T cell proliferation. Further assessment of accessory cell activity in Ia antigen-independent Con A-induced proliferation assays showed TBH Mφ could support T cell proliferation and that alterations in accessory and stimulatory function were not due to generalized suppression phenomenon. Mφ Ia expression was assessed using Mφ as allogeneic stimulators in the MLR. A progressive reduction in MLR stimulation (Ia antigen-dependent) by TBH splenic and thioglycollate-elicited peritoneal Mφ was observed as the tumor developed. Loss of Ia antigen expression by TBH Mφ was confirmed by: (i) blockade of normal but not TBH Mφ MLR (allogeneic T cell) stimulatory capacity using anti-Ia^dantibodies and (ii) *in vitro* culture of peritoneal Mφ for 24 hr. Following either of these treatments, reductions in the stimulatory capacity of normal but not TBH Mφ were noted.

Indomethacin, an inhibitor of arachidonic acid metabolism, was added to the MLR cultures concomitant with the addition of splenic Mφ. The ineffectiveness of indomethacin treatment showed that *in vitro* PGE_2 production was not the cause of reduced MLR reactivity or loss of Ia antigen expression. In summary, we demonstrated a coordinate loss of Mφ Ia antigen surface expression and Ia antigen-dependent functions during tumor development.

Materials and Methods

Animals

Eight to 12 wk-old male BALB/c (H-2^d) and C3H (H-2^k) mice were obtained from Dominion Laboratories, Dublin, VA. Tumor induction was achieved by intramuscular injection with a single-cell suspension of a methylcholanthrene-induced transplantable nonmetastatic fibrosarcoma (3 x 10^5 tumor cells into the left hind leg) (68, 69). Varying degrees of tumor growth were achieved by using TBH that were initiated at 7-day intervals.

Medium

All cells, unless stated otherwise, were grown in RPMI-1640 medium supplemented with 5% heat-inactivated FCS (Flow Laboratories, McLean, VA) and 4 x 10^{-5} *M* 2-mercaptoethanol (complete medium). All medium also contained 50 mg/l Gentamicin (Grand Island Biologicals Co., Grand Island, NY), 2 g/l $NaHCO_3$, and 25 mM HEPES (Sigma, St. Louis, MO).

Mφ Preparation

Peritoneal Mφ were obtained from BALB/c and C3H mice 4 days after intraperitoneal injection of 2.0 ml of sterile thioglycollate (48, 69). Splenic Mφ were obtained from single cell suspensions of whole spleen cells (WSC) that had been treated with a 0.85% w/v solution of pH 7.4 NH_4Cl to remove erythrocytes. Both peritoneal and splenic Mφ were selected for by plating cells on plastic dishes for 2-4 hr in serum-free medium. Nonadherent cells were removed by rinsing.

Adherent cells were harvested by scraping with a rubber policeman, washed, centrifuged at 500 x *G*, and resuspended in serum-free medium. Adherent cells (> 97% esterase positive) were counted with trypan blue dye and resuspended to 4 x 10^6 viable cells/ml for later dilution.

Detection of Ia Antigens with mAb

Monoclonal anti-Ia antibodies were prepared as previously described for anti-Mac antibodies (89, 90). Hybridoma cell line HB-3 producing mouse IgG anti-$Ia^d$was purchased from the American Type Culture Collection, Rockville, MD. Anti Ia^k.17 was a gift from Dr. Lawrence B. Schook, Medical College of Virginia, Richmond, VA. Mφ from 6 mice were pooled and treated with either anti-Ia^dor anti-Ia^k(8 μg/ml) for 45-60 min at 37C, washed with RPMI medium 3 times, and resuspended to 4 x 10^6 cells/ml. mAb labeled Mφ were treated with rhodamine-labeled $(Fab')_2$anti-mouse IgG (Jackson Immunoresearch Laboratories, Avondale, PA) for immunomicroscopy. Labeled cells were smeared and air dried onto glass slides. The smears were fixed with 95% ethanol for 5 minutes, overlayed with 50% glycerol/RPMI medium and observed with an Olympus IMT-2 phase- fluorescence microscope. At least 200 cells per slide were examined and the percentage of fluorescing cells was determined.

Assessment of Mφ Stimulatory Activity in the MLR

MLR reactivity was initiated in C3H (H-2^k) responder splenic T cell populations using allogeneic normal or TBH BALB/c (H-2^d) Mφ as stimulator cells (69, 69). Spleens from 4-6 C3H mice were excised and passed through a wire sieve to yield a single cell suspension. Splenocyte preparations were washed and resuspended in medium supplemented with 5% heat-inactivated FCS. Responder cells were placed on nylon wool columns and incubated for 45 min at 37C. Nonadherent cells were then eluted, centrifuged, and placed on Sephadex G10/glass bead columns

for 45 min at 37C. Nonadherent cells were eluted as per nylon wool columns, centrifuged, and placed on plastic tissue culture dishes for 2 hr. These plated and column purified nonadherent C3H T cells were counted with a Coulter counter and dispensed in complete RPMI medium at 2 x 10^5 T cells per well in 96 well "U" bottom microculture dishes (Flow Laboratories). To obtain C3H splenic or peritoneal Mφ, spleens or peritoneal cells were plated and washed according to the procedure described for Mφ preparation. Adherent cells were counted, resuspended to 2 x 10^7 cells/ml, treated with Mitomycin-C (final concentration of 25 μg/ml) for 20 min at 37C, and washed 4 times. Mitomycin C-treated C3H stimulator Mφ were added to syngeneic T cells at varying dilutions as a background proliferation and cell addition control. BALB/c and C3H Mφ did not proliferate in the MLR with or without Mitomycin C treatment (data not shown). Allogeneic BALB/c Mφ stimulator cells were added to quadruplicate wells at time of initiation. Where indicated, indomethacin (Sigma) was added to the MLR at a final concentration of 1 x 10^{-7} *M*. This concentration of indomethacin inhibits PGE_2 production but has no effect on proliferation in the MLR (data not shown). The reaction proceeded for 96 hr at 37C and was pulsed with 1 μCi tritiated thymidine (^{3}H-TdR; spec. act. 6 Ci/mM, Amersham Corp., Arlington Heights, IL) per well 6 hr before termination and harvest. Cells were harvested onto glass fiber filters (Whatman 934-AH) and counted in a Beckman LS230 liquid scintillation counter.

Blockage of Mφ Ia Surface Antigens

Mφ were added to the MLR as alogeneic stimulator cells at initiation (0 hr). Anti-Ia antibodies were added at 0, 24, and 48 hr after initiation of MLR. To each MLR reaction well 0.3 μg (10 μl per 200 μl well volume) of anti-Ia (k or d specific) antibody was added. Control wells received RPMI medium. Cultures were incubated for 90 hr, pulsed for 6 hr with ^{3}H-TdR, harvested, and counted.

Assessment of Mφ Accessory Cell Activity in the MLR

BALB/c (H-2^d) responder T cells were prepared as per C3H (H-2^k) responder T cells. T cell stimulation was achieved with C3H (H-2^k) WSC that were twice depleted of Mφ with Sephadex G10/glass bead columns. Stimulator and responder cell preparations were mixed at a 2:1 ratio. Enough splenic Mφ were added to yield a final Mφ density of 1 x 10^5 cells/well or a 1:2 accessory to responder cell ratio. Cultures were incubated for 90 hr, pulsed for 6 hr with ^{3}H-TdR, harvested, and counted.

Assessment of Mφ Accessory Cell Activity in Con A-induced T Cell Proliferation

BALB/c splenic T cells were prepared as described above for C3H T cells. T cells from normal and TBH BALB/c mice were dispensed at 2 x 10^5 cells per well in flat-bottom microculture plates (Flow Laboratories) in medium with 10% heat-inactivated FCS. 2-mercaptoethanol was omitted because of its ability to enhance Mφ functions. To assess Mφ Ia antigen-dependent accessory cell function, 1 x 10^5 splenic BALB/c Mφ from normal or TBH were added at time of initiation, along with 0.8 μg of Con A (Difco Laboratories, Detroit, MI) per well. Cultures were incubated for 66 hr, pulsed with ^{3}H-TdR for 6 hr, harvested, and counted.

Statistics

Experiments were done in triplicate, with representative experiments reported. Comparisons between data points were performed using Student's *t* test (at $P \leq 0.05$) on data from 4-6 replicate samples. In each preparation, cells were obtained from at least 4 animals and pooled.

Results

Tumor-induced Alterations in Mφ Ia Antigen Expression and Dependent Accessory Cell Function

Immunofluorescence was used to determine the number of Ia antigen positive Mφ. Staining of anti-Ia^d-treated splenic Mφ with $(Fab')_2$-rhodamine conjugated goat anti-mouse IgG showed that the percentage of Ia positive cells in normal mouse splenic Mφ preparations was 50% (Table 2). A steady decrease in Ia antigen expression was observed with each of the successive stages of tumor growth, reaching background (11%) by Day-21 post tumor cell inoculation (PTI). Thioglycollate-elicited peritoneal Mφ from Days 0, 7, 14, and 21 PTI were 7, 7, 3, and 4% Ia^dpositive, respectively, by immunofluorescence.

To determine their accessory cell capabilities, splenic Mφ preparations from BALB/c mice 0 to 21 days PTI were added to Con A-stimulated normal host T cells in the absence of 2-mercaptoethanol (Table 2). 2-mercaptoethanol was omitted because of its ability to enhance Mφ functions, resulting in a clearer assessment of Ia antigen-mediated accessory activity. Con A-induced T cell proliferation in the absence of Mφ was approximately 70% less than Con A-induced WSC proliferation (data not given). Normal host Mφ demonstrated a lesser degree of accessory cell function (605% increase in T cell proliferation) than did TBH Mφ. Day-14 TBH Mφ demonstrated the highest accessory cell activity (1394% increase) in the mitogen assay. In another assessment of Ia antigen-mediated activities, splenic Mφ were added as accessory cells to the BALB/c (syngeneic responder) versus C3H (stimulator) MLR. The addition of 2×10^5 splenic Mφ per well caused a reduction in blastogenesis ranging from 56% (Day-0 PTI) to 89% (Day-21 PTI).

Table 2. Relationship Between Splenic Mφ Ia Antigen Expression and Regulatory Activities During Tumor Development

Days PTI[a]	% Ia antigen positive splenic Mφ[b]	% Change in T Cell Response[c]	
		Con A	MLR
0	50	605 (↑)	56 (↓)
7	31	1068 (↑)	69 (↓)
14	26	1394 (↑)	80 (↓)
21	11	915 (↑)	89 (↓)

[a]Days PTI represented groups of animals in different stages of tumor development.

[b]Percentage of splenic Mφ expressing Ia antigens were determined as the number of rhodamine-fluorescing cells versus total number of cells observed. Background labeling of cells was determined to be 12% using anti-Ia^kantibody with normal host BALB/c Mφ.

[c]Percentage change in T cell response after addition of 2×10^5 Mφ per well was determined as a ratio of T cell response with Mφ versus T cell response in the absence of Mφ.

[d]"↑" in parenthesis represents enhancing activity whereas "↓" represents inhibitory activity.

Normal and TBH Peritoneal Mφ MLR Stimulation

Since MLR are dependent on stimulator cell Ia antigen expression (225), responder cell proliferation reflects the extent of Ia antigen expression by the stimulating cells. Therefore, we measured the MLR stimulatory capacity of normal and TBH Mφ to identify differences in Ia antigen expression between the two host conditions. Figure 12 depicts the efficiency of different numbers of peritoneal Mφ from different sources (normal and TBH) as stimulators in allogeneic MLR. The data showed that thioglycollate-elicited peritoneal Mφ from BALB/c normal (Day-0) and Day-7 TBH stimulated C3H T cells to proliferate above background levels (C3H T cells plus 1 x 10^5 syngeneic WSC = 5139 cpm). Using normal host peritoneal Mφ, optimal stimulation was observed at cell concentrations of 1 x 10^5 Mφ/well (a 1:2 stimulator:responder ratio) for all peritoneal BALB/c Mφ sources.

Figure 12 also illustrates the stimulatory capacity of Mφ from normal and TBH with respect to host conditions (days PTI). At Day-7 the stimulatory activity of Mφ significantly decreased and continued to decrease until Day-14. However, at 21 days PTI, the stimulatory activity of Mφ began to increase as these Mφ caused increased T cell blastogenesis relative to their Day-14 counterparts.

Effect of 24-hr plating on Mφ stimulatory capacity

To further establish the correlation between Ia antigen expression, tumor growth, and MLR stimulation, thioglycollate-elicited BALB/c peritoneal Mφ (stimulators) from normal, Day-14 and -28 TBH were plated for 4 and 24 hr, then added to the MLR at time of initiation (Table 2). C3H Mφ addition to syngeneic T cells in the MLR represented background proliferation for both plating intervals. Since *in vitro* culture of Mφ is known to decrease Ia antigen expression (22, 122), loss of stimulatory activity was assessed. In Table 3 the cpm values of MLR stimulation after 4-hr plating illustrated that Ia antigen expression decreased with tumor development through Day-14.

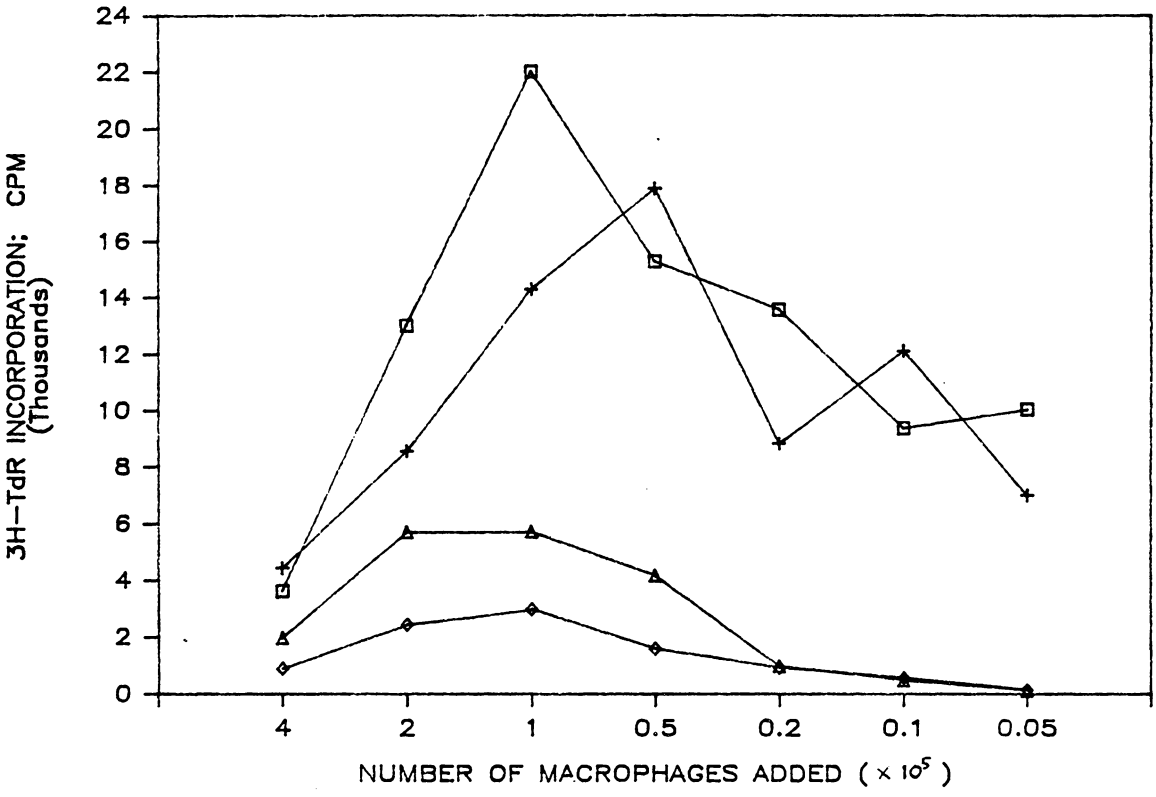

Figure 12. Dilution profiles of peritoneal Mφ stimulatory capacities: Thioglycollate-elicited peritoneal H-2^d adherent Mφ from animals at different days PTI were titrated from 4 to 0.05 x 10^5 per well as stimulators in the MLR (with H-2^k responder T cells). TBH peritoneal Mφ from Day-0 (□), Day-7 (+), Day-14 (◇), and Day-21 (△) PTI were added as stimulators at time of initiation of the MLR. The degree of proliferation was measured by ^{3}H-TdR incorporation and reported as cpm.

By Day-28, Mφ stimulatory capacity had returned to normal levels. The ability of both normal and Day-28 TBH Mφ to stimulate the MLR was significantly diminished after 24-hr *in vitro* culture. No significant difference was observed in Day-14 TBH Mφ stimulatory capacity on 24-hr plating.

Normal and TBH splenic Mφ MLR stimulation

BALB/c splenic Mφ were used as stimulators in the MLR to determine the extent of tumor-induced Ia antigen modulation (Fig. 13). Dilutions of BALB/c normal (Day-0) through Day-21 TBH Mφ were used as stimulators of C3H responder T cell proliferation in the MLR. Normal and Day-7 TBH splenic Mφ preparations were similar in their stimulatory capacity. Day-14 and -21 TBH Mφ were less stimulatory than either normal or Day-7 TBH Mφ. Day-21 TBH Mφ were significantly less stimulatory at the two highest concentrations than any other splenic Mφ preparations.

To assess the contribution of PGE_2 in the MLR, indomethacin was added to replicate samples of splenic Mφ preparations from normal (BALB/c and C3H) and Day-21 BALB/c TBH. In Fig. 14, the different stimulatory capabilities of Mφ from different sources is illustrated with respect to Mφ number. Splenic Mφ from normal BALB/c hosts were significantly (4-fold) better stimulators than TBH splenic Mφ. The contribution of PGE_2 produced by Mφ during the MLR was not significant when normal (allogeneic or syngeneic) or TBH Mφ were used as stimulators, as shown by the ineffectiveness of *in vitro* indomethacin treatments.

Masking of Mφ Stimulatory Capacity with Anti-Ia Antibodies

Because MLR are dependent on Ia antigen presentation to responder cells, anti-Ia antibodies are capable of blocking the reaction and thereby preventing T cell proliferation (210). Mφ

Table 3. Alterations in Mφ MLR Stimulatory Capacity Following *in vitro* Culturing

Mφ source	MLR reactivity	
	Length of Mφ culturing before adding to MLR	
	4 hr	24 hr
C3H (syngeneic)	10,927 ± 711	12,667 ± 788
Normal (allogeneic)	30,074 ± 2983	17,613 ± 1398
Day-14 TBH (allogeneic)	17,839 ± 2242	21,092 ± 1048
Day-28 TBH (allogeneic)	29,903 ± 788	14,037 ± 2203

[a]^{3}H-TdR incorporation by C3H T cells was expressed as cpm ± SEM.

[b]Thioglycollate-elicited peritoneal Mφ obtained from C3H mice (H-2k; syngeneic) and BALB/c mice (H-2d; allogeneic) were used to stimulate C3H T cells. Day-14 and -28 TBH represent Mφ harvested 14 or 28 days after inoculation of tumor cells into BALB/c mice.

[c]Culturing Mφ *in vitro* was performed in the absence of FCS.

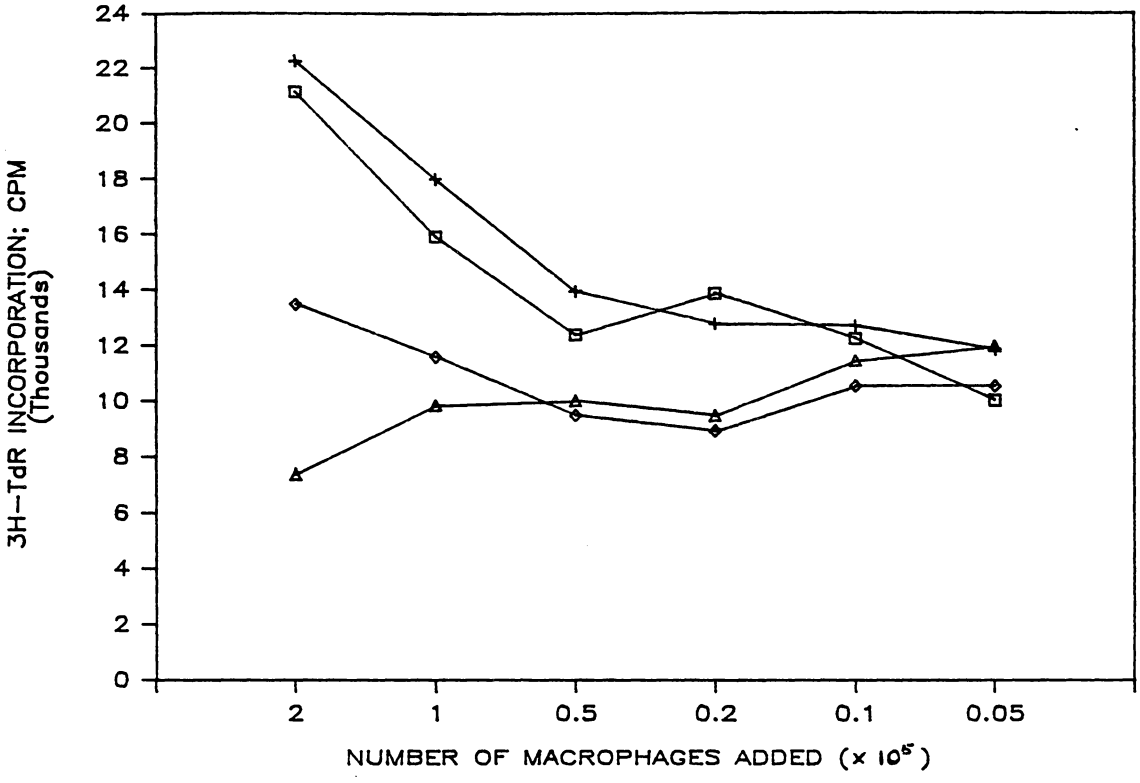

Figure 13. Dilution profiles of TBH splenic Mφ stimulatory capacities: TBH H-2^d splenic Mφ from Day-0 (□), Day-7 (+), Day-14 (◇), and Day-21 (△) PTI were titrated from 2 to 0.05 x 10^5 per well as stimulators of C3H (H-2^k) responder cells in an MLR. The proliferative response was measured by ^{3}H-TdR incorporation and reported as cpm.

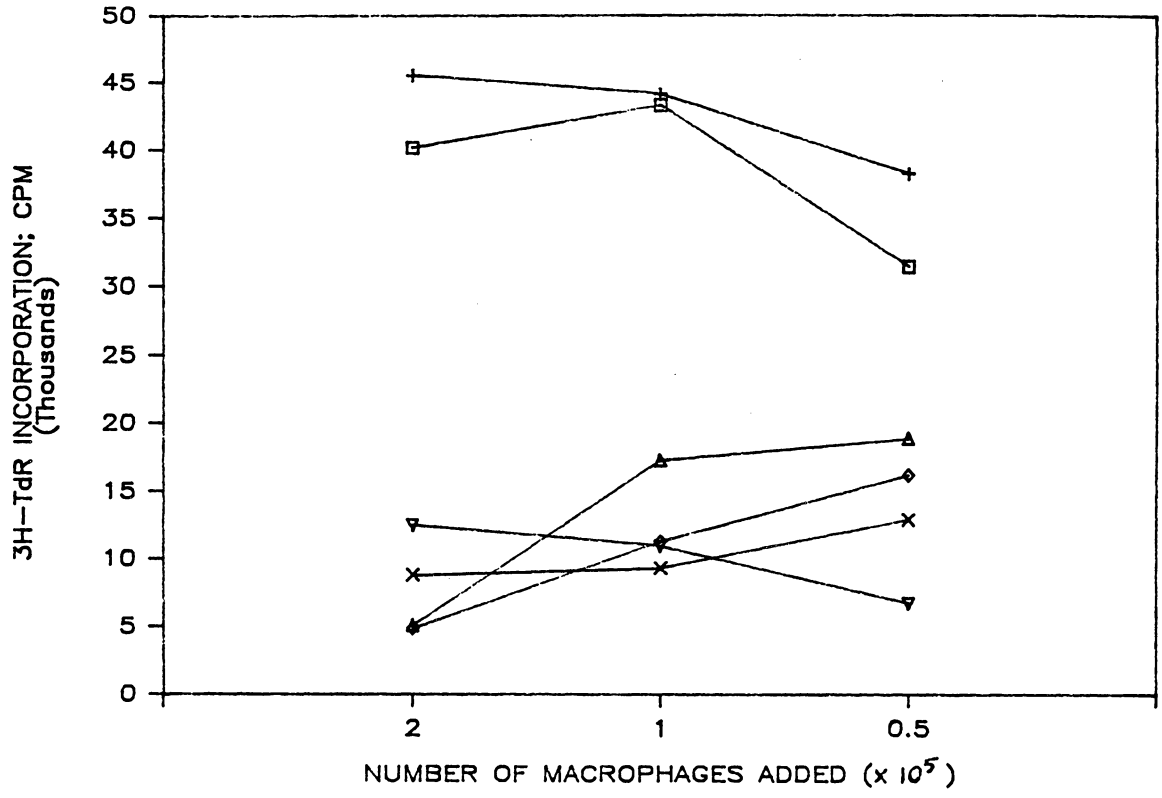

Figure 14. **Effect of indomethacin on MLR stimulation by Mφ:** Varying dilutions of BALB/c (H-2^d) Mφ were added to C3H (H-2^k) responder T cells in the presence of 10^{-7} *M* indomethacin. All additions were made at time of initiation of the MLR. TBH splenic Mφ from Day-0 (□; no treatment and +; indomethacin-treated) and Day-21 (◇; no treatment and △; indomethacin-treated), and non-TBH C3H (X; no treatment and ∇; indomethacin-treated) Mφ were used as stimulators in the MLR. The proliferative response was measured by ^{3}H-TdR incorporation and reported as cpm.

were treated with anti-Ia three times (at 0, 24, and 48 hr) during the MLR to maintain an effective dose of antibodies. Figure 15 shows that the addition of anti-Ia^d to splenic BALB/c Mφ before and during the MLR significantly reduced the stimulatory capacity of normal and Day-7 TBH Mφ but was less effective with Day-14 and -21 TBH Mφ. Anti-Ia^k treatments were ineffective at altering the stimulation by BALB/c Mφ (equivalent to RPMI-treatment). Thus, the anti-Ia^ktreatment was used as the control treatment in calculations of percentage reduction in MLR stimulation at each cell number. Day-21 TBH Mφ were not significantly affected by anti-Ia^d, as the stimulation decreased only by 13% at a cell density of 2×10^5 Mφ per well. The greater percentage reduction in MLR stimulation seen at lower Mφ numbers probably results from the antibody being in relative excess, and therefore more effective, at lower cell numbers.

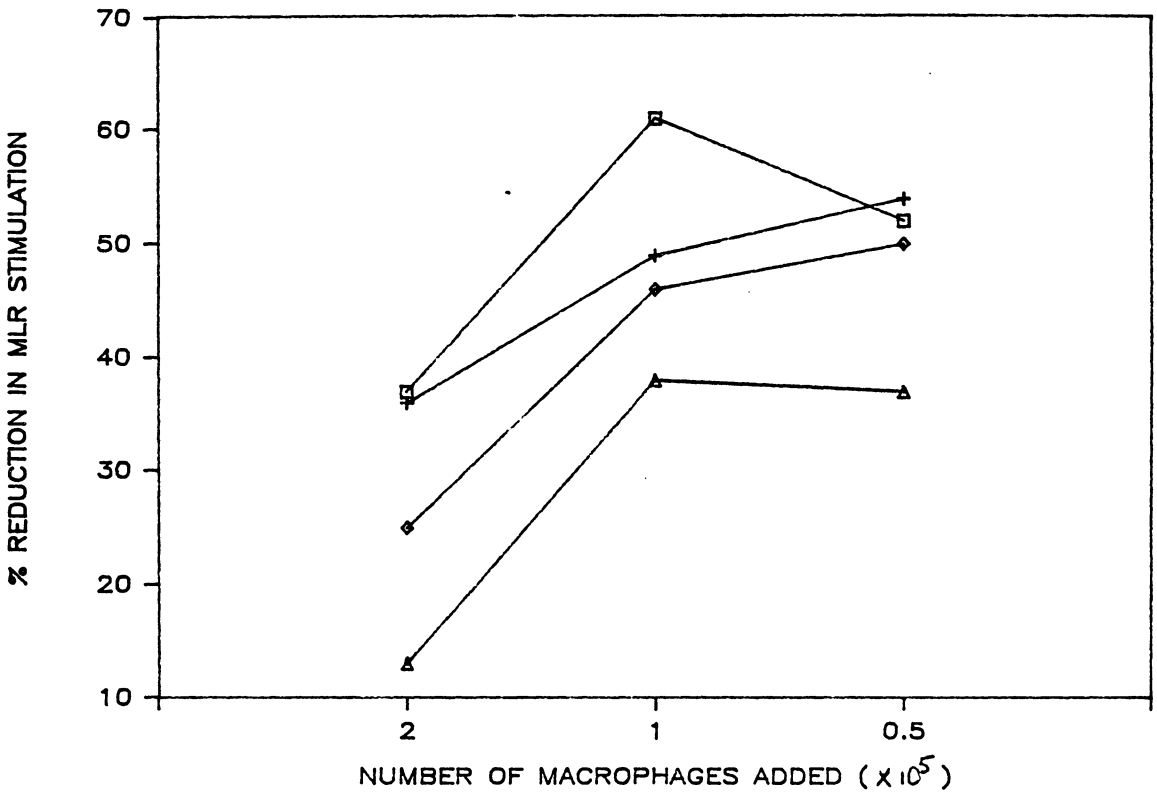

Figure 15. Masking of MLR reactivity with anti-Ia antibody: Varying dilutions of Mφ (H-2^d), which had been treated with 0.3 μg of anti-Ia^dmonoclonal antibody per 10^5 cells, were added as stimulators to C3H (H-2^k) responder T cells. Antibody treatments were continued *in vitro* daily for 3 days to maintain blocking effect during the MLR. TBH splenic Mφ from Day-0 (□), Day-7 (+), Day-14 (◇), and Day-21 (△) PTI were used as stimulators. The proliferative response was measured as ^{3}H-TdR incorporation and reported as percentage reduction in MLR stimulation relative to their appropriate controls. Control (100%) proliferation levels (raw data not shown; used in calculation of % reduction) were MLR responder cells stimulated with anti-Ia^k-treated H-2^d Mφ from each time point PTI.

Discussion

Stimulation of allogeneic T cell proliferation is an established assay for Ia antigen expression (47, 102, 174 210, 266). Mφ express Ia antigens on their membranes constitutively or transiently as a function of different subpopulations, maturation levels or activation states (22, 122, 151, 284). Mφ Ia antigen expression has been implicated, both directly and indirectly, as a contributing factor in the regulation of T cell responses (73, 156, 231, 247, 298) In previous reports (68, 69, 90), we describe functional and phenotypic Mφ differences associated with tumor growth. These findings demonstrate that *in vivo* environmental conditions (tumor burden) alter Mφ regulatory activities and prompted the present study.

Diminished Mφ Ia antigen expression during tumor growth was shown using immunofluorescence. By Day-21 PTI, a significant (39%) reduction in the number of Ia antigen positive cells was seen using anti-$Ia^d$and rhodamine-labeled $(Fab')_2$ anti-IgG. This degree of reduction in Mφ Ia antigen expression is comparable to human Mφ DR antigen reductions in Hodgkin's Disease (185). In our murine system, low Ia antigen levels were not a nonspecific effect of the injection, as others have shown BALB/c mice maintain high Ia antigen levels 28 days after BCG injection, and that this is subsequently lost on *in vitro* culture (122). Loss of Ia antigen expression as the tumor developed was corroborated by blocking studies. Anti-Ia^d, but not anti-Ia^k, was capable of blocking and thereby reducing the stimulatory capacity of normal BALB/c ($H\text{-}2^d$) splenic Mφ. As the tumor developed, anti-Ia^d treatment was less effective at reducing Mφ stimulatory capacity in the MLR. This reflected the progressive loss of Mφ Ia antigen expression. Since Ia antigens are essential for Mφ-T cell communication (20, 21, 52, 71, 140, 303), tumor-induced reduction in Mφ Ia antigen expression plays a role in both the cause and result of TBH hyporesponsiveness. This reduction in Ia antigen expression occurs simultaneously with elevated *in vivo* PGE_2 levels (57), reduced delayed type hypersensitivity response (92) and low interleukin 2 (39) and interleukin 3 levels (38). These observations prompted an analysis of tumor-induced impairment of Ia antigen-mediated Mφ activities.

Plating of Mφ for 24 hr *in vitro* reduces the expression of Ia antigens on Mφ (22, 122, 210). When peritoneal Mφ from normal (Day-0) or Day-28 PTI were plated for 24 hr their MLR stimulatory capacity was significantly decreased. However, Mφ from Day-14 PTI did not decline in stimulatory capacity following 24 hr culture. Loss of stimulatory activity in Day-14 PTI Mφ (4-hr plating) was interpreted as resulting from an *in vivo*-induced reduction in Ia antigen expression. This lowered stimulatory capacity of Day-14 TBH Mφ could not be further reduced by 24-hr plating.

Stimulation of T cells in the MLR (Ia antigen-dependent, as shown by loss upon *in vitro* plating) by TBH peritoneal Mφ decreased until some time between Day-14 and -21. From that time Mφ stimulatory capacity increased, reaching normal host Mφ levels by Day-28. An alternative explanation of the same data was that Mφ suppression decreased with chronic tumor growth (> 14 days) and that this decreased inhibition manifested itself as a high stimulatory capacity. If one speculates that the inhibitory activity may be induced by PGE_2 *in vivo*, then both explanations become plausible. Since PGE_2 causes a reduction in Ia antigen expression (230, 231), PGE_2 production *in vivo* could indirectly inhibit the MLR by reducing the stimulatory capacity of Mφ. Indomethacin failed to significantly alter the stimulatory capacity of normal or TBH splenic Mφ. This suggested that (i) the contribution of *in vitro* PGE_2 production in the MLR was minimal and (ii) the effects of high *in vivo* levels of PGE_2 (57) resulting in low Ia antigen expression could not be reversed by indomethacin treatment.

Previously published work using Mφ as accessory cells (57) demonstrates that TBH MLR responder cells (14-21 days PTI) are less sensitive to down-regulation by exogenous PGE_2 than their normal counterparts. This can now be interpreted in light of the present data on Ia antigen expression by normal and TBH Mφ. A loss of Ia antigen expression by TBH Mφ accounts for the ineffectiveness of the exogenous PGE_2. Addition of PGE_2 to normal host responder cells (containing normal host Mφ) results in a pronounced down-regulation (or increased susceptibility) due to the presence of Ia antigens which can be modulated by the PGE_2. High *in vivo* PGE_2 levels in the TBH explain this phenomenon (57).

Peak MLR stimulation was generally seen with 1 x 10^5 peritoneal Mφ. Except for high density-associated peritoneal Mφ inhibition, similar results were observed with splenic Mφ. Increased MLR stimulation and lack of inhibition by the addition of > 1 x 10^5 splenic Mφ could have been due to differences in cell population composition. Splenic Mφ are richer in Ia positive cells than are thioglycollate-elicited peritoneal Mφ and contain a population of dendritic cells. Since dendritic cells express Ia antigens, they could also have been a factor in MLR stimulation by splenic Mφ preparations. Some studies (189, 190, 243) describe the splenic dendritic cell as the major stimulator in the MLR. The diminished stimulatory activity of the TBH spleen cell preparation in the MLR could imply differences between normal and TBH dendritic cell populations. The assumption of alterations in Ia antigen expression by both Mφ and dendritic cells is unlikely because Ia antigen expression by dendritic cells is believed to be constitutive (268). An alternative hypothesis is that in the TBH spleen there was a reduction in the number of dendritic cells or a change in their relative proportion to other cell types. The present series of experiments were designed to compare normal and TBH Mφ populations. There is a well documented splenomegaly in the TBH that is accompanied by a 2-fold increase in adherent Mφ (69). Normal and TBH Mφ stimulator and accessory cells were resuspended to equivalent numbers to compensate for this 2-fold increase in TBH Mφ numbers. A 2-fold dilution can not account for a 5-fold decrease in Ia antigen expression (by immunofluorescence) nor the loss of Ia antigen-dependent functions seen with both splenic and peritoneal Mφ as the tumor develops.

To further characterize Mφ from different stages in tumor development, we examined their accessory cell function (using syngeneic T cells) during Con A- and MLR-induced T cell proliferation. Mφ regulation of mitogenesis occurs via soluble factors (5, 60). Both positive and negative Mφ regulation may require Ia antigen expression. The coculture of normal BALB/c Mφ with syngeneic normal T cells (in the absence of 2-mercaptoethanol) resulted in enhanced Con A-induced proliferation compared to normal host T cells without Mφ. Normal T cells supplemented with TBH Mφ were more proliferative than T cells supplemented with normal host Mφ. More specifically, an inverse relationship was noted between MLR stimulatory capacity (Ia antigen expression) and accessory cell function in Con A-induced T cell mitogenesis. This was in sharp

contrast to the suppression associated with lack of TBH Ia positive Mφ stimulators in the allogeneic MLR. Li and Miller (156) have identified an Ia^+ suppressor Mφ in cultured normal host splenocytes. Another study implicates an Ia positive Mφ in the induction of T suppressor cells during Con A stimulation (248), which could be a factor during tumor development. The observed increase in accessory cell function could be interpreted as a decrease in Ia^+ Mφ suppressor activities. Similar Mφ regulatory kinetics are seen with other tumor models using mitogen stimulation (73, 148). Addition of accessory splenic Mφ to syngeneic T cells caused a reduction in the response to allogeneic stimulator cells. This decrease in responsiveness became more pronounced with advanced tumor development. Mφ could have initiated this reduction in two ways: (i) reduced Mφ Ia antigen expression during tumor development may interfere with Mφ accessory functions, or (ii) Mφ suppressor activities in the MLR may have increased concomitant to reductions in Ia antigen expression.

In conclusion, Mφ Ia antigen expression varied during tumor growth. The environmental influence of tumor burden caused a reduction in the stimulatory capacity of both thioglycollate-elicited peritoneal and splenic Mφ. This tumor-induced reduction in MLR stimulation was not attributable to *in vitro* PGE_2 production by Mφ (indomethacin treatment was ineffective) and was paralleled by an increase in TBH Mφ ability to support Con A-induced T cell proliferation. A similar regulatory phenomenon is reported by others (73, 247, 248, 298) and may reflect a requirement for Ia antigen in both immunosuppressing and immunoenhancing Mφ functions.

Chapter V

NORMAL AND TBH PERITONEAL Mφ PHENOTYPES AND SOLUBLE SUPPRESSOR FACTORS

Introduction

Mφ serve many diverse functions in the immune response. However, a precise correlation of Mφ function with differentiation remains elusive. The delineation of this relationship is particularly critical in studies involving altered host immune systems, such as in TBH. Many studies have implicated Mφ as contributors to tumor-induced immune dysfunction (68, 69, 95, 249, 258, 261, 277). Of these, a limited number define functional subsets of Mφ based on phenotypic variations (249, 258). The correlation of function with specific cell phenotypes, as with T cell subsets, has progressed significantly over the past decade due primarily to the production and application of mAb. The recent availability of anti-Mφ mAb allows for similar characterizations of Mφ phenotypes (82, 113, 219, 221, 233, 257, 272). Springer *et al.* (234, 272) have produced a series of

mAb directed against Mφ surface antigens. These antigens are designated Mac-1, -2, and -3 and are described as glycoproteins of 190 and 95, 32, and 110 relative molecular mass (M_r), respectively. After their identification, other studies examine the induction (114, 208), distribution (147), and molecular structure (117) of these antigens. Of the three, only the dipeptide Mac-1 has been functionally defined and is now accepted as the C receptor type 3 (116, 147). The variable expression of Mac-2 among Mφ from different tissues or from Mφ elicited by different inflammatory agents (114) suggests that Mac-2 antigens may represent different levels of Mφ differentiation and/or activation. In contrast, the continuous expression of Mac-1 on murine Mφ and other related cell types suggests that it is an early differentiation antigen (115, 208, 235). This marker of differentiation is further illustrated by a notable increase in Mac-1 expression as monocytes mature. Although variable expression of Mac-2 and heterogeneous expression of Mac-3 is described (114, 117), no functional significance has been ascribed to either.

Mφ membrane proteins undoubtedly play active roles in various Mφ functions, such as cellular interactions and the reception or sending of biological signals (62, 63, 64, 65, 124, 175, 183). To fully characterize the involvement of these molecules in certain Mφ activities, it is advantageous to examine Mφ populations and assess their associated functional activities. In this study, we compared Mφ derived from normal and TBH. Analysis of supernatants from populations selected by anti-Mac mAb allowed for the correlation of antigen expression with cellular function. We and others (50, 68, 69, 276, 277, 287) have reported differences in the regulatory activity exhibited by normal and TBH Mφ-derived factors. Since differences in the phenotypic composition of Mφ may correspond to variable regulatory activities, we chose to fractionate whole peritoneal Mφ based on their expression of Mac antigens and to assess the remaining cells (those with or without limited expression of the Mac antigens) for corresponding changes in regulatory activities.

Initial characterization of normal and TBH peritoneal exudate Mφ showed that they contained greater numbers of peroxidase-positive cells than their normal counterparts. Indirect immunofluorescence analysis of mAb binding suggested that Mac-2 expression decreased while Mac-3 expression increased as a result of tumor growth. Assessing the percentage reduction in Mφ after mAb + C treatment further illustrated the decreased expression of Mac-2 and showed a

correlation between endogenous peroxidase activity and Mac-1^+, 3^+ Mφ. This relationship was absent in normal host-derived Mφ. Fluorescence analysis of Mac antigen expression, after mAb + C depletion/enrichment, showed that anti-Mac-1 and -2 treatments selected for Mac-3^+ cells. Thus, populations of Mφ that differ in Mac antigen expression can be evaluated for differences in functions following mAb + C treatment.

Anti-Mac plus C-treated Mφ were all resuspended to original numbers to enrich for the remaining subpopulations. Supernatants from mAb-selected peritoneal Mφ were collected after four days culture in serum-free medium. Monokine-mediated immunoregulatory activity was assessed by their ability to alter MLR responder T cell blastogenesis. A titratable suppressor activity was detected. Of the three mAb tested, only enrichment of Mac-3^- Mφ from normal hosts abrogated the usual inhibitory activities found in supernatants of nontreated or NRIgG + C-treated controls. In contrast, enrichment of Mac-2^- but not Mac-3^- Mφ from TBH decreased the inhibitory activities found in Mφ culture supernatants. The reduction noted in inhibitory activity from TBH Mφ factors was not correlatable with protein concentrations of those supernatants but correlated with arachidonic acid metabolites (manuscript in preparation). Indirect immunofluorescence showed altered Mac antigen expression in TBH Mφ populations. Among TBH Mφ Mac-2 expression was reduced and Mac-3 expression was increased as compared to normal host Mφ populations.

Materials and Methods

Animals

Eight to 12 wk-old male BALB/c and C3H mice were obtained from Dominion Labs, Dublin, VA. BALB/c mice, designated TBH, received intramuscular injections of a single-cell suspension of 3 x 10^5 methylcholanthrene-induced transplantable fibrosarcoma cells into the left hind leg. This resulted in palpable tumors by days 10-14 (68, 69). Mice with 21-day old tumors were used throughout. Allograft controls were initiated by injecting 3 x 10^5 normal C3H fibroblast cells into the left hind leg of BALB/c mice. In addition to the observations made in the Results section, allografted mice did not demonstrate splenomegaly or immunosuppression and yielded Mφ populations similar to normal host controls. Immunosuppression in TBH mice was not due to LDH virus contamination. Normal and TBH tested negative for LDH virus (Microbiological Associates, Bethesda, MD).

Medium and Cell Lines

All cells, unless stated otherwise, were grown in RPMI-1640 medium supplemented with 5 or 10% heat-inactivated fetal calf serum (FCS; Flow Laboratories, McLean, Va.) and 4 x 10^{-5} *M* 2-mercaptoethanol (called complete medium). All media contained 50 mg/l Gentamicin (GIBCO, Grand Island, NY) 2 g/l $NaHCO_3$, and 25 mM HEPES. 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.* (234, 272) were obtained from the American Type Culture Collection, Rockville, MD. Initially, cells were cultured in 75 cm^2 tissue culture flasks. For larger scale production of mAb, hybridoma cells were transferred to 2 L roller bottles and cultured in 250 ml

RPMI medium supplemented with 10% FCS at 37°C for 3 days. 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%.

Mφ Preparation

Peritoneal Mφ were obtained from BALB/c mice 4 days (unless otherwise indicated) after intraperitoneal injection of 2.0 ml sterile 0.5% thioglycollate. The use of thioglycollate allowed us to obtain greater numbers of Mφ than was possible without an eliciting agent. The effect of thioglycollate on Mφ antigen expression is described by Ho and Springer (114) and was not pursued in these experiments. We were only interested in the alteration that tumor growth may impart to the expression of Mφ surface antigens, but were aware that thioglycollate elicits Mφ expressing Mac antigens. There was no appreciable difference in the number of cells present in the peritoneal exudate population of normal and TBH (0.8-1.5 x 10^7 and 1-2 x 10^7 cells per mouse, respectively). These populations were composed of 57% and 68% Mφ (as defined by plastic adherence) for normal and TBH, respectively. Similar values were obtained by latex bead uptake. Mφ were selected for by plating, lavaged peritoneal cells on plastic dishes for 4-8 hr. Nonadherent cells were removed by rinsing with RPMI medium. Adherent cells were removed by scraping with a rubber policeman, washed with medium, centrifuged at 500 x *G*, and resuspended to 4 x 10^6 cells/ml in RPMI medium. The collected adherent cells were > 97% esterase positive (68, 69) and, at the 4 x 10^6 cell density, produce a high molecular weight factor(s) that inhibits MLR reactivity, is noncytotoxic, and by virtue of resistance to dialysis and lack of significant competition with radioactive thymidine is not a "thymidine-artifact" (10, 164, 255, 287).

Analysis of "young" (i.e., recently immigrated) Mφ was performed by staining for peroxidase activity (kit #391-A, Sigma Chemical Co., St. Louis, MO). Unfractionated or the adherent fraction of peritoneal exudate cells (PEC) were obtained from normal and TBH by peritoneal lavage. The PEC were resuspended to 4 x 10^6 cells/ml and 200 µl was spread over a glass slide and air dried for

15 min. The cell smear was fixed in a 75% glutaraldehyde 25% acetone solution for 90 sec. Fixed cells were examined for peroxidase activity.

Untreated Mφ were >75% viable before, during, and after *in vitro* culture. Stable *in vitro* viability was confirmed by measuring the accumulation of Lactate Dehydrogenase (LDH; kit # LD-L-10, Sigma) in the culture supernatant at initiation of and at 1-day intervals during *in vitro* culture. Cell-free supernatants from normal and TBH Mφ contained low levels of LDH (< 20 U/L at each time point) as compared to a standard LDH source, FCS (241 U/L). One-hundred percent lysis of 5 x 10^6 Mφ/ml yielded 96 U/L of LDH. Trypan blue exclusion data confirmed LDH viability estimates for untreated Mφ.

For modification of Mφ populations, cells were treated with either mAb or NRIgG (Cappel Laboratories, Malvern, PA) with and without C and incubated at 37°C for 90 min. Complement (Low Tox rabbit C, Accurate Biochemicals, Westbury, NY) was used at a 1:10 final dilution. When NRIgG + C-treated controls were not available C-treatments were used as substitutes. After rinsing 3 times with RPMI medium, the treated cells were recounted with trypan blue, resuspended to 4 x 10^6 viable cells per ml (enriched for remaining cells), and incubated in serum-free RPMI medium at 37°C for 4 days. Differences in the total number of remaining viable cells were determined after treatment with NRIgG or mAb + C. Percentage removal (cytotoxicity) was determined by comparing total number of cells remaining after mAb treatment to the total number of cells remaining after treatment with RPMI medium. Mφ supernatants were collected after incubation, sequentially centrifuged at 500 x *G* for 10 min and 10,000 x *G* for 30 min, and stored at -70°C.

Monoclonal Antibodies

Supernatants from hybridoma cultures were harvested every 3 days and remaining cells replenished with fresh complete medium. The harvested medium was centrifuged as described for Mφ supernatants. Cell-free supernatants were either stored at -70°C for later use or immediately

purified by ammonium sulfate precipitation and DEAE chromatography. 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). Positive fractions were pooled and concentrated 20-fold by ultrafiltration using a YM-10 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 NRIgG by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and radial immunodiffusion. All protein determinations were performed using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA) using a bovine albumin standard (Sigma).

Mixed Lymphocyte Reaction

MLR reactivity was initiated between BALB/c (H-2^d) Mφ-depleted responder T cell populations and C3H (H-2^k) stimulator cells (10). 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% 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. Mφ supernatant dilutions of undiluted, 1:5, 1:10, and 1:20 were used because in other replicate experiments (data not given), dilutions of > 1:20 had no inhibitory activity. 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 (^{3}H-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.

Statistics

Pooled spleen cells or peritoneal Mφ from 4-18 mice were used in each experiment and each data point represents the mean of 4-6 replicate samples. Any significant differences ascribed between data points were Student's *t* tested at $P \leq 0.05$. Results given in Fig. 16, 17, and 18 were obtained from a representative experiment performed with normal and TBH Mφ supernatants and tested in the same MLR to allow for direct comparisons. The entire experimental procedure including antibody treatments, supernatant generation, and MLR testing was repeated and showed similar activities for the various treatment groups.

Results

Peroxidase and Antigen Characterization of Normal and TBH PEC

Peritoneal exudate cells derived from normal, TBH, and mice administered cellular allografts were compared for differences in intracellular cytoperoxidase activity (Table 4). The PEC were obtained from mice at different times after thioglycollate injection, to assure that putative differences were not attributable to differences in general inflammatory responses. No difference in the number of peroxidase-positive PEC were noted between normal or TBH at each of the time points. In general, both normal and TBH PEC populations decreased in their peroxidase activity between 24 and 48 hr after thioglycollate injection. Also, both normal and TBH PEC demonstrated a static level of peroxidase activity between 48 and 96 hr after thioglycollate injection. Mice which received allograft injections were also examined 96 hr after thioglycollate injection and were found to have percentages of peroxidase-positive PEC similar to those observed in normal and TBH. Thus, no differences in PEC were attributable to allograft responses. Plastic adherent PEC populations (Mφ that were collected simultaneously) were also examined for peroxidase activity. Adherent PEC from normal host and allograft recipients contained 27 and 23% peroxidase-positive cells, respectively. Alternatively, TBH-derived adherent PEC were composed of 60% peroxidase-positive cells. Repetition of these experiments confirmed that TBH Mφ populations contain twice as many peroxidase-positive cells as do normal host-derived Mφ.

Mac antigen expression was assessed on normal and TBH Mφ with primary mAb and subsequently detected with a secondary rhodamine-labeled Fab'_2 mouse-anti-rat IgG. The results in Table 5 show the percentage of Mφ that were positive for the Mac antigens based on indirect immunofluorescence. Normal host-derived Mφ populations contained 80% Mac-3^+, 53% Mac-2^+, and 66% Mac-3^+ cells. In TBH Mφ populations the predominant cell type appeared to be Mac-1^+, -3^+, based on fluorescence values of 77% and 100% for those respective antigens. In

Table 4. Percentage of Peroxidase$^+$ Thioglycollate-elicited PEC

Hours after injection	Total PEC[a]		
	PEC source[b]		
	Normal	TBH	Allograft
24	84[c]	82	N.D[d]
48	59	65	N.D.
96	67	59	68
	Adherent PEC[e]		
96	27	60	23

[a]Total PEC were exudate cells collected directly from the peritoneal cavity without plating.

[b]PEC were obtained from nontumor-bearing (Normal) mice, or TBH mice which received 2 x 10^5 fibrosarcoma cells into the hind leg, or allografted mice which received 2 x 10^5 C3H ($H\text{-}2^k$) fibroblasts in the place of tumor cells.

[c]Values represent percentage of PEC staining positive for peroxidase. Staining was performed on glutaraldehyde fixed cells (see Materials and Methods).

[d]N.D. designates not determined, since allografted mice were equivalent to normal.

[e]Adherent PEC were exudate cells that were plated in serum-free medium for 3-4 hr on plastic culture dishes. Normal, allografted, and TBH-derived cells were all collected, plated, and stained on the same day.

contrast, detectable levels of Mac-2 antigens were found on only 37% of TBH Mφ. Comparisons of normal and TBH Mφ populations based on their Mac antigen expression showed that a decrease in Mac-2 expression occurred concomitant with increased Mac-3 antigen expression during tumor development. Observed differences between normal and TBH Mφ with respect to NRIgG treatments might be interpreted as nonspecific Fc binding and in this context could represent another phenotypic difference between normal and TBH.

Modification of Mφ Populations with Antibody-induced Cytotoxicity

To confirm the C fixing abilities of each of the mAb, ^{51}Cr-labeled normal host Mφ were treated with mAb + C and the percentage release of isotope was examined. In these assays, maximum lysis (54-55%) for each mAb was achieved at mAb concentrations of 12.0 μg/10^5 cells which corresponded to 1.3 x 10^7 antibody molecules per cell. Two parameters are presented in Table 6 which show and compare the sensitivity of normal and TBH peritoneal Mφ to mAb + C treatment: (i) percentage reduction in total Mφ population (expressed as specific lysis) and (ii) percentage reduction in the peroxidase-positive subpopulation (expressed as specific lysis). Differential reductions in total Mφ numbers were noted between normal and TBH Mφ following treatment with either anti-Mac-2 or -3 + C. Both treatments removed more Mφ from normal than TBH Mφ populations; anti-Mac-2 + C removed 67% of the normal host and 33% of the TBH Mφ. In contrast, depletion of Mφ by anti-Mac-1 + C was equivalent for both normal and TBH. The remaining data in Table 6, percentage reduction in peroxidase-positive Mφ, clearly suggested a correlation between Mac-1, -3 but not Mac-2 antigen expression and endogenous cytoperoxidase activity in TBH Mφ populations. In normal Mφ peroxidase activity was not correlated with any particular Mac antigen expression.

Table 7 depicts the percentage expression of Mac antigens following anti-Mac plus C treatment. The data represent the number of Mφ that are positive for a particular antigen (detected by mAb binding and indirect immunofluorescence) following enrichment with the same or another

Table 5. Percentage of Mac-1, -2, and -3 Positive Normal and TBH PEC

PEC source	mAb treatment[a]			
	NRIgG	Mac-1	Mac-2	Mac-3
Normal	22[b]	80	53	66
TBH	7	77	37	100

[a]The amount of mAb binding to adherent peritoneal Mφ was determined by treating cells first with either NRIgG, anti-Mac-1, -2, or -3 and subsequently washing and treating with rhodamine-labeled $F(ab')_2$ anti-rat antibodies. Cells were counted using an Olympus IMT-2 inverted phase-fluorescence microscope. At least 200 cells were counted from each treatment group in random fields to arrive at the percentage.

[b]Values represent percentage of total population fluorescencing after labeling with rhodamine-labeled secondary anti-rat $F(ab')_2$ antibodies.

Table 6. Depletion of Mφ from Adherent PEC by mAb + C

	Mφ source[a]			
	Normal		TBH	
Antibody used[b]	Total cells	Peroxidase$^+$	Total cells	Peroxidase$^+$
	(% specific lysis)			
Anti-Mac-1	12[c]	0[d]	14	46
Anti-Mac-2	67	0	33	0
Anti-Mac-3	38	0	27	40

[a]PEC were obtained and treated as described in Materials and Methods.

[b]Concentrated mAb were used as described in Materials and Methods. Complement was used at a final concentration of 1:10.

[c]Values represent the percentage lysis of Mφ and were calculated by the following equation:

$$\frac{\text{Cells removed by mAb plus C - cells removed by NRIgG plus C}}{\text{Cells remaining after NRIgG plus C treatment}}$$

[d]Values represent the percentage change in peroxidase$^+$ cell populations after mAb treatment and were calculated by the following equation:

$$\frac{\%\ \text{peroxidase}^+\ \text{cells after NRIgG plus C - \% peroxidase}^+\ \text{cells after mAb plus C}}{\%\ \text{peroxidase}^+\ \text{cells after NRIgG plus C}}$$

mAb. Because this analysis involved two mAb treatments, high fluorescence levels were produced and for the most part high background was observed. Despite the background levels several consistent observations were possible. For example, both anti-Mac-1 and anti-Mac-2 plus C treatments yielded populations of normal and TBH Mφ that were predominantly Mac-3^+. In contrast, anti-Mac-3 plus C treatments selected populations of Mφ that contained equivalent numbers of Mac-1^+ and -3^+ Mφ irrespective of normal or TBH. Depletion was observed in Mφ populations following anti-Mac-2 or -3 plus C treatments. In normal and TBH the number of Mac-2^+ Mφ was diminished following anti-Mac-2 plus C treatment as was the number of Mac-3^+ Mφ in the TBH following Mac-3 plus C treatment. Reductions in Mac-3^+ Mφ number were particularly noticeable because with other treatments of normal and TBH Mφ Mac-3 mAb labeling was generally higher than Mac-1 or -2 labeling. Thus, alterations in Mφ populations resulted from mAb plus C treatments and these alterations demonstrated differential Mφ heterogeneity between normal and TBH.

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

It has been demonstrated in other studies (255) that Mφ grown *in vitro*, in the absence of serum, produce and secrete protein products that generally increase the protein content of the medium. Furthermore, medium conditioned by Mφ has been shown to contain factors with immunoregulatory activity (10, 164, 166). To determine the role(s) of Mac-defined Mφ subpopulations in monokine production, we examined the conditioned culture supernatant of mAb + C modified Mφ populations. After treatment with mAb + C, normal and TBH Mφ cultures were established by resuspension to 4 x 10^6 cells/ml, incubated for 4 days in serum-free medium, and Mφ factor-mediated modulation was assessed using MLR cultures. The effect of mAb + C treatment on production of suppressor factors and the protein content (μg/ml) of the respective

Table 7. Selection for Mφ subsets

mAb + C selection[a]	Percentage indirect fluorescence after treatment with anti-Mac-1, -2, or -3 + C			
	NRIgG	Mac-1$^+$	Mac-2$^+$	Mac-3$^+$
Normal Host				
Mac-1 + C	25[b]	60	59	86
Mac-2 + C	6	65	14	79
Mac-3 + C	46	73	55	75
TBH				
Mac-1 + C	44[b]	63	66	91
Mac-2 + C	55	46	38	71
Mac-3 + C	35	58	45	51

[a]Peritoneal Mφ were obtained by plating. They were resuspended to 4 x 10^6 cells/ml, treated with anti-Mac-1, -2, or -3 + C, washed, and subsequently treated with the same mAb without C to determine if selection of a particular cell subset occurred. The secondary antibody, rhodamine-labeled anti-rat F(ab′)$_2$, was added to visualize Mφ binding mAb.

[b]Percentage fluorescence was determined by the following equation:

$$\frac{\text{Number of cells positive for indirect fluorescence x 100\%}}{\text{Total number of cells}}$$

At least 200 cells were counted for each treatment.

supernatants is illustrated in Table 8. One-hundred percent suppression of MLR reactivity was defined with supernatants derived from C alone treated normal and TBH Mφ. Zero percent was defined as a complete removal of inhibitory activity. (As demonstrated in Fig. 16-18, supernatant dilutions of 1:5 were used to compare normal and TBH Mφ-derived regulatory factors, because the 1:5 dilution consistently reflected the most linear portion of the undiluted to 1:20 titration curve for both normal and TBH Mφ supernatants.) Each treatment group yielded supernatants that were consistently less suppressive in the MLR than controls.

Comparison of Normal and TBH Mφ-derived Supernatants

Mφ supernatants were harvested and added at varying dilutions to MLR cultures. Supernatants from mAb and control treated normal or TBH Mφ were all inhibitory to some extent. The peak proliferative response (representing 100% reactivity) was observed in MLR cultures receiving no Mφ supernatants (NONE) (Fig. 16-18). Variations were observed in the down-regulation attributed to normal or TBH Mφ. To examine the correlation of Mac antigen expression and Mφ-mediated immunoregulation during tumor growth, suppressive activity was determined for dilutions of Mφ supernatants from mAb-treated normal or TBH Mφ. Other studies (50, 68, 69) demonstrate that at supernatant dilutions ≥ 1:100 all suppressive activity is lost, therefore we used undiluted, 1:5, 1:10, and 1:20 dilutions to test for differences in supernatant inhibitory activities.

Supernatants from anti-Mac-1 + C-treated normal host Mφ had a significantly decreased ability to suppress MLR reactivity compared to control Mφ supernatants, at all dilutions (Fig. 16). Supernatants from anti-Mac-1 + C-treated TBH Mφ had a significant decrease in suppressive activity only at the highest supernatant concentration (undiluted). Supernatants from anti-Mac-1 + C-treated normal host Mφ showed a 36% decrease in suppression of MLR reactivity (Table 6). This was associated with a 67% increase in protein concentration (42 to 70 μg/ml). Supernatants

Table 8. Percentage suppression and protein content of supernatants derived from anti-Mac-treated Mφ

Antibody[b] used + C	Mφ supernatant source[a]			
	Normal		TBH	
	% MLR[c] suppression	protein[d] (μg/ml)	% MLR suppression	protein (μg/ml)
NRIgG	100	42	100	42
Anti-Mac-1	64	70	102	60
Anti-Mac-2	102	12	20	80
Anti-Mac-3	22	60	111	50

[a]Mφ supernatants were obtained from Mφ cultured in serum-free RPMI medium for 4 days. Both normal and TBH were used as Mφ sources.

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

[c]Suppression of 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:

$$1 - \frac{\text{anti-Mac + C cpm}}{\text{NRIgG + C cpm}} \times 100$$

[d]Protein concentrations were determined using a Bio-Rad protein assay kit (see Materials and Methods). Protein concentrations are expressed in μg/ml.

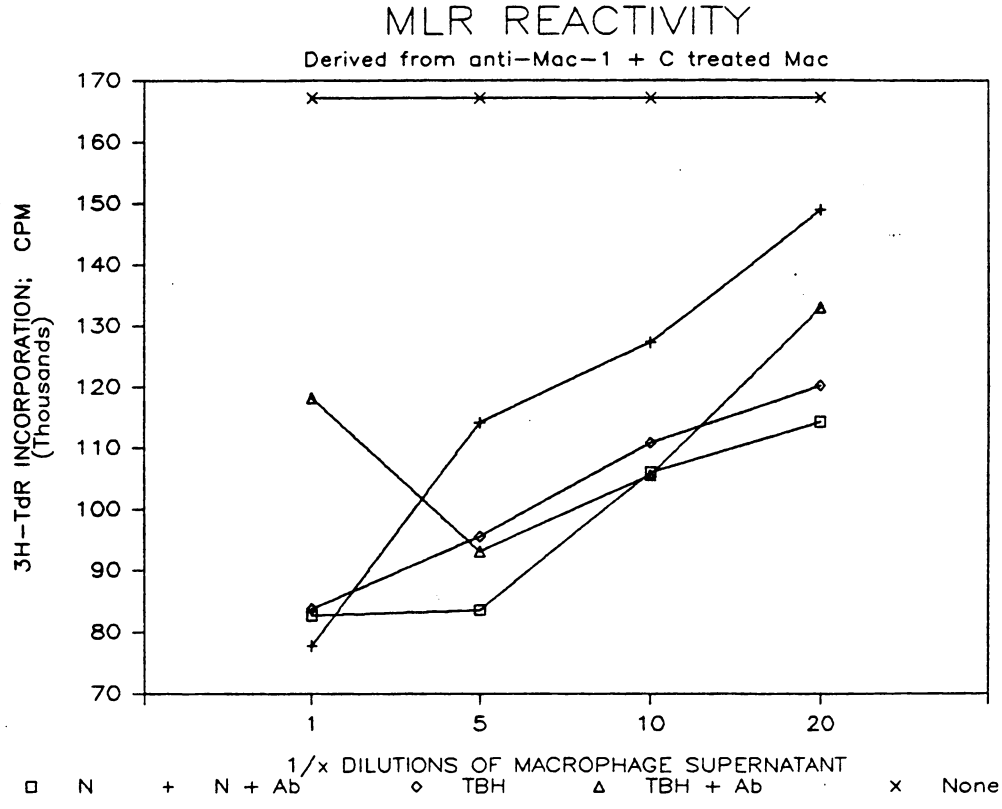

Figure 16. **Anti-Mac-1 + C modification of normal and TBH Mφ regulatory activity:** Mφ from either normal (□; N + NRIgG and +; N + anti-Mac-1) or TBH (◇; TBH + NRIgG and △;TBH + anti-Mac-1) were treated with NRIgG or anti-Mac-1 + C and subsequently resuspended to 4 x 10^6 cells/ml (enrichment of remaining cells). The 4-day culture supernatants of the treated cells were added at various dilutions to MLR cultures. MLR cultures that did not receive Mφ supernatants are represented by "X--X". Regulatory activity of the supernatants was determined by assessing the degree of ^{3}H-TdR incorporation due to T cell blastogenesis.

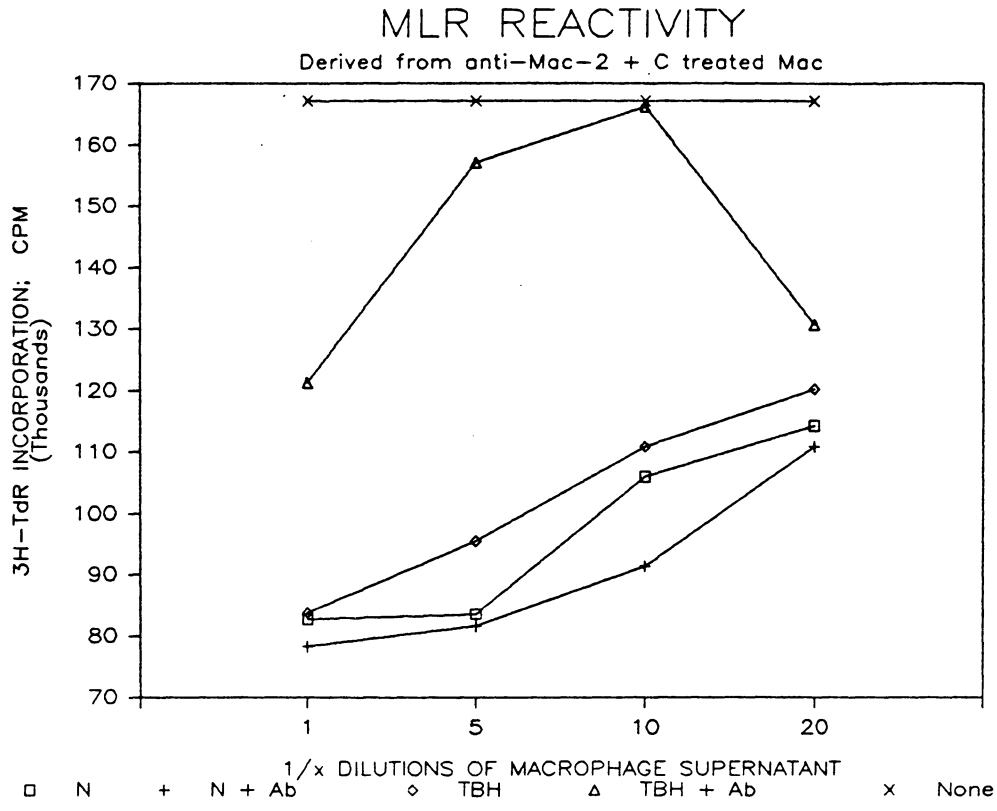

Figure 17. **Anti-Mac-2 + C modification of normal and TBH Mφ regulatory activity:** Treatment protocol as described in Fig. 16 legend for anti-Mac-1, except using anti-Mac-2 mAb.

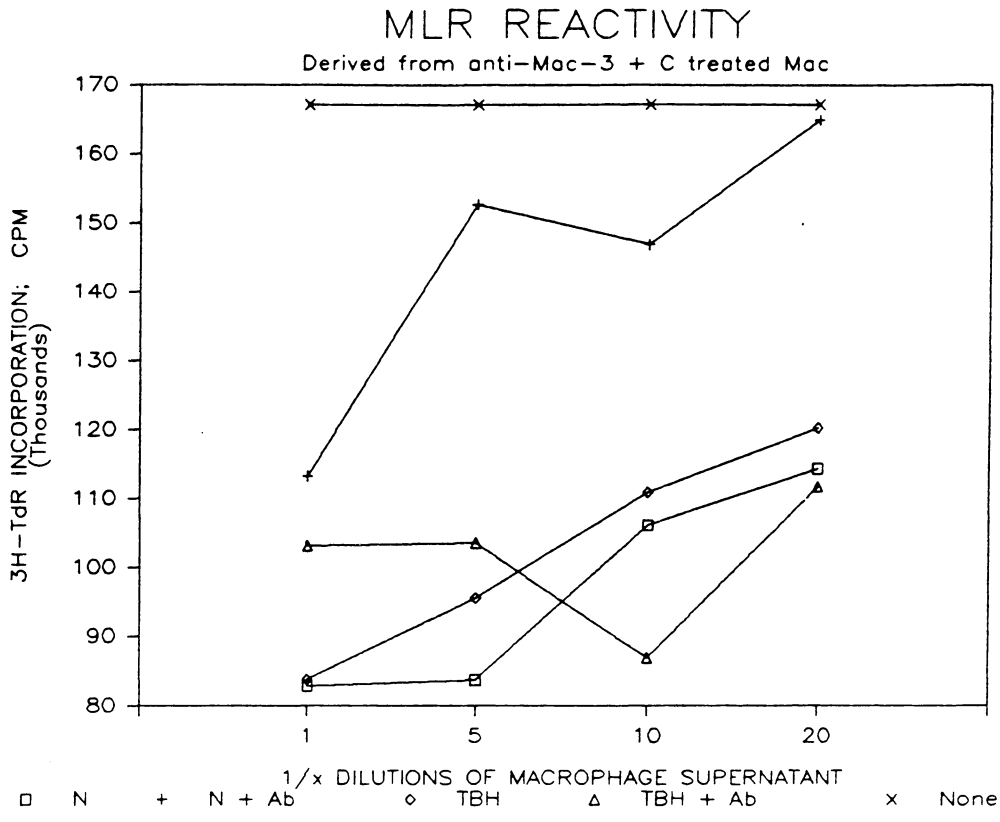

Figure 18. **Anti-Mac-3 + C modification of normal and TBH Mφ regulatory activity:** Treatment protocol as described in Fig. 16 legend for anti-Mac-1, except using anti-Mac-3 mAb.

from treated (anti-Mac-1 + C) and control TBH Mφ were unchanged in suppressive activity but protein content significantly increased (43%).

A significant decrease in suppressive activity was observed for TBH Mφ that had been treated with anti-Mac-2 + C (Fig. 17). The increase in suppressive activity at the 1:20 dilution of Mφ supernatant was assumed to be an artifact since a > 1:20 dilution (not shown) returned activity to nontreated levels repeatedly. Normal host Mφ-mediated suppressive activity was unaffected by anti-Mac-2 + C treatment. As described in Table 5, inhibitory activity of supernatants from treated TBH Mφ was reduced by 80%. The significant decrease in suppression was associated with a 90% increase in protein content (42 to 80 μg/ml).

Normal host Mφ treated with anti-Mac-3 + C resulted in culture supernatants which had significantly reduced suppressive activity (Fig. 18). A significant (at the undiluted and 1:10 dilution) but lesser modulation of TBH Mφ by anti-Mac-3 + C was demonstrated. Table 5 shows that the protein concentrations of the supernatants from anti-Mac-3 + C-treated normal and TBH Mφ were altered differentially. Anti-Mac-3 + C-treated normal host Mφ demonstrated a significant decrease (100 to 22%) in suppressive activity, but the protein content of the supernatants increased by 43% (42 to 62 μg/ml). In contrast, the anti-Mac-3 + C treatment of TBH Mφ did not significantly alter their suppressive activity or the protein content (42 to 50 μg/ml).

Discussion

The removal of Mφ using specific mAb demonstrated that variations in the expression of Mac-2 and Mac-3 during tumor growth correlated with compromised immunoregulatory activities. Because Mφ populations were recounted and enriched for remaining cell types, the differences in total number of Mφ did not cause the variations in regulatory activity. The activities were the result of qualitative changes in the composition of the treated Mφ populations. Since these changes differed with respect to Mφ source (normal versus TBH), they were indicative of alterations in Mφ antigen expression (phenotype) due to tumor growth. Furthermore, we have confirmed these aberrations in Mφ populations by fluorescent microscopy and endogenous-peroxidase activity. Analysis of mAb labeling was performed with rhodamine-conjugated anti-rat IgG rather than fluorescein-conjugated anti-rat antibodies because less autofluorescence was observed within the wavelength ranges used for rhodamine. Using this approach portions of normal and TBH Mφ populations were nonfluorescing even after mAb and anti-rat-rhodamine labeling. TBH Mφ populations contained more Mac-2$^-$ adherent cells than their normal counterparts, whereas normal Mφ contained fewer Mac-3$^-$ cells than TBH populations. Equivalent Mac-1 expression by normal and TBH Mφ was observed by both immunofluorescence and mAb-mediated lysis. This confirmed our earlier predictions of equivalent Mφ content in the two populations. However, Mac-1 expression by both normal and TBH Mφ was lower than expected based on other parameters such as adherence and esterase staining but was acceptably high (77-80%).

Following mAb plus C treatment, trypan blue dye exclusion was performed with both normal and TBH Mφ and demonstrated that differential levels of C-mediated lysis resulted, depending on mAb and Mφ source. Anti-Mac-1 + C lysed equivalent proportions of normal and TBH populations and caused the least modulation of Mφ supernatant suppressor activity. Even though changes in regulation by normal host Mφ were caused by treatment with anti-Mac-1 + C, and were significant, these were not as severe as changes observed after anti-Mac-2 + C treatment of TBH Mφ or anti-Mac-3 + C treatment of normal host Mφ. These results may have been

due to the minimal percentage lysis observed with anti-Mac-1 in normal and TBH populations. Although anti-Mac-1 removed the same number of cells in normal and TBH Mφ populations, the remaining cell population's differed in peroxidase and regulatory activities (i.e., Mac-1$^-$ cells). In this context, enrichment procedures were effective but the altered Mφ populations did not vary greatly in their production of soluble inhibitors.

Anti-Mac-2 + C treatment differentially reduced cell numbers in normal and TBH Mφ preparations. Although the expression of this antigen may be induced by thioglycollate, tumor growth imposed additional influences on its expression. Correlations between Mac-2 expression and stimulatory agent are described (114). The Mac-2 antigen is implicated as a possible marker for differentiation. The loss of suppressive activities following the removal of TBH Mac-2$^+$ Mφ and the ineffectiveness of this treatment on altering suppressor secretion by normal host Mφ might be explained in terms of remaining cell population composition. Since anti-Mac-2 + C treatment removed more Mφ from normal hosts than from TBH, one might predict that either more Mac-2 sites were expressed on each of the normal host Mφ or that more Mac-2$^+$ cells are present in normal host populations. The immunofluorescence data supports the second suggestion, since more Mφ were labeled among normal Mφ. Nonetheless, it appeared that the major inhibitory Mφ in the TBH was Mac-2$^+$. The observed correlation between peroxidase activity and the expression of Mac-1 and -3 and the lack of any correlation between Mac-2 expression and peroxidase activities supports our hypothesis of selective removal of Mφ subpopulations and suggested that the source of the inhibitory compound(s) is not a peroxidase positive TBH Mφ.

Suppressive activity from anti-Mac-3 + C-treated normal host Mφ was significantly less than similarly treated TBH Mφ. This would be expected if the inhibitory Mφ population in normal hosts was Mac-3$^+$ and, as a subpopulation, represented only a portion of the total number of Mφ. The immunofluorescence data supports this idea, since Mac-3 expression was lower among normal host Mφ than TBH Mφ. Accounting for this alteration in suppressive activity as a reduction in cell number is unsatisfactory because all Mφ populations were recounted and resuspended to equal cell numbers. Furthermore, anti-Mac-2 + C treatment which removed large numbers of normal host Mφ but did not cause noticeable changes in the suppressive activity me-

diated by the remaining cells. This implied that the modulation was not due to a quantitative change in total Mφ number. Thus, one might speculate that the major inhibitory Mφ in the normal host was Mac-3^+ and Mac-2^-. This phenotype implies a negative correlation between Mac-3 and Mac-2 expression in Mφ populations, thus anti-Mac-3 treatment should enrich for Mac-2^+ Mφ or anti-Mac-2 treatment should select for Mac-3^+ Mφ. This phenomena was demonstrated in TBH Mφ populations, where anti-Mac-1 and -2 treatments selected for Mac-3^+ Mφ, but was not clearly observed in normal host cell populations. An alternative explanation of anti-Mac-3-mediated depletion might be found in comparing the percentage of normal host Mφ that are Mac-3^+ with the percentage of cells removed by anti-Mac-3 treatments. In this respect, the level of observed lysis equals approximately half of the proportion of cells that expressed Mac-3 in normal host populations but corresponds to only 27% of the total Mac-3^+ Mφ population observed in TBH populations. Since anti-Mac-3 depletion or enrichment did not alter TBH Mφ inhibitor production this might explain the different sensitivities of suppressor secretion between normal and TBH. Although these ideas are contrary to work by Ho and Springer (117), which suggests that Mac-3 is expressed on all Mφ, others (81, 283) have also detected variable Mac antigen expression on Mφ and other related cell types. Differential susceptibility to mAb + C treatment has not been previously addressed with respect to Mac-3 heterogeneity and may be a contributing factor in our findings.

The nature of our Mφ-derived regulators has not been completely characterized (50, 164, 166). Because these supernatants were not dialyzed, the soluble modulator could have been either of low molecular weight (PGE_2) or high molecular weight (protein monokines). Investigations focusing on the role of PGE_2 were pursued by biochemical analysis of Mφ supernatants and by indomethacin treatment of isolated Mφ populations. Preliminary PGE_2 analysis of supernatants from mAb + C-treated normal and TBH Mφ by radioimmunoassay showed that while the levels of PGE_2 fluctuated with respect to Mφ source and treatment, the estimated levels of PGE_2 were below 3.5 *nM* in the test cultures except for anti-Mac-3 + C-treated normal host Mφ. A putative association between TBH Mac-2^+ Mφ and PGE_2 production has been detected and will be presented elsewhere (manuscript in preparation).

The protein content of Mφ supernatants varied among mAb treatments. Reductions of 36 and 78% in normal host Mφ-mediated suppression following anti-Mac-1 and -3 treatment were paralleled by 67 and 43% increases in protein concentration, respectively. The reverse was true for anti-Mac-2-treated normal host Mφ. Treatment of TBH Mφ with anti-Mac-1 and -3 left suppression unaltered, but again protein content increased. Loss of TBH Mφ-mediated suppression after anti-Mac-2 treatment was associated with a 90% increase in protein content. Thus, an inverse relationship was demonstrated between reduction in suppression and increases in protein content. Since the relationship was not equivalent in degree of reduction in suppression, both a quantitative and qualitative difference in Mφ supernatants was suggested.

The data strongly suggest that there is a relationship between tumor-induced suppression and appearance of different Mφ populations expressing Mac-1, -2, or -3 antigens. The finding that the composition of Mφ populations was altered by tumor growth is reasonable, since Mφ and other types of immune cell populations demonstrate alterations in their phenotypic composition because tumor growth (57, 120, 126, 182, 184, 242). Furthermore, we have shown altered Ia, Mac-1, -2 and -3 expression in TBH-derived splenic Mφ populations (90, 91). In the present, study peroxidase analysis of peritoneal Mφ from normal and TBH suggested that a significant proportion of Mφ elicited in the TBH were immature. This may be interpreted as a tumor-induced *in vivo* or environmental alteration in Mφ differentiation that results in altered Mφ populations. Although a strict assignment of function to Mφ subsets should be approached cautiously, we can tentatively implicate tumor growth as an inducer of Mφ heterogeneity and further speculate that this heterogeneity was responsible for different regulatory activities displayed by the respective Mφ populations, normal versus TBH. There remain unresolved problems of considerable importance. Is there a difference in quantitative and qualitative expression of these antigens on Mφ from normal versus TBH and does expression vary during different stages of tumor growth? Analysis of TBH Mφ for surface expression (immunofluorescence) and metabolic production (Western Blot) of Mac-2 and -3 (preliminary findings) suggested that altered expression may be both qualitative and quantitative. Since an indirect involvement of Mac-2 and -3 was suggested by this study, we are pursuing the possibility that these antigens play a direct "functional" role in regulation. The delineation of

functional involvement is imperative before we can understand the Mφ and its repertoire of immunological activities.

Chapter VI

INVERSE CORRELATION BETWEEN Ia ANTIGEN EXPRESSION AND PGE_2 SECRETION IN NORMAL AND TBH

Introduction

Mφ are able to control lymphocyte activities by producing soluble immunoregulatory molecules (178, 213, 298). These molecules (monokines) can be isolated from Mφ conditioned culture media (40, 77, 254) and thus can be derived *in vitro*. In previous studies (89, 90), we demonstrated that the production or expression of enhancing and suppressing activities by *ex vivo* Mφ populations may be attributable to antigen-defined subpopulations of Mφ. Other investigators have associated Mφ effector functions with the expression of certain Mφ antigens (249, 257). Therefore, Mφ subpopulations exist which perform specific functions. A recent study on human peripheral blood monocytes identified four monocyte populations based on their different buoyant densities. Each of these populations varied in their production of PGE_2 and IL-1 (36). mAb can

be applied to Mφ populations, in the presence of C, and may be used to select for or against certain antigen-defined Mφ populations. The resulting modified populations can subsequently be examined for their immunoregulatory activities. In this study, we have extended our earlier work reported in Chapter 4, on Ia expression in normal and TBH to examine the correlation that Ia antigen may have with the secretion of Mφ-derived immunosuppressive compounds. In our earlier studies (Chapter 4), we showed that alterations occur in the expression of Ia antigens on Mφ that are isolated from TBH. These alterations were correlated with changes in Mφ stimulatory capacity and accessory function. In the present study, we investigated the possible role of soluble immune regulators as mediators of altered Mφ function. Furthermore, we attempted to correlate Mφ Ia antigen expression with the production of Mφ-derived soluble immune regulators. Peritoneal and splenic Mφ from normal or TBH were treated with anti-Ia mAb in the presence of C and the resulting populations were cultured for four days. The culture supernatants from these Mφ were added to syngeneic T cells in a MLR. Immunoregulatory activity of the Mφ culture supernatants was determined by measuring changes in T cell proliferation. We show here that Ia^- Mφ produce supernatant that are more suppressive than Ia^+ TBH Mφ and that this suppression is at least in part due to increased PGE_2 production. These phenomena were most pronounced in splenic Mφ populations and confirm previous findings (57).

Materials and Methods

Animals

Twenty-four BALB/c (H-2^d) mice were used as a source for peritoneal Mφ. Half of the mice used were inoculated with nonmetastatic methylcholanthrene-induced fibrosarcoma cells into the left hind leg (3 x 10^5 tumor cells/mouse). Similar mice numbers and inoculation procedures were performed to obtain normal TBH splenic Mφ.

Cell Culture Medium

As previously described (89) RPMI medium was used to culture all cells. For Mφ culture, FCS supplementation was omitted. In T cell cultures (MLR), 5% FCS supplementation was used as was $10^{-5}M$ 2-mercaptoethanol.

Mφ Harvesting and Treatment

Peritoneal Mφ were obtained as previously described (89) using thioglycollate as an eliciting agent. Four days after intraperitoneal injection of thioglycollate, peritoneal lavage was carried out with two 10 ml washes with cold (10°C) RPMI medium. Peritoneal cells were washed with 2 changes of cold RPMI medium, suspended in 10 ml of RPMI and added to plastic culture dishes. The adherent cell population was removed by scraping after 3-4 hr of incubation at 37°C. Dislodged adherent peritoneal cells were washed with RPMI medium and resuspended to 4 x 10^6 cells/ml in the same medium. Splenic Mφ were obtained from single cell suspensions of whole

spleen cells that had been forced through a wire sieve. The splenic cells were treated the same as the peritoneal cells and adherent cell populations were collected.

Treatment of either peritoneal or splenic Mφ was performed as previously described (89). Briefly, adherent cells were treated with anti-Ia^d or anti-Ia^k in the presence of "low-tox" rabbit complement (Accurate) (prepared at a 1:10 dilution). The treatment was carried out at 37°C for 90 min after which the cells were washed three times with cold RPMI medium. The treated Mφ were resuspended to 4 x 10^6 cells/ml and cultured for 4 days at 37°C in serum-free RPMI medium. Preparation of Mφ supernatants was performed as previously stated (Chapter 5).

MLR

MLR were initiated between BALB/c (H-2^d) and C3H (H-2^k) splenocytes. Responder cultures were BALB/c T cells while stimulators were mitomycin-D inactivated C3H splenocytes. MLR cultures were carried out with 2 x 10^5 responders/well and 4 x 10^5 stimulators/well in a 200 µl/l of 5% FCS supplemented RPMI medium contained in each of 96 wells on a microculture plate (Flow Laboratories). Degree of response was assessed by ^{3}H-TdR incorporation as previously described (89).

Mφ culture supernatants were obtained from mAb plus C-treated normal or TBH peritoneal or splenic Mφ. Fifty µl of these supernatants were added to each of 4-6 MLR cultures at the initiation of the reaction. The degree of immunoregulatory activity was assessed by comparing MLR cultures which received supernatants with those that only received RPMI medium.

PGE_2 Analysis

The amount of PGE_2 contained by Mφ supernatants was determined by radio-immune assay. The procedure is described elsewhere (165).

Results

Modifications in Immunoregulation After mAb + C Treatment of Mφ

Culture supernatants from either anti-Ia^k or anti-Ia^d plus C-treated Mφ were collected after 4 days of *in vitro* culture. Cleared and filter sterilized Mφ supernatants were added to allogenically stimulated T cell cultures in a MLR. Figure 19 illustrates the effect of the supernatants on T cell proliferation, as assessed by ^{3}H-TdR incorporation. The control Mφ supernatants were derived from Mφ that were treated with anti-Ia^k plus C, which is an irrelevant antibody since the treated Mφ are Ia^d. Comparison of Mφ supernatants derived from either normal or TBH spleens showed that TBH Mφ-derived supernatants were more immunosuppressive. Furthermore, the production of suppressive factors was increased by the removal of Ia^+ cells from the splenic adherent cell populations (SAC). Normal and TBH peritoneal Mφ supernatants were roughly equivalent in terms of suppressive activities but in both cases the removal of Ia^+ Mφ yielded a more suppressive supernatant, as compared to their respective controls. These results suggested that, although Ia antigen expression may not be directly involved in secretion of suppressor factors their may be an association between presence of the antigen and secretion.

Evaluation of PGE_2 Produced by Anti-Ia Treated Mφ

Since removal of Ia^+ Mφ from peritoneal or splenic Mφ populations yielded supernatants that were more suppressive of T cell proliferation, the PGE_2 content (a major immunosuppressant produced by Mφ) was determined. In Fig. 20, the PGE_2 concentrations of the various Mφ supernatants is given in picograms. In each pair of samples, TBH versus normal, more PGE_2 was produced by the TBH-derived Mφ. Both normal and TBH Mφ produced more PGE_2 after Ia^+

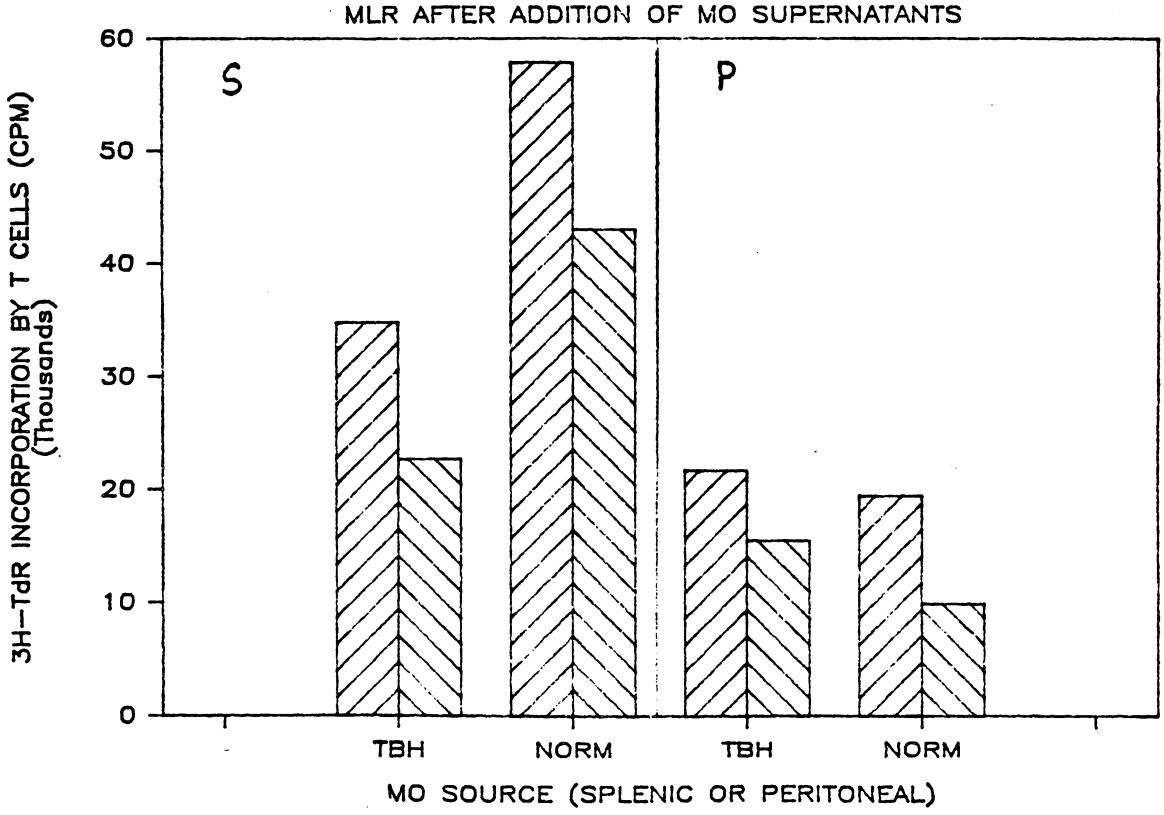

Figure 19. T cell proliferation after the addition of mAb-treated Mφ-derived culture supernatants: T cell proliferation is shown as the amount of ^{3}H-TdR incorporation occurring in a MLR. Treatments of Mφ shown are, anti-Ia^{k} (///) and anti-Ia^{d} (\\\) for splenic (S) or peritoneal (P) Mφ Mφ were derived from either normal or TBH.

Mφ were removed by mAb + C treatments. Collectively, it appeared that increased PGE_2 production was more pronounced in TBH peritoneal Mφ populations, where a two-fold increase was observed. This coincidence is viewed as a correlation between tumor-induced alterations in Mφ phenotypes and regulatory function.

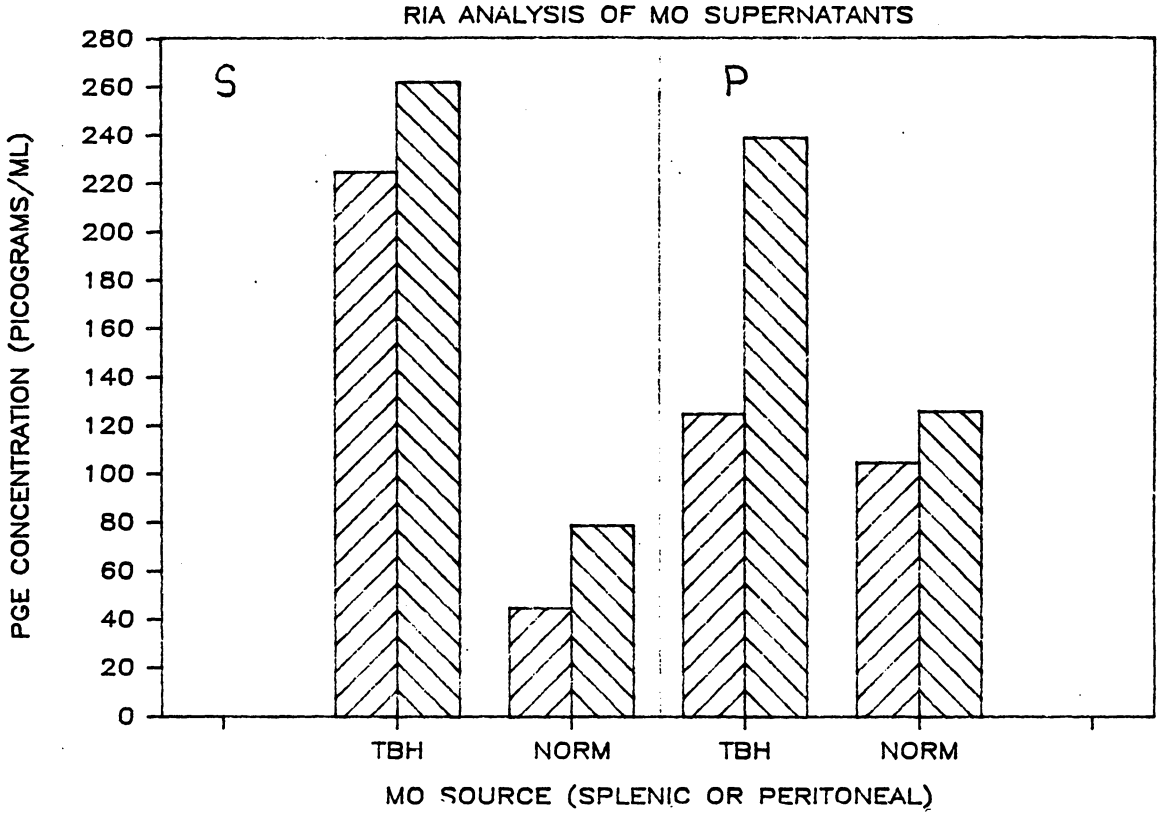

Figure 20. **PGE_2 produced by mAb-treated Mφ:** PGE_2 content of Mφ culture supernatants was determined for each of the different Mφ groups and treatments. Anti-Ia^k (///) and anti-Ia^d (\\\) treated Mφ are shown. Two sources of Mφ were used, splenic (S) or peritoneal (P).

Discussion

The possibility that Mφ phenotypic heterogeneity could be a source for Mφ functional heterogeneity is suggested in several studies (89, 249, 257). We showed that altered Ia antigen expression by Mφ populations occurs during tumor burden and may in part be associated with altered TBH immune responses (91). The alteration in Ia antigen expression occurs in both the spleen and in peritoneal Mφ populations. In this study, we have shown that there is an apparent association between Ia antigen expression and the production of immunosuppressive monokines. Enrichment of Ia^- Mφ in either normal or TBH Mφ populations increased the suppressive activity of the resulting culture supernatants. This observation was not a direct result of decreased Mφ numbers since: (i) Mφ from each source and treatment were resuspended to equal cell densities (4×10^6 cells/ml), (ii) more Mφ would be removed from normal host Mφ populations because of greater number of Ia^+ Mφ. Instead, these results were most likely the result of altering the Ia^+/Ia^- cell ratio of the treated populations.

To account for the immunoregulatory activity as a decrease in enhancing activities or an increase in suppressive activities, we examined the PGE_2 content of the supernatants. Reports by others (153) show that PGE_2 (suppression) and IL-1 (enhancement) production can be separated into two distinct populations of activated murine Mφ based on their buoyant density. Our results also show a correlation between Mφ phenotype and PGE_2 production. The observation that the PGE_2 content of TBH splenic Mφ-derived supernatants was higher than normal and was also in agreement with previous work by our laboratory (56). Collectively, from the regulatory activity results and from the PGE_2 content of the Mφ supernatants it seems that Ia^- Mφ are the principle producers of PGE_2 and that this compound represents one of the modes of Ia^- Mφ suppression of T cell proliferation. In a separate report by our laboratory, we (165) describe the correlations between Mac antigen expression on peritoneal Mφ and the production of PGE_2. In this study distinct differences in anti-Mac-2 and -Mac-3 sensitivities of normal and TBH Mφ were associated with PGE_2 production. Segregation of Mφ subsets by other procedures has also demonstrated that

a particular subpopulation of Mφ that are bone marrow-derived and not resident may be responsible for PGE_2 secretion among splenic Mφ

Cumulatively, results here and elsewhere (7, 136, 192) indicate that specialized Mφ regulatory functions may be divided among different Mφ subpopulations. During periods of immunological stress or challenge subpopulations ratios may change such that the overall regulatory function of the total Mφ population is also changed. Perhaps, further studies dealing with Mφ heterogeneity in aberrant immune systems will link immune dysfunctions to altered altered Mφ function.

Summary

Collectively, these studies support the hypothesis of Mφ heterogeneity, both according to functional and phenotypic pleomorphism. But more important this research showed that immunocompromized TBH possess radically altered populations of Mφ. Previous studies (187, 202, 267, 278, 360) indicate that the Mφ populations of TBH are functionally different from normal host, but they fail to correlate the functional abnormality with phenotypic characteristics. As stated earlier our goals were to examine variations in Mφ regulatory function and to correlate these changes with alterations in Mφ phenotypes, because of tumor burden. Initially, we anticipated having to produce our own mAb against Mφ surface antigens and to use these mAb as markers and selective parameters in the identification of functionally independent Mφ phenotypes. However, the recent popularity of mAb production and cloning of hybridomas that produce antibody specifically for Mφ greatly facilitated our endeavors by eliminating the necessity of creating our own hybridomas. The success of other laboratories (233, 235) and their generosity in sharing of their hybridomas provided us with a variety of mAb against Mφ surface antigens.

The mAb that we chose to use in these studies were specific for Ia, Mac-1, -2, and -3 Mφ surface antigens. Because of the undefined nature of the anti-Mac antibodies preliminary testing of their cytotoxicity and ligand-receptor binding effects were examined. In addition, we had to determine whether depletion or enrichment procedures were appropriate for modifying the ratios of

putative Mφ subpopulations. Our initial testing of the two techniques, depletion and enrichment indicated that enrichment was the better of the two approaches, because the total cell number could be controlled and thus quantitative differences in Mφ number was not a factor in the assignment of Mφ regulatory activities. For example, previous work in our laboratory (49, 50, 56) defined a Mφ density of 4 x 10^6 cells/ml for secretion of a high molecular weight inhibitor of T cell proliferation. When Mφ cultures were treated with anti-Mac-1, -2, or -3 plus C and the remaining cells were cultured without resuspension to 4 x 10^6 cells/ml, a loss of inhibitory activity in the resulting *in vitro* culture supernatants was observed only with anti-Mac-3-treated Mφ. Since all of the anti-Mac antibodies were cytotoxic in the presence of C, the depletion results suggested that anti-Mac-1 and -2 were not removing the source of the inhibition, even though they were effective at removing Mφ. Thus, there appeared to be a correlation between Mac-3 expression and regulatory functions. By comparing the depleted Mφ populations with enriched populations we were able to demonstrate that selected Mac-1^- and -3^- (enriched) Mφ cultures had lost part of their ability to produce suppressive factors if the cell density of the treated Mφ was restored to 4 x 10^6 Mφ/ml. This inferred that the lack of suppressor activity was due to qualitative changes in the total cell population. For example, if more than one population of cell is present enrichment procedures produce changes in the ratios of the different cells. Since both depletion and enrichment of anti-Mac-3 treated Mφ resulted in decreased suppressor activity, it appeared that this antigen might represent a restrictive marker for a Mφ subpopulation that produced inhibitory monokine(s). This led us to believe that enrichment procedures were more relevant to analysis of suppressive Mφ functions, since cell densities in the final cell cultures were controlled.

We also had to consider the consequences of mAb binding to Mac antigens and either mimicking or blocking primary regulatory signals. Soluble immunoregulators can effectively modulate cellular functions and in some cases mAb to cellular antigens may block (53, 161) or induce (250) Mφ functions. In this context, we analyzed the immunoregulatory of Mφ culture supernatants after treating the Mφ with mAb alone or mAb plus C. Both normal and TBH were examined, since we intended later to use mAb plus C-mediated lysis to differentiate between Mφ populations derived from these host conditions. After treating normal and TBH-derived Mφ with

anti-Mac-1 or -3, we observed differential increases in the suppressor activity of their respective supernatants. However with anti-Mac-2 both normal and TBH-derived Mφ produced more suppressive supernatants than their controls. This, in fact, was a good sign since these results differed from the decreases in suppressive activity noted with mAb plus C. Thus, the mAb plus C-induced changes were probably not a result of ligand-receptor interaction, but rather a direct consequence of Mφ removal.

The next step in our approach was to repeat the mAb plus C treatment of peritoneal Mφ derived from either normal and TBH and to compare the suppressive activities of the enriched populations, in the same MLR assay. In other words, we were looking for differences in the immunoregulatory activities of Mac^- populations between the two host conditions. Variations in the amounts of the supernatants added to MLR were performed to detect differences in titration of the inhibitors. This turned out to be an important parameter because at higher concentrations of Mφ-derived suppressor factors a threshold effect might obscure differences in the actual concentrations of the molecules causing the changes. Usually, we noticed this phenomenon as a less defined difference in suppressor activity at high concentration while a greater degree of difference between treated and untreated Mφ was observed with diluted supernatants. More important, these procedures allowed us to ascribe suppressor production to a $Mac\text{-}3^+$ cell in normal Mφ preparations and a $Mac\text{-}2^+$ cell in TBH Mφ preparations. In order to relate these differences to alterations in Mφ phenotypes, we examined the expression of the Mac antigens by indirect immunofluorescence in both normal and TBH Mφ populations. We found correlations between the functional aspects of Mac antigen expression and the physical expression of the Mac antigens. A notable decrease in Mac-2 expression in the TBH indicated that the observed differences in Mφ function may be attributable to changes in the immigration of Mφ during thioglycollate elicitation. In light of the work of other laboratories which are presently assigning a Mac-2 phenotype to elicited Mφ and a Mac-3 phenotype to resident Mφ (personal communication with Carlton Stewart), it would appear that tumor burden may alter the maturation pattern of Mφ in the peritoneum and possibly functional activities of these Mφ. To further illustrate this idea, we examined the peroxidase activity within normal and TBH Mφ. Endogenous peroxidase has been used as a

marker for immature Mφ (59, 128, 220). In TBH Mφ populations we observed a direct correlation between Mac-1 and -3 expression and peroxidase activity but observed a lack of correlation between Mac-2 and peroxidase activity. Since there was no correlation between any of these antigens and peroxidase activity in the normal host Mφ populations, we concluded once more that *in vivo* environmental factors associated with tumor growth may alter the maturation pattern of TBH Mφ. The exact influence of tumor growth is as yet unknown but in some manner it causes an increase in peroxidase and Mac-3 expression and a drop in Mac-2 expression by Mφ migrating into the peritoneum.

Following the hypothesis that Mφ maturation was, in some way, disturbed in the TBH we examined the expression of Ia on normal and TBH-derived peritoneal Mφ. To further illustrate the relevance of the disturbance in terms of resident Mφ populations, we also examined splenic Mφ Ia expression and correlated the physical expression of the antigen with aberrant immunoregulatory activities. Ia antigens expressed on Mφ are involved in the presentation of antigens to T cells (35, 96, 149, 232). Since the MLR functions according to class II MHC differences, we were able to use allogeneic BALB/c ($H\text{-}2^d$) Mφ as stimulator cells in a MLR that contained C3H ($H\text{-}2^k$) responder cells. Thus, differences in Ia antigens triggered a response by C3H T cells which encountered different (altered) Ia molecules on the BALB/c Mφ. Reductions in Ia expression on the BALB/c Mφ could then be observed as decreased stimulation of C3H T cells. Based on these assumptions, we used various dilutions of Mφ in an MLR as stimulator cells and were able to observe Mφ number dependent stimulation. Since the presentation or interaction of cellular Ia antigens with T cells is the driving force behind T cell stimulation (5, 21, 61, 228), we concluded that decreased proliferation by T cells in the presence of low numbers of Mφ reflected a progressive dilution of Ia^+ stimulator cells. On the basis of these observations and conclusions we attributed reduced stimulation by TBH-derived Mφ to reduced numbers of Ia^+ Mφ in these cell populations.

Analysis of Ia expression by mAb (anti-Ia) binding demonstrated that the reduction of Ia expression on TBH Mφ yielded a higher percentage of Ia^- cells in TBH-derived Mφ populations and was not merely attributable to fewer Ia antigens per Mφ. The decrease in number of Ia^+ Mφ

was not only observed in peritoneal Mφ populations but also in splenic Mφ populations. This illustrated that the tumor-derived effect on Mφ maturation and/or differentiation was not limited to Mφ that were immigrating to the peritoneal cavity as a result of thioglycollate injections, but was also observable in resident splenic populations. Furthermore, decreases in Ia^+ Mφ numbers in the spleen were even more noticeable, since the splenic Mφ generally contains Mφ populations with 50% Ia Mφ (as opposed to 10% in the peritoneum because of thioglycollate injection). To again dismiss any arguments between per cell Ia expression and number of Mφ expressing the Ia antigens, mAb was used as a blocking agent on stimulator Mφ in MLR. Although anti-Ia blocked the proliferation of T cells in response to normal host-derived Mφ, further reduction of T cell stimulation with mAb treated TBH Mφ was not observed. If reduced numbers of Ia molecules per Mφ had been responsible for reduced MLR stimulation with TBH Mφ one would expect to see further reduction by blockade of the existing Ia molecules with anti-Ia. Since mAb did not reduce TBH Mφ stimulatory capacities, the idea of decreased Ia^+ cells was supported. This result also confirmed that the effector that was examined in the MLR was the Ia molecule since anti-Ia blocked the stimulation of T cells by normal Mφ.

Another factor which could not be overlooked was the suppressive role that PGE_2 might have in reduced MLR. PGE_2 production by Mφ is higher in Mφ derived from TBH (118, 200, 288) and could account for decreased T cell stimulation, by increased suppressive. The addition of indomethacin to Mφ cultures reduces the production of PGE_2. By adding indomethacin to MLR that contain Mφ, PGE_2 induced suppression was avoided. Comparison of MLR with and without PGE_2 demonstrated that PGE_2-mediated suppression was not a factor in these experiments, since no differences in T cell proliferation were observed in either normal or TBH-derived Mφ.

The hypothesis of reduced Ia^+ cell numbers in Mφ derived from TBH is consistent with work by others who demonstrate that Mφ Ia expression is transient (22) and is controlled by PGE_2 and IFN (23, 204). Since reports (230) show that PGE_2 can cause a reduction in Ia expression even in the presence of IFN (a molecule that normally increases Ia expression), it might be hypothesized that although PGE_2 did not alter *in vitro* MLR, it may play a role *in vivo*. PGE_2

is known to be an immunosuppressive factor *in vivo* during tumor growth and part of immune hyporesponsiveness might be due to PGE_2-induced changes in Mφ Ia expression. In human cancer patients similar decreases in HLA-DR antigens have been observed which supports our findings of reduced Ia (185). The mechanism by which neoplasia causes changes in Mφ Ia expression has been eluded to by Poutsiaka *et al.* (204, 205). In these studies a direct correlation between shed melanoma membrane vesicles, inhibition of IFN activity, and reduce Mφ Ia expression was determined. In further agreement with our study was the observation that the reduction in Ia expression was independent of PGE_2.

The consequences of alterations in Ia expression by Mφ were obvious in MLR stimulation which is an Ia-dependent phenomenon. However, the role of Ia antigens in other T lymphocyte reactions are controversial. For example, Con-A lectin stimulation of T cells can proceed only with Mφ or the Mφ-derived monokine, IL-1 (60). No direct correlation has been definitively shown between Ia antigen expression and accessory cell activities in this type of assay. In our hands, TBH-derived splenic Mφ were more capable accessory cells than normal host-derived splenic Mφ. This finding might represent an inverse relationship between Ia antigen expression Mφ and their ability to produce IL-1. Alternatively, it may also represent a requirement of Ia^+ Mφ for negative control of T cell proliferation. Although these hypotheses were not pursued by this project, others have shown that in fact an Ia^+ Mφ is involved in the induction of T suppressor cells during polyclonal stimulation with Con-A lectin (206). This implies that functional heterogeneity of splenic Mφ populations could demonstrate or explain specialized Mφ immunoregulatory functions.

To assess the hypothesis of functionally specialized splenic Mφ, anti-Mac mAb were once again employed with C to remove phenotypically distinct Mφ populations based on their expression of the Mac antigens. Initially, we examined the expression of these antigens by mAb binding and indirect immunofluorescence. This showed that changes occur in the splenic Mφ population with respect to Mac antigen expression. While Mac-1^+ cell numbers increased Mac-3^+ cell numbers decreased as a result of tumor growth. Although relating these changes in Mac antigen expression to Ia antigen expression is beyond the limits of this research, one might speculate and predict that an influx of Ia^- Mac 1^+ 3^- Mφ occurs in the spleen during the course of tumor de-

velopment. However, the direction of these studies was to relate immunoregulatory functions to antigen expression and the course of experiments did not correlate or expound on the connections between Ia expression and Mac antigen expression. Instead, the accessory role of Mac^+ or Mac^- Mφ was examined. Following depletion of WSC with anti-Mac-1 or -3, reductions in the response of the WSC to Con A were observed. This was to be expected since Mφ are implicated by some as the major accessory cell in these reactions (103, 119, 214). However, the incomplete removal of accessory function by these antibodies might reflect the existence of Mac^- cells which may also serve as accessory cells or it might reflect the incomplete removal of Mac^+ cells. Since percentage reduction in SAC numbers as a consequence of anti-Mac treatments corresponded with percentage of cells labeled with mAb, the idea of reduced or incomplete removal of Mac^+ cells was not an accurate assumption. This leads one to agree with the proposal of increased or decreased numbers of Mac^- cell types. If this were the case then negative selection of the Mac^- cell types could further delineate or assign Mφ accessory functions to antigenically defined phenotypes.

Dissection of the splenic Mφ populations into Mac^+ or Mac^- cell types required the Mφ population to be isolated, treated with anti-Mac mAb, and then added back to enriched T cell populations. To do this, Mφ were adhered to plastic and the remaining T cells were enriched for by nylon wool, G-10 sepharose filtration. Only normal host T cells were used to prevent introduction of tumor-induced T cell abnormalities. Thus, normal or TBH SAC (containing Mφ) were added to normal host T cells. The SAC populations were treated with each of the anti-Mac mAb or NRIgG plus C and resuspended to 4×10^4 Mφ/2×10^5 T cells. Changes that might be attributable to cell number were controlled by selecting SAC/T cell ratio established by others (212). The results of these experiments demonstrated that a $Mac\text{-}1^+$ SAC mediated a negative control cell in normal SAC populations but was the major accessory cell in TBH-derived SAC populations. In contrast, normal host $Mac\text{-}2^-$ SAC were incapable of accessory function in the normal host SAC populations but were very capable accessory cells in the TBH SAC populations.

Taking these results into the context of increased accessory function by splenic Mφ during tumor growth, one might speculate that the change in Mφ phenotypes observed during tumor growth are responsible for changes in regulatory activity. For example, $Mac\text{-}1^+$ cell numbers in-

creased in the spleen because of tumor growth, concomitant to anti-Mac-1 sensitivity of accessory cell function. This correlation indicated that Mac-1^+ Mφ accumulate in the spleen during tumor growth and serve as the dominant accessory cell to T cell proliferation. This line of reasoning is in agreement with other studies which show increased Mφ numbers in the spleen during tumor (79) and increased splenic accessory cell activity during tumor growth (73). In the present study, we have shown that these two events are connected, by using Mφ specific reagents.

Admixture of separately treated (anti-Mac treatments) SAC yielded results that are difficult to explain. For example Mac-1^- normal host SAC accessory functions could not be reversed by the addition of anti-Mac-2- or anti-Mac-3-treated SAC and thereby demonstrated that the Mac-2^- population was not suppressive. Therefore, we may assume that anti-Mac-2 treatment is removing a cell group from SAC populations. that is a positive regulator of T cell proliferation. A problem with this explanation is defined in work by Ho and Springer (117) which describes the splenic Mφ population as having very low or no Mac-2 expression. In this context, we found Mac-2 expression to be the lowest of all of the Mac antigens in normal SAC populations and also observed only modest lysis of SAC by the mAb plus C. A solution to this dilemma might be provided if a Mac-2^+ accessory cell exists in the SAC population but at very low cell numbers. In this situation accessory activity could be provided by small number of cells but go undetected in cell enumeration assays. An excellent example that might have some relevance in this phenomena is the dendritic cell. These cells are found in the spleen (72), have been putatively identified as adherent Mac-2^+,-3^+ cells, and are excellent accessory cells in other assays (80, 81). Since dendritic cells fulfill many of the requirements as the accessory cell in these experiments (72, 129), one might propose that not only changes in Mφ populations occur during tumor development but also changes in the dendritic cell populations. However, one important aspect of accessory cell function in lectin-stimulated T cell proliferation prevents an overall acceptance of this hypothesis. That is, dendritic cells do not produce IL-1. IL-1 has been shown by others to be the major accessory factor needed in T-cell proliferation (196). The only defense that can be made in light of this point is that IL-1 and Mφ may not be the only factor(s) that serves to support T cell proliferation. Despite the argument of what cell is responsible for the normal host SAC admixture results, it was obvious

from the TBH SAC admixtures that the observed shift in Mac-defined phenotypes and functions was accurate. Furthermore, the addition of Mac-3$^-$ SAC could only partially overcome the reduction in accessory function caused by anti-Mac-1 treatment and that Mac-2$^-$ cells were incapable of restoring Mac-1$^-$ accessory function. In this context, Mac-1$^+$ and Mac-2$^-$ cell populations may represent two phenotypical distinct populations that interact to maintain homeostasis. Alternatively, one can speculate that in the TBH, Mac-1$^-$ SAC may inhibit T cell proliferation and that this activity when selected for can overwhelm the supportive activities of either Mac-2$^-$ or -3$^-$ SAC.

In an effort to identify the monokines secreted by the various Mφ phenotypes, *in vitro* culture supernatants from anti-Mac plus C-treated Mφ were subjected to preliminary biochemical analysis. Protein analysis of anti-Mac plus C-treated peritoneal Mφ populations showed that an inverse correlation existed between quantity of secreted protein and inhibitory activity. This might represent autoregulation on the part of the Mφ, that is the inhibitor operates not only on T cells but also regulates the Mφ population from which it is derived. This possibility was also proposed by Connolly (48) and was based on studies in this laboratory. Since then other studies have either referred to or demonstrated this phenomenon (14). PGE_2 is produced by Mφ and exhibits similar characteristics (29, 144). As previously mentioned PGE_2 down regulates the expression of Ia antigens on the Mφ membranes (230). Therefore, this substance could be a factor in the regulation of both Mφ and T cells by Mφ culture supernatants.

Although we found the concentration of PGE_2 in these supernatants to be below minimum concentration of exogenous (commercial) PGE_2 treatments required to yield suppression in the MLR, synergistic effects between PGE_2 and other Mφ-derived factors could not be overlooked. Therefore, we have examined more closely the levels of PGE_2 present in cultured anti-Mac-treated Mφ cultures and found that a correlation existed between Mac-2$^+$ TBH Mφ and PGE_2 production. More specifically, if Mac-2$^+$ TBH Mφ are are moved by mAb + C treatment, PGE_2 production is negated. This finding correlated nicely with the loss of suppressor activity in TBH Mφ culture supernatants after anti-Mac-2 plus C treatment. Inasmuch as this phenomenon was not observed in normal host Mφ populations, it implied once more that immigration and maturation of Mφ into

the peritoneum is susceptible to tumor-derived alterations. To pursue the lead of PGE_2 involvement, anti-Ia plus C treatment was performed on splenic and peritoneal Mφ. What was noted, was an increase in PGE_2 production by enriched Ia^- Mφ populations. This observation was consistent with both splenic and peritoneal and normal and TBH Mφ populations. Therefore, connection between Mac-2^+ Mφ and PGE_2 may be paralleled by a connection with Ia^- Mφ. But at this time with only the present data this can not be explained.

It may be somewhat over ambitious to propose a model system for Mφ immunoregulatory circuits with respect to phenotype at this time, but a modest diagram was derived that seemed to fit the existing data. Figure 21 illustrates what might occur in normal peritoneal Mφ populations (panel A) as opposed to TBH peritoneal Mφ populations (panel B). In normal host, Mφ either already exist in the peritoneum (Mac-1^+, 3^+) or are elicited there by thioglycollate (Mac-1^+, 2^+). Within this population three types of regulation are described: (i) production of T cell enhancing monokines, (ii) production of PGE_2, and (iii) production of high molecular weight inhibitors (HMW INH) ($>$ 10,000 daltons). On the basis of the presented and ongoing research on these functions, the regulatory activities may be divided among three Mφ cell types: (i) Mac 1^+, 2^+ enhancer production, (ii) Mac-2^+ PGE_2 production, and (iii) Mac-1^+,-3^+ inhibitor production. All three of the parameters are capable of affecting T cell proliferation and provide immunoregulatory interactions among the Mφ sources. These interactions among the Mφ groups are essential to the overall regulatory effect imposed on the T cells because of a need for self regulation. Each side of the model is balanced so that changes in the production of one activity are controlled by the production of another. In this model a central Mφ is the transducer or the coordinator for the regulatory balance. The central factor in this control is PGE_2. The assignment of PGE_2 to this important post as a mediator of homeostasis is based on its pivotal role as an effector on Mφ (29) and T cells (94). More important is the assignment of Mφ phenotypes, which is based on sensitivity of normal host Mφ inhibitor production to anti-Mac-3 plus C treatment and the preliminary findings that anti-Mac-1 and -3 treatment enhanced PGE_2 production in normal Mφ populations.

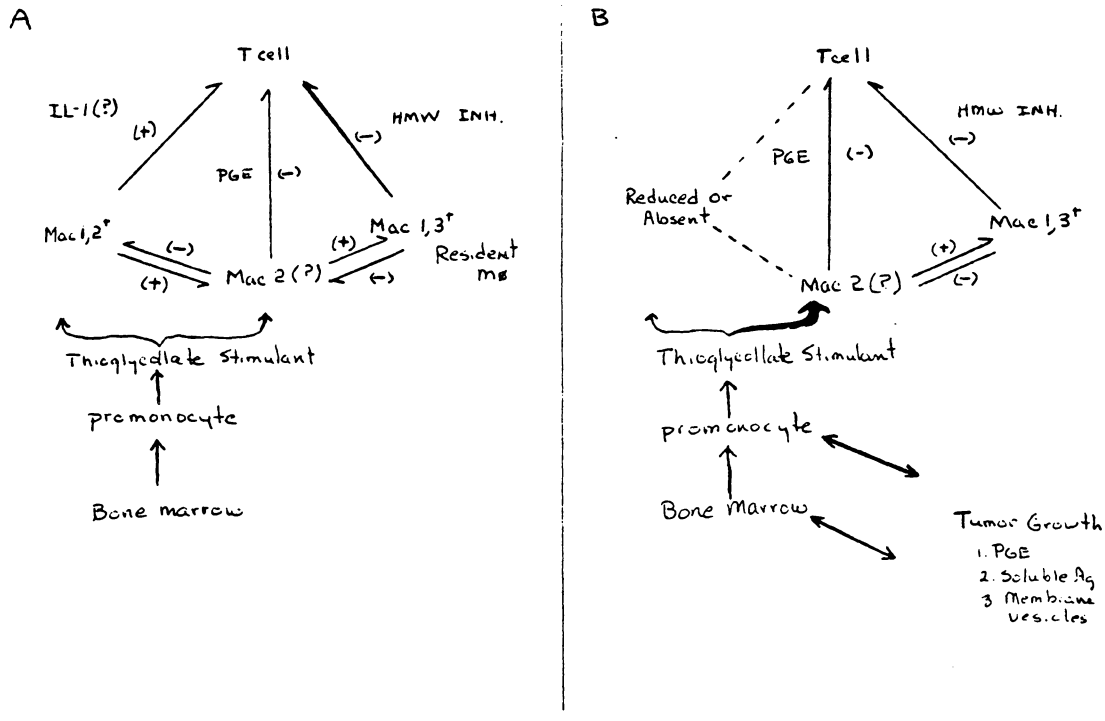

Figure 21. Model of Mφ subpopulation interactions: These diagrams illustrate a possible regulatory circuit in Mφ populations derived from normal (panel A) and TBH (panel B). The cells are represented by phenotype descriptions and the regulatory functions are shown as arrows. Positive (+) and negative (-) activities are designated for soluble factors that may be involved (such as, high molecular weight inhibitor [HMW INH]).

In the TBH model similar roles are proposed for PGE_2 and HMW INH, but one side of the model was omitted. The omission of this parameter was on the basis of the findings that peritoneal Mφ populations change during tumor burden and show a general increase in the percentage of Mac-3^+ Mφ, an increase in inhibitory activity in TBH Mφ-derived supernatants, and a decrease in the percentage of Mac-2^+ cells in TBH Mφ populations. In these contexts, aberrations in the regulatory abilities of TBH Mφ was viewed as a shift in peritoneal populations that "short circuited" the balance of the Mφ population.

The model may be tested in several ways, by hypothetically imposing the effect of anti-Mφ antibodies on the populations and subsequently deriving the overall immunoregulatory effects. For example, if normal Mφ were treated with anti-Mac-3 plus C, Mac-3^- Mφ were enriched. The observed results of this was decreased inhibitory activity. In this model the same results would be predicted based on the removal of the Mac-1^+, 3^+ producer of the HMW INH. Alternatively, recent results in our laboratory have shown that this event is also paralleled by an increase in PGE_2 production. The model accounts for this parameter by predicting that the Mac-1^+, 3^+ population negatively controls PGE_2 production and when these cells are removed increased PGE_2 production occurs. Anti-Mac-2 plus C treatment does not alter the inhibitory activity of normal host peritoneal Mφ and therefore might be best represented as a separate arm in the control circuit. By assigning a positive function to this subpopulation and connecting it to the Mac-3^+ population by positive interactions via a PGE_2 producing cell then an account can be made for the observed inhibitory activities in these supernatants.

In the TBH model (Fig. 21, panel B) the reductions in the Mac-2^+ enhancing populations may account for the extreme susceptibility of PGE_2 production to anti-Mac-2 plus C treatment. Under most depletion and enrichment conditions complete removal of cell groups is not observed, therefore the effects that are seen generally represent enrichments of cells and their functions by selecting against (or reducing) other populations. Therefore, treatment with anti-Mac-2 plus C may have served to enrich for Mac-1^+, 3^+ Mφ which produce the HMW INH and negatively regulate the remaining PGE_2 producers. Since PGE_2 production was negated by this treatment and because suppressor activity in the supernatants was maintained, this line of reasoning seems plausible. If

tumor growth causes changes in Ia^+ Mφ percentages (79), it is feasible that changes in Mac antigens may also occur.

These hypotheses may also be applied to other observations made in the course of this study. For example, if Mac-2^+ Mφ are the major cells elicited to the peritoneum by thioglycollate injection, then the observation that there are more peroxidase-positive (immature) Mφ in TBH population and fewer Mac 2^+ Mφ agrees with the proposal of an alteration in a Mac 2^+ regulatory cell. Other researchers (146, 163, 184, 187, 204, 205) have described mechanisms and tumor factors which alter immunocyte differentiation and maturation. In light of these reports, a tumor-derived factor or effect may cause alterations in the migration and/or maturation of pro-Mφ immigrating to the peritoneum. Furthermore, the assignment of Mac-antigen phenotypes to certain regulatory functions can be applied to observations in splenic Mφ. Examination of SAC showed that removal of Mac-2^+ SAC negated the accessory function of normal SAC populations but was only partially effective on TBH SAC populations. In the TBH, Mac-1^+ SAC were the predominant accessory cell. Thus, as in the peritoneal populations, simultaneous changes in Mφ phenotype and function were seen as a consequence of tumor growth.

Without a doubt this study has both answered and generated questions about Mφ diversity, Mφ function, and immunological effects of tumor growth. In response to the initial questions that prompted this study we can say that: (i) Mφ antigen expression (phenotypes) change during tumor growth, (ii) Mφ immunoregulatory functions change as the tumor grows, and (iii) that changes in Mφ phenotype and function are correlated. A summary of these changes in Mφ phenotype are provided in Table 9. However, these findings did not explain "how" the event of tumor growth caused these change, nor did the research expound on the interactions between different Mφ sub-populations. In response to these ideas it would appear that the direction of future studies in this area should pursue some of the following lines of investigation.

1. Is the expression of Mφ antigens (Mac-1, -2, and -3) susceptible to known immunomodulators of Mφ function such as PGE_2 and interferon λ? This could be accomplished by exposing either normal or TBH-derived Mφ to PGE_2 or interferon *in vitro* and measuring Mac-antigen

Table 9. Summary of Changes in Peritoneal-derived Mφ During Tumor Growth.

	Mφ Antigen							
	Normal				TBH			
	Ia	Mac-1	Mac-2	Mac-3	Ia	Mac-1	Mac-2	Mac-3
Change in 1st Mac Ag expression	--	--	--	--	↓	NC[b]	↓	↑
MLR suppression after mAb treatment	↑	↓	NC	↓	↑	NC	↓	NC
Px activity after mAb treatment	ND[c]	NC	NC	NC	ND	↓	NC	↓

[a]Condition of host.

[b]NC refers to no change.

[c]ND refers to not done.

expression by mAb binding and indirect immunofluorescence. Other studies might use populations of WSC that are stimulated by Con-A and examine the expression of Mac-antigens on Mφ that are removed from the WSC populations after stimulation. The premise for this experiment lies in the relationship between λ-interferon-secreting T cells and Mac-antigen expression.

2. Positive collection of functionally diverse Mφ subpopulations from normal and TBH is now possible with the availability of fluorescence activated cell sorting (FACS). Isolation of subpopulations that are implicated by the present study could lead to homogeneous Mφ populations and thereby augment the collection, purification, and identification of Mφ-derived monokines.

3. Finally, because tumor growth was the implied source of modulatory effects on Mφ, it would be appropriate to examine more closely the cause of these effects. The use of promonocytes from the bone marrow is now a routine method for generating mature Mφ. The extraction and then culture of bone marrow cells in the presence of TBH-derived body fluids could reveal the presence of tumor-derived modulators of Mφ differentiation. Briefly, one could obtain normal host promonocytes and culture them *in vitro* with or without TBH factors and subsequently define the Mac-antigen phenotypes of the cultured cells. This line of study could also be followed *in vivo* where the same maturation could be initiated in the peritoneum of either normal or TBH. Normal or TBH pro-monocytes could be added to the peritoneum of normal hosts. At various time periods the peritoneum could be lavaged and the populations examined for their expression of Mφ-surface antigens. Perhaps in this way the effect of TBH could be identified and chemotherapeutic agents could be used to counteract its effect on the immune system.

Because proposal number 3 is rather involved, a guideline for possible protocols is given below:

Part I. *In vitro* manipulation of Mφ populations is performed to identify the modulatory capability of TBH-derived factors and immunomodulatory agents. Bone marrow cells

from normal and TBH are cultured in the presence of CSF-1 and evaluated for Mac-antigen expression, endogenous enzyme activities, and regulatory functions.

Part II. *In vivo* manipulation of Mφ populations is performed to assess the environmental factors in TBH. Bone marrow cells from normal mice will be injected into the peritoneum of normal and TBH. The resulting populations after various time periods is examined for the previously described parameters.

Part III. *In vivo* control of TBH Mφ maturation is assessed with various immunotherapeutic agents. For instance those agents which were useful *in vitro* would be of primary interest.

Although the direction of these new proposals diverges from the dogma of phenotypical characterizations in defined Mφ populations, it is aimed at defining the cause of aberrations in Mφ phenotypes and function during tumor growth. By understanding the course of natural events occurring in immune system during cancer growth the immunological role of the Mφ may be clarified. Finally, armed with the knowledge gained by trial and error the enigma of immune control may be unlocked, perhaps only to find another impenetrable barrier.

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Appendix 1

Chromium Cytotoxicity

1. Obtain a single cell suspension of Mφ.

2. Resuspend cells to 4 x 10^6 cells/ml in RPMI medium. Add enough ^{51}Cr to yield 100 μCi/10^7 cells. Incubate cells for 1 hr at 37°C with occasionally gentle mixing (we used 5000 μCi/ml).

3. Centrifuge at 500 x G for 5-10 min and rinse cells 3-4 times or until ^{51}Cr release is absent.

4. Recount cells by trypan blue exclusion using 'hot' hemocytometer and resuspend cell suspension to 2 x 10^6/ml (or desired concentration). Aliquot in 50 μl portions to a microtiter plate (2 x 10^5/well).

5. Add various mAb and/or C combinations to each well and incubate for 60-90 min. Add a 1% solution of the detergent Triton-X 100 to one group of samples to give total lysis.

6. Remove supernatant from cells with a Titertech cytotoxicity sampler.

Appendix 2

Mφ Collection

1. Inject 4-6 mice with 2 ml of sterile thioglycollate intraperitoneally.

2. Four days after injection kill mice by cervical dislocation and lavage with cold (10°C) RPMI medium with Fungizone (1 ml/100 ml medium). Use 25 ml per mouse with a 10cc syringe and 18-20 gauge needle.

3. Spin at 500 x G for 5-10 min. Resuspend pellets into a single 50 ml centrifuge tube and bring volume up to 50 ml with cold RPMI medium. Repeat the centrifugation and wash procedures.

4. Plate peritoneal cells at approximately 1 mouse per plate (i.e., if 4 mice were used bring volume in tube to 40 ml and plate on 4 large plastic dishes.

5. Plating time may vary but should proceed for at least 3 hr to yield maximal numbers of Mφ.

6. Remove nonadherent cells by washing plates with (4) 10 ml volumes of warm 37°C RPMI medium.

7. After final washing step cover plate with 10 ml of cold (10°C) RPMI medium. Scrape plates with a rubber policeman and rinse off dislodged cells with 20 ml of cold RPMI medium.

8. Centrifuge the Mφ at 500 x G and pool the pellets into a single tube. Count the viable cells by trypan blue exclusion (25 µl of cells, 100 µl of trypan blue and 375 µl of Isoton).

9. The collected Mφ may be treated with antibody and used directly in add back experiments or for Mφ supernatant production. For treatments, add 1 mg of antibody to 10^7 Mφ with or without 1:12 dilution of low tox rabbit C. Incubate the cells for 90 min at 37°C, collect, and rinse with RPMI medium 3 times.

10. Count the final cell preparation and resuspend the cells to 4 x 10^6 Mφ/ml. From these cell culture supernatants can be produced or the cells can be diluted and added back to T cell proliferation assays.

Appendix 3

Western Blot Analysis of Mφ Cell Lysates

1. Collect 4 x 10^7 - 1 x 10^8 Mφ and resuspend them in 1 ml of PBS. Add 100 µl of 10% SDS to yield 1% SDS. Sonicate the cell lysate for about 30 sec to fragment the DNA. The solution should now be less viscous. Store the solution at -80°C with PMSF protease inhibitor.

2. When ready for electrophoresis, thaw the samples and add 1 part sample to 5 parts acetone. Store the samples on ice for 30 to 60 min and then spin them at 10,000 x G for 30 min in the cold. Decant the supernatant and set the tubes in the flow hood to allow for evaporation of the remaining supernatant.

3. Resolubilize the samples in 100-200 µl of PAGE sample buffer (1x). This should yield a (10-20 x) concentrated cell lysate. Go through normal gel preparation procedures such as boiling and add 5 µl of Pyronin Y to each lysate. Electrophorese the samples into a 10% polyacrylamide gel.

4. Remove the gel after Pyronin Y has almost reached the bottom. Place the gel onto a piece of pre-wet nitrocellulose and force out the air bubbles between the gel and the paper by rolling a test tube across the gel. Assemble the supports and immerse the sandwiched gel-paper unit into the transfer buffer (80% methanol and 20% Tris buffer [pH 8.0]). Make sure that the paper is on the positive side of the transfer unit. Run the transfer overnight at 200 mA or in 2 hr at 900 mA.

5. Remove the nitrocellulose from the gel and cut into 2 parts. One part is stained in amido black for 20 min, the other is immersed in 20% milk/TBS (pH 7.4) for 1 hr. Rinse off the milk with 3 washes of TBS and resuspend nitrocellulose in anti-Mac antibody for 1 hr (with constant shaking). Rinse off the unbound antibody with 3 washes of TBS and finally add the labeled antibody (1:500 dilution). Allow the peroxidase-labeled anti-Rat antibody to react for 1 hr with the nitrocellulose and then wash 3 times with TBS. React the washed gel with 4-chloro-1-napthol reagent and stop color reaction when proper intensity is reached by washing with distilled H_2O.

Appendix 4

Fluorescence Analysis of Mφ

1. Obtain 4 x 10^7 Mφ and wash them thoroughly with RPMI medium. Divide the cells between the number of tests required by using at least 8 x 10^6 Mφ per sample. React the cells with 1 ml of hybridoma supernatant (unconcentrated antibody) or dilute concentrated antibody 1:10 in PBS. Allow the cells to bind antibody for 45 min in the cold (4°C).

2. Wash the labeled cells with PBS (pH 7.4) 3 times, by centrifugation and resuspension. To the washed cells add 1 ml of Rhodamine-labeled anti-rat IgG antibody (Fab' fragment). Incubate the cells in the cold for 30 min and consequently wash them again 3 times with ice cold PBS.

3. Count the cells and resuspend them to at least 2 x 10^6 cells/ml in PBS. Apply 200 µl of the cell suspension to a clean microscope slide and allow it to air dry (this works faster in the flow hood). After the slide is dry, fix the cells to the slide by immersion

in ice cold methanol for 5 min. Once the slides are removed allow them to dry and overlay with 50% glycerol/PBS and store for later viewing.

Appendix 5

(Alternate) Fluorescence Procedure

1. Obtain 4 x 10^6 Mφ/ml from each source and apply 100 µl to the surface of a microscope slide. Allow the cell smear to air dry and then fix the cells in ice cold methanol for 5 min. The fixed smears can be stored at 4°C or treated right away for viewing.

2. For anti-Mac treatment, immerse the cell smears into a 50 ml tube containing anti-Mac mAb (100 µg/ml). After 30 min in the antibody remove and rinse by immersion in 3 other tubes with fresh RPMI medium.

3. Repeat the previous procedure with the second antibody Rhodamine-labeled anti-rat IgG (Fab' portion) and wash again in RPMI medium.

4. If cell smears are to be stored, cover each slide with 50% glycerol/RPMI medium and place in the refrigerator.

Appendix 6

Cell Culture and Monoclonal Antibody Preparation

1. Hybridoma cells producing anti-Mac-1, -2, and -3 were purchased from ATCC. Frozen cells were quickly thawed in a 37°C water bath. The vial was etched with a diamond pencil and was thoroughly with 70% ethanol.

2. The vial of cells was opened aseptically in the hood and the cell suspension was placed into two small 3 x 5 cm flasks. The one ml cell suspension in each flask was brought to 2.5 ml by the addition of 1.5 ml of 20% FCS in RPMI medium (also containing 10^{-5} M β-mercaptoethanol). The growth vessels were angled such that the cell suspension was kept at one end of the flask.

3. The cells were cultured at 37°C in a CO_2 incubator and were left undisturbed for 5 days. The cells were kept in fresh 20% FCS/RPMI medium by adding 1 culture volume of 20% FCS/RPMI medium every 3-5 days. Once the flask volume reached 6 ml, one half of the culture was removed and a new flask was started.

4. Once 100 ml of cell suspension was obtained in culture flasks 50 ml of the suspension was used to start a roller bottle culture of the hybridoma cells.

5. Monoclonal antibody containing supernatants were harvested from roller bottles by removing 3 culture supernatants. The hybridoma cells were removed by centrifugation at 500 x G for 5 min. The remaining particulate material was removed by centrifugation at 10,000 x G for 30 min and filtration through at 0.22 μm filter. Supernatants were stored at -80°C until used.

6. Concentration of the mAb from the culture supernatants was performed by precipitation of protein in 45% ammonium sulfate (pH 7.4). The precipitate was allowed to collect overnight at 4°C and later collected by centrifugation at 10,000 x G for 30 min. The precipitate was resuspended in a minimal volume of 0.05 M Tris buffer (pH 8.0) to yield a 10-20 X concentrate. Remaining salts were removed by dialysis of the preparation against two 2 liter volumes of Tris pH 8.0.

7. Monoclonal antibody preparations were further purified by anion exchange chromatography. Ten ml of the antibody preparation was placed onto 30-40 ml of DEAE-cellulose that had been previously equilibrated with 0.05 M Tris buffer pH 8.0. Once the antibody preparation had completely entered the column elution of unbound protein was started with 0.05 M Tris buffer pH 8.0. After 100 ml of Tris buffer pH 8.0 had entered the column, elution with 100 ml of 0.05 M Tris buffer containing .05 M NaCl was performed and was followed by elution with 100 ml of 0.05 M Tris with 0.1 M NaCl. The eluant was collected from all of the washes in 100 3 ml fractions. The fractions were stored covered at 4°C.

8. Analysis of the fractions for the presence of mAb was performed by Ouchterlony tests and ELISA. Once the antibody containing fractions were identified, they were pooled and the protein was precipitated in 60% ammonium sulfate (at 4°C overnight). The

precipitates were collected by centrifugation at 10,000 x G for 30 min and solubilized in RPMI medium (to yield 10-20 times the whole culture supernatant concentration). The concentrated antibody solutions were finally dialyzed against two 2 liter volumes of RPMI medium, filter sterilized and stored at -80°C.

9. Previous column elution patterns can be used to predict where a particular mAb is located. Anti-Mac-1 and -3 elute at the end of the 0.05 M NaCl wash and anti-Mac-2 elutes at the end of the 0.0 NaCl or the beginning of the 0.05 M NaCl wash.

10. Concentrations of mAb are determined by Mancini diffusion. One hundred µl of anti-rat IgG antibody are added to molten agarose (45°C). After hardening wells are cut into the agarose and either standardized IgG samples are added or the unknown samples are added. By computing a standard curve based on diffusion pattern diameters, the unknown quantities can be estimated.

Appendix 7

Cellular ELISA

1. Obtain 1 x 10^7 Mφ or P388 D_1 cells and wash with cold PBS. Adhere the cells to flat bottom microtiter plates at a concentration of 5 x 10^4-1 x 10^5 per well by incubating the cells at 37°C for 3 hr on the plastic plates. The time can be shortened by initially spinning the plates at 500 x G. After adherence carefully decant the supernatant and allow them to air dry.

2. Dried cell suspensions can be fixed to the plastic by added either cold ethanol or methanol to the wells for 15 min. After fixing the cells to the plastic, the plates should be flicked to remove the fixative. One hundred µl of 9:1 glycerol/RPMI plus 100 µl of 10% FCS and 0.03% NaN_3 are added to each well.

3. Antigen or antibody detection is performed by removing the overlay and adding 100 µl of various anti-Mac antibody dilutions. Incubate the plates for 1 hr at 4°C and then flick out the antibody. Rinse the plates three times (5 min each) with PBS (no tween). Repeat the antibody binding procedure with peroxidase-labeled anti-rat IgG.

4. Rinse out the second antibody as was done with the primary antibody and prepare the substrate. ABTS 1 μg/ml of Citrate buffer (pH 4.0) with 0.05% H_2O_2 is applied to each well (100 μl). The reaction is read on the ELISA reader at 600nm.

Appendix 8

T Lymphocyte Proliferation Assays (MLR)

1. Need at least 3 BALB/c mice for responder cells and possibly 5 C3H mice for stimulator cells.

2. Heat inactivated 4 tubes (10 ml ea) of FCS 30 minutes (if frozen allow to thaw and then time the heat inactivation). Warm two bottles of RPMI medium to 37°C. Begin soaking nylon wool columns with warmed RPMI medium. Place warming nylon wool columns in 37°C incubator.

3. While columns are warming cervicaly dislocate the mice and remove their spleens. Homogenize the spleens in a wire sieve and resuspend the cells in a 50 ml centrifuge tube. Centrifuge the cells at 500 x G for 5-10 min.

4. Resuspend the cells in 8.7% NH_4Cl to remove the RBC. Centrifuge immediately at 500 x G for 5 min and resuspend in 20 ml of RPMI medium with 5% FCS. Wash

cells twice more with RPMI medium and plate the cells onto plastic dishes (1 plate: mouse).

5. After 2 hr of plating remove the nonadherent cells, centrifuge, resuspend in RPMI plus 5% FCS medium and place them onto a washed nylon wool (37°C) column. Place the column back in the 37°C incubator for 45 min. Rinse the adherent cells twice with warm RPMI medium and remove by scraping. Resuspend the dislodged cells in RPMI medium and place them in ice (to be used if desired in mAb treatments and or add back to T cell cultures).

6. After 45 min incubation remove the nonadherent cells from the nylon wool column, centrifuge and place resuspended cells onto the G-10 Sephadex-Glass bead column. Incubate at 37°C for 45 min.

7. After 45 min drain and wash the column and centrifuge the nonadherent cells. Re-suspend the cells and count with Coulter counter. Record values for responder cells and remove 1 ml aliquot for preparation of inactivated R responder control cells.

8. Separately resuspend an aliquot of responders and all of stimulator cells to 1 x 10^7 cells/ml and treat with 25 µl of mitomycin C per 10^7 cells. Incubate at 37°C for 20 min. Immediately wash the treated cells 3 times with RPMI + 5% FCS medium. Recount the treated cells and resuspend them to 8 x 10^6 cells/ml in RPMI + 5% FCS medium.

9. Add 50 µl of stimulators (4 x 10^5 cells) to each well in a microtiter plate (except for 4-6 wells to which 50 µl of the treated responder cells are added). Add 50 µl of the untreated responder cells to each well (2 x 10^5 cells/well). Complete the wells by adding 50 µl of test sample (monokines or Mφ cells) and 50 µl of RPMI medium with 10% FCS and 10^{-5} M β-mercaptoethanol.

10. Incubate the plates for 4 days at 37°C. Six hr prior to harvesting add 50 µl containing 1 µCi of tritiated thymidine per well.

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