Macrophage Tumor Killing: Influence of the Local Environment

Abstract. *Tumor killing by activated macrophages is not a highly determined biologic event, but a relative capability influenced by the local environment. An intrinsic macrophage cytotoxic effector system is modulated by serum and other environmental factors that can either enhance or suppress tumor killing. Activated macrophages kill tumor cells only when a regulating threshold drops to a critically low level.*

Activated peritoneal macrophages from mice with chronic infections with bacillus Calmette-Guérin (BCG) or *Toxoplasma gondii* can selectively kill syngeneic, allogeneic, or xenogeneic tumor cells in vitro by a nonphagocytic mechanism that requires intimate contact (*1*). Normal peritoneal macrophages or macrophages stimulated by intraperitoneal injection of 2 ml of 10 percent peptone broth into normal mice 72 hours before macrophage harvest are not tumoricidal. We report here that tumor killing by activated macrophages can be modulated by factors in the environment such as serum factors, macrophage activating factors, endotoxin, and proteinases. (i) Activated macrophages from mice with chronic infection generally kill tumor cells when the culture medium contains fetal bovine serum (FBS), but they do not kill when the culture medium contains other types of serum. Other studies (*2*) show that serum contains separate factors capable of either inhibiting or enhancing tumor killing by activated macrophages. (ii) Mice with chronic BCG or toxoplasma infection have a continuing stimulus for the production of tumoricidal activated macrophages as a result of high levels of macrophage activating factor (MAF) produced by the interaction of sensitized lymphocytes with persisting specific microbial antigens in the tissues (*1*). In this study we show that activated macrophages from BCG- or toxoplasma-infected mice kill tumor cells in all serums if low concentrations of MAF-containing serum from BCG-infected mice are added to the culture medium. Also, when exposed to high concentrations of MAF, stimulated macrophages in all serums kill tumor cells, while normal macrophages tested under identical conditions do not. (iii) Small quantities of endotoxin (1 to 5 ng/ml, and in some experiments as little as 10 pg/ml), will make activated macrophages kill tumor cells in all serums tested. These low amounts of endotoxin do not make normal or stimulated macrophages kill tumor cells. (iv) Prior treatment of activated macrophages with trypsin inhibits tumor killing induced by FBS, MAF, or endotoxin.

Macrophage tumor killing is affected by chemical signals from two separate environments: first, host factors that influence the activated macrophage tumor-killing potential before they are harvested from mice with chronic infection, and second, serum factors that further modulate activated macrophage tumor killing in vitro. Individual experiments were carried out with the same group of activated macrophages in culture medium containing either 10 percent FBS or 10 percent adult bovine serum (ABS) (Fig. 1). Experiments conducted over a period of several years yielded three patterns of results. (i) Activated macrophages killed 3T12 cells in culture medium containing 10 percent FBS but not 10 percent ABS (> 85 percent of experiments). (ii) Activated macrophages killed 3T12 cells in culture medium containing both types of serum (< 10 percent of experiments). (iii) Activated macrophages did not kill tumor cells in either type of serum (< 5 percent of experiments). These three patterns suggest that modulation in vivo by host factors resulted in the production of activated macrophages with different potentials for tumor killing. In addition, the first pattern of interaction suggests that serum factors also modulate and can be the determining influence in the expression of activated macrophage tumor killing. The modulation of toxoplasma-activated macrophage tumor killing by serum is shown in Fig. 1, rows A and G. In row A, the culture medium contained 10 percent ABS and the 3T12 cells grew up to a thick multilayer over the activated macrophages. In row G, the culture medium contained 10 percent FBS, and activated macrophages from the same group of toxoplasma-infected mice destroyed 3T12 cells. These results suggest that the intrinsic macrophage cytotoxic effector system is modulated by serum factors and is primed or perhaps triggered when a regulating threshold reaches or drops below a critically low level. Furthermore, if this tumoricidal threshold remains above the critical level, activated macrophages do not kill tumor cells.

Macrophage activating factor (*3*) and endotoxin (*4*), two factors known to induce nonspecific tumor killing by macrophages, were next examined separately and in combination to determine their ability to make macrophages kill tumor cells in 10 percent ABS. (i) For MAF alone, we used a technique developed by Salvin *et al.* (*5*) to obtain control serums and to obtain serum with a high level of MAF activity from BCG-infected mice. Experiments showed that the control serums had no MAF activity, but that toxoplasma- or BCG-activated macrophages killed tumor cells in ABS if MAF-enriched serum (0.005 to 2.0 percent, by volume) was added to the culture medium. Small amounts of MAF (0.05 percent) did not make stimulated or normal macrophages kill tumor cells (Fig. 1, row B). However, a higher concentration of MAF (1.0 to 2.0 percent) in the culture medium made activated and stimulated but not normal macrophages kill tumor cells (Fig. 1, row C). Normal macrophages from CBA mice developed responsiveness to MAF after 72 hours of culture in vitro in medium with 10 percent ABS. If exposed to MAF (1.0 percent) at this time, they became tumoricidal in a manner similar to stimulated macrophages elicited in vivo by 10 percent peptone. (ii) Picogram or nanogram quantities of endotoxin alone made BCG- or toxoplasma-activated macrophages kill tumor cells when the culture medium contained 10 percent ABS (Fig. 1, rows D and E). These quantities of endotoxin alone did not make stimulated or normal macrophages kill tumor cells. (iii) When MAF and endotoxin were used together, we found that the small amounts of MAF (not effective alone) were now sufficient to make stimulated macrophages kill 3T12 cells (Fig. 1, row F), suggesting a synergistic effect of endotoxin and MAF in making stimulated macrophages tumoricidal. The mechanism of action of endotoxin is unclear. It may induce activated macrophages to kill tumor cells by amplifying MAF; it may itself produce an MAF-like signal in the macrophage membrane; it may induce a few contaminating B lymphocytes in the macrophage monolayer to secrete a lymphokine with MAF activity (*6*); or it may act by a combination of these actions.

We next examined the effect of other adult, newborn, and fetal serums on activated macrophage tumor killing. Results showed that BCG- or toxoplasma-activated macrophages were not cytotoxic for tumor cells or were only inconsistently so when cultured in medium with 10 percent human, mouse, rat, guinea pig, rabbit, swine, goat, horse, chicken, human umbilical cord, newborn rat (collected within 4 hours of birth), or fetal rabbit serum. However, as was the case with ABS, small quantities of MAF (0.005 to 0.1 percent) or endotoxin (0.5

to 5.0 ng/ml) induced BCG- and toxoplasma-activated macrophages to kill 3T12 cells in all of these serums. There was no difference in the amount of MAF or endotoxin needed to make activated macrophages tumoricidal in fresh unheated or heat-inactivated (56°C, 30 minutes) guinea pig or mouse serum, suggesting that complement activation is not a critical factor in the modulation of tumor killing. In addition, under the conditions we used, the presence or absence of plasmin in the tissue culture environment did not appear to be responsible for the modulation of tumor killing by activated macrophages. In cytotoxicity assays with a serum whose plasminogen is not converted to plasmin by mouse plasminogen activator [chicken serum (*7*)], the results were comparable to assays with serums where plasmin is formed (mouse and human serums). However, prior treatment of activated macrophages with a related serine proteinase, trypsin (Worthington, 209 units per milligram), raised the threshold for induction of tumor killing. This suggests that under certain physiologic or pathologic conditions, plasmin may affect tumor killing by macrophages. Tumor killing is completely inhibited, and the 3T12 cells grow to a thick multilayer over the trypsin-treated macrophages, if activated macrophages are treated for 2 hours with trypsin (50 μg/ml) in serum-free medium and then cultured in medium containing 10 percent FBS or 10 percent ABS plus low concentrations of MAF (0.005 to 0.5 percent), or 10 percent ABS plus low concentrations of endotoxin (0.5 to 25 ng/ml). However, if the same procedure is followed with a higher concentration of trypsin (500 μg/ml), tumor killing induced by all concentrations of MAF is completely inhibited and only the highest concentrations of endotoxin (500 ng/ml) induce a partial tumoricidal effect. Macrophage monolayers were greater than 95 percent viable after a 2-hour incubation period with trypsin as determined by trypan-blue exclusion. These observations show that trypsin, like a serum factor (*2*), can raise the threshold for activated macrophage tumor killing. Trypsin probably raises the tumoricidal threshold by removal of macrophage membrane receptors for activating or regulating chemical signals, or both, while the serum factor raises the tumoricidal threshold by a different mechanism (*2*).

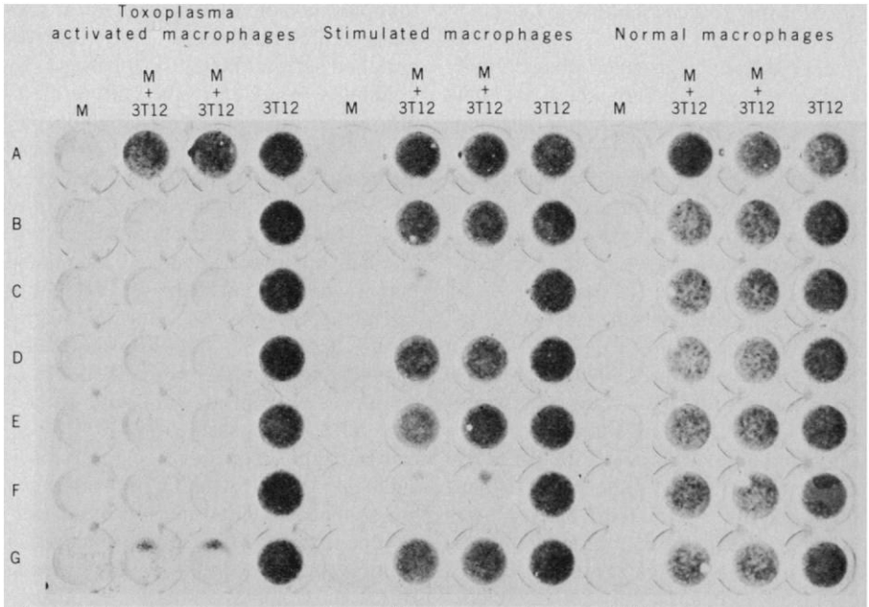

Fig. 1. Modulation of macrophage tumor killing by serum factors, MAF, and endotoxin. Female mice (strain ICR) were used in these studies. Peritoneal cells were harvested from normal mice, peptone (Difco)-stimulated normal mice, or mice with chronic infection (*1*). Peritoneal cells were collected 17 to 22 days after infection with the Paris strain of BCG (0.2 mg, intraperitoneally) or 8 to 20 weeks after infection with the C56 strain of *Toxoplasma gondii* (*1*). Peritoneal cells (4×10^5) in 0.1 ml of Dulbecco's modified Eagle medium supplemented with 1.0 g of glucose per liter, 0.02*M* Hepes buffer, streptomycin (100 μg/ml), and penicillin (100 units per milliliter) (DMM) were added to chambers of microtiter plates (Falcon 3040). After 1 hour at 37°C in a humidified atmosphere (5 percent CO_2 in air) to allow for adherence of macrophages, each chamber was washed three times with 0.15*M* NaCl to remove nonadherent cells with the use of a sterile Pasteur pipette and gentle vacuum suction. Tumorigenic 3T12 cells (6×10^3 per chamber) were added in 0.1 ml of DMM and allowed to adhere for 30 minutes. An additional 0.1 ml of DMM with 20 percent of the indicated serum (to give a final concentration of 10 percent serum in DMM) was then added with or without MAF or endotoxin (*Escherichia coli* 0128:B12, Sigma). The microtiter plates were incubated for 60 hours; then the cell preparations were fixed in methanol and stained with Giemsa. Serum containing MAF was obtained as follows. Mice were inoculated intravenously with 0.2 mg of BCG; after 2 weeks they were given 0.2 ml (intravenously) of old tuberculin (Jensen-Salsbery) (*5*) and exsanguinated 3 hours later. Control serum was obtained from mice infected with 0.2 mg of BCG (intravenously) 2 weeks earlier but not inoculated with old tuberculin prior to exsanguination as well as from normal uninfected mice that were inoculated intravenously with 0.2 ml of old tuberculin and exsanguinated 3 hours later. Serums from all animal species used in these studies were collected by sterile technique. Chloroform-extracted portions of each lot of serum were tested by limulus amoebocyte lysate assay (*15*). A lot was not used in the cytotoxicity assay unless it was endotoxin-free (FBS obtained commercially is invariably positive in the limulus assay for endotoxin). Serum was inactivated by heat (56°C for 30 minutes). The figure is a low-power photograph of a Giemsa-stained microtiter plate. Row A, 10 percent ABS; row B, 10 percent ABS with 0.05 percent MAF; row C, 9 percent ABS with 1.0 percent MAF; row D, 10 percent ABS with endotoxin (0.5 ng/ml); row E, 10 percent ABS with endotoxin (5.0 ng/ml); row F, 10 percent ABS with 0.05 percent MAF and endotoxin (5.0 ng/ml); row G, 10 percent FBS. Columns M, M + 3T12, and 3T12 represent chambers with macrophages alone, macrophages plus 3T12 cells, or 3T12 cells alone, respectively. The 3T12 cells are stained darkly and macrophages are not discernibly stained. The experiments in this microtiter plate were prepared at the same time with the same pool of each type of macrophage and the same dilution of each additive. Tumor cells (6×10^3) added in 0.1 ml of DMM attach evenly to the chamber surface. When stained immediately after attachment, 33 to 37 cells are seen per microscopic field at ×300. At the end of the 60-hour assay period, three or fewer 3T12 cells are seen per field (at ×300) in all chambers which show gross evidence of macrophage-mediated cytotoxicity. In several experiments in which macrophages were induced to kill tumor cells, there is an area of localized 3T12 cell growth at the 12 o'clock position. The Pasteur pipette used to wash the macrophage monolayer by gentle suction prior to adding 3T12 cells was inserted here, producing a localized decreased density in the monolayer sufficient to permit circumscribed progressive growth of 3T12 cells. However, in the rest of the chamber where the macrophage monolayer was not disturbed, the cytotoxic effect was marked; that is, three or fewer 3T12 cells remain per field. Growth of 3T12 cells in an area of decreased macrophage density occurred in chambers where the stimuli that induced tumor killing were relatively weak.

We also evaluated the effects of several classes of synthetic low molecular weight inhibitors of proteolytic and esterolytic activity on the modulation of tumor killing by stimulated or activated macrophages using three different procedures. (i) Macrophage monolayers were exposed to the desired dose of synthetic inhibitor in serum-free culture medium during a 2-hour incubation period prior to exposure to target cells, complete culture medium, and other additives. (ii) Macrophage monolayers were exposed to the synthetic inhibitor during a 2-hour incubation period in serum-free culture

medium, the inhibitor also being present in the complete culture medium during the cytotoxicity assay. (iii) The 2-hour incubation period was omitted, but the synthetic inhibitor was maintained in the complete culture medium throughout the cytotoxicity assay.

Regardless of the procedure used, the following inhibitors, in a dose range of nontoxic concentrations (*8*), had no effect on tumor killing by activated macrophages: two inhibitors of plasminogen activation (*9*), ϵ-aminocaproic acid and tranexamic acid; two synthetic ester substrates that are competitive inhibitors of plasmin and other serine proteinases (*10*), tosyl lysine methyl ester and tosyl arginine methyl ester; and two inhibitors that covalently and irreversibly label the active enzymatic site of serine proteinases (*11*), diisopropylfluorophosphate and phenylmethylsulfonyl fluoride. These synthetic inhibitors were not themselves inducers of tumor killing by activated macrophages, nor did they interfere with the ability of low doses of MAF (0.005 to 0.5 percent) or endotoxin (1 to 5 ng/ml) to make activated macrophages tumoricidal in 10 percent ABS, mouse serum, horse serum, or human serum. These inhibitors did not interfere with activated macrophage tumor killing induced by 10 percent FBS. In contrast, tosyl lysine chloromethyl ketone (TLCK) (*8, 12*), a third inhibitor that covalently labels the active site of susceptible serine proteinases and also inhibits certain sulfhydryl-dependent enzymes, in nontoxic concentrations did prevent activated macrophage tumor killing induced by 10 percent FBS or by MAF (0.005 to 2.0 percent), and endotoxin (0.5 to 50,000 ng/ml) when the culture medium contained 10 percent ABS, mouse serum, horse serum, or human serum. Also, TLCK completely suppressed activated macrophage tumor killing when it was included in experiments with MAF or endotoxin in any of the following sequences: (i) when TLCK was added during the 2-hour incubation period in serum-free culture medium, MAF or endotoxin being added to the complete culture medium for the assay; (ii) when MAF or endotoxin was added during the 2-hour incubation period in serum-free culture medium, TLCK being added to the complete culture medium for the assay; (iii) when TLCK plus MAF were added to the complete culture medium for the assay; and (iv) when TLCK plus endotoxin were added to the complete culture medium for the assay. Unlike serum factors (*2*), MAF, endotoxin, and trypsin, all of which appeared to modulate a tumoricidal threshold. TLCK completely and irreversibly disrupted transductive interactions linking expression of macrophage tumoricidal function to chemical signals in the microenvironment.

The results reported here suggest the following interpretation. There appears to be a biologic "resistor" that controls the expression of activated macrophage tumor killing by means of a variable threshold [perhaps functioning by modulation of fluidity (*2, 13*), receptor density,

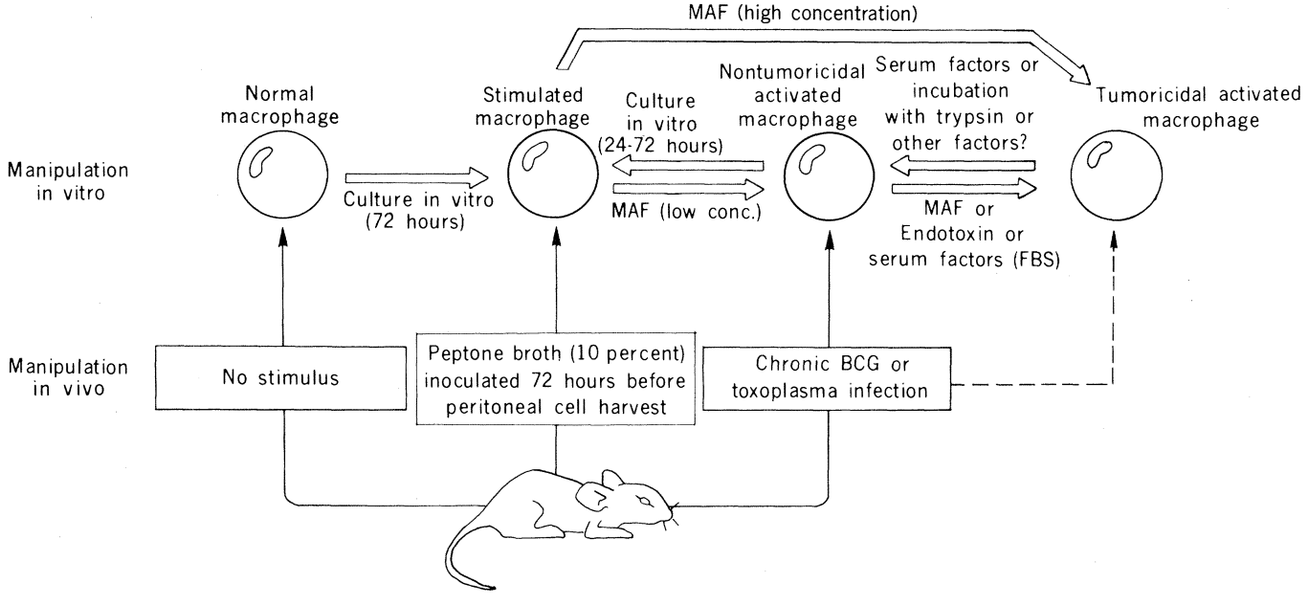

Fig. 2. Modulation of activated macrophage tumor-killing potential. The experimental results suggest that in vitro, and probably in vivo, a dynamic state exists among the environmental chemical signals that can either increase or decrease the probability for expression of macrophage-mediated tumor killing. Macrophages representing four different functional states (within a continuum of macrophage differentiation) can be isolated from appropriate groups of mice and can also be generated in vitro by exposing the macrophages to appropriate stimuli. (i) Normal macrophages are obtained from the peritoneal cavities of mice that have not received peptone or been infected with BCG or toxoplasma. They are monocyte-like and do not respond effectively to chemical signals that induce the expression of tumor killing. (ii) Stimulated macrophages are obtained from mice injected intraperitoneally with 10 percent peptone. In vitro, normal macrophages acquire the stimulated state after culture for 72 hours. Stimulated macrophages do not kill tumor cells in culture medium containing FBS or other serums but become tumoricidal when exposed to MAF. (iii) Nontumoricidal activated macrophages are obtained from the peritoneal cavity of mice with chronic BCG or toxoplasma infection, or are produced in vitro by exposure of stimulated macrophages to low concentrations of MAF. They are generally tumoricidal when cultured with 10 percent FBS but not when cultured with other types of serum. However, they have a markedly lowered threshold for induction of tumoricidal effect because picogram or nanogram quantities of endotoxin or low concentrations of MAF induce tumor killing regardless of the kind of serum in the medium. (iv) Tumoricidal activated macrophages are also obtained from the peritoneal cavity of mice with chronic BCG or toxoplasma infection, but they kill tumor cells regardless of the type of serum in the culture medium and without added MAF or endotoxin. Such macrophages were only sporadically obtained in our experiments, hence the broken line in the diagram. The probability of these highly activated macrophages being obtained is increased by using toxoplasma-infected mice within 4 to 6 weeks after infection with the microorganism or within 48 to 96 hours after administering an intraperitoneal booster dose of BCG or toxoplasma. Tumoricidal activated macrophages can also be generated from stimulated macrophages exposed in vitro to high concentrations of MAF. In addition, we have observed that treatment with trypsin greatly increases the threshold for acquisition of tumor killing by macrophages. Under physiologic or pathologic conditions in vivo, it is possible that plasmin could produce a similar effect on the macrophage tumoricidal threshold.

or both in macrophage membranes] that can be regulated by environmental chemical factors. Therefore, the term activation, when used in the context of macrophage tumor killing, may reflect a relative lowering of tumoricidal threshold from a preexisting baseline level. These studies also suggest that a macrophage may be activated; that is, its tumoricidal threshold may be lowered, but it may not actually kill tumor cells unless the threshold falls to a critically low level necessary for expression of cytotoxic effect against the tumor cell with which it is in contact. Our experiments also suggest that nonimmunologically derived factors could prevent the final triggering step of the biologic program for macrophage-mediated tumor killing by raising the tumoricidal threshold of activated macrophages above this critical level. It is significant that nonimmunologic mechanisms that inhibit the final triggering step of macrophage tumor killing such as those described herein could operate locally in tumor tissue and could be initiated by factors elaborated by tumor cells. Such nonimmunologic modulation of macrophage tumor killing could function independently of all immunologic events preceding it and in a fully immunocompetent host. A relevant example may be the observation that many tumors contain large numbers of macrophages but continue to grow progressively (*14*). Within neoplastic tissue the balance between opposing signals that affect macrophage tumor killing (see Fig. 2) may be shifted toward inhibition of tumor killing. This could be important in the pathogenesis of progressive tumor growth.

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8. Sources and range of molar concentrations of inhibitors used: from Lederle, ϵ-aminocaproic acid (7.6×10^{-5} to $1.9 \times 10^{-2}M$) and tranexamic acid (6.4×10^{-5} to $1.3 \times 10^{-2}M$); from Calbiochem, tosyl lysine methyl ester (2×10^{-5} to $5 \times 10^{-4}M$); from Sigma, tosyl arginine methyl ester (1×10^{-4} to $3 \times 10^{-3}M$), diisopropylfluorophosphate (1×10^{-6} to $5 \times 10^{-4}M$), phenylmethyl sulfonylfluoride (3×10^{-4} to $3 \times 10^{-3}M$), and tosyl lysine chloromethyl ketone (4.1×10^{-5} to $6.8 \times 10^{-5}M$).
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16. Supported by the Veterans Administration and NIH grants CA 14045 and CA 15811. We thank C. C. Moore, J. E. Brisbay, M. S. Knowlton, and R. Christensen for assistance.

1 February 1977; revised 5 April 1977

Modulation of Macrophage Tumoricidal Capability by Components of Normal Serum: A Central Role for Lipid

Abstract. *The tumoricidal capabilities of macrophages can be reversibly inhibited by a lipoprotein of high molecular weight, and the inhibition appears to be reproduced by enrichment of macrophage plasma membranes with cholesterol. A second serum component of lower molecular weight enhances macrophage cytotoxicity. The presence of these components in normal serums suggests a physiological role for such factors in the regulation of macrophage function.*

Activated macrophages can kill tumor cells in vitro (*1, 2*). The acquisition of tumoricidal potential seems to parallel enhanced bactericidal activity (*3*). Aside from macrophage-activating factors secreted by lymphocytes, the endogenous factors that regulate these abilities remain largely unknown. That such factors may exist, however, is suggested by the ability of macrophages to modify a number of metabolic and secretory functions in response to alterations in their microenvironment (*4*). We have analyzed fractionated serums from humans, mice, and bovine fetuses in an attempt to define some of the biochemical determinants that modulate macrophage tumoricidal capability. Our results indicate that the acquisition of macrophage cytotoxic activity can be reversibly inhibited by a serum component of high molecular weight that is isolated with a lipoprotein of low density, and that this inhibition can be reproduced by temporarily enriching macrophage plasma membranes with cholesterol. In addition, a serum component of lower molecular weight appears to enhance macrophage tumoricidal potential. The potency of these opposing factors seems nearly balanced in human and mouse serums, and accounts for the neutral or slightly inhibitory nature of these serums in assays of macrophage cytotoxicity in vitro. The ability of cholesterol and perhaps other lipids to modulate the expression of macrophage tumoricidal activity suggests that tumor cells themselves may be able to modify macrophage function by transferring lipids to macrophages.

Peritoneal cells were harvested from ICR female mice with chronic infection with bacillus Calmette-Guérin (BCG). The cells adhered to Falcon microtiter chambers (4×10^5 cells per chamber) as described (*5*). The cells were incubated for 2 hours at 37°C in 10 percent whole serum or various serum fractions (see Figs. 1 and 2) in a humidified atmosphere containing 5 percent CO_2. After incubation, the adherent cells were washed twice with 0.15*M* saline and then incubated for 60 hours with 3T12 cells, initially 6×10^3 cells per chamber. The cultures were then fixed with methanol and stained with Giemsa.

Macrophages from BCG-infected mice are tumoricidal when cultured in fetal bovine serum (FBS) but kill tumor cells only variably or not at all when cultured in other serums (*5*). These macrophages can be induced to kill tumor cells in other serums, however, by the addition of nanogram amounts of endotoxin to the assay. In the experiments described here, all of the cultures (60-hour assays) were supplemented with 10 percent adult bovine serum. Data are shown in Fig. 2. Macrophage monolayers incubated for 2 hours at 37°C in 10 percent whole human serum (row A) or in fraction 2 (row C) and subsequently cultured in 10 percent adult bovine serum did not inhibit 3T12 growth but became markedly cytotoxic upon the addition, during the 2-hour incubation period only, of endotoxin (50 ng/ml; *Escherichia coli* 0128:B12, Sigma). In contrast, macrophages first incubated in fraction 1 (high molecular weight serum components) (row B) were not detectably cytotoxic either with or without added endotoxin. Finally, macrophages first incubated in fraction 3 (lower molecular weight components)