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.

JOHN B. HIBBS, JR.
READ R. TAINTOR
HAROLD A. CHAPMAN, JR.
J. BRICE WEINBERG

Veterans Administration Hospital and
Department of Medicine,
Divisions of Infectious Diseases and
Hematology-Oncology,
University of Utah Medical Center,
Salt Lake City 84132

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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 $0.15M$ 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)

(row D) were markedly cytotoxic without added endotoxin.

We next determined that fraction 1 would prevent the effect of fraction 3 (see Fig. 2, rows E to G). When mixed with equal volumes of tissue culture medium (DMM) fraction 3 still enhanced (row E) and fraction 1 still inhibited (row G) cytotoxic activity. However, the enhancing effect of fraction 3 was largely eliminated when fraction 3 was mixed with fraction 1 (row F). Similar findings were observed with G-200 fractions of unheated normal ICR mouse serum and FBS, except that the high molecular weight fraction of FBS was clearly less inhibiting. This difference probably accounts, at least in part, for the relative facility of expression of macrophage cytotoxicity in cultures supplemented with FBS. Thus the acquisition of cytotoxic capability by macrophages on exposure to endotoxin or other activating factors as occurs in mice with chronic infection can be clearly modified, either potentiated or inhibited, by components of normal serum. Other macrophage regulatory factors not tested in these experiments may also exist in blood and, indeed, several have been previously described (*6*).

Previous data indicate that macrophages obtained from BCG-infected mice kill neoplastic but not normal cells (*1*). In experiments similar to those described above we found that macrophages obtained from BCG-infected mice and cultured in vitro in medium supplemented with 10 percent adult bovine serum did not kill either normal or neoplastic (SV40 transformed) WI-38 fibroblasts. After the addition, during a 2-hour incubation period, of either endotoxin (50 to 200 ng/ml) or fraction 3 (total protein, 8 to 20 mg/ml), the macrophages expressed a marked cytotoxic effect for neoplastic WI-38 cells but did not kill the normal WI-38 fibroblasts. These observations, which indicate that the cytotoxic potential of macrophages that is enhanced by either endotoxin or serum fraction 3 is selective for neoplastic cells, are consistent with the results of earlier experiments in vitro in which the medium was supplemented with commercially acquired FBS (*1*).

In attempting to explain our findings, we considered the following.

1) When macrophages were incubated for 2 hours with fraction 1 either before or while being incubated with endotoxin (10 to 100 ng/ml) the enhancing effect of endotoxin on macrophage cytotoxicity was abolished. However, the subsequent addition (during the 60-hour array) of endotoxin (5 to 10 ng/ml) fully restored the tumoricidal activity. Thus, both the enhancing effect of endotoxin and the inhibitory effect of fraction 1 are reversible. This reversibility has implications for the study of macrophage cytotoxicity as, for example, macrophages isolated from normal or neoplastic tissues and cultured in vitro may well function differently from macrophages in vivo.

2) The inhibitory effect was not limited to endotoxin-stimulated macrophages. Macrophages from mice with chronic BCG infection were incubated in a medium containing serum (1 percent by volume) rich in macrophage activating factors (MAF) [prepared according to Salvin (*7*)] that inhibits macrophage migration and contains no detectable endotoxin when examined by our limulus assay (*8*). These macrophages were markedly cytotoxic to 3T12 cells. However, if serum fraction 1 was added to this medium the enhancing effect was abolished.

Other evidence also supports the interpretation that the inhibitor directly affects macrophage function. Macrophages from mice with chronic infection

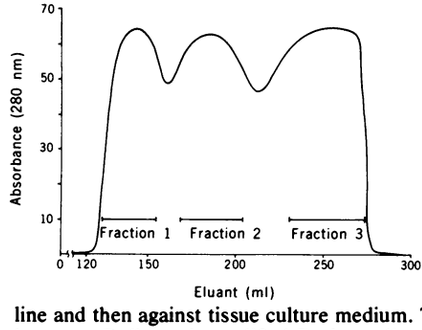

Fig. 1. Gel chromatography of whole human serum. Heat-inactivated (56°C, 30 minutes) human serum (20 ml) was applied to a Sephadex G-200 column equilibrated in 5 m*M* tris, *p*H 7.4, 0.9 percent NaCl, and 0.02 percent sodium azide and eluted with the same buffer at 4°C. Fractions of the eluted protein were pooled as indicated. Fractions 1 to 3 contain proteins of decreasing molecular weight. Molecular weight markers of fractions 1, 2, and 3 are, respectively, immunoglobulin M, immunoglobulin G, and albumin. Pooled fractions were dialyzed exhaustively against 0.15*M* saline and then against tissue culture medium. The medium used in all the experiments reported here was Dulbecco's modified Eagle medium (DMM) supplemented with 0.02*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) and 1 g of glucose per liter. All dialysis solutions were filtered, the dialysis tubing was autoclaved, and the column effluent was periodically cultured to test for sterility and to prevent contamination with endotoxin in the preparation of serum fractions. The serum fractions were then filtered through 0.22-μm Millipore filters and used directly in the cytotoxicity assay as described (see text and Fig. 2). The average protein contents of fractions 1, 2, and 3 were 2.0 mg/ml, 4.0 mg/ml, 16 mg/ml, respectively, by biuret assay. Assays for contaminating endotoxin by limulus assay of dialysis solutions and chloroform-extracted fractions were consistently negative (*8*). Although not shown, protein eluting from the G-200 column in the trough regions between the major serum fractions was also pooled and similarly tested in the cytotoxicity assay. Protein eluting between fractions 1 and 2 inhibited and protein eluting between fractions 2 and 3 enhanced macrophage tumoricidal capabilities.

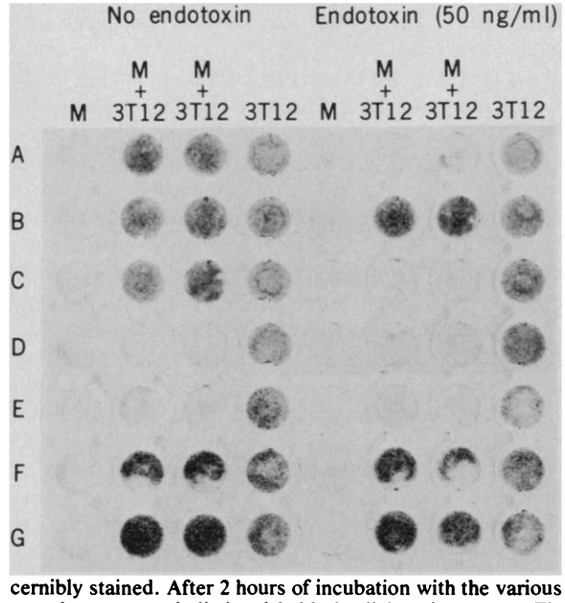

Fig. 2. A low-power photograph of a Giemsa-stained microtiter plate in which each circle represents an individual culture. Macrophage monolayers were incubated for 2 hours with: (row A) 10 percent whole human serum; (row B) fraction 1; (row C) fraction 2; and (row D) fraction 3 of heat-inactivated human serum applied to a Sephadex G-200 column; (row E) fraction 3 with DMM (1 : 1 by volume); (row F) fraction 3 with fraction 1 (1 : 1 by volume); (row G) fraction 1 with DMM (1 : 1 by volume). Columns titled *M*, *M + 3T12*, and *3T12* only represent control macrophage cultures, macrophages plus 3T12 cells, or control 3T12 cultures, respectively. The 3T12 cells are stained darkly and macrophages are not discernibly stained. After 2 hours of incubation with the various serum fractions, the macrophage monolayers were indistinguishable by light microscopy. The gross evidence of macrophage-mediated cytotoxicity in these experiments is also confirmed by light microscopy. The 3T12 cells (6×10^3) added in 0.1 ml of DMM attach evenly to the chamber surface, so that 33 to 37 cells can be seen in the microscopic field (at × 300) when they are stained immediately after attachment. In all of the cultures in which 3T12 growth was inhibited, there were three or fewer 3T12 cells per field (at × 300) at the end of the 60-hour assay period.

are occasionally tumoricidal without added endotoxin and regardless of the type of serum they are cultured in. Prior incubation of these macrophages with fraction 1 totally reverses their tumoricidal activity.

3) The high molecular weight inhibitor is isolated with low density lipoprotein. In early attempts to explain the inhibitory character of the high molecular weight fraction, immunoglobulin M was isolated by dialysis against H_2O and α-2 macroglobulin was isolated by the method of Song *et al.* (*9*). No inhibitory activity could be found in these components. Low density lipoprotein, also a component of our serum fraction 1, was isolated by ultracentrifugation of serum under a discontinuous salt gradient (*10*). By immunoelectrophoretic methods, the isolated low density lipoprotein reacted with antiserum to β lipoprotein but not with antiserum to α lipoprotein and revealed one line of identity with antiserum to whole human serum. Inhibitory activity was recovered in the low density lipoprotein band; fraction 1 from which the low density lipoprotein had been removed by ultracentrifugation prior to G-200 gel chromatography was usually devoid of inhibiting activity.

4) The inhibitory effect of the low density lipoprotein band can be reproduced by temporarily enriching macrophage plasma membranes with cholesterol. Low density lipoproteins have a relatively high content of free cholesterol and cholesterol ester (*11*) and have been shown to transfer free cholesterol to mycoplasmal plasma membranes (*12*) and to L cells (*13*). We attempted, therefore, to reproduce the inhibitory activity of low density lipoproteins by enriching macrophage plasma membranes with cholesterol. Cholesterol-phospholipid liposomes [40 mg of cholesterol (Sigma, 99+ percent pure) plus 40 mg of dipalmityl phosphatidyl choline (Sigma) in 10 ml of DMM and 4 ml of bovine serum] were prepared according to Cooper *et al.* (*14*). These sonicated lipid dispersions transfer free cholesterol to erythrocyte membranes, the membrane cholesterol showing a net increase with 50 percent equilibration being reached in 2.3 hours at 37°C. In experiments similar to those already described herein, macrophages incubated for 2 hours at 37°C in free cholesterol-phospholipid liposomes were clearly inhibited in their ability to respond to endotoxin or to express cytotoxic effect in FBS. To a lesser extent, cholesterol-dilinoleate phosphatidyl choline dispersions were also inhibitory. Oleate, palmitate, or myristate esters of cholesterol (Sigma) mixed with phosphatidyl choline or dipalmityl phosphatidyl choline dispersions without cholesterol were noninhibitory.

These experiments suggest that the inhibitory effect of the low density lipoproteins is probably dependent on the influx of free cholesterol into macrophage plasma membranes. This suggestion seems plausible both because of the importance of cholesterol in membrane functions and because macrophages synthesize little or no cholesterol (*15*). Macrophage membrane cholesterol is thus necessarily subject to modulation by the microenvironmental determinants of cholesterol flux. Although other components of the low density lipoproteins may also be inhibitory, these observations suggest that, in addition to participating in transport, some species of low density lipoproteins may have regulatory functions influencing a number of cell types; for example, Curtiss and Edgington have described a subspecies of the low density lipoproteins that reversibly inhibits lymphocyte proliferation (*16*).

Among the described abnormalities of lipid metabolism in neoplastic cells, one of the most consistent findings has been increased rates of cholesterol synthesis (*17*). This abnormality in cholesterol metabolism could be translated by the tumor cell into inhibition of macrophage tumoricidal capability, either by direct transfer of cholesterol or other lipids during the intimate contact that occurs between macrophages and tumor cells or by transfer via a soluble exchange protein.

5) The nature of the enhancing factor in serum fraction 3 is unclear. This factor is not a classical lipoprotein because fraction 3 obtained from gel chromatography of serum previously depleted of lipoprotein by ultracentrifugation (*10*) is still clearly enhancing. Furthermore, delipidation of fraction 3 by isopropyl ether and butanol solvents (*18*) does not abrogate its effect. Although stable to exposure to 56°C for 30 minutes, the activity of fraction 3 is destroyed after heating to 60°C for 1 hour. Thus, the heat sensitivity, molecular weight range, and tumoricidal promoting activity of fraction 3 are consistent with the known physiochemical and biological properties of lymphocyte-derived MAF (*2*), and indicate the presence in normal serums of a similar factor or its precursor.

Our data indicate that the acquisition of cytotoxic capability by macrophages is not the acquisition of a discrete functional state but of a relative capability subject to modification by factors present in normal serums. Further, the presence in normal serums of inhibiting and enhancing determinants of macrophage tumoricidal potential suggests a role for such factors in the regulation of macrophage functions in addition to tumor cell destruction. Indeed, our preliminary experiments indicate that the ability of macrophages to inhibit mitogen-induced lymphocyte proliferation (*19*) can also be modulated, either inhibited or enhanced, by these same components of normal serum. This modulation seems to depend in part on cholesterol. Previous studies with artificial and natural membranes have demonstrated the importance of cholesterol in modulating the activity of membrane-bound enzymes such as adenosine triphosphatase, the distribution of membrane proteins, membrane permeability, and membrane fusion (*20*). Our demonstration of a serum-derived regulatory system that directly affects macrophage function and depends partly on cholesterol flux suggests that the structural importance of cholesterol first recognized in these earlier observations may be realized in vivo in the modulation of the cellular mechanisms that control cell growth.

HAROLD A. CHAPMAN, JR.
JOHN B. HIBBS, JR.
Veterans Administration Hospital and
Department of Medicine,
Division of Infectious Diseases,
University of Utah Medical Center,
Salt Lake City 84132

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Binocular Differences in Cortical Receptive Fields of Kittens After Rotationally Disparate Binocular Experience

Abstract. *Kittens were afforded visual experience only while wearing goggles fitted with prisms that rotated the inputs to the two eyes equally but in opposite directions about the visual axes (16° for experimental subjects, 0° for control subjects). Subsequently, receptive-field organization of the visual cortex was studied, special attention being given to the preferred orientations of binocular cells. For each group, the distribution of interocular differences in preferred orientation centered about the prism rotation experienced during early development. Thus, for moderate amounts of relative rotation, the development of interocular matching of orientation specificity in binocular cells of the visual cortex reflects the correspondence of early visual input between the two eyes.*

The two functional characteristics of visual cortical neurons that have received perhaps the most attention are binocularity (or ocular dominance) and orientation specificity. For most cells, a distinct, optimally oriented stimulus evokes a maximal response, and most cells are activated by stimuli to either eye. Each of these properties is susceptible to the effects of early experience: complete visual deprivation during early development substantially reduces the number of orientation-selective cells present in the adult, and monocular visual deprivation or profoundly anomalous early binocular experience (such as experimental strabismus) can severely disrupt the normal development of binocularity (*1*).

For binocular cells in the normally reared adult cat, the preferred stimuli for the two eyes are similar, particularly receptive-field location and orientation. Correspondence in receptive-field location is largely provided by the innate topographic mappings of the retina within the visual system, although visual experience has a crucial role in the final adjustment of this correspondence (*2*).

Although receptive-field orientations of binocular cells in the normally reared adult cat are well matched (*3*), kittens that are visually inexperienced (*4*) or binocularly deprived by reverse suturing (*5*) lack such matching. We have further examined the role of visual experience in the development of interocular matching of receptive-field orientations. We raised kittens whose only visual experience was with a visual field optically rotated 16° between the left and right eyes, and found that the subsequent distribution of interocular differences in the preferred orientations of cells was centered about the rotational disparity experienced during early development. To our knowledge, the results reported here are the first to show that the binocular matching of this fundamental aspect of the functioning of visual cortical cells develops so as to correspond to the visual environment.

Three litters of kittens and their mothers were placed in a darkroom at ages 7 to 10 days and kept there except for daily periods of 1 to 2 hours of visual experience between the ages of 4 and 12 weeks. During the daily exposure periods, the kittens wore goggles molded from silicone rubber sealer (Dow Corning) and held in place by Velcro straps behind the head and under the chin. Each eyepiece was fitted with a pair of small right-angle prisms cemented together. Such an arrangement optically rotates the visual field around its center without mirror reversals; the angle of rotation is exactly twice the angle by which the two prisms are misaligned. For three kittens, the visual fields were rotated 8° counterclockwise in the left eye and 8° clockwise in the right eye (the +16° condition). For one kitten, the rotations were 8° clockwise in the left eye and 8° counterclockwise in the right eye (the −16° condition). Three control kittens wore goggles fitted with prisms that did not rotate the visual fields (the 0° condition).

The kittens had visual experience while wearing the goggles in a well-illuminated room containing toys and obstacles suitable for climbing, in the presence of littermates and an experimenter. Frequent checks were made to ensure that the kittens' eyes were open and clearly visible to the experimenter. Occasionally the prisms became fogged; when this happened, the kitten was placed briefly in a lightproof box while the prisms were cleaned. For each kitten, the total of visual experience over the 8-week period was between 50 and 100 hours.

In general, all kittens showed good visual behavior while wearing their goggles. The kittens played with each other and with toys, climbed on and jumped off furniture, avoided obstacles, stalked moving targets, and followed the experimenter to explore other rooms. Visual orientation and depth discrimination were evident in these behaviors and confirmed in informal testing (for example, visual cliff and visual placing).

Subsequently, receptive-field organization of visual cortex was studied in all kittens using conventional techniques of extracellular unit recording (*6*). Each kitten was studied during from one to three recording sessions separated by at least 3 weeks, at ages ranging from 5 months to 1 year. Kittens remained in the darkroom between recording sessions. For recording, subjects were prepared under ketamine hydrochloride and ether anesthesia. Small craniotomies were made 4 mm posterior to instrument zero and 2 to 3 mm lateral to the midline. Subjects were then immobilized with gallamine triethiodide (Flaxedil) (20 mg/ml) administered at a rate of about 1 ml/hr and placed in an atraumatic head holder facing a translucent gray tangent screen at 46 cm. Pupils were dilated with ophthalmic atropine sulfate (1 percent), nictitating membranes were retracted with ophthalmic phenylephrine hydrochloride (2.5 percent), wound margins were infiltrated with a long-lasting local anesthetic [procaine in peanut oil, 15 mg/ml (Zyljectin)], and ophthalmic proparacaine hydrochloride (0.5 percent) was applied topically on the corneal surfaces. Each eye was focused on the tangent screen by corneal contact lenses, and the optic disk and area centralis were projected onto the screen and mapped separately for each eye. Artificial pupils (3-mm diameter) were used to improve depth of field. Glass or metal microelectrodes were aimed at the posterolateral gyrus, usually so as to traverse the medial bank. Moving visual stimuli consisting of slits, dark bars, and edges were back-projected on the tangent screen during record-