Attributable mainly to genetic factors, these large interindividual variations are responsible for some cases of drug toxicity arising when "usual" doses of these drugs are administered to all subjects without appropriate modification (*12*). For these drugs, the assays employed exclude metabolites (*12*). Although warfarin, like the aforementioned drugs, is metabolized extensively by hepatic mixed function oxidases, warfarin plasma half-lives and apparent volumes of distribution are very similar in unrelated, nonmedicated, healthy subjects. To test whether the 12 subjects with similar warfarin half-lives were also homogeneous with respect to the elimination of other drugs, we determined at a later time their plasma antipyrine half-lives after a single oral dose of antipyrine (18 mg/kg). Plasma antipyrine half-lives varied more than threefold, ranging from 6.5 to 21.0 hours; the intraindividual correlation between plasma antipyrine and warfarin half-lives was .56, not quite attaining significance ($.10 > P > .05$). The currently widely accepted tenet that in healthy, nonmedicated subjects all drugs metabolized primarily by this hepatic microsomal system exhibit large interindividual variations in rates of elimination should be reexamined.

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Heterocytolysis by Macrophages Activated by Bacillus Calmette-Guérin: Lysosome Exocytosis into Tumor Cells

Abstract. *The cytotoxic activity of activated macrophages against tumorigenic target cells appears to be mediated by lysosomal enzymes of activated macrophage origin. Lysosomes of activated macrophages are secreted directly into the cytoplasm of susceptible target cells, which subsequently undergo heterolysis. This reaction can be inhibited by agents which prevent the exocytosis of macrophage lysosomes (hydrocortisone) or which interfere with the action of lysosomal enzymes (trypan blue).*

Mouse peritoneal macrophages, activated in vivo by chronic infection by BCG (bacillus Calmette-Guérin) or toxoplasma, or in vitro by endotoxin, destroy tumorigenic cells by a nonphagocytic mechanism requiring direct contact. This cytotoxic reaction is based on a system of nonimmunologic discrimination at the effector level; in this reaction activated macrophages have little or no cytotoxic activity for nontumorigenic cells and normal macrophages are not cytotoxic (*1*, *2*).

Phase contrast microscopy and lysosomal markers were used to show that lysosomes are transferred from activated macrophages to the cytoplasm of susceptible target cells that subsequently undergo heterolysis; a similar transfer of lysosomal markers was not noted with normal macrophages. It was observed that the cytotoxic effect of activated macrophages is inhibited by hydrocortisone, a membrane stabilizer (*3*) which interferes with the transfer of lysosomal markers to tumorigenic target cells. This suggests that hydrocortisone prevents membrane fusion reactions required for lysosome exocytosis into susceptible target cells. In addition, the cytotoxic effect of activated macrophages can be inhibited with trypan blue, an inhibitor of lysosomal enzyme action (*4*). Activated macrophages readily transfer the trypan blue lysosomal marker to susceptible target cells but the target cells continue to undergo rapid mitosis. These results suggest that the cytotoxic action of activated macrophages can be inhibited either by preventing exocytosis of lysosomes or by directly inactivating lysosomal enzymes secreted into target cells. Therefore, it appears that lysosomal enzymes of activated macrophage origin are the effectors of target cell destruction.

These studies were performed with macrophages activated by chronic infection with BCG or toxoplasma. Macrophages activated by these two infections had identical cytotoxic activity. The cytotoxicity tests were prepared as described (Table 1) unless otherwise indicated.

Figure 1A shows a thick multilayer of tumorigenic 3T12 cells surrounding a central monolayer of BCG-activated macrophages. The destruction of 3T12 cells occurred only where they were in contact with activated macrophages. This is evidence that the cytotoxic mechanism does not involve a soluble cytotoxic mediator (SCM). To further test for a possible SCM elaborated by activated macrophages, we removed culture medium every 3 hours and each time replaced it with fresh medium warmed to 36.5°C. The cytotoxic effect was identical to that of control cultures (where the medium was not changed), suggesting that destruction was independent of an SCM whose effect could be diminished by dilution. To promote the thorough distribution of an SCM, we mixed the culture medium with a sterile Pasteur pipette every 60 minutes during the 72-hour incubation period. This did not interefere with 3T12 cell destruction among activated macrophages nor did it produce inhibition of 3T12 cell growth on the periphery of the cover slip which was free of activated macrophages. It was also possible that an SCM was present but active only in cooperation with macrophages. To test this, we removed the supernatant medium after 24, 48, and 72 hours of incubation from monolayers of activated macrophages or from activated macrophages that had been challenged with 3T12 cells. Both types of activated macrophage-conditioned medium were added to normal macrophages that had been challenged with 3T12 cells 1 hour earlier, and the cultures were evaluated for cytotoxic effect after a 72-hour incubation period. Activated macrophage-conditioned medium did not render normal macrophages cytotoxic. These experiments and those of earlier studies (*1*, *5*) failed to demonstrate SCM activity associated with the activated macrophage cytotoxic reaction.

Nonenzymatic agents that accumulate

and are stored in the vacuolar system of macrophages were used to study the nonphagocytic contact–dependent mechanism or mechanisms of target cell destruction. Normal and activated macrophages readily take up dextran sulfate (Pharmacia), which is concentrated in secondary lysosomes (*6*). Dextran sulfate is indigestible and nontoxic, and it stains metachromatically with toluidine blue O (*6*). Normal and activated macrophages were labeled with dextran sulfate (Table 1). The transfer of the dextran sulfate secondary lysosome marker to target cells paralleled their susceptibility to destruction by activated macrophages. For example, after a 24-hour incubation period, 68 ± 8 percent of 3T12 cells but only 10 ± 9 percent of BALB/c mouse embryo fibroblasts (MEF) in contact with BALB/c BCG-activated macrophages had metachromatic cytoplasmic vacuoles. Similar results were obtained with the same target cells with the use of C3H/He macrophages activated by chronic toxoplasma infection. Transfer of dextran sulfate to 3T12 cells from BCG- or toxoplasma-activated macrophages could be detected at 6 hours and was increased at 12 hours. Normal BALB/c macrophages transferred dextran sulfate to 5 ± 2 percent of 3T12 cells and 2 ± 2 percent of BALB/c MEF. Similar results were obtained with normal C3H/He macrophages and the same target cells. It was found that 3T12 cells growing on the cover slip periphery and not in contact with macrophages did not contain metachromatic granules. To test whether there was significant loss of dextran sulfate from cytoplasmic vacuoles of macrophages into the culture medium during the course of the cytotoxicity test, the following experiments were performed. Supernatant medium was removed from normal and activated macrophages or from mixed monolayers of activated macrophages and 3T12 cells after 24, 48, and 72 hours of incubation and was added to normal macrophages, to 3T12 cells, and to monolayers of activated macrophages that had been challenged with 3T12 cells 1 hour previously. In no case did medium from monolayers of macrophages that had dextran sulfate–laden vacuoles render sentinel macrophages or 3T12 cells metachromatic. Results similar to those with dextran sulfate were obtained with normal and activated macrophages labeled in the dark with neutral red (Gibco), which also accumulates in lysosomes (*7*).

Our results suggest that activated macrophages directly transfer the contents of secondary lysosomes into susceptible target cells. Such a process of exocytosis involves membrane fusion which should be partially inhibited by membrane stabilization (*8*). Hydrocortisone (Upjohn) was added to normal and activated macrophages for 6 to 24 hours before the target cells were challenged. Preliminary treatment of activated macrophages with hydrocortisone inhibited their cytotoxic effect (Table 1). Maximum inhibitory effect was obtained with $3.6 \times 10^{-5}M$ hydrocortisone, and a slight inhibitory effect was seen with doses as low as $2.8 \times 10^{-7}M$ if maintained in the culture medium throughout the 72-hour incubation period. Hydrocortisone also inhibited the transfer of dextran sulfate to 3T12 target cells. After a 24-hour incubation period 23 ± 7 percent of 3T12 cells in contact with labeled BALB/c BCG-activated macrophages had metachromatic cytoplasmic vacuoles. The reduction in the transfer of dextran sulfate to 3T12 cells was significant at $P \leq .001$. These results provide evidence that transfer of the contents of activated macrophage lysosomes is associated with target cell destruction.

Experiments were done to determine whether lysosomal hydrolases of activated macrophages could be the effectors of target cell heterolysis. Trypan blue (Gibco), a specific inhibitor of

Table 1. Effects on target cell destruction of introduction of nonenzymatic agents into the vacuolar system of activated macrophages, and the effect of stabilization of membranes of activated macrophages with hydrocortisone. The culture of target cells and the preparation of primary cultures of mouse embryo fibroblasts (MEF) have been described (*2*). The MEF used in the cytotoxicity test were from the third to the eighth in vitro passage. Mouse peritoneal cells were harvested as described (*2*). Peritoneal macrophages from BALB/c female ($H\text{-}2^d$) mice were collected 30 to 60 days after the mice were infected with the Paris strain of bacillus Calmette-Guérin (BCG) (0.2 mg intraperitoneally) or from C3H/He female ($H\text{-}2^k$) mice 6 weeks to 3 months after they had been infected with the C56 strain of *Toxoplasma gondii* (*2*); these were used as the source of effector cells. Peritoneal cells (6×10^5) in 0.1 ml of Dulbecco's modification of Eagle medium with 20 percent fetal calf serum, streptomycin (100 μg/ml), and penicillin (100 unit/ml) (complete medium) were added to the center of 25-mm diameter glass or plastic (Lux) cover slips for 2 to 3 hours at 36.5°C in air; with 5 percent CO_2 to allow for adherence of macrophages. Each cover slip was then washed with Hanks' balanced salt solution to remove nonadherent cells so that the central monolayer of adherent macrophages was restricted to the size of the 0.1-ml drop in which they were added to the cover slip. The peripheral portion of the cover slip remained free of macrophages. The cover slips were then placed in tissue culture dishes (35 by 10 mm; Lux) and flooded with 2 ml of complete medium or of complete medium plus appropriate additive. The agents stored in secondary lysosomes were added to macrophages in complete medium as indicated. After 18 hours, the cover slips were washed to remove extracellular labeling agent and placed in fresh culture dishes. Target cells (3T12, 1×10^5; MEF, 1.2×10^5; and SV-3T3, 1.3×10^5) were added in 2 ml of complete medium and attached evenly to the entire cover slip surface. This resulted in an average of 10 to 11 target cells on the cover slips per microscopic field, viewed at × 400. The cover slips were then incubated at 36.5°C in air with 5 percent CO_2 for 72 hours unless otherwise indicated. When used, hydrocortisone was added to the macrophage monolayers for 6 to 18 hours and then removed by washing before target cell challenge. The cell preparations on the cover slips were fixed in methanol and stained with Giemsa or toluidine blue O. Symbols used: 0, multilayer of tumorigenic target cells or confluent monolayer of normal target cells among activated macrophages (AM); 1+, patchy areas of confluent target cell growth among AM (slight cytostasis); 2+, 10 to 20 target cells per field among AM (cytostasis); 3+, 3 to 9 target cells per field among AM (cytotoxic effect); 4+, 2 or less target cells per field among AM (marked cytotoxic effect).

Treatment of activated macrophages used as effector cells*		Target cells and cytotoxic effect†			
		Nontumorigenic		Tumorigenic	
Lysosomal label	Concentration (*M*)	BALB/c MEF	C3H MEF	BALB/c 3T12	BALB/c 3T3 SV40 transformed
Dextran sulfate‡	2×10^{-8} or 4×10^{-8}	0–1+	0–1+	4+	4+
Dextran sulfate + hydrocortisone‡	2×10^{-8} or 4×10^{-8} + 3.6×10^{-5}	0	0	0–1+	0–1+
Neutral red	1.2×10^{-4}	0–1+	0–1+	4+	4+
Trypan blue	4.2×10^{-4}	0	0	0–1+	0–1+
Sucrose	2.9×10^{-2}	0–1+	0–1+	4+	4+
Ficoll	2.5×10^{-5}	0–1+	0–1+	4+	4+
None		0–1+	0–1+	4+	4+
None + hydrocortisone	3.5×10^{-5}	0	0	0–1+	0–1+

* Control normal BALB/c and C3H/He macrophages were exposed to the same lysosomal markers as activated macrophages in each of the above experiments. Normal macrophages never inhibited the growth of or were cytotoxic for target cells. † The results are given as the range obtained from four separately performed experiments. ‡ Macrophages from C3H/He female and BALB/c female mice activated in vitro with endotoxin as described (*2*) produced similar results to those listed above except that the cytotoxic effect and the transfer of dextran sulfate markers was not as great as in equivalent experiments when macrophages activated in vivo by immunologic mechanisms were used.

lysosomal hydrolases (*4*), is readily taken up by macrophages, is nontoxic, and is stored in secondary lysosomes (*9*). Normal and activated macrophages were vitally stained in vitro by incubation with $4.2 \times 10^{-4} M$ trypan blue in culture medium for 18 hours to challenge with target cells. Peritoneal macrophages were also labeled in vivo by inoculation of 4 mg of trypan blue intraperitoneally 48 hours before the mice were used as a source of macrophages for the cytotoxicity test. The cytotoxic effect of BCG- and toxoplasma-activated macrophages containing trypan blue in their vacuolar system was inhibited (Table 1). However, there was abundant transfer of trypan blue from activated macrophages to 3T12 cells. For example, after a 24-hour incubation period 62 ± 10 percent of 3T12 cells in contact with BCG-activated macrophages had vacuoles containing trypan blue when viewed in Sykes-Moore chambers (Bellco) with bright-field microscopy. These results suggest that lysosomal enzymes of activated macrophage origin may be the final molecular effectors of target cell destruction in this cytotoxicity system.

The inhibitory effect of trypan blue is not shared by the other tested non-enzymatic agents stored in secondary lysosomes. Dextran sulfate and neutral red had no inhibitory effect on the cytotoxic reaction, and similar results were found with sucrose (Mallinckrodt) and Ficoll (average molecular weight, 400,000; Pharmacia)—carbohydrates not digested by macrophages—that are taken up by macrophages and stored in secondary lysosomes (*10*). Normal and activated macrophages were exposed to sucrose and Ficoll as described in the caption to Table 1. Normal macrophages with large secondary lysosomes containing sucrose or Ficoll were not cytotoxic for 3T12 cells. Activated macrophages containing sucrose and Ficoll were cytotoxic for 3T12 cells (Table 1).

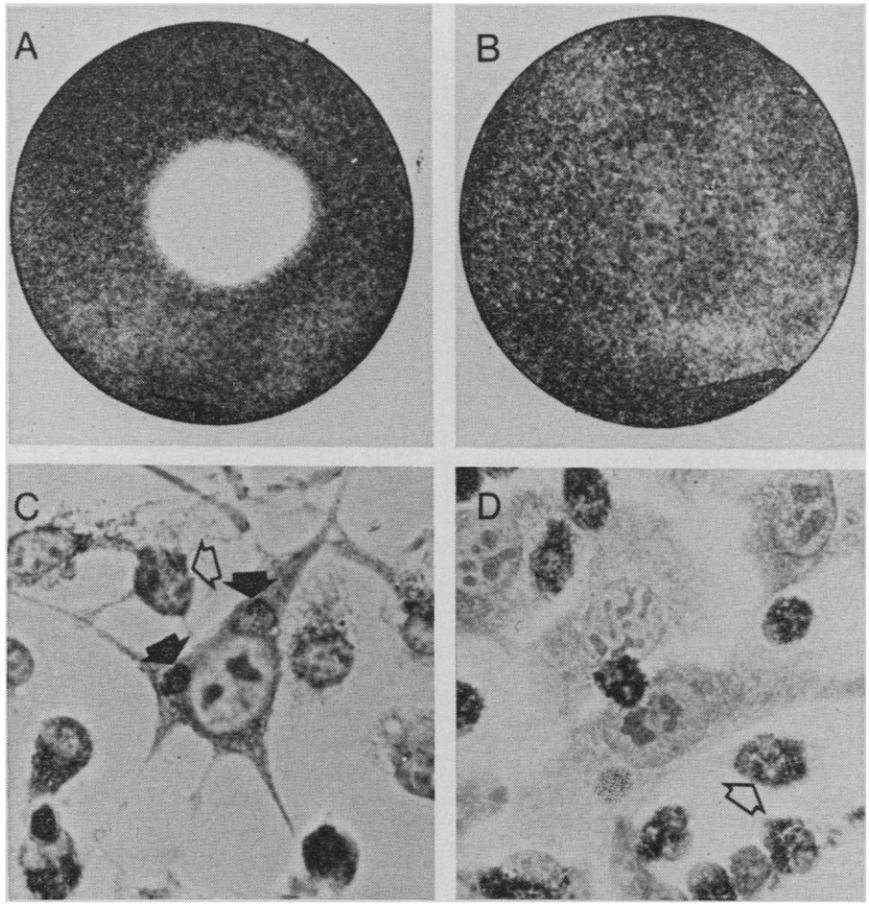

Fig. 1. Macroscopic and microscopic views of the interaction of BCG-activated and normal BALB/c macrophages with 3T12 target cells. The cytotoxicity test is described in the caption to Table 1. (A) and (B) were stained with Giemsa 72 hours after 3T12 cell challenge. In (C) and (D), macrophages were first labeled with dextran sulfate, challenged with 3T12 cells, and 24 hours later were stained with toluidine blue O. Macrophages are marked with white arrows. (A) A thick multilayer of 3T12 cells grows to the immediate edge of a central monolayer of activated macrophages, which have destroyed the 3T12 cells (0.4 3T12 cells per field at $\times$ 400) with which they were in initial contact. (B) The central monolayer of normal macrophages has been completely overgrown by a thick multilayer of 3T12 cells. (C) A 3T12 cell in contact with activated macrophages is undergoing degenerative changes which include clumping of nuclear chromatin, vacuolation, and partial retraction of cytoplasm. The vacuoles (black arrows) of the 3T12 cell contain large dark dextran sulfate particles, which were strongly metachromatic when viewed with bright-field microscopy. (D) Healthy 3T12 cells, which are in contact with normal macrophages, contained no metachromatic granules when viewed with bright-field microscopy. Darker staining normal macrophages were strongly metachromatic [(A) and (B), $\times$ 2; (C) and (D), $\times$ 790].

The interaction of macrophages and target cells in Sykes-Moore chambers maintained at 37°C was observed by phase contrast microscopy. Initial contact between macrophages and target cells began as the target cells were spreading on the cover slip among the macrophages. Long thin extensions of macrophage cytoplasm (pseudopods) made contact with the target cell surface (Fig. 1C). Activated macrophages were more active in extending pseudopods than normal macrophages were. Phase-dense granules could be seen to move from the macrophage perinuclear region to the pseudopod which they entered and slowly traversed centrifugally toward the target cell. Phase-dense granules were observed to be transferred from activated macrophages into the cytoplasm of 3T12 cells. Activated macrophages vitally stained with neutral red or trypan blue were observed to transfer secondary lysosomes containing dye to 3T12 cells. The cytoplasmic bridges between activated macrophages and target cells were always temporary, lasting several minutes to many hours. Activated macrophages were only rarely seen to transfer phase-dense granules or vitally stained secondary lysosomes to normal target cells. Normal macrophages were not observed to transfer phase-dense granules or vitally stained secondary lysosomes to either normal or tumorigenic target cells.

The findings suggest that the critical modification underlying the destruction of tumorigenic cells by activated macrophages may be local or general membrane destabilization in both cells, which favors focal and temporary membrane fusion (*8, 11*). The apparent universal susceptibility of tumorigenic cells to destruction by activated macrophages (*1, 2, 5, 12*) suggests that decreased

membrane stability (increased membrane fluidity) may be fundamental to expression of the neoplastic phenotype. In addition to morphologic (*13*) and biochemical modifications (*14*), the results suggest, and subsequent experiments have confirmed (*15*), that destabilization of macrophage membranes occurs in parallel with activation. Therefore, normal macrophages may not be cytotoxic because their stable membranes do not participate in the fusion reactions required for target cell heterolysis. Likewise, activated macrophages may have little or no cytotoxic effect for normal target cells because the stability of the latter's membranes does not favor the temporary cell fusion reaction required for heterolysis.

The interaction of activated macrophages with tumorigenic cells can result in target cell death. Four hours after challenge, there were 11 3T12 cells per field (at a magnification of $\times 400$) among BCG-activated macrophages, but after 72 hours there was an average of 0.1 3T12 cell per field. The cellular events leading to target cell death were followed by phase contrast microscopy in Sykes-Moore chambers maintained at 37°C: (i) lysosomes of activated macrophage origin accumulated in the cytoplasm of target cells; (ii) cytoplasmic vacuoles in the target cell cytoplasm dilated; (iii) the target cell periphery often began to manifest blebbing (*16*); (iv) the target cell rounded up and often developed violent blebbing, which resulted in target cell fragmentation by pinching off of blebs to form spherical structures identical to those described as apoptotic bodies by Kerr *et al.* (*17*); and (v) all cytoplasmic movement ceased and the target cell eventually disappeared from the cover slip surface. Cell death can occur without blebbing or cell fragmentation. When there was evidence of target cell injury, the activated macrophage or macrophages that were the source of the heterolysosomes have often moved out of the microscopic field and may have made contact with another tumor cell. Target cell destruction in this system could be misinterpreted as an autolytic rather than a largely heterolytic process unless this sequence was followed in situ during an 18- to 48-hour period.

This cytotoxicity system may be an in vitro model with broad biological significance. Certain types of cell death which occur during normal embryonic development may involve macrophage participation (*18*). Trypan blue (*19*) and hydrocortisone (*20*) have been found to be teratogenic in some model systems, and in our studies they inhibit the activated macrophage cytotoxic reaction. This is circumstantial evidence that macrophages could be effector cells during some types of tissue remodeling which occur in normal embryogenesis. In invertebrates, macrophages function in the absence of lymphocytes, plasma cells, and immunoglobulins (*21*). Phylogenetically as well as ontogenetically, macrophages may be a fundamental surveillance system on which specific immunologic mechanisms are superimposed (*22*).

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22. Our studies show that host resistance to tumor development and certain intracellular pathogens is related and mediated at the effector level by activated macrophages (*1, 2*). This suggests that membrane destabilization may also promote membrane fusion reactions within the vacuolar system of activated macrophages resulting in enhanced delivery of lysosomal contents to pathogen containing cytoplasmic vacoules.
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Lectin Binding Saccharides on a Parasitic Protozoan

Abstract. Leishmania donovani *promastigotes were specifically agglutinated by concanavalin A and phytohemagglutinin P. Somatic-somatic, flagellar-somatic, and flagellar-flagellar type agglutination was observed with the lectins. Enzyme-treated promastigotes gave reduced lectin agglutination reactions. The results suggest that complex saccharide moieties are randomly distributed on the surface of this organism.*

Leishmania donovani, a protozoan pathogen of humans, has a life cycle which includes a flagellated extracellular promastigote stage in the gut lumen of sandfly vectors, and a proliferative intracellular amastigote stage in macrophages of vertebrate hosts. Recently, several cytochemical techniques (*1*) were used jointly with electron microscopy to demonstrate polysaccharide-like materials on the surface membranes of *L. donovani* promastigotes (*2*). Further, isolated and solubilized promastigote pellicular membranes separated by disc-gel electrophoresis contain at least three major glycoproteins (*3*).

No report exists on specific terminal saccharide residues on the surfaces of any parasitic protozoan. Inasmuch as plant agglutinins (lectins) have provided highly specific means for studying terminal residues of polysaccharides and glycoproteins on the surfaces of diverse cell types (*4*), several lectins were used to ascertain what, if any, were their effects on promastigotes of *L. donovani*.

Chromatographically pure concanavalin A (Con A; Miles-Yeda), specific for terminal residues similar to α-D-glucose and α-D-mannose (*5*), was used in the agglutination experiments. Unpurified phytohemagglutinin M (PHA-M) and phytohemagglutinin P (PHA-P, Difco), both having an affinity for *N*-acetyl-D-galactosamine–like residues (*6*), also were used.

L. donovani strain 1-S promastigotes were obtained in primary culture from amastigotes isolated from infected hamster spleens. Promastigotes from the