

UC Irvine

UC Irvine Previously Published Works

Title

The in vitro induction and release of a cell toxin by immune C57B1/6 mouse peritoneal macrophages

Permalink

<https://escholarship.org/uc/item/2tj4t616>

Journal

Cellular Immunology, 3(1)

ISSN

0008-8749

Authors

Kramer, JJ
Granger, GA

Publication Date

1972

DOI

10.1016/0008-8749(72)90229-8

Copyright Information

This work is made available under the terms of a Creative Commons Attribution License, available at

<https://creativecommons.org/licenses/by/4.0/>

Peer reviewed

The *In Vitro* Induction and Release of a Cell Toxin by Immune C57B1/6 Mouse Peritoneal Macrophages¹

J. J. KRAMER² AND G. A. GRANGER

*Department of Molecular Biology and Biochemistry, University of California, Irvine
Irvine, California 92664*

Received May 14, 1971

Peritoneal macrophages from Sarcoma 1 (SA1)-sensitized C57B1/6 mice release a toxic factor(s), termed macrophage toxic factor (MTF), into the medium when exposed to allogeneic target L cells *in vitro*. Medium containing MTF is cytotoxic to cultures of allogeneic L cells, syngeneic C57B1/6 fetal fibroblasts and xenogenic HeLa cells. The cell toxin(s) is insensitive to the effects of nuclease, neuraminidase and trypsin, but is partially abrogated by treatment with pronase. Two fractions of toxic activity are eluted from Sephadex G-100, one associated with a 150,000 mol wt marker (IgG), and the other associated with a 47,000 mol wt marker (ovalbumin). Goat anti-serum prepared against PHA-induced mouse lymphotoxin (MLT) is capable of neutralizing the toxicity of both MTF and MLT, indicating that the factors may be similar.

INTRODUCTION

Gorer proposed that the macrophage is the primary effector cell in the destruction of allografts of ascites cells, while it plays a lesser role in the rejection of allografts of slowly growing vascularized tissue and leukotic cells (1). Sarcoma 1 (SA1) is an ascites tumor of A/JAX mouse (H-2^k/d) origin which is consistently rejected within a period of 10-12 days, upon intraperitoneal transplantation into C57B1/6 (H-2 d) mice (2). Studies by a number of workers have demonstrated that the peritoneal macrophage appears to be the dominant cell involved in the rejection of this tumor (2-4). While these *in vivo* studies have not revealed the mechanism by which cell destruction occurs, contact between aggressor and target cells is considered to be the first step in the destructive reaction. Actual tumor cell cytolysis apparently occurs by a nonphagocytic mechanism resulting in destruction of both the aggressor and the target cell (2).

In vitro studies have demonstrated that purified peritoneal macrophages obtained from C57B1/6 mice immunized against SA1 are capable of destroying cell monolayers containing target A strain antigens (5). As in the previously mentioned *in vivo* system, cell destruction occurs by a nonphagocytic mechanism which requires cell contact. It has also been demonstrated that the immune macrophage possesses

¹ This work was supported by Grant AI 09460-01, from the National Institutes of Health.

² Supported by a predoctoral fellowship, 5-F01-GM 45558-02, from the National Institutes of Health.

a surface factor, specific for A strain antigens, which promotes contact with the target cell. This factor has the biological properties of an immunoglobulin (5). While aggressor cell metabolism is necessary for the destructive reaction, the mechanism by which killing occurs has not been elaborated (5). The present report suggests that the immune peritoneal macrophage causes *in vitro* target cell destruction by release of nonspecific cytotoxic factors.

MATERIALS AND METHODS

Mouse—tumor system. Female A strain and C57B1/6 mice were obtained from Simonsen Laboratories (Gilroy, Ca). Sarcoma 1 (SA1), an ascites tumor indigenous to the A strain mouse, was maintained in our laboratory by weekly intraperitoneal (IP) injections of 0.1 ml of whole ascites fluid containing approximately $15\text{--}20 \times 10^6$ cells (2).

Culture medium and cell lines. Culture medium used in all experiments was Eagle's Minimal Essential Medium supplemented with 10% or 15% fetal calf serum (FCS), 0.29 mg/ml glutamine, 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 25 units/ml mycostatin (MEM). Medium concentrates were diluted with serum-free MEM. Hypotonic medium fractions were raised to physiologic levels by adding a concentrate of salts, amino acids, vitamins and antibiotics (STP), which has been described elsewhere (6). Mouse L 929 cells and HeLa cells (Flow Laboratories, Los Angeles, Ca) were maintained in 32 oz. prescription bottles and passed twice weekly. Primary fetal fibroblasts were obtained from minced, trypsinized mouse embryos.

Immunization and peritoneal cell collection. Immune peritoneal macrophages were obtained from C57B1/6 mice immunized by the IP injection of $5\text{--}10 \times 10^6$ SA1 tumor cells. These cells were obtained from A strain mice 7 days after the IP injection of SA1. The kinetics of tumor rejection was followed by monitoring the number of viable tumor cells in the peritoneum of the recipient C57B1/6 mice. Ascites fluid was removed aseptically from the mice after most of the tumor had been destroyed (9–13 days) and diluted 1:2 with MEM supplemented with 10% to 15% FCS. Control cells were obtained from the peritoneal washings of untreated animals or from animals 3 days after the injection of 1 ml of 1% starch (Connaught Laboratories, Toronto, Canada) solution. To collect the peritoneal cells, each animal was injected with 10.0 ml MEM + 7% fetal calf serum. The washings were collected, pooled, and the cells sedimented by centrifugation at 175g in an International Pr-2 refrigerated centrifuge. Differential cell counts of the resuspended cells revealed that immune suspensions were composed of 80% medium-small to medium-large macrophages, 15% lymphocytes, 1–2% polymorphonuclear cells and 1–4% tumor cells; starch-induced preparations were typically 95% medium-sized macrophages; normal macrophage suspensions were typically 90–95% medium-small macrophages.

Reverse plaque technique. This technique is a modification of the plaque test described by Granger and Weiser (5). Immune peritoneal macrophages (7×10^6 cells/bottle) were allowed to adhere to the surface of milk dilution bottles for 30 min. To remove all nonadhering cells, the monolayers were washed three times with a total of 15 ml of MEM. Then 10 ml of MEM was added to each bottle

followed by two drops (0.1 ml) of a suspension of target cells (10^6 cells/ml), which were carefully placed at two premarked loci on the monolayer. The cultures were left undisturbed for 2 hr at 37°C . Additional culture medium was carefully added to bring the volume to 20 ml, and the bottles sealed in an atmosphere of 95% air, 5% CO_2 . After 48 hr incubation at 37°C , the cultures were removed and the monolayers washed with 0.15 M NaCl and stained with a 1% crystal violet solution.

Production and Collection of Toxic Media. Macrophage monolayers were established in 32 oz. prescription bottles or 250 ml plastic culture flasks (Falcon Plastics, Oxnard, Ca.) at a density of $5\text{--}15 \times 10^6$ cells per culture vessel. After incubation at 37°C for 1 hr, the adherent monolayers were washed three times with a total of 30 ml of culture medium to remove all nonadhering cells. Cell counts on May-Grunwald-Giemsa-stained coverslip monolayers revealed them to consist of 99% medium to large macrophages and 1% small lymphocytes. Target L cells, which share the same allele at the H-2 locus (H-2^k) as SA1 tumor cells, were added to the monolayers at concentrations of 6×10^6 cells per 32 oz. bottle and 3×10^6 cells per 250 ml flask. The volume was adjusted with MEM to 40 ml per 32 oz. bottle and 20 ml per flask. The cultures were incubated at 37° for 48 hr in a 5% CO_2 , 95% air atmosphere. Following incubation, the media were collected and cells and debris were removed by sedimentation at $300g$ in an International Pr-2 refrigerated centrifuge. The media were then filter-sterilized and stored at 4°C . Certain batches of pooled media were concentrated $10\times$ in an Amicon ultra-filtration unit using a #3 membrane (30,000 mol wt filter) at 25 PSI pressure, and stored at -18°C . Greater than 90% of the activity was recovered in the concentrated sample. When pressures of greater than 25 PSI were employed, however, there was a reduction in recovered activity. Control media were obtained from parallel cultures containing normal or starch-induced macrophages substituted in place of the immune macrophages, and/or HeLa or fetal fibroblasts (C57B1/6) in place of L cells. These media were similarly collected and stored at 4°C .

Assay System. Cell-free media were tested for toxicity on 1 ml tube cultures of various target cells containing 5×10^4 cells established 24 hr prior to testing as previously described (7). Levels of toxicity were determined by exposing 1.0 ml tube cultures of target cells to various dilutions of test media. These dilutions are expressed as ml of toxic medium per 1.0 ml culture medium. The cells were incubated in the presence of the test medium for 48–72 hr. During this time, the cultures were periodically examined with the light microscope. After incubation, cell viability was assayed by measuring their ability to incorporate ^{14}C amino acids into trichloroacetic acid precipitable protein, as previously described (7). This data is expressed as % destruction, where % destruction = $[(\text{CPM Control} - \text{CPM Exp.})/\text{CPM Control}] \times 100$.

Measuring Macromolecular Synthesis

Suspensions of starch and SA1 induced peritoneal macrophages from C57B1/6 mice were collected and purified by a differential centrifugation technique or adsorption to glass as previously described (5). Macrophage suspensions were adjusted to either 2.5×10^6 or 8×10^6 cells/ml and 1 ml cultures were pulse labeled with

medium containing one of the following: (a) 0.3 $\mu\text{Ci/ml}$ [^{14}C] AA hydrolysate, (b) 1 $\mu\text{Ci/ml}$ [^3H] uridine or (c) 1 Ci/ml [^3H] thymidine (Schwartz Bio Research, Orangeburg, NJ). After 20–60 min at 37°C, the cells were sedimented and the nucleic acid or protein extracted and measured for radioactivity as previously described (8). In several experiments, 2×10^6 cells were allowed to establish themselves as monolayers for 24 hr in plaque bottles. The washed monolayers were then labeled and extracted as described above for suspensions.

Characterization of the macrophage toxin

Heat stability. The heat stability of the cell toxin was assessed in the following manner. Test and control media (3.0 ml aliquots) were placed in individual screw-capped tubes (16 \times 125 mm). The tubes were exposed for 15 min to various temperatures in a water bath. After heating, the tubes were plunged into a 4°C ice bath and subsequently tested on indicator L cells for toxicity.

Enzyme stability. Three milliliter aliquots of toxic medium containing 10% FCS (5 mg/ml protein as determined by the Folin–Lowry method) were incubated separately with 30 $\mu\text{g/ml}$ DNAase (Worthington, Freehold, NJ, electrophoretically purified), 30 $\mu\text{g/ml}$ RNAase (Worthington, electrophoretically purified), trypsin (1 mg enz/5 mg medium protein, Difco, Chicago, Ill), pronase (1 mg enz/5 mg medium protein, Cal. Biochem., Los Angeles, Ca), or neuraminidase (10 units/ml, Cal. Biochem.). The media were incubated for 48–72 hr at 37°C and the enzymes inactivated by boiling for 15 min prior to testing for toxicity. Control tubes containing MEM + 10% FCS were tested simultaneously with the same levels of enzymes.

Sephadex column chromatography. The approximate size of the macrophage toxin(s) was estimated by molecular sieving on a 1.4 \times 35 cm G-100 Sephadex bed in a siliclad (Clay Adams, Parsippany, NJ) glass column. The column was equilibrated with 0.01 M Tris–HCl, 0.025 M NaCl, 10^{-5} M EDTA, pH 7.0, and calibrated by applying a 0.7 ml sample containing the following molecular weight markers (Pentex, Kankakee, Ill.): (a) bovine serum albumin (BSA), 67,000 daltons, (b) human gamma globulin, 150,000 daltons, and (c) ovalbumin, 47,000 daltons. Then the flow rate was adjusted to 18 ml per hour and 1 ml fractions were collected at 4°C. The absorbancy of each 1.0 ml fraction was measured at 280 nm. Toxic media and control MEM were concentrated 10 \times in an Amicon (Lexington, MA) ultrafiltration unit using a PM 10 membrane and were then fractionated in a similar manner. Adjacent fractions were then pooled, reconstituted with STP, filter-sterilized and tested for toxicity on L cells.

Antiserum production. Medium containing phytohemagglutinin (PHA)-stimulated Swiss Webster mouse lymphotoxin (MLT) was concentration 10 \times by Amicon ultrafiltration, then fractionated and concentrated by sequential ammonium sulfate precipitation (9). Toxicity was found in the precipitate which formed between the 50–80% salt saturation level. The precipitate was sedimented, resuspended in distilled water and passed through a G-25 Sephadex column or dialyzed against 0.15 M NaCl to remove the ammonium sulfate. This solution contained 15–30 mg/ml protein. It was then emulsified with an equal volume of Freund's Complete Adjuvant. Two Swiss goats were each injected with 0.5 ml of the emulsion sub-

cutaneously on each side and 0.5 ml intramuscularly in the adductor magnus. After 14 days, the goats were reinjected in a similar fashion and bled from the jugular vein 7 days after the second injection. The blood was allowed to clot at 37°C for 2 hr. The serum was then collected, cleared of red cells by centrifugation, heat-inactivated at 56°C for 30 min, and stored at -18°C. Normal goat serum (NGS) was collected from the same goats prior to injection.

RESULTS

Specificity of Macrophage-induced in vitro target cell destruction

A drop containing either HeLa cells, syngeneic C57B1/6 fibroblasts or allogeneic L cells was carefully placed at two separate loci on three different monolayers of immune C57B1/6 macrophages as described in Methods. The plaque bottles were sealed and incubated at 37°C. The cell monolayers were examined at regular intervals with the light microscope and stained with crystal violet after 48 hr to develop the plaques. The absence of a dark staining area of target cells against the background of monolayer macrophages indicates the absence of target cells. It is assumed these cells have been destroyed, since viable cells were not present in the medium. The results of a typical experiment can be seen in Fig. 1. Bottles A and B received an overlay of xenogeneic HeLa cells and syngeneic C57B1/6 fibroblasts, whereas bottle C received an overlay of allogeneic L cells. Upon microscopic examination, it was observed that both the target L cell and aggressor macrophage were undergoing simultaneous destruction.

Release and specificity of macrophage toxins

Several authors have reported that lymphocytes may cause target cell destruction by secreting a nonspecific cell toxin(s) (10-14). Pincus has shown that normal macrophages release a cell toxin when exposed *in vitro* to soluble antigens (15). It seemed plausible that immune peritoneal macrophages might release a cell toxin(s) during this process of *in vitro* target L cell destruction. To test the possibility, test medium was collected from monolayers of immune macrophages cultured for 48 hr in the presence of specific target L cells. The medium was cleared of cells and debris by centrifugation at 300g for 15 min and tested for toxicity on fresh target L cell monolayers. The indicator cultures were examined microscopically at various intervals and after 48 hr assayed for viability by the ¹⁴C amino acid incorporation technique (7). The first indication of medium effects became evident at 12-24 hr, when bipolar vacuoles appeared in the target cells. Between 24-48 hr the process of vaculation continued until the entire target cell cytoplasm was involved. Finally, the cells ruptured leaving only a pyknotic nucleus and cell debris attached to the glass. The changes observed in Macrophage Toxic Factor (MTF)-treated L cells were very similar to those previously described for MLT-treated L cells (8). There was variability in the capacity of different batches of test media to cause target cell destruction. Generally, macrophages taken from immune C57B1/6 mice which had totally rejected the tumor caused complete target cell destruction, and the cell-free media had high levels of cytotoxic activity. In contrast, macrophages taken from C57B1/6 mice with a low macrophage to tumor cell ratio caused less target cell destruction, and the cell-free media had little or no

EFFECTS OF SAI IMMUNIZED C57BL/6 PERITONEAL MACROPHAGES ON XENOGENEIC, SYNGENEIC AND ALLOGENEIC CELLS IN VITRO

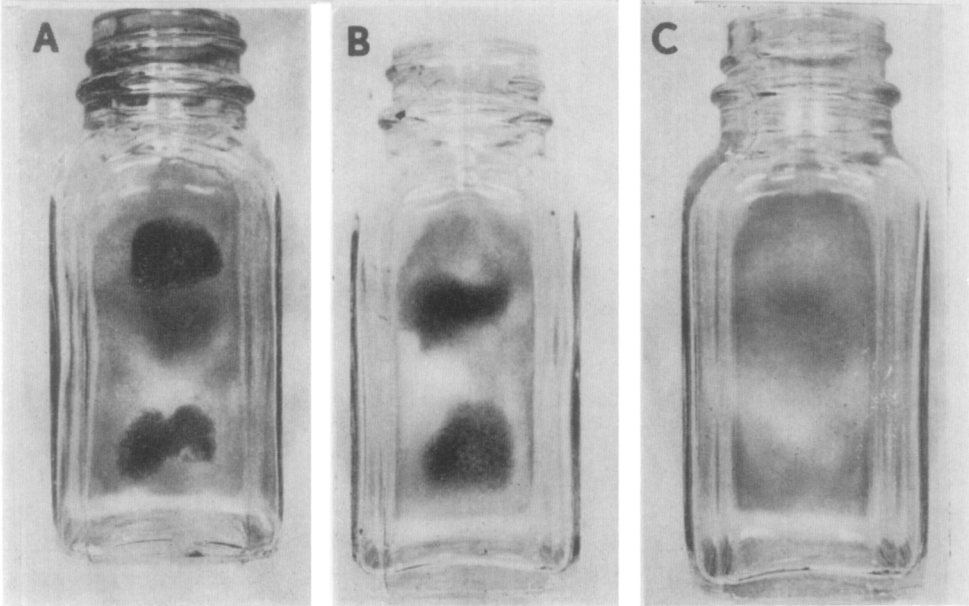


FIG. 1. Effects of SAI immunized C57B1/6 peritoneal macrophages on xenogeneic, syngeneic, and allogeneic cells *in vitro*. The dark staining spots are overlay target cells on the macrophage monolayer. The absence of the spots are indicative of target cell destruction. (A) Xenogeneic HeLa Cells, (B) Syngeneic C57B1/6 Fetal Fibroblasts, (C) Allogeneic L Cells.

cytotoxic activity. Certain animals did not apparently respond and were killed by the tumor. Cell-free media from control preparations, fresh MEM and MEM from L cell monolayers demonstrated no cytotoxic activity. Control media from immune macrophage monolayers cultured in the absence of target cells, however, caused early vacuolation similar to that observed with toxic medium, but 50%–100% of the cells recovered and were not killed. Additional studies were performed to determine the specificity of the toxic medium. Figure 2 shows the results of one of several such experiments. It is apparent that the medium was cytotoxic for allogeneic L cells, xenogeneic HeLa cells and syngeneic C57B1/6 fetal fibroblasts. In many cases we observed that lower dilutions stimulated cellular protein synthesis above control cultures. A series of extensive experiments verified that medium toxicity was not due to microbial contamination and could not be reversed by adding fresh serum or essential nutrients.

Induction of cell toxin release

Experiments were next performed to determine the nature and specificity of the release of the cell toxin. Pure monolayers of immune, starch-induced and normal

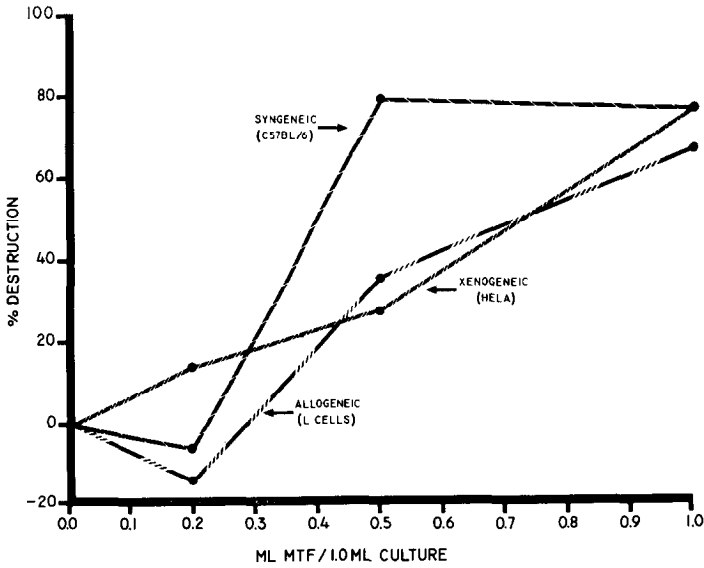


FIG. 2. The Effect of Dilutions of Medium Containing MTF on Syngeneic C57B1/6 Fetal Fibroblasts, Allogeneic L Cells and Xenogeneic HeLa Cells.

peritoneal macrophages from C57B1/6 mice were established in 250 ml plastic flasks. The monolayers were either overlaid with a ratio of 5 L cells to 1 macrophage or left untreated. Then the culture vessels were placed at 37°C. After 48 hr of incubation, the medium was collected and cleared of cells and debris. The toxic media was diluted with fresh MEM and tested on L cell monolayer tube cultures. In one experiment HeLa cells were used in place of L cells as the target cell. Figure 3 illustrates results which were characteristic of these experiments. Medium

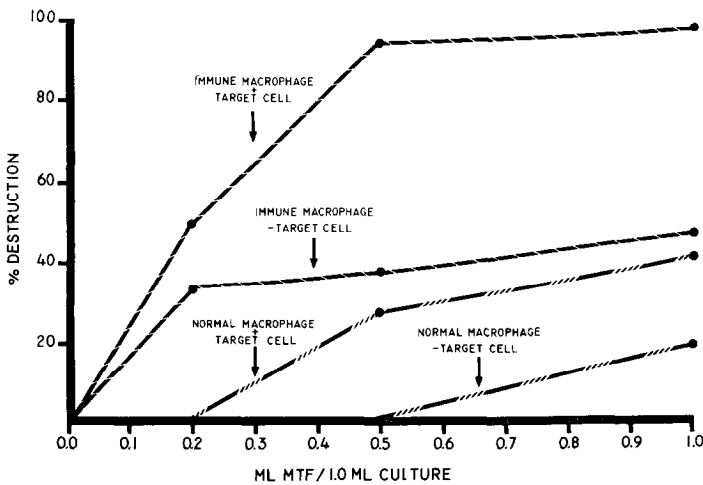


FIG. 3. Induction *in vitro* of MTF from Peritoneal Macrophages obtained from SA1-Immunized and Normal C57B1/6 Mice in the presence or absence of target L cells.

from cultures of immune macrophages + L cells was highly cytotoxic, even at a dilution of 1:5. Control cultures of immune cells alone, however, had a reproducibly low level of activity. Medium from cultures of normal cells + target cells or normal cells alone had a low level of toxicity. The data obtained with starch-induced macrophages paralleled that shown for normal macrophages. While it is not shown, the amount of medium toxicity detectable when HeLa cells were used as targets was parallel to that found when immune cells were cultured in the absence of target cells.

Macromolecular Synthesis of "immune" and "nonimmune" peritoneal macrophages

Experiments were designed in an attempt to compare the relative amounts of DNA, RNA and protein synthesized by starch-induced and SA1 tumor-"activated" peritoneal macrophages. These cells were collected from C57B1/6 mice and pulse-labeled with various radioactive precursors as described in Materials and Methods. The results of a typical experiment are visualized in Table 1. These experiments were repeated with the cells from three separate sets of animals and were highly reproducible. It is clear that levels of nuclei acid synthesis were only slightly elevated in immune cells as compared to starch-induced cells; however, on a per cell basis, the level of immune macrophage protein synthesis was approximately 12 ± 2 times that of a starch-induced macrophage.

Characterization of the cell toxin

Heat stability and Enzyme Susceptibility. Samples of toxic and control media were heated at various temperatures for 15 min and subsequently tested for toxicity on L cell monolayers. As shown in Table 2, the cytolytic activity was stable to heating at 100°C for 15 min. Control tubes containing MEM, which were subjected to the same protocol showed no toxicity. Neither DNAase (30 µg/ml), RNAase (30 µg/ml), trypsin nor neuraminidase treatment destroyed or reduced the toxic effect of the medium. Pronase treatment, however, partially reduced the toxic effect of the medium.

Sephadex column chromatography. Initial experiments revealed toxicity was nondialyzable. Therefore, studies were performed using small 1.4 × 7.0 cm G-50, G-75 and G-100 Sephadex columns. All Sephadex chromatography was performed on 10× concentrated test and control normal MEM. Toxicity for L cells was excluded with the blue dextran marker in the case of the G-50 and G-75 columns.

TABLE 1

COMPARISON OF MACROMOLECULAR SYNTHESIS IN SA1-IMMUNIZED AND STARCH-INDUCED C57B1/6 PERITONEAL MACROPHAGES

	Cell No.	DNA(³ HTdr)	RNA(³ HUdr)	Protein(¹⁴ C A.A.)
SA1-Immunized	2.5×10^6	2097 ^a	61,683	13,527
	8.0×10^6	3000	156,373	64,491
Starch-induced	2.5×10^6	1617	64,050	1029
	8.0×10^6	2220	144,561	4689

^a Extracted counts per minute.

TABLE 2
THE ABILITY OF HEAT AND VARIOUS ENZYME TREATMENTS
TO ABLATE MTF ACTIVITY IN VITRO

	MTF	MEM
Untreated	85 ^a	0.0
100°C(15min)	88	0.0
DNAase(30µg/ml)	97	0.0
RNAase(30µg/ml)	98	4.0
Neuraminidase(10units/ml)	95	6.0
Trypsin(1mg/5mg)	92	13
Pronase(1mg/5mg)	12	28

$$^a \% \text{ Destruction} = \left(\frac{\text{CPM Control} - \text{CPM Experimental}}{\text{CPM Control}} \right) \times 100.$$

It was retarded, however, on the G-100 column and eluted in the fraction following the blue dextran marker. Toxic and control media were then fractionated on a 1.4 × 30 cm G-100 Sephadex column. Figure 4A shows the elution profile of three protein markers and the toxicity observed with control preparations. Figure 4B shows the elution profile of test medium. One peak of toxicity eluted from the column with γG globulin (= 150,000 ml wt) while the second peak eluted between the BSA and OA markers (= 47,000 ml wt).

Neutralization of toxicity by goat anti-serum. Goats were immunized with ammonium sulphate fractionated MLT as described in Methods. The ability of this antiserum to neutralize the macrophage toxin was tested *in vitro*. A varying amount of test and control sera was added to 2.0 ml cultures of L cells (1.5 × 10⁵ cells/

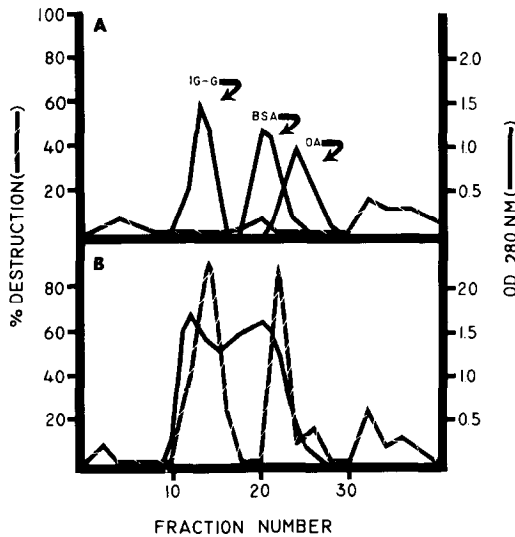


Fig. 4. Fractionation of MTF Activity by Sephadex Chromatography. (A) Solid line: Optical Density A²⁸⁰ Profile of Human IgG (IG-G), Bovine Serum Albumin (BSA), and Ovalbumin (OA) from a 1.4 × 30 cm Sephadex Column. Broken line: Elution Profile of Toxicity of Control 10× MEM from same column. (b) Optical Density A²⁸⁰ Profile (Solid line) and Elution Profile of Toxicity (Broken line) of 10× MTF Medium.

tube), containing either toxic medium from macrophage or lymphocyte cultures, or control MEM. Figure 5 shows the results of these experiments. The MTF employed in these experiments was toxic at a 1:2 dilution, while the MLT was toxic at a 1:5 dilution under the same conditions. This may account for the apparent difference in the ability of the antiserum to neutralize the MTF more effectively than the MLT. The antiserum obtained from the MLT-immunized goat completely protected the target cells from the cytolytic action of both MLT and the macrophage factor(s). In contrast, normal serum from the same goat was unable to neutralize the toxic effect. A second control experiment was performed to test the specificity of the antiserum. The highest level of antiserum employed in the previous experiment was used in an attempt to neutralize the LT secreted by PHA-activated human lymphocytes *in vitro*. Medium containing HLT was diluted 1:1 with fresh MEM before testing in order to bring the toxic level of this medium to that of the MLT-containing medium. Table 3 shows the outcome of these experiments. The antiserum was unable to neutralize the toxic effects of the HLT at the levels used.

DISCUSSION

It is clear that purified monolayers of peritoneal macrophages obtained from SA1-immunized C57B1/6 mice release toxic substances into the culture medium upon interaction with specific target cells *in vitro*. Furthermore, these materials act nonspecifically, since they destroy cultures of syngeneic, allogeneic and xenogeneic target cells. Cell destruction is not synchronous, although the majority of the target cells are killed in a period of 24–72 hr after initial exposure to the toxic medium. The cytotoxic effect is an active process and not due to microbial contami-

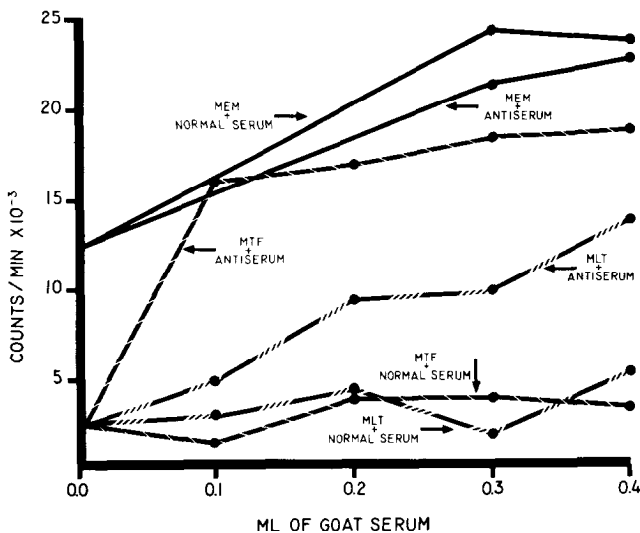


FIG. 5. The effects of normal goat serum and goat antiserum to PHA-induced mouse lymphotoxin (MLT) on MLT and MTF activity *in vitro*. Cell viability is expressed as CPM incorporated into target cell protein. The amount of serum employed is expressed as ml serum added per 2.0 ml test or control medium.

TABLE 3
EFFECTS OF NORMAL GOAT SERUM AND GOAT ANTISERUM TO MOUSE LYMPHOTOXIN
ON PHA-INDUCED HUMAN LYMPHOTOXIN ACTIVITY IN VITRO

Medium ^a	Serum ^b	% Destruction
HLT	NORMAL GOAT	88
HLT	GOAT ANTI-MLT	91
HLT	NONE	90
MEM	NONE	0.0

^a HLT diluted twofold with MEM.

^b 0.4ml goat serum + 2.0ml of medium.

nation or depletion of essential medium nutrients. The cytotoxins are not present in the expended medium in high concentration, for they quickly lose activity upon dilution with fresh medium. A series of experiments revealed that maximum amounts of the toxic materials are recovered from culture media containing 10–15% Fetal Calf Serum. It has been demonstrated that polymorphonuclear neutrophils (PMN) release toxic substances upon disintegration in culture (16). Our preparations did not, however, contain significant numbers of PMN. Although these cell preparations contain 1% contaminating lymphocytes, the possibility that they are releasing the factors in this study is very remote. For detectable levels of factor release requires 10^3 times this number of lymphocytes per milliliter in cultures of PHA-activated splenic lymphocytes, and this stimulation is more potent than specific antigen stimulation.

It became apparent that monolayers of normal and starch-induced C57B1/6 mouse peritoneal macrophages also release low levels of cytolytic substance(s) into the culture media. We found that the levels of cell toxin increase when the macrophage monolayers are incubated with cellular antigens, various soluble antigens or PHA. This phenomenon has been previously described *in vitro* by Pincus (15). We have no firm explanation for factor release under these conditions.

Whether the appearance of the cytotoxin(s) in the culture medium is due to active macrophage protein biosynthesis and secretion subsequent to contact with the target cell, or simply due to the release of toxic intracellular materials into the medium upon cell death is, at present, unknown. The former situation is suggested by the following three observations. It has been shown that cell extracts from 10^8 macrophages/ml are needed to destroy target cell monolayer cultures *in vitro* (17). Since the cell concentration in these extracts is several orders of magnitude higher than those used in our studies, the medium toxicity we have observed would not appear to be due to macrophage destruction. Secondly, immune macrophages have an elevated rate of protein biosynthesis. Finally, one series of experiments indicates that the cell toxins are present in culture media before any evidence of target-aggressor cell destruction can be observed (prior to 24 hr).

Fractionation of the toxic medium on Sephadex G-100 columns reveals that the activity is associated with at least two macromolecules of approximately 50–80,000 and 100–150,000 mol wt. The cytotoxic activity is stable for long periods at 4°C in high serum-containing medium and to exposure to 100°C for 15 min. The macro-

molecule(s) is insensitive to DNAase, RNAase and neuraminidase, indicating that it is not nucleic acid or protein containing *N*-acetyl neuraminic acid. While the activity is not destroyed by treatment with the proteolytic enzyme trypsin, it is partially ablated by pronase digestion. (These experiments have proven difficult to evaluate for the pronase digestion of control medium apparently generates toxic peptides.) Because of the amount of serum present, the interpretation of the results with trypsin and neuraminidase may be questionable. The macrophage and lymphocyte cytotoxic factors are apparently antigenically related, since both are inactivated by the antiserum made against the lymphocyte factor. This is a surprising result, for while the two share certain physical properties, i.e., heat stability and enzyme resistance, the macrophage factor(s) differs in molecular weight from the lymphocyte factor (9). These observations suggest that if they are indeed the same, they may under certain conditions, be able to complex with themselves or other serum proteins.

The large cell toxin released by target cell-stimulated immune C57Bl/6 mouse peritoneal macrophages is similar in size to the toxin secreted *in vitro* by PPD-stimulated immune guinea pig alveolar macrophages reported by Heise and Weiser (12). The latter cell toxin is, however, more heat sensitive, which may reflect the fact that they come from different animal species. Several articles by Pincus have described the *in vitro* release of a low molecular weight cell toxin by both antigen-stimulated immune and normal guinea pig macrophages (15, 18-20). This material has been recently identified as a phospholipid of a molecular weight of less than 1,000 daltons (20). The cytotoxin(s) described in the present report appears to be unrelated to the factor described by Pincus, since the activity is clearly associated with macromolecules. Additional physical studies are required to either affirm or negate this relationship.

It has been previously demonstrated that immune macrophages from SA1-immunized C57Bl/6 mice cause specific nonphagocytic contact destruction of target cells carrying SA1 antigens *in vitro* (5). Yet the present results indicate the immune C57Bl/6 mouse peritoneal macrophages are capable of releasing non-specific cytotoxic substances into the culture medium upon exposure to specific cellular antigens. These data taken together suggest that the specificity of these reactions may lie at the level of recognition. Recognition probably occurs via a previously described specific heat-elutable material (possibly cytophilic antibody) on the surface of the immune macrophage (5). It is probable that the cytophilic antibody (CAB) provides the receptor which permits the macrophage to both recognize and attach to the target cell antigens. Subsequent to cell contact, cell destruction may then occur by a nonspecific mechanism, namely the release of nonspecific cell toxins by the macrophage. There is also a report that mouse macrophages can release a specific toxin into the culture medium upon interaction with target cells *in vitro* (21). The relationship of this specific toxin to MTF is at present unknown. While destruction does not involve phagocytosis, one might envision a somewhat similar process where the cell releases the cell toxins (lysosomal enzymes?) at the junction of the macrophage-target cell contact points, as suggested by the study of Journey and Amos (22).

At present, there is no evidence to support the *in vivo* role of macrophage cyto-

toxic factors in these reactions. Preliminary studies, however, have indicated that the ascites fluid taken from C57B1/6 animals which have just rejected the tumor are nonspecifically cytotoxic when tested on cells in tissue culture. Whereas cell-free ascites fluid from tumor bearing A/JAX mice or C57B1/6 mice, prior to tumor rejection, was not toxic. Ascites fluid toxicity has also been reported previously in mice undergoing an immune response to Erlich's Ascites Tumor (23). In this system, the cell-free ascites fluid would cause cell damage to these cells *in vitro*. It remains to be demonstrated that the factors from the ascites fluid are the same as those released *in vitro* from the activated macrophage. In addition, recent studies have demonstrated that nonspecific target cell destruction can occur *in vivo* in cutaneous delayed hypersensitivity reactions (24, 25). The main effector cell in these reactions appears to be a monocytic cell. The specificity of these reactions then may be attributed to CAB on the immune macrophage, and the mechanism of cellular destruction may occur by release of a nonspecific cell toxin(s).

REFERENCES

1. Gorer, P. A., *Advances in Canc. Res.* **4**, 149, 1956.
2. Baker, D., Weiser, R. S., Jutila, J., Evans, C. A., and Blandau, R. J., *Ann. N.Y. Acad. Sci.* **101**, 46, 1962.
3. Tsoi, M., T. and Weiser, R. S., *J. Nat. Canc. Inst.* **40**, 23, 1968.
4. Bennett, B., *J. Immunology*, **95**, 656, 1965.
5. Granger, G. A. and Weiser, R. S., *Science* **151**, 97, 1966.
6. Kolb, W. P. and Granger, G. A., *Proc. Nat. Acad. Sci. U.S.A.* **61**, 1250, 1968.
7. Granger, G. A. and Kolb, W. P., *J. of Imm.* **101**, 111, 1968.
8. Williams, T. W. and Granger, G. A., *J. of Imm.* **102**, 911, 1969.
9. Kolb, W. P. and Granger, G. A., *Cellular Immun.* **1**, 122, 1970.
10. Granger, G. A. and Williams, T. W., *Nature* **218**, 1253, 1968.
11. Ruddle, N. H. and Waksman, B. H., *Science* **157**, 1060, 1967.
12. Hiese, E. R. and Weiser, R. S., *J. Immun.* **103**, 570, 1969.
13. Dumonde, D. C., Wolstencroft, R. A., Panoyi, G. S., Matthow, M., Morley J., and Hawson, W. T., *Nature* **224**, 38, 1969.
14. Pincus, W. B., Wood, W. W. and Pang, R., *J. Retic. Soc.* **7**, 220, 1970.
15. Pincus, W. B., *J. Reticuloendothel. Soc.* **4**, 140, 1967a.
16. Falk, R. E., Falk, J. A., Möller, E., and Möller, G., *Cellular Immunology* **1**, 150, 1970.
17. Granger, G. A., "Studies on *in vitro* homograft rejection in mice." Ph.D. thesis. University of Washington Library, Seattle, WA, 1965.
18. Pincus, W. B., *J. Reticuloendothel. Soc.* **4**, 122, 1967b.
19. Pincus, W. B., *J. Amer. Rev. Resp. Dis.* **98**, 250, 1968.
20. Sintek, D. E. and Pincus, W. B., *J. Retic. Soc.* **8**, 508, 1970.
21. McIvor, K. L. and Weiser, R. S., *Immunology* **20**, 315, 1971.
22. Journey, L. J. and Amos, D. B., *Canc. Res.* **22**, 998, 1962.
23. Hartveit, F., *Acts. Path. et. Microbiol. Scandinav.* **58**, 10, 1963.
24. Morton, D., Eilber, F. R., Malingren, R. A., and Wood, W., *Surgery* **68**, 158, 1970.
25. Williams, A. C. and Klein, E., *Cancer.* **25**, 450, 1970.