EVIDENCE FOR A CYTOLYTIC FACTOR RELEASED
BY MACROPHAGES

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After 8 h in vitro cultivation mouse peritoneal macrophages acquire the ability to
lyse syngeneic or allogeneic erythrocytes (1). The lytic process is extracellular
and energy-requiring (2, 3).

Under ordinary tissue culture conditions with serum added to the medium, the
lysis demands contact or close proximity between effector cells and target cells.
Under these conditions one can observe morphological characteristics typical of
effector/target cell interaction.

However, by modifying the incubation conditions it is possible to demonstrate
a soluble lytic factor released from the macrophages. This factor is very unstable
and therefore not detected under ordinary conditions. The present report de-
scribes the spatial relationship between target and effector cells, and the con-
ditions under which the soluble lytic factor can be found in the medium from
macrophage cultures.

Materials and Methods

Cells and Culture Condition. Normal 4–6-wk old female B6D2F1/BOM (C57BL/6x DBA/2)
hybrid mice and athymic nude mice were killed with ether. Cultures of 5 x 10⁶ peritoneal macrophages
were established in circular wells in Linbro plastic plates (FB-16-24 TC, Linbro Chemical Co., Inc.,
New Haven, Conn.) with or without cover slips and grown at 37°C in 20 mM Hepes-buffered minimal
essential medium (HEMEM) (Biocult Laboratories, Glasgow, Scotland) (320 mM) containing 20%
fetal calf serum (Difco Laboratories, Detroit, Mich.) and antibiotics (crystalline penicillin, streptomycin, 50 IU/ml of each). For investigation by
transmission electron microscopy (TEM) 2 x 10⁶ macrophages were cultivated in 35 mm Nunclon
plastic petri dishes (Nunclon, Denmark). For details, see (1, 3). Cystein (CSH) and 2-mercaptoetha-
nol (MSH) (both obtained from Sigma Chemical Corp., St. Louis, Mo.) were dissolved in HEMEM
immediately before use, and the agents were added in a vol of 100 µl or less to the macrophage
cultures. Syngeneic erythrocytes served as target cells. The cells were collected in a 320 mosM sucrose
solution with 10 IU/ml heparin from the cut tail of a prewarmed mouse (40°C/15 min). The cells were
washed once in the same solution without heparin and isotope labeled with ¹⁴Cr as previously
described (1).

Abbreviations used in this paper: CSH, cystein; HEMEM, Hepes-buffered minimal essential
medium; MCF, macrophage cytolytic factor; MM, cell-free macrophage medium; MSH, 2-mercaptop-
ethanol; MSH-MM, cell-free macrophage medium containing 5 x 10⁻¹ M 2-mercaptoethanol;
NBCS, newborn calf serum; SEM, scanning electron microscopy; TEM, transmission electron
microscopy.
Cytotoxicity Experiments. Three types of cytotoxicity experiments were performed in the presence or absence of heat-inactivated (56°C, 30 min) 10% newborn calf serum (NBCS) (Grand Island Biological Co., Grand Island, N. Y.).

Conventional cytotoxicity study. Labeled target cells in 1 ml medium were added to the macrophage cultures which had been thoroughly rinsed.

Contact/proximity cytotoxicity studies. The effect of separating effector/target cells was studied by using wells in Linbro plates equipped with perspex rings (diameter: 12 mm, wall thickness: 1/10 mm, height: ½ mm, 2 mm, 4 mm). The wells were filled with 1 ml target cells in medium, and the erythrocytes were left to sediment. 45 min later cover slips with rinsed macrophages were mounted on top of the rings with the macrophage layer underneath facing the sedimented target cells.

Cell-free cytotoxicity studies. Macrophages were rinsed and reincubated with or without MSH or CSH in serum-free medium. This macrophage medium was collected and centrifuged (1,000 rpm/5 min) and added as cell-free macrophage medium (MM) to target cells in 5-ml sterile plastic centrifuge tubes for cytotoxicity tests. The effect of heat on MM cytotoxicity was investigated by heating MM in Leighton tubes (Belco Glass, Inc., Vineland, N. J.) to 60°C and 100°C. After 30 min the tubes were removed, cooled on ice, and target cells added for the cytotoxic assay at 37°C.

The ratio of target cells to macrophages either present during cell interaction or present in cultures from which MM was obtained was always 10:1. After 2-4 h of incubation samples were taken for quantitation of cytotoxic effect as previously described in detail (1). Quantitation of pinocytosis and phagocytosis was performed by the use of colloidal 111Au- and 51Cr-labeled human erythrocytes as previously described (4).

Morphological Studies. Cover slips with washed macrophages were fixed in 2% glutaraldehyde in 0.1 M phosphate-buffered saline (pH 7.4), rinsed and inverted over a drop of water on glass slides for examination in the phase contrast microscope at × 1,000 linear magnification. Material for electron microscopy was fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) with 0.1 M sucrose. Some of the material was processed for demonstration of the glycocalyx with the ruthenium red method (5) and with the concanavalin A-peroxidase method (6). Specimens were postfixed in 1% OsO4 in 0.1 M cacodylate buffer and dehydrated in ethanol of increasing concentration. For TEM studies the cells were embedded in Epon either as a pellet after centrifugation, or in situ in the culture dish. Sections were made with a LKB microtome III and examined in a Hitachi HU-12 electron microscope (Hitachi Ltd., Tokyo, Japan) after staining with lead. Some were also stained "en bloc" with 2% uranyl acetate in absolute ethanol as the last step in dehydration.

Material for scanning electron microscopy (SEM) was transferred to amylacetate after ethanol dehydration, desiccated by critical point drying in a Hitachi HSU-6 apparatus, and coated with carbon and gold in a Hitachi evaporation unit. The specimens were examined in a Hitachi HHR-2 microscope.

Results

Cytotoxicity Experiments Performed in Medium with Serum. When isotope-labeled target cells in HEMEM with 10% NBCS were sedimented on rinsed 18-h old macrophage monolayers there was a rapidly increasing isotope release reaching 60% in 2 h. The spontaneous release from target cells alone was only 10% (Table I). As already described (1) assays performed in the presence of 10% NBCS showed no lysis when: (a) MM was transferred to target cells, (b) the homogenate of macrophages (cell disruption obtained by homogenization, sonication, osmotic stress, and rapid decompression of an inert gas) were incubated with target cells, or (c) macrophages were separated from target cells by dialysis membrane or Millipore filter. However, macrophage monolayers separated only ½ mm from the sedimented target cells by a perspex ring caused 40% isotope release while a separation distance of 2 mm gave only 20% release in the presence of serum (Table I).

By phase contrast microscopy well spread macrophages were found. In close
The Influence of Effector/Target Cell Separation on Mouse Macrophage Cytotoxicity in Medium with 10% NBCS as Demonstrated by Chromium Release from Isotope-Labeled Syngeneic Erythrocytes after 4-h Incubation

| Degree of effector/target cell separation | Cytotoxicity | Range |
|-----------------------------------------|-------------|-------|
| Erythrocytes sedimented on macrophage layer | 80          | 65-95 |
| Erythrocytes separated from effector cells by a 1/2 mm perspex ring | 45          | 30-60 |
| Erythrocytes separated from effector cells by a 2 mm perspex ring | 20          | 15-25 |
| Erythrocytes incubated in macrophage supernate or homogenate | 10          | 5-15  |
| Spontaneous lysis from erythrocytes in cultivation medium | 5           | 5-15  |

All data are average values from a minimum of three determinations at each point.

contact with the macrophages, numerous erythrocyte ghosts and erythrocytes with abnormal configuration—some obviously spherocytic, were seen (1).

TEM revealed close proximity and sometimes contact between macrophages and target cells (Fig. 1). This was the case both in the pelleted preparations and in the material fixed in situ. There was no obvious specialization in the macrophage cell membrane at the point of closest proximity. However, the pictures suggested that the macrophages have a more irregular outline with small projections around the contact zone. Some of the erythrocytes had a clearly abnormal form (droplet, spherocytic) and ghosts were observed. A prominent coat was found on the surface of both the macrophages and the red blood cells, and this was essentially the same with the two different methods of coat-demonstrations (ruthenium red, concanavalin A-peroxidase)(Fig. 2). In contact areas the coats of the two cells seemed to blend with each other resulting in a total thickness equal to the thickness of only one coat alone. The ghosts also showed coats at their surface (Fig. 2).

SEM displayed a great irregularity of the macrophage surface (Fig. 3) with numerous small projections. At the periphery, ruffling and slim projections were seen. In the perinuclear area depressions possibly related to endocytosis, were seen. Ghosts appeared as irregular heaps of membranes (Fig. 3). The red cell contact was invariably on one of the small projections, almost without exception in the perinuclear area, and never occurred on the flat peripheral surface. The red cells were frequently drawn out to a point at the site of contact (Fig. 4).

Cytotoxicity Experiments Performed in Absence of Serum. Macrophages incubated with target cells in medium without serum caused 80% isotope release in the course of 2 h, while the spontaneous lysis under these conditions was variable and ranged between 5 and 15% (Table II). The cytolytic effect of the macrophage monolayer separated from target cells by a perspex ring was 50%, and equally efficient irrespective of whether the separation was 1/2 mm or 4 mm. Transfer of a single 1 ml MM portion to target cells failed to cause lysis in 2 h.
Fig. 1. TEM micrograph of a mouse peritoneal macrophage (M) and a syngeneic erythrocyte
(E) incubated in medium with 10% NBCS. Close proximity between the two cells is observed
with no apparent macrophage membrane specialization. Note a small projection of the macro-
phage surface (arrow). x 19,500.

Fig. 2. TEM micrograph of a mouse peritoneal macrophage and syngeneic erythrocyte
ghosts (G) incubated in medium with 10% NBCS which show cell coats as demonstrated by the
concanavalin-A-peroxidase method. x 6,200.
FIG. 3. SEM micrograph of a mouse peritoneal macrophage (M) and syngeneic erythrocytes (E) incubated in medium with 10% NBCS. Erythrocyte ghosts (G) are seen in the perinuclear region of the macrophage surface which displays great irregularity with numerous microvilli. × 3,000.

FIG. 4. SEM micrograph of a mouse peritoneal macrophage (M) and a syngeneic erythrocyte (E) incubated in medium with 10% NBCS. Point-like contact between the two cells is observed. × 24,000.
However, when 100 µl aliquots of the medium from macrophage cultures (1 h previously rinsed and reincubated in 1 ml HEMEM) were successively transferred each 5 min to a 100 µl concentrated solution of target cells, to yield a final vol of 1 ml there was a 30% lysis after a further 2-h incubation (Table II).

MM from macrophage cultures previously rinsed and resuspended in 1 ml HEMEM with MSH or CSH for 2 h, greatly enhanced the lytic activity of transferred medium (Table III). Thus, the presence of $5 \times 10^{-4}$ M CSH caused

### Table II

**The Influence of Effector/Target Cell Separation on Mouse Macrophage Cytotoxicity in Medium Without Serum as Demonstrated by Chromium Release from Isotope-Labeled Syngeneic Erythrocytes After 2-h Incubation**

| Degree of effector/target cell separation | Cytotoxicity | Range   |
|-----------------------------------------|--------------|---------|
| Erythrocytes sedimented on macrophage layer | 75           | 65-85   |
| Erythrocytes separated from effector cells by a 4 mm perspex ring | 60           | 45-75   |
| Erythrocytes incubated in macrophage supernate transferred each 5 min in 100-ml aliquots to yield a final vol of 1 ml | 30           | 20-40   |
| Erythrocytes incubated in macrophage supernate transferred in one dose of 1 ml | 10           | 5-15    |
| Spontaneous lysis from erythrocytes alone in medium | 10           | 5-15    |

All data are average values from a minimum of three determinations at each point.

### Table III

**The Influence of Reducing Substances on Transferable Cytotoxic Activity in MM Without Serum from Macrophage Cultures as Demonstrated by Chromium Release from Isotope-Labeled Syngeneic Erythrocytes after 2-h Incubation**

| Effect of macrophage treatment with reducing substance | Cytotoxicity | Range   |
|--------------------------------------------------------|--------------|---------|
| Erythrocytes incubated in MM from macrophage culture preincubated with $5 \times 10^{-4}$ M cysteine for 2 h | 80           | 75-85   |
| As above with MSH                                       | 60           | 50-70   |
| Erythrocytes incubated in MM from macrophage cultures without previous exposure to cysteine or MSH | 10           | 5-15    |
| Spontaneous lysis from erythrocytes alone in medium with $5 \times 10^{-4}$ M cysteine | 20           | 5-35    |
| Spontaneous lysis from erythrocytes alone in medium with $5 \times 10^{-4}$ M MSH | 10           | 5-15    |

All data are average values from a minimum of three determinations at each point.
80% isotope release and 60% lysis occurred with $5 \times 10^{-4}$ M MSH, while the spontaneous lysis in test tubes with target cells alone was 20% and 5% respectively. There was no lysis when target cells were incubated in the presence of MSH in MM from macrophage cultures without previous exposure to MSH. Subsequent experiments employed $5 \times 10^{-4}$ M MSH because of the low rate of spontaneous lysis. The degree of macrophage medium cytotoxicity was dependent on the duration of MSH incubation time, and Fig. 5 shows the linear relationship obtained between MM cytotoxicity and the time of macrophage exposure to MSH when the experiments were performed at 37°C. At 4°C the cytotoxic reaction did not occur (Fig. 5).

MM incubated for 2 h with $5 \times 10^{-4}$ M MSH (MSH-MM) was selected for experiments dealing with the influence of heat, storage, dilution, dialysis, and absorption with unlabeled target cells on cell-free cytotoxicity. The cytolytic effect of MSH-MM was completely abolished by treatment at 100°C for 30 min, but not at 60°C (Table IV). Storage of MSH-MM for 6 h at 4°C and 5 h at 25°C also completely abolished cytolytic activity (Table IV). When MSH-MM was diluted with HEMEM containing $5 \times 10^{-4}$ M MSH, there was no lysis at a dilution of $\frac{1}{6}$ while at $\frac{1}{2}$ 50% lysis occurred (Table V). MSH-MM dialysed against 100 vol of HEMEM with $5 \times 10^{-4}$ M MSH caused no isotope release from the target cells while there was a 50% cytolysis when the dialysis procedure was performed with a 1:1 vol. In this case the dialysate also caused 50% isotope release (Table V). When MSH-MM was incubated with $10^8$ unlabeled target cells for 30 min, and then centrifuged, the supernate was not cytolytic against labeled target cells (Table V).
TABLE IV
The Effect of Heating and Storage on Cytotoxicity Mediated By MM-MSH as Demonstrated by Chromium Release from Isotope-Labeled Syngeneic Erythrocytes After 2 h Incubation

| MSH-MM treatment                  | Cytotoxicity | Range |
|-----------------------------------|--------------|-------|
| Untreated                         | 60           | 50-70 |
| Heated, 100°C, 30 min             | 10           | 5-15  |
| Heated, 60°C, 30 min              | 60           | 50-70 |
| Storage at 4°C, 4 h               | 60           | 55-65 |
| 7 h                               | 10           | 5-15  |
| Storage at 25°C, 3 h              | 60           | 50-70 |
| 5 h                               | 10           | 5-15  |
| Spontaneous lysis from target cells alone in MSH medium | 10 | 5-15 |

All data are average values from a minimum of three determinations at each point.

TABLE V
The Effect of Dilution, Dialysis, and Absorption with Unlabeled Target Cells on Cytotoxicity Mediated by MM-MSH as Demonstrated by Chromium Release from Isotope-Labeled Syngeneic Erythrocytes After 2-h Incubation

| MSH-MM treatment                  | Cytotoxicity | Range |
|-----------------------------------|--------------|-------|
| Untreated                         | 60           | 50-70 |
| MSH-MM dilute, with MSH-medium    | 50           | 45-55 |
| ¼                                 | 20           | 10-30 |
| ½                                 | 10           | 5-15  |
| Dialysis against MSH-medium       | 10           | 5-15  |
| 1:100                             | 50           | 45-55 |
| 1:1                               | 50           | 45-55 |
| Dialysis solution when dialysed   | 1:1          | 5-15  |
| Previous absorption with 10⁴ unlabeled target cells | 10 | 5-15 |
| Spontaneous lysis from target cells alone in MSH-medium | 10 | 5-15 |

All data are average values from a minimum of three determinations at each point.

Supernates of macrophage cultures from athymic nude mice were equally lytic when compared to the normal B6D₂ strain. The cytolytic effectiveness was not enhanced when the number of contaminating lymphocytes was increased. The presence of MSH did not increase the capacity of macrophage endocytosis judged by the use of colloidal ¹⁹⁸Au as a marker of pinocytosis and the use of ⁵¹Cr-labeled human erythrocytes as a marker of phagocytosis.
Discussion

Mouse peritoneal macrophages in vitro develop a strong capacity to lyse syngeneic and allogeneic erythrocytes (1). The action is extracellular, energy-requiring, and occurs in the absence of the complete complement sequence (2, 3).

It is known that polymorphonuclear neutrophils release toxic substances upon disintegration in culture (7). Contamination with granulocytes is negligible in 18-h old macrophage cultures while there may be quite a number of such cells in cultures of freshly seeded macrophages. Since no cytotoxic activity was obtained in cultures of freshly seeded macrophages (1) granulocytes are excluded as a possible source of lytic molecules in our system. The possibility that contaminating T lymphocytes release lytic lymphotoxins (8) is also remote, because there was no correlation between the number of contaminating lymphocytes and cytolytic effectivity. Moreover, macrophage cultures from athymic nude mice were equally efficient producers of lytic medium, as the thymic BJD2 strain. This leaves us with the macrophage as the only possible source of the lytic factor.

In experiments performed under ordinary tissue culture conditions with serum present in the medium isotope release was only obtained when the target cells were sedimented on the macrophage layer. When effector and target cells were separated by the slightest distance a drastically reduced cytotoxic effect could still be demonstrated. We have previously shown (1) that macrophages separated from target cells by a Millipore filter or dialysis membrane failed to cause target cell lysis in the presence of 20% serum. These experiments suggest that the macrophage cytotoxic effect is mediated by soluble factors that do not easily diffuse and are quickly inactivated by serum or other components in a normal macrophage culture medium.

The results obtained with serum-free medium further demonstrate the inhibitory effect of serum on the MM cytotoxicity. Under this condition there was a marked cytotoxic effect in spite of 4-mm separation between effector/target cells. However, in the absence of serum the cytotoxic factor appeared to be extremely unstable since transfer of MM in a single portion caused no lysis, whereas the stepwise transfer of MM in small volumes every 5 min caused significant lysis.

MM obtained from macrophage cultures incubated with low concentrations of reducing substances like MSH retained its lytic effect. Moreover, experiments with MM from cultures to which MSH had been added at different time points before the collection of the medium indicated that the MSH protected the lytic factors from degradation and thereby resulted in an accumulation of this factor during the incubation period. It thus appeared that the lytic factor was easily degradable possibly through oxidation. Inactivation caused by serum may be explained along these line, however, other possibilities should not be excluded. Preliminary experiments with MM from macrophages grown in a modified serum-free medium supplemented with SH-reacting reagents indicate that the macrophage-mediated lytic molecules contain thiol groups necessary for cytotoxic activity2 and that the oxidation of the SH-groups will render the molecules

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2 Melsom, H., T. Sanner, and R. Seljelid. 1974. Characterization of a macrophage cytotoxic factor. Manuscript in preparation.
ineffective. MM from noncytolytic freshly seeded macrophages (1) exposed to MSH caused no target cell lysis. This indicates that lysis cannot be attributed to microbial contamination or depletion of essential medium nutrients or to products from a normal cell coat or from cell metabolites by cleavage or chemical reaction by MSH treatment. The presence of MSH caused no increase in the process of endocytosis and an effect of MSH as an inducer of improved cellular metabolic activity therefore seems unlikely. Experiments designed to characterize the macrophage cytolytic factor (MCF) obtained from MSH-MM revealed that it is an easily dialysable low molecular weight substance. The factor resisted mild heating (60°C) for 30 min, but activity was removed by treatment at 100°C for 30 min. MCF was stable to storage for 3 h at 25°C and for 5 hours at 4°C. These results demonstrate that MCF is different from the macrophage cytotoxins previously described by Kramer and Granger (high molecular weight) (9), and Sintek and Pincus (stable at 100°C) (10).

In experiments where MM was preincubated with unlabeled erythrocytes, centrifuged, and the supernates tested against labeled target cells no further lytic activity was recorded. This indicates that the cytolytic activity could be removed from the supernate by absorption with target cells, and suggests an interaction between MCF and the red cell membrane. The molecular nature and site of action of MCF activity on target cells is unknown. Several processes may be involved, such as derangement of membrane molecules by detergent-like activity and enzymatic degradation, or chemical alteration of the erythrocyte membrane. An effect of MCF as metabolic inhibitor with subsequent interference with erythrocyte metabolism and ATP generation as well as interference with ion transport must also be considered. The experiments do very little to distinguish between these possibilities, but since the lytic process did not occur at 4°C (Fig. 5) it is tempting to speculate that the lysis involves enzymatic reactions. However, the inhibition of lysis by decreased temperature may also be explained on the basis of alterations in the erythrocyte membrane properties at lower temperatures.

The addition of MSH has previously been reported to improve lymphocyte viability in vitro (11) and to facilitate lymphocyte transformation (12). In the Mischell-Dutton system the presence of MSH increased the immune response (13) and MSH has also been reported to enhance the mixed leukocyte culture reaction in absence of supplemented proteins (14). The possibility that the addition of MSH in these systems act by stabilizing easily degradable substances similar to the ones operating in our experiments should be kept in mind.

As stated above, the contact requirement of lysis with serum present is most easily explained as a consequence of MCF inactivation by serum. Only when target cells are on or very close to the macrophages will the concentration of active MCF be effective. Thus the adherence of target cells to macrophages is probably a process separate from the lytic event. It is noteworthy that the target cells always were attached to small projections on the macrophage surface. This peculiar distribution of contact points may be related to the localization of phagocytic receptors on the macrophage membrane. So far very little is known about the precise distribution of such receptors (15), but the problem is currently under study in our laboratory.
There was no membrane specialization at the site of contact, in contrast to what has been found in macrophage/red cell interaction after opsonization (16) and in T lymphocyte/target cells contacts (17). The cell coat appeared, moreover, normal. These findings are in harmony with the fact that true contact is nonessential for the erythrocyte lysis by macrophages in our system.

Summary

Mouse peritoneal macrophages cultivated in vitro acquire a strong extracellular cytotoxic activity towards isotope labeled syngeneic erythrocytes as demonstrated by isotope release to the medium. This lytic process is mediated by an extremely labile macrophage cytolytic factor (MCF) which is not detected under ordinary tissue culture conditions with serum present in the medium. By the use of serum-free medium containing low doses of 2-mercaptoethanol MCF is stabilized and found to be an easily dialysable, low molecular substance which resists heating at 60°C for 30 min.

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