# CYTOTOXIC ACTIVITY OF MOUSE MACROPHAGES STUDIED BY VARIOUS INHIBITORS

By HAAKON MELSOM

*(From Norsk Hydro's Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, Oslo 3, Norway)*

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We have recently reported that mouse peritoneal macrophages can exert strong extracellular cytotoxic effect against allogeneic and syngeneic erythrocytes (1–3). This phenomenon was studied by isotope techniques, determination of hemoglobin release, as well as time-lapse phase contrast microcinematography. By these methods the cytotoxic reaction was found to require proximity between "activated" macrophages and target cells. Activation of the macrophages was achieved by prolonged in vitro cultivation, while immunization did not prove necessary. The killer reaction was found to involve some sort of osmotic lysis of the target cells (3).

This report will deal with the influence of various agents such as enzymes, metabolic inhibitors, membrane stabilizers, temperature change, and anaerobiosis on the cytotoxicity of macrophages. The results demonstrate that the cytotoxic activity of macrophages requires intact cell membrane and energy production while the process is independent of DNA, RNA, and protein synthesis. The killer mechanism seems to involve a cyanide-sensitive reaction.

## *Materials and Methods*

*Cells and Culture Condition.*—Macrophages were obtained from normal 4–6-wk old female $B_6D_2F_1$/BOM ($C_{57}BL/6_q \times$ DBA/2) hybrid mice. The cells were cultivated overnight in 1 ml Eagle's minimal essential medium (MEM) (Biocult, Glasgow, Scotland) with 20% fetal calf serum (FCS)[1] (Difco Laboratories, Detroit, Mich.) and antibiotics (crystalline penicillin/streptomycin, 50 IU of each) (Biocult). The osmolarity of the medium was adjusted from 290 mosM to 320 mosM either with sodium chloride or sucrose. All sera used were complement inactivated by heating (56°C for 30 min). The macrophages were incubated in silicone-stoppered Leighton tubes (Bellco Glass, Inc., Vineland, N.J.) or in circular wells in Linbro plastic plates (FB-16-24-TC, Linbro Chemical Co., Inc., New Haven, Conn.) at 37°C in 5% $CO_2$ and 95% air. In some experiments flying cover slips were used for morphological examination. The number of macrophages per well was $3 \times 10^5$ and in the Leighton tube $10^6$ cells. For details, see references 1 and 4.

Syngeneic erythrocytes served as target cells. These were obtained and isotope labeled with $^{51}Cr$ as earlier described (1). All washing procedures of erythrocytes as well as macrophages were performed in 20 mM Hepes-buffered MEM (HEMEM) with 2.5% FCS.

Experiments were started by adding isotope-labeled red cells in 1 ml MEM with 10%

[1] *Abbreviations used in this paper*: FCS, fetal calf serum; HEMEM, Hepes-buffered minimal essential medium; NBCS, newborn calf serum.

gamma globulin-free newborn calf serum (gg-free NBCS) (Grand Island Biological Co., Grand Island, N.Y.) to the macrophage cultures which had been thoroughly rinsed. The ratio of macrophages/erythrocytes was 1:10. Cytotoxic quantitation was determined after incubation for 2–3 h as previously described in detail (1). The effect of inhibitors on the macrophage cytotoxic activity was calculated from the equation

$$I = \frac{(R^o - S) - (R^i - S) \times 100}{R^o - S}$$

where $I$, inhibition ratio; $R^o$, isotope release from labeled target cells in the presence of macrophages not exposed to inhibitor; $R^i$, isotope release from labeled target cells in the presence of macrophages treated with inhibitor; and $S$, isotope release from labeled target cells alone.

*Enzymes, Pharmacia, and Chemicals.*—Unless otherwise stated macrophages cultivated in Linbro plates were preincubated with inhibitors before the addition of target cells. Macrophages were cultivated with papain (pure, E. Merck AG, Darmstadt, W. Germany), trypsin (Difco) and phospholipase-A (Bee Venom, Sigma Chemical Co., St. Louis, Mo.) in HEMEM without serum. In these experiments macrophages preincubated in serum-free HEMEM served as controls.

Chlorpromazine hydrochloride, cinkain, tetracain, hydrocortisone acetate, dexamethasone and sodium thioauromalate were used as pure substances obtained from various pharmaceutical companies. The other chemicals were of commercially available analytical reagent grade. Antimycin-A and rotenone were dissolved in absolute alcohol and oligomycin was dissolved in aceton. These agents were added to the macrophage cultures in a volume of less than 20 μl. This concentration of the solvent was not found to influence the cytolytic potential of the macrophages. The other substances were dissolved in HEMEM immediately before use and the agents were added in a volume of 100 μl or less to the macrophage cultures. The effect of temperature (4° and 45°C) was studied with macrophages cultivated in Leighton tubes containing Hepes-buffered cultivation medium during the period of temperature deviation.

*Set-up for Preparation of Extremely Hypoxic Cells.*—A small nitrogen cylinder (purified gas, Union Carbide Corp., New York) was connected with oxygen diffusion-tight plastic pipes (Argyle, Sherwood Medical Industries, Northern Ireland) to the Leighton tube with the effector/target cell in the incubator. The gas was led into the silicone stoppered tube through a Wasserman needle and escaped through another pipe-connected needle under water. Before the addition of target cells the whole system was flushed with nitrogen for 15 min and during the experiment the system was continuously flushed with gas. The oxygen concentration in the gas phase was determined with a Hersch oxygenmeter (Mark II, Engelhard Ind. Ltd., England). Hepes-buffered cultivation medium was used for these experiments.

*Morphological Examination.*—The cultures were gently washed with HEMEM at 37°C before the cover slips were fixed with 2% phosphate-buffered glutaraldehyde, rinsed, and inverted over a drop of water on glass slides. The slides were studied with phase contrast at X1,000 magnification. Observations were also made in Sykes-Moore tissue culture chambers (Bellco Glass, Inc.) to evaluate the extent of red cell attachment to macrophages. A final concentration of $10^7$ erythrocytes in 1 ml HEMEM with 10% gg-free NBCS was injected into these chambers with the macrophage layer at the bottom. After 30 min the chambers were turned upside down. 100 macrophages were counted and cells with at least five erythrocytes attached were considered as rosette-forming cells. Some cultures were studied by time-lapse phase contrast microcinematography as previously described (3). In all experiments viability by the trypan blue exclusion test remained over 90%.

## RESULTS

When macrophages were incubated with $^{51}Cr$ labeled erythrocytes in cultivation medium containing 10% gg-free NBCS there was a rapidly increasing

isotope release from the target cells reaching consistently about 60% in 2–3 h. For the same time period the isotope release from the target cells alone in the absence of macrophages was about 10%. By the use of inhibitors macrophage cytotoxicity was decreased to various degrees. In order to avoid influence on target cell integrity the inhibitors were mainly applied to the macrophages before the addition of target cells. However, in some cases, as stated in the text, inhibitors were present during effector/target cell interaction.

*The Influence of Membrane-Active Agents on Macrophage Cytotoxicity.*—The cytotoxic activity was drastically decreased when macrophages were pretreated with agents acting on macrophage surface before the addition of target cells (Table I). Thus Triton X-100, phospholipase-A, and papain almost completely inhibited the cytotoxic reaction while a less marked effect was obtained with trypsin. The effect of the proteolytic enzymes was reversible (Fig. 1), but the recovery process was sensitive to inhibitors of protein synthesis. Thus the continuous presence of cycloheximide (20 μg/ml) during the preincubation period with proteolytic enzymes as well as during effector/target cell interaction abolished the recovery of the cytolytic activity. Pretreatment of macrophages with the "membrane stabilizers" chlorpromazine hydrochloride, propanolol, cinkain, and tetracain (1), employed in concentrations previously shown to have membrane stabilizing activities (5–7), also reduced isotope release to 60% (Table II). In contrast hydrocortison acetate and dexamethasone did not affect cytotoxicity. To assess the requirement of divalent ions EDTA was applied. $10^{-3}$ M EDTA present during effector/target cell incubation completely impaired the cytotoxic reaction.

*The Influence of Inhibitors of Glycolysis on Macrophage Cytotoxicity.*—Sodium iodoacetate (IA) and sodium fluoride (NaF) employed in concentrations previously shown to inhibit macrophage glycolysis (8), both decreased the cytotoxic reaction when the target cells were added to pretreated macrophages (Table III). The effect of IA was most pronounced and macrophages exposed to $10^{-4}$ M IA lost 55% of their cytolytic activity while $10^{-5}$ M caused 25% inhibition.

TABLE I

*The Effect of Enzymes and Nonionic Detergent on Macrophage Cytotoxicity*

| Inhibitor | Concentration of inhibitor | Preincubated with the macrophages before the addition of target cells | Inhibition of cytotoxic activity |
|---|---|---|---|
| | μg/ml | h | % |
| Papain | 1,000 | 1 | 91 |
| Papain | 1,000 | ½ | 63 |
| Trypsin | 1,000 | 1 | 72 |
| Phospholipase-A | 30 | ½ | 90 |
| Phospholipase-A | 10 | ½ | 33 |
| Triton X-100 | 0, 1% (vol/vol) | ½ | 91 |

Data are average values from a minimum of four determinations at each point.

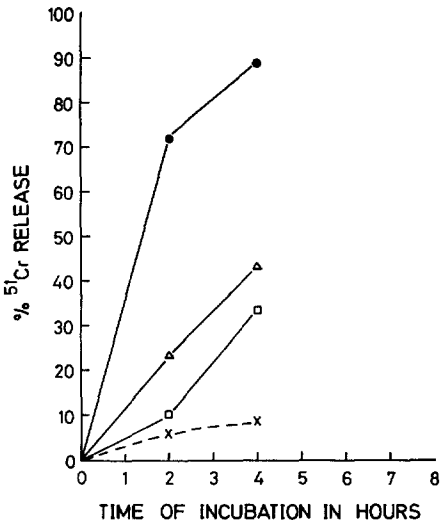


FIG. 1. Chromium release from labeled mouse erythrocytes in the presence (——) or absence (------) of mouse peritoneal macrophages pretreated with proteolytic enzymes before red target cells were added. ●, Macrophages without enzyme treatment; □, Macrophages pretreated 1 h with papain 1 mg/ml; △, Macrophages pretreated 1 h with trypsin 1 mg/ml. All data are average values from a minimum of four determinations at each point.

TABLE II

*The Effect of Membrane Stabilizers and Chelating Agents on Macrophage Cytotoxicity*

| Inhibitor | Concentration of inhibitor | Preincubated with the macrophages before the addition of target cells | Concurrent with the target cells | Inhibition of cytotoxic activity |
|---|---|---|---|---|
| | *M* | *h* | | % |
| Chlorpromazine | $10^{-4}$ | ½ | | 70 |
| Chlorpromazine | $7.5 \times 10^{-5}$ | ½ | | 32 |
| Chlorpromazine | $5 \times 10^{-5}$ | ½ | | 20 |
| Chlorpromazine | $2.5 \times 10^{-5}$ | ½ | | 0 |
| Propanolol | $3 \times 10^{-4}$ | 1 | | 54 |
| Propanolol | $2 \times 10^{-4}$ | 1 | | 36 |
| Propanolol | $10^{-4}$ | 1 | | 0 |
| Cinkain | $5 \times 10^{-5}$ | 1 | | 41 |
| Tetrakain | $5 \times 10^{-5}$ | 1 | | 53 |
| EDTA | $10^{-3}$ | | + | 100 |

Data are average values from minimum of four determinations at each point.

NaF ($10^{-3}$ M) resulted in 30% reduction of cytotoxicity. This was also the case when macrophages were preincubated with 5-thio-D-glucose ($10^{-2}$ M, 3 h) which inhibits glucose transport across the cell membrane (9) (Table III).

*The Influence of Inhibitors of Respiration and Oxidative Phosphorylation on Macrophage Cytotoxicity.*—The influence of inhibitors is shown in Table IV.

TABLE III

*The Effect of Glycolytic Inhibitors on Macrophage Cytotoxicity*

| Inhibitor | Concentration of inhibitor | Preincubated with the macrophages before the addition of target cells | Inhibition of cytotoxic activity |
|---|---|---|---|
| | *M* | *h* | % |
| NaF | $10^{-3}$ | 1 | 31 |
| Iodoacetate | $10^{-4}$ | 1 | 56 |
| Iodoacetate | $5 \times 10^{-5}$ | 1 | 31 |
| Iodoacetate | $10^{-5}$ | 1 | 23 |
| 5-Thio-D-glucose | $10^{-2}$ | 3 | 32 |

Data are average values from minimum of four determinations at each point.

TABLE IV

*The Effect of Inhibitors of Respiratory Chain and Oxidative Phosphorylation on Macrophage Cytotoxicity*

| Inhibitor | Concentration of inhibitor | Preincubated with the macrophages before the addition of target cells | Concurrent with the target cells | Inhibition of cytotoxic activity |
|---|---|---|---|---|
| | | | + | % |
| Anaerobiose | | | | 95 |
| KCN | $10^{-3}$ M | 1 | | 25 |
| KCN | $10^{-4}$ M | 0 | + | 98 |
| KCN | $10^{-5}$ M | 0 | + | 45 |
| KCN | $10^{-6}$ M | 0 | + | 20 |
| Antimycin-A | $10^{-4}$ M | 1 | | 23 |
| Antimycin-A | $10^{-4}$ M | 0 | + | 93 |
| 2,4-dinitrophenol | $10^{-3}$ M–$10^{-5}$ M | up to 3 | | 25 |
| | | 0 | + | 30 |
| Oligomycin | 20 μg/ml | 2 | | 25 |
| Rotenone | $5 \times 10^{-6}$ M | 1 | | 31 |
| | | 0 | + | 33 |

Data are average values from a minimum of four determinations at each point.

Extreme hypoxy with oxygen tension below 23 ppm during effector/target cell interaction, completely abolished macrophage cytotoxicity. The importance of a complete respiratory chain was evaluated with the inhibitors rotenone (which blocks transfer of electrons from NAD to cytochromes), antimycin-A (which blocks transfer of electrons from cytochrome *b* to *c*) and potassium cyanide (KCN) (which blocks the terminal cytochrome A–$A_3$ step) (10). When KCN was present during incubation, $10^{-6}$ M was found sufficient to influence cytotoxicity and $10^{-4}$ M inhibited it completely. However, macrophages pretreated for 1 h with $10^{-3}$ M KCN and rinsed before incubation with target cells lost only 30% of their lytic activity. Similar results were obtained with antimycin-A ($10^{-4}$ M). Rotenone ($5 \times 10^{-6}$ M), however, inhibited the isotope release by 30% whether the inhibitor was used in preincubation experiments or in the

presence with the target cells. 2–4 dinitrophenol and oligomycin were used to study the influence of oxidative phosphorylation (12). 2–4-dinitrophenol preincubated with the macrophages or applied during incubation with target cells equally reduced cytotoxicity by 30%. Pretreatment of macrophages with oligomycin caused similar decrease in cytolytic reactivity, but this agent could not be employed in the presence of target cells because of the hemolytic effect of its dissolvent.

Since the inhibition of electron transport by oligomycin can be reversed by 2,4-dinitrophenol which does not restore phosphorylation (11), the three inhibitors, IA, oligomycin, and 2,4-dinitrophenol employed in combination may markedly influence ATP formation while substrate oxidation still occurs.

TABLE V

*The Effect of Suppressed ATP Production with the Use of Combined Inhibitors on Macrophage Cytotoxicity*

| Inhibitor | Concentration of inhibitor | Preincubated with the macrophages before the addition of target cells | Inhibition of cytotoxic activity |
|---|---|---|---|
| | | *h* | % |
| Employed in combination: | | | |
| Iodoacetate | $5 \times 10^{-5}$ M | 1 | 92 |
| 2,4-Dinitrophenol | $5 \times 10^{-5}$ M | 1 | 92 |
| Oligomycin | 20 μg/ml | 2 | 92 |
| Each agent by itself: | | | |
| Iodoacetate | $5 \times 10^{-5}$ M | 1 | 31 |
| 2,4-Dinitrophenol | $5 \times 10^{-5}$ M | 1 | 25 |
| Oligomycin | 20 μg/ml | 2 | 25 |

Data are average values from a minimum of four determinations at each point.

This effect was investigated with macrophages preincubated with oligomycin (20 μg/ml, 2 h), 2,4-dinitrophenol ($5 \times 10^{-5}$ M, 1 h) and IA ($5 \times 10^{-5}$ M, 1 h) (Table V). Macrophages exposed to this inhibitor cocktail lost completely their cytotoxic activity while each agent by itself suppressed cytotoxicity only by 20–30%. Under these conditions macrophages were found to retract their pseudopods and become rounded, and judged by time-lapse microcinematography the cell movement was markedly reduced. However, only 10% of the cells were stained by trypan blue.

*The Influence of Inhibitors of Nucleic Acids and Protein Synthesis.*—Inhibitors of DNA (mitomycin-C) and RNA (actinomycin-D) synthesis had no influence on macrophage cytotoxicity. This was also the case when inhibitors of mitochondrial (ethidium bromide, chloramphenicol) and extramitochondrial (cycloheximide, puromycin) protein synthesis were applied.

*Miscellaneous Inhibitors.*—Pretreatment of the macrophages with 100 μg/ml sodium thioauromalate before the addition of target cells completely impaired

the cytotoxic reaction, and this agent was still inhibitory at 25 μg/ml (Table VI). When effector/target cell incubation took place in a medium with osmolarity raised from 290 mosM to 320 mosM with sucrose insead of sodium chloride, the cytotoxicity was reduced by 25% (Fig. 2).

*The Influence of Temperature.*—The cytotoxic effect was completely abolished by pretreatment of the macrophages at 45°C for 30 min even though viability was unchanged as judged by the trypan blue exclusion test. Similarly, the cytotoxic reaction did not occur at 4°C. The effect of low temperature was reversible since reincubation of the effector/target cells in the incubator at 37°C brought isotope release back to control value after 2 h (Fig. 3).

TABLE VI

*The Effect of Sodium Aurothiomalate on Macrophage Cytotoxicity*

| Inhibitor | Concentration of inhibitor | Preincubated with the macrophages before the addition of target cells | Inhibition of cytotoxic activity |
|---|---|---|---|
| | *μg/ml* | *h* | % |
| Aurothiomalate | 100 | 1 | 80 |
| Aurothiomalate | 50 | 1 | 69 |
| Aurothiomalate | 25 | 1 | 49 |

Data are average values from a minimum of four determinations at each point.

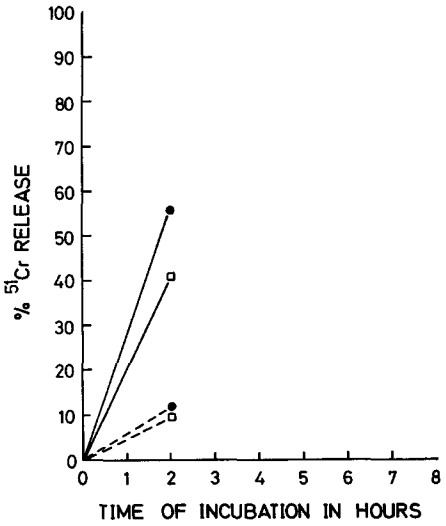


FIG. 2. Chromium release from labeled mouse erythrocytes in media with or without sucrose in the presence (——) or absence (------) of mouse macrophages. ●, Osmolarity of medium raised from 290–320 mosM with sodium chloride; □, Osmolarity of medium raised from 290–320 mosM with sucrose. All data are average values from a minimum of four determinations at each point.

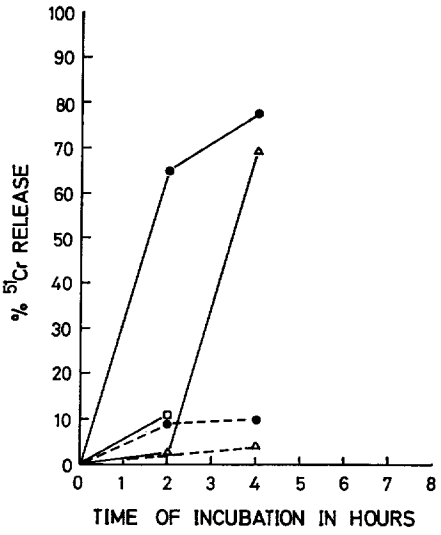


FIG. 3. Effect of temperature change on chromium release from labeled mouse erythrocytes in the presence (——) or absence (-----) of mouse peritoneal macrophages. ●, Experiments performed at 37°C; □, Macrophages pretreated at 45° C/30 min before the addition of target cells; △, Experiments performed at 4°C for the first 2 h, then effector/target cells were re-incubated at 37°C for further 2 h. All data are average values from a minimum of four determinations at each point.

*Rosette Formation.*—When studied in Sykes-Moore chambers about 80% of the macrophages in normal cultures were found to form rosettes with erythrocytes in the course of 30 min at 37°C. The treatment of macrophages with selected inhibitors influenced their binding capacity to various degrees (Table VII). Attachment of erythrocytes to macrophages pretreated with Triton X-100, chlorpromazine hydrochloride, sodium thioauromalate, and trypsin was decreased. However, rosette formation was not significantly reduced by treatment of macrophages with papain and KCN. Considerably more erythrocytes were attached to each macrophage at 4°C than at 37°C.

## DISCUSSION

The lysis of syngeneic erythrocytes by mouse macrophages in vitro occurs extracellular and is not mediated by phagocytosis with subsequent lysosomal breakdown of target cells (1, 3). The present investigation was undertaken to characterize the molecular basis of this phenomenon in more detail.

The results clearly show that the cytotoxic activity of macrophages was strongly temperature dependent. The inability of effector cells to lyse target cells at 4°C was completely restored by reincubation at 37°C while pretreatment of the macrophages at 45°C irreversibly abolished their cytolytic potential. This demonstrates that intact macrophage metabolism is essential for full

TABLE VII

*The Influence of Selected Inhibitors on the Ability of Macrophages to Form Rosettes with Erythrocytes*

| Inhibitor | Concentration of inhibitor | Preincubated with the macrophages before the addition of target cells | Concurrent with the target cells | Inhibition of rosette formation |
|---|---|---|---|---|
| | | *h* | | % |
| 4°C | | | | 0 |
| KCN | $10^{-4}$ M | 1 | | 25 |
| KCN | $10^{-4}$ M | 0 | + | 25 |
| Oligomycin | 20 μg/ml | 2 | | 75 |
| Iodoacetate | $10^{-4}$ M | 1 | | 80 |
| Chlorpromazine | $10^{-4}$ M | 1 | | 60 |
| Triton X-100 | 0.1% (vol/vol) | ½ | | 70 |
| Aurothiomalate | 100 μg/ml | 1 | | 90 |
| Trypsin | 1,000 μg/ml | 1 | | 90 |
| Papain | 1,000 μg/ml | 1 | | 5 |
| Combined: | | | | |
| Iodoacetate | $5 \times 10^{-5}$ M | 1 | | 80 |
| 2,4-Dinitrophenol | $5 \times 10^{-5}$ M | 1 | | 80 |
| Oligomycin | 20 μg/ml | 2 | | 80 |

Data are average values from a minimum of four determinations at each point.

cytolytic activity. The results obtained with effector cells pretreated with various metabolic inhibitors before the addition of target cells demonstrate that the cytotoxic activity of macrophages requires energy while the process does not involve concomitant DNA transcription, translation, or protein synthesis. Macrophages treated with combined blockers of glycolysis and oxidative phosphorylation completely lost their cytotoxic capacity while separate inhibition of these metabolic pathways alone reduced lytic activity to a much lesser degree. This indicates that ATP from both mitochondrial respiration and glycolysis is the high energy intermediate utilized during the subsequent steps leading to target cells lysis.

Pretreatment of the macrophages with cyanide and antimycin-A, inhibitors which are firmly bound to the two terminal cytochromes (13), reduced cytotoxicity to equal levels as obtained in experiments performed with inhibitors of only oxidative phosphorylation and not electron flow. This rest cytotoxicity, however, was completely blocked with conditions of anaerobiosis during effector/target cell interaction. Since chemical blocking of the respiratory chain alone did not prevent lysis (probably because glycolysis will furnish sufficient energy) one can assume that molecular oxygen is required for the reaction leading to target cell lysis. This is in keeping with previous investigation of metabolic events during other activities of leukocytes such as phagocytosis and bactericidal activity (14, 15). The reaction was strongly suppressed by the

presence of cyanide below $10^{-4}$ M in a reversible manner. This indicates that an essential step in the lytic process is inhibited rather specifically by cyanide, since blockers of oxidative phosphorylation did not mimic the effect of cyanide in this respect. It is tempting to speculate that this reflects the activity of a cyanide-sensitive enzyme.

A dramatic reduction in cytolytic potential was caused by preincubation of macrophages with various lytic enzymes, membrane stabilizers and a nonionic detergent. Several factors may be involved such as removal of cell-surface components, dearrangement of membrane molecules, inactivation of certain membrane transport enzymes, interference with ion transport, and effects on the transport of enzymes necessary for the cytotoxic action to occur. The assay system used does not give information of the exact changes that these diverse agents might cause, but stresses the significance of macrophage membrane integrity in this cytotoxic reaction. However, it is interesting to note that the effect of proteolytic enzymes is reversible. This suggests that a surface substance is removed which later can be resynthesized by the cell. It is moreover tempting to speculate that such a membrane substance is directly involved in the lysis of the erythrocytes, or is essential for the transport of lytic molecules to the target cells. Since the reaction is dependent on proximity between target cells and macrophages, this indicates that the effector molecules are either integrated in the cell membrane or are released into the microenvironment of the cells. Preliminary electronmicroscopic evidence may favor the latter view.[2] The lysis is dependent on the presence of divalent ions. Whether this is so because of the necessity of the ions to preserve a normal membrane structure or because the actual effector molecules have specific ionic requirements is not known.

Several of the metabolic inhibitors employed are substances which effectively block endocytosis (16), but in our system they did not prevent, only decreased, lysis of the target cells. These experiments therefore are in accordance with our earlier findings (1), and exclude phagocytosis as the mechanism of lysis. We have shown (1) that cytochalasin-B (10 $\mu$g/ml) did not diminish the cytotoxicity of macrophages in our system. These results are in keeping with recent findings of Temple et al. (17), who suggest that this reflects the effect of cytochalasin-B on the release of lysosomal enzymes as demonstrated by Goldstein et al. (18). This is further supported by the blocking effect of aurothiomalate since this agent is reported to inhibit a number of lysosomal enzymes (19). However, the specificity of this inhibition can be questioned as one cannot exclude other effects of aurothiomalate on macrophage cell metabolism.

Whatever the nature of the effector molecules, some sort of osmotic shock is involved in the red cell lysis. This was clearly brought out by time-lapse recording of the lytic events (3) as well as the present demonstration that sucrose in

[2] Gruca, S., and R. Seljelid. Observation to be published.

the medium during the effector/target cell interaction protected the erythrocytes from lysis. This may be due to the raised effective extracellular osmotic pressure caused by sucrose (20) which will delay erythrocyte swelling, membrane rupture, and isotope release from the disintegrated target cells to the medium.

Most of the treatments found to suppress cytotoxicity also abolish adherence between macrophages and target cells. This indicates again the important role of closeness between effector/target cells and also demonstrates the active role of the macrophages in establishing these contacts. Notable exceptions from the above generalization are the presence of cyanide below $10^{-3}$ M and low temperature. In these two instances the contacts were as in the controls, or more numerous, whereas lysis did not occur. The finding demonstrates the separateness of the two processes, the attachment, and the effector mechanism which lyse the target cells.

## SUMMARY

The influence of various inhibitors on the cytolytic potential of mouse macrophages against syngeneic erythrocytes has been investigated in vitro by isotope techniques. Intact macrophage membrane and cell metabolism was essential for full cytotoxic activity. The process was completely blocked by anaerobiosis and cold. ATP from both mitochondrial respiration and glycolysis seems to be the high energy intermediate which is utilized during the cytotoxic activity of macrophages leading to target cell lysis. The process did not depend on concomitant DNA transcription, translation, or protein synthesis.

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