

## THE CYTOTOXIC EFFECT OF MOUSE MACROPHAGES ON SYNGENEIC AND ALLOGENEIC ERYTHROCYTES

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The ability of lymphoid cells to exert cytotoxic effects *in vivo* and *in vitro* has been documented by numerous workers (1). However, the nature of the events during the interactions between effector cells and target cells is largely unknown. Also, the identity of the effector cells has not always been established satisfactorily. Although it is known that the involvement of thymus-derived (T) lymphocytes<sup>1</sup> is essential in cell-mediated cytotoxic reactions, recent papers indicate that the cytotoxicity may be mediated by other lymphoid cells (2-6).

The present communication supplies evidence that macrophages may exert strong extracellular cytotoxicity *in vitro*, and also gives clues to some of the mechanisms that may be involved. A preliminary report has appeared elsewhere (7).

### *Materials and Methods*

*Animals.*—The experiments were carried out with inbred (A/J/BOM<sub>f</sub> and C3H/Tif/BOM<sub>f</sub>) and hybrid (B<sub>6</sub>D<sub>2</sub>F<sub>1</sub>/BOM [C57BL/6<sub>q</sub> × DBA/2]) female mice. B<sub>6</sub>D<sub>2</sub> and C3H mice were about 6 wk old while A/J mice were around 4 mo of age. The mice were all killed by overdoses of ether.

*Cells and Culture Conditions.*—Macrophages were obtained by peritoneal washings (8). The cells were grown in Eagle's minimum essential medium (MEM), pH 7.45, with penicillin (Proca-penicillin AL, final concentration 0.15 g/liter medium) and streptomycin (Streptomycin Novo, final concentration 0.05 g/liter medium). Experiments were performed with media of 320 mosM (approximately the value for mouse plasma) as determined by freeze point depression. In some cases media of 290 and 260 mosM were used for experiments. The osmolarity was increased or reduced by addition of sodium chloride and sterile, triply distilled water. Heat-inactivated (56°C, 1 h) fetal calf serum (FCS) (Difco Laboratories, Detroit, Mich.), newborn calf serum (NBCS) (Grand Island Biological Co., Grand Island, N. Y.), gamma globulin-free NBCS (Grand Island Biological Co.), and mouse serum produced from B<sub>6</sub>D<sub>2</sub> hybrid mice were added to 20 or 1%. The macrophages were cultivated in silicone-

<sup>1</sup> *Abbreviations used in this paper:* Con-A, concanavalin A; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; MEM, minimum essential medium; NBCS, newborn calf serum; PBS, phosphate-buffered saline; PHA-M, phytohemagglutinin; T lymphocyte, thymus-derived lymphocyte.

stoppered Leighton tubes (Bellco Glass, Inc., Vineland, N. J.), some with flying cover slips. In some cases 35 mm plastic Petri dishes (Falcon Plastics, Oxnard, Calif.) were used. Incubation took place at 37°C in an atmosphere of 95% air and 5% carbon dioxide. Experiments were performed with either freshly seeded macrophages or with macrophages collected the day before the experiment. These types of cultures were established in two slightly different ways.

*Macrophages cultivated overnight:* These were obtained between 1 and 3 p.m. the day before the experiment by washing the peritoneal cavity with 2 ml of phosphate-buffered saline (PBS) with heparin (25 IU/ml) at 0–4°C. The fluid was then collected and centrifuged (1,000 rpm for 5 min) after which the cells were resuspended in MEM with 20% serum and placed in Leighton tubes. In some experiments the macrophages were harvested without heparin by injecting MEM with 20% serum into the peritoneal cavity. The fluid was then collected and directly seeded into Leighton tubes without previous centrifugation. After incubation for 1½–2 h, the tubes were rinsed with warm MEM with 2.5% FCS to remove unattached cells and reincubated with 1 ml fresh MEM containing 20% serum until the following day. The experiments were performed between 7 and 9 a.m.

*Freshly seeded macrophages:* These were collected 1 and 1½ h before the start of the experiments by injecting MEM with 20% serum at room temperature directly into the peritoneal cavities. The contents were removed with Pasteur pipettes and directly seeded into Leighton tubes for incubation as previously described.

To remove nonadhering cells all cultures were washed four times with MEM containing 2.5% FCS immediately before the experiments were started. In some experiments adherent cells were scraped off the cover slips with a silicone stopper and counted in a Bürker hemacytometer to estimate the number of macrophages which participated in the cytotoxic reactions. In our experiments this was approximately  $10^6$  cells per Leighton tube. In another experiment detached macrophages were ground in a tissue grinder (Bellco Glass, Inc.) and homogenized. Under the microscope no intact cells were seen after this treatment. For quantitation of cytotoxic effects the homogenate of  $10^6$  macrophages cultivated overnight was transferred to Leighton tubes and added with target cells to a final concentration of  $10^7$  target cells/ml MEM with 20% FCS. Trypan blue in a final concentration of 0.5% was used for viability tests. Modified Karush type dialysis chambers (Bellco Glass Inc.) were used for experiments with Millipore filters (pore size 0.025  $\mu$ m, Millipore Corp., Bedford, Mass.) and dialysis membranes (Union Carbide Corp., New York). In some experiments the macrophage layer was made to cover only one part of the tissue culture tube by tilting the Leighton tube while the cells were gravitating. Thus the cells were attached to the bottom of approximately one-half of the tube. The cytotoxic activity of macrophage medium was tested by adding  $10^7$  target cells to 1 ml of the supernatant from macrophages cultivated overnight with MEM containing 20% FCS or 20% NBCS.

*Target cells:* Erythrocytes from C3H or B<sub>6</sub>D<sub>2</sub> mice were used. The mouse tail was cut and bled into tubes with sucrose citrate solution of 320 mosM, pH 7.4. The washed erythrocytes were used for experiments 1–1¼ h later. For cytotoxicity studies the erythrocytes were labeled with either <sup>51</sup>Cr or <sup>59</sup>Fe (Statens Institutt for Atomenergi, Kjeller, Norway). Chromium labeling was carried out as described by Perlmann and Holm (9) incubating erythrocytes in MEM containing 2.5% FCS with the chromium isotope for 1 h at 37°C. Iron labeling was performed by injecting the mice intraperitoneally with 100  $\mu$ Ci <sup>59</sup>Fe on the 4th, 3rd, and 2nd days before the collection of the erythrocytes. The erythrocytes were washed three times in MEM with 2.5% FCS before the cytotoxic reaction was induced by adding  $10^7$  labeled target cells in 1 ml of MEM with serum to the macrophages. During some experiments the pH of the incubation fluid was determined at intervals and never found below 7.2.

*Cytotoxic Quantitation.*—After appropriate time intervals, samples of the incubation mixture were removed for the measurement of the cytotoxic effect. Cytotoxic activity was evaluated by measuring the release of isotope or free hemoglobin from the target erythrocytes to the

medium. This was expressed as the percentage of total radioactivity present or of the total amount of hemoglobin present. When carrying out isotope determinations, 0.1 ml samples were taken and placed on top of 0.9 ml MEM with 2.5% FCS. After centrifugation (1,000 rpm for 10 min) the upper 0.5 ml was aspirated and counted in an automatic gamma spectrometer (Nuclear Enterprises Ltd., England). Two or three determinations were performed from each culture during the experiment.

The release of free hemoglobin to the incubation fluid was measured spectrophotometrically as described by Flatmark and Sjøbstad.<sup>2</sup> With this method it is possible to measure amounts of hemoglobin down to  $10^{-8}$  M. This method is based on the principle that haptoglobin enhances the peroxidatic activity of hemoglobin. For these measurements 0.2 ml samples of the incubation fluid were removed and layered on 0.2 ml of MEM containing 2.5% FCS and haptoglobin (Kabi, Stockholm, Sweden) in a conical centrifuge tube. After centrifugation (1,000 rpm for 10 min) the upper 0.2 ml was aspirated and added to a spectrophotometric cuvette filled with 2.5 ml pyrogallol ( $19.2 \times 10^{-3}$  M solution in a citrate buffer,  $40 \times 10^{-3}$  M, pH 3.5) and 0.5 ml hydrogen peroxide ( $24 \times 10^{-2}$  M). The reaction was followed at 430 nm (Hitachi spectrophotometer, model 101, Hitachi, Ltd., Tokyo, Japan) and the reaction rate was determined from the initial slope of the progress curve, i.e.  $\Delta 430$  nm within the period of 30–150 s. Cytotoxicity was expressed as percentage of total hemoglobin in the added erythrocytes.

*Chemicals.*—The minimal concentration of DEAE-dextran (250  $\mu$ g/ml) (Sigma Chemical Co., St. Louis, Mo.), and concanavalin A (Con-A) (100  $\mu$ g/ml) (Sigma Chemical Co.) having microagglutinating effect upon erythrocytes (in concentration  $10^7$  erythrocytes/ml MEM with 20% FCS) were used.  $ZnCl_2$  (analytical grade, Merck Chemical Div., Merck & Co., Rahway, N. J.) was added at a final concentration of  $1.25 \times 10^{-4}$  M and phytohemagglutinin (PHA-M) (100  $\mu$ g/ml) (Difco Laboratories) was used. Cytochalasin B (Imperial Chemical Industries, Pharmaceutical Division, Cheshire, England) was dissolved in dimethyl sulfoxide (DMSO) and diluted in medium containing 20% FCS giving a final concentration of 10  $\mu$ g cytochalasin and 0.1% vol/vol DMSO. The chemicals were all added to macrophages together with the target erythrocytes at the start of the experiment.

*Irradiation.*—A 220 kV apparatus supplied with a Thoreus filter was used. The dose rate was 500 rad/min and the duration was 10 min. The irradiation was applied at various time points before the addition of target cells.

*Immunizing Procedures.*—Immunization was carried out according to Evans and Alexander (10). At 10-day intervals A/J mice were twice injected intraperitoneally with  $2 \times 10^{-7}$  erythrocytes from C3H mice. These cells were suspended in 1 ml saline. Immune macrophages were harvested 10 days after the second immunization. 0.1 ml guinea pig serum (Statens Institutt for Folkehelse, Oslo, Norway) was added as complement source in some of the experiments.

*Morphological Examinations.*—Cover slips were fixed in 2% glutaraldehyde, rinsed, and mounted on glass slides for examination in the phase-contrast microscope at  $\times 1000$  linear magnification. Some cultures were prepared in Sykes-Moore tissue culture chambers (Bellco Glass Inc.). A final concentration of  $10^7$  erythrocytes/ml MEM containing 20% serum was injected into these chambers with the macrophage layer at the bottom. 1 h later the chamber was turned upside down and studied at intervals under the phase microscope.

## RESULTS

*Quantitation of Cytotoxicity and the Kinetics of the Cytotoxic Reactions.*—With macrophages cultivated overnight and with 20% NBBS present there was a

<sup>2</sup> Flatmark, T., and G. Sjøbstad. Personal communication.

rapidly increasing chromium release from the target cells reaching 100% in 5 h. This was the same whether syngeneic or allogeneic target cells were used. In the presence of freshly collected macrophages the chromium release did not exceed 6% during the first 5 h, and was not significantly different from the spontaneous lysis in test tubes containing target cells alone (Fig. 1). From 5 h on there was a rapidly increasing cytotoxic effect of the freshly seeded macrophages as well. When macrophages cultivated overnight were incubated with target cells in 20% FCS the chromium release was 50% in 5 h. Tubes with

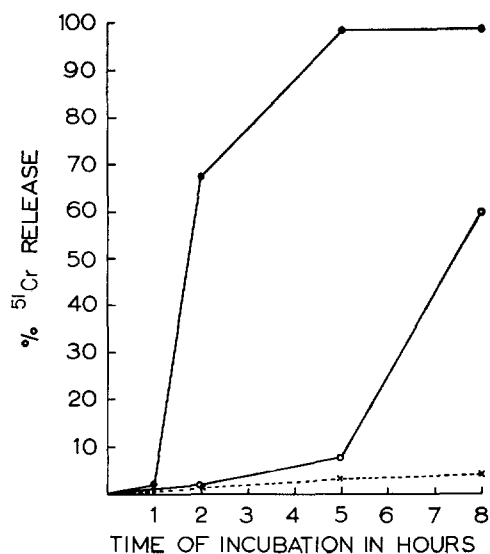


FIG. 1. Chromium release from labeled mouse erythrocytes in medium with 20% NBCS in the presence (—) or absence (----) of mouse peritoneal macrophages. ●, macrophages cultivated overnight; ○, freshly seeded macrophages; ×, erythrocytes only.

Note: All the data presented in the graphs are average values from a minimum of four determinations at each point.

freshly seeded macrophages did not cause chromium release exceeding the controls (Fig. 2). Similar results were obtained when release of <sup>59</sup>Fe isotope (Figs. 3-4) and free hemoglobin (Fig. 5) was determined. Experiments performed with mouse serum and with gamma globulin-free NBCS gave approximately the same results as with FCS (Fig. 6). There was no appreciable difference in isotope release between experiments performed with macrophages cultivated on glass surface and on plastic surface. The cytotoxic activity was equally efficient whether the macrophages cultivated overnight were obtained with or without heparin in the washings. The addition of DEAE-dextran, Con-A, and Zn<sup>++</sup> to freshly seeded macrophages increased the isotope release to various degrees (Fig. 7). The presence of PHA-M had no appreciable effect on the cyto-

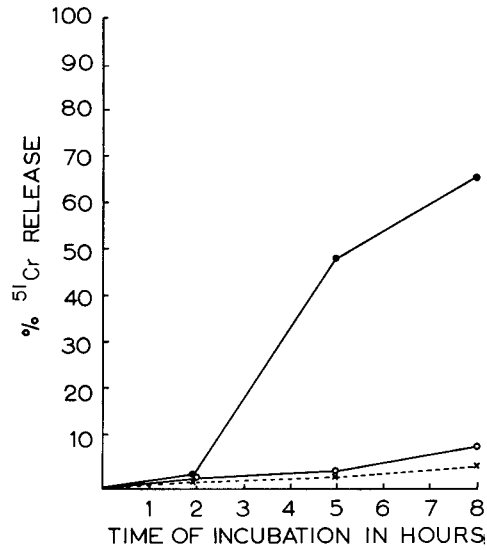


FIG. 2. Chromium release from labeled mouse erythrocytes in medium with 20% FCS in the presence (—) or absence (-----) of mouse peritoneal macrophages. ●, macrophages cultivated overnight; ○, freshly seeded macrophages; ×, erythrocytes only.

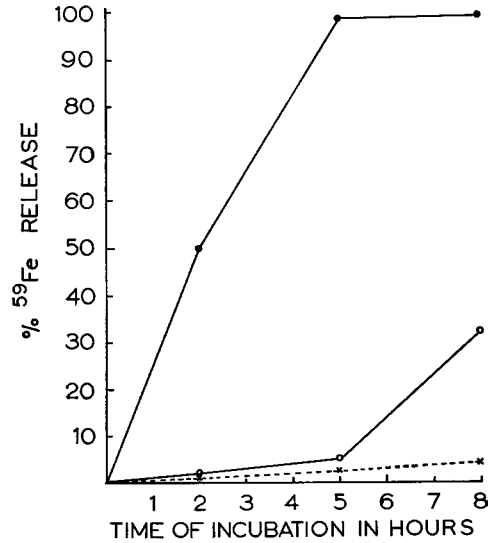


FIG. 3. Iron release from labeled mouse erythrocytes in medium with 20% NBCS in the presence (—) or absence (-----) of mouse peritoneal macrophages. ●, macrophages cultivated overnight; ○, freshly seeded macrophages; ×, erythrocytes only.

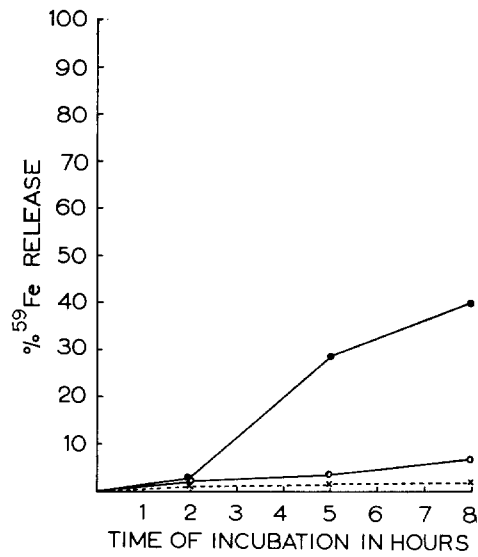


FIG. 4. Iron release from labeled mouse erythrocytes in medium with 20% FCS in the presence (—) or absence (----) of mouse peritoneal macrophages. ●, macrophages cultivated overnight; ○, freshly seeded macrophages; ×, erythrocytes only.

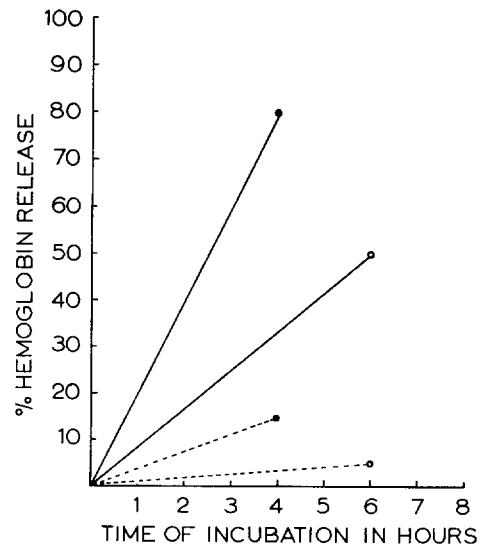


FIG. 5. Hemoglobin release from mouse erythrocytes in the presence (—) or absence (----) of mouse peritoneal macrophages. ○, medium with 20% FCS; ●, medium with 20% NBCS.

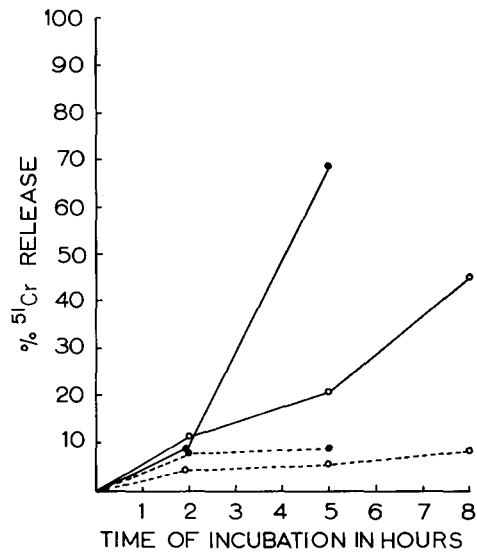


FIG. 6. Chromium release from labeled mouse erythrocytes in the presence (—) or absence (----) of mouse peritoneal macrophages. ○, medium with 20% mouse serum; ●, medium with 20% gamma globulin-free NBCS.

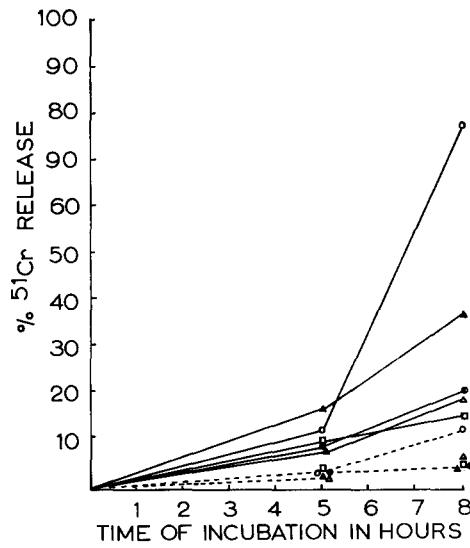


FIG. 7. Chromium release from labeled mouse erythrocytes in the presence (—) or absence (----) of freshly seeded macrophages. □, in medium with 20% FCS; ●, when DEAE-dextran added; ○, ZnCl<sub>2</sub>; ▲, Con-A; △, PHA-M.

toxic reaction (Fig. 7). Changing the osmolarity of the medium from 260 to 320 mosM made the cytotoxic reaction more rapid and more pronounced (Fig. 8). However, spontaneous target cell hemolysis increased in the low osmolarity experiment. When we incubated with 1% serum instead of the usual 20% serum concentration, we observed a similar effect (Fig. 8).

The cytotoxic effect was minimal when macrophages were separated from target erythrocytes by a Millipore filter or by a dialysis membrane (Fig. 9). Medium from macrophage cultivation did not cause target cell lysis. Also, the

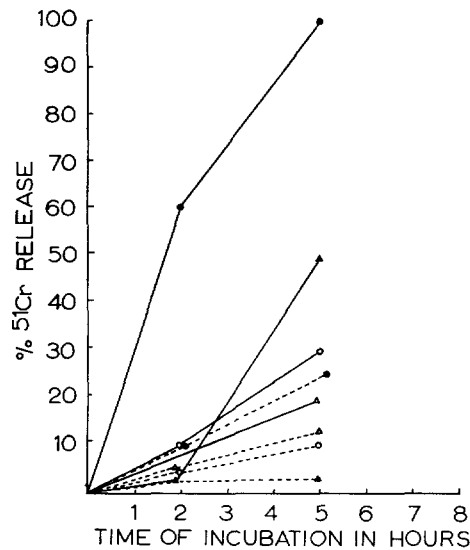


FIG. 8. Chromium release from labeled mouse erythrocytes in media of varying osmolarities and different serum concentrations in the presence (—) or absence (----) of mouse peritoneal macrophages cultivated overnight. ●, 1% FCS and medium, 290 mosM; ○, 20% FCS and medium, 290 mosM; ▲, 20% FCS and medium, 320 mosM; △, 20% FCS and medium, 260 mosM.

lytic effect was found to require intact cells. Thus the isotope release did not exceed the controls when target cells were incubated with homogenized macrophages (Fig. 9). Effector cells cultivated in only one part of the tissue culture tube caused a much less cytotoxic effect compared with the results obtained with the usual extended layer of macrophages (Fig. 10). Fig. 11 shows the results obtained when macrophages cultivated overnight were irradiated. X rays given 1 and 2 h before target cells were added did not reduce the isotope release, while irradiation 4 h before the experiments abolished the cytotoxic activity. Less than 5% of the macrophages were dead 2 h after the irradiation as judged by trypan blue exclusion. 4 h later more than 60% were found dead. The experimental results with freshly seeded A/J immune macrophages were the same as with nonimmune macrophages.



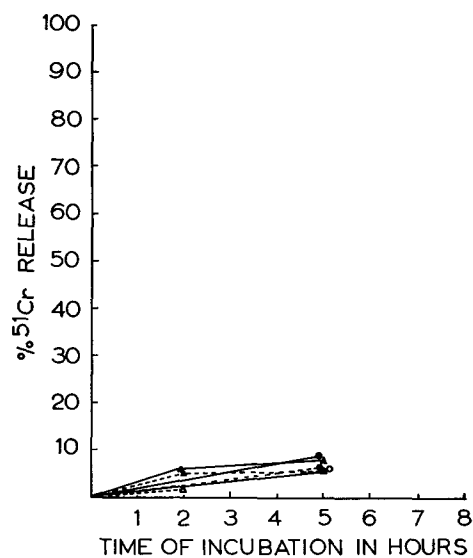


FIG. 9. Chromium release from labeled mouse erythrocytes in medium with 20% FCS (—) or 20% NBCS (-----). ●, erythrocytes separated from macrophages cultivated overnight by a Millipore filter; ○, erythrocytes separated from macrophages cultivated overnight by a dialysis membrane; △, erythrocytes incubated with homogenate from macrophages cultivated overnight; ▲, erythrocytes incubated with supernatants from macrophages cultivated overnight.

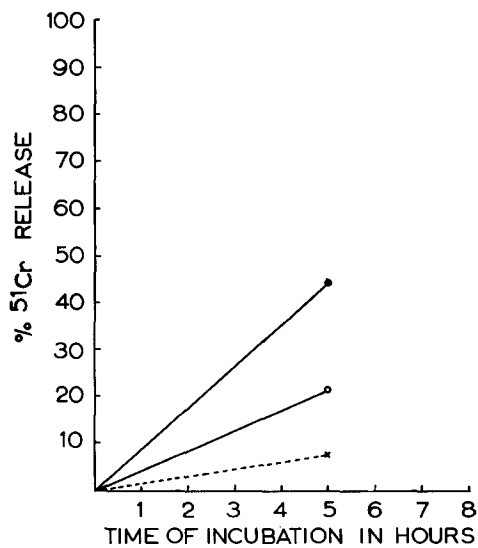


FIG. 10. Chromium release from labeled erythrocytes in medium with 20% FCS in the presence (—) or absence (-----) of mouse peritoneal macrophages cultivated overnight. ●, macrophages covering the bottom of the whole tissue culture tube; ○, macrophages covering the bottom of only half of the tissue culture tube; ×, erythrocytes only.

Cytochalasin B did not inhibit the isotope release. Complement added to medium with FCS had no significant effect upon the isotope release. Addition of complement to erythrocytes in NBCS brought immediate and complete hemolysis. A low titer of congenitins was found in the NBCS. Conglutinins were not demonstrated in the gamma globulin-free NBCS, FCS, or in the mouse serum. L cells cultivated under similar conditions as the macrophages did not show any cytotoxic activity against erythrocytes, as judged by chromium release.

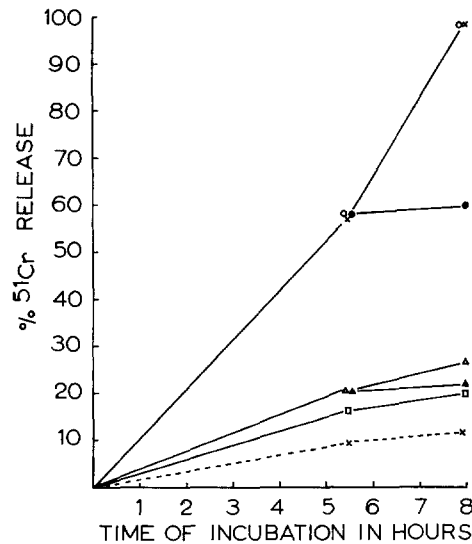


FIG. 11. Chromium release from labeled mouse erythrocytes in medium with 20% FCS in the presence (—) or absence (----) of mouse peritoneal macrophages cultivated overnight and irradiated at various time intervals before the addition of erythrocytes. ○, macrophages irradiated 1 h before the addition of erythrocytes; ●, macrophages irradiated 2 h before the addition of erythrocytes; ▲, macrophages irradiated 4 h before the addition of erythrocytes; ▲, macrophages irradiated 5 h before the addition of erythrocytes; □, macrophages irradiated 6 h before the addition of erythrocytes; ×, macrophages not irradiated; ×---×, erythrocytes only.

*Morphological Observations.*—Phase-contrast micrographs of macrophages cultivated overnight with subsequent addition of target erythrocytes showed well spread macrophages after 5 h of incubation. In close contact with these macrophages were numerous erythrocyte ghosts together with erythrocytes displaying abnormal configurations, some obviously spherocytic (Fig. 12). There was very little apparent erythrophagocytosis.

Micrographs of cultures with freshly seeded macrophages showed erythrocytes attached to macrophages but there were no ghosts to be seen. The erythrocyte attachment was most pronounced in cultures incubated with NBCS, and

in this case aggregates of erythrocytes in contact with the macrophages were often seen. Similar observations were made on the interactions in Sykes-Moore tissue culture chambers. In spite of the gravitational forces when the chamber was turned upside down the erythrocytes were held in close contact with the macrophages. Phase-contrast micrographs of L cells cultivated in the presence of erythrocytes did not show erythrocytes attached and there were no ghosts to be seen.

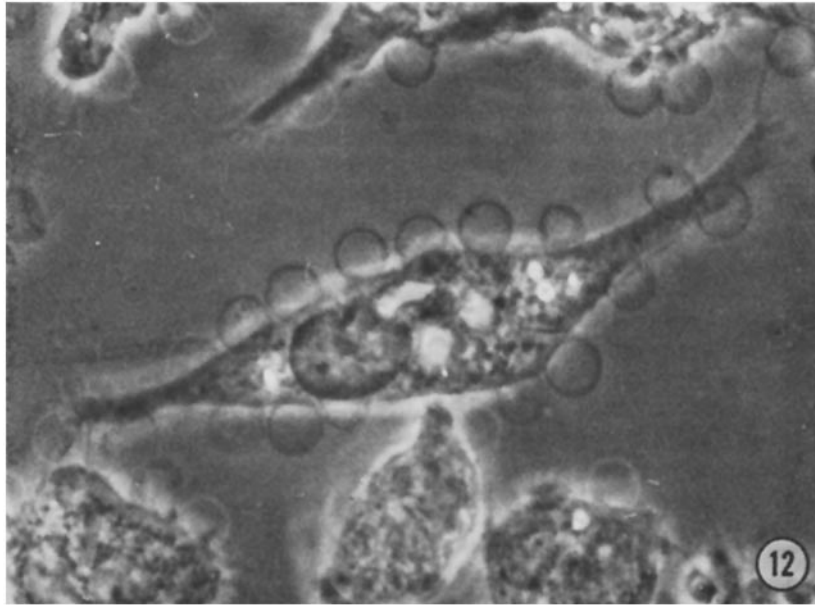


FIG. 12. Phase-contrast micrograph of mouse peritoneal macrophages cultivated overnight and incubated for 5 h with 20% NBCS in the presence of syngeneic erythrocytes. The picture shows numerous ghosts attached to the macrophages.  $\times 5,000$ .

#### DISCUSSION

The cytotoxic effect of macrophages on syngeneic and allogeneic erythrocytes is demonstrated by the release of isotopes. From the presence of intact hemoglobin molecules in the medium, it appears that the effect is mainly extracellular and not mediated by phagocytosis with subsequent lysosomal breakdown of the erythrocytes (11). This is also clearly borne out in the micrographs.

The isotope release in experiments with macrophages separated from target cells by a dialysis membrane or Millipore filters did not exceed the controls. This was also the case in experiments where target cells were incubated with supernatant fluids from macrophage cultures and where target cells were added

to homogenized macrophages. This indicates that the cytotoxic effect depends on contact with cells and is not mediated by effector molecules released into the incubation medium (12, 13, 19). The result of the experiments performed with irradiated macrophages proves the indispensibility of living effector cells. The experiment also shows that the cytotoxic effect is not caused by leakage of toxic substances from dead or dying macrophages.

Besides being contact dependent, the cytotoxic reaction seems to require some sort of activation of the macrophages. This is in keeping with recent observations made by Evans and Alexander (10). However, in our experience it has not proved essential to immunize the animals to obtain macrophages with cytotoxic potential. On the contrary, the phenomenon is fully operative in a syngeneic situation provided that the macrophages have been activated in other ways. In our system this activation was apparently brought about by cultivating the macrophages on glass and plastic surfaces overnight. Judged by the isotope release, a more rapid activation seems to be brought about in the presence of Con-A and  $Zn^{++}$ . These substances are both known to render lymphocytes cytotoxic in vitro (14, 15). The possibility of similar effects on macrophages should therefore be considered, although the lack of effect of PHA-M indicates that the mechanism of action is different.

The degree of cytotoxicity depends on the type of serum present. The greatest isotope release was found in the presence of NBCS. The cytotoxic interaction can be conceived as a two-phase phenomenon, the sticking of the erythrocytes to the macrophage surface and the actual lysis of the target cells. It is not yet clear in which phase the various chemical components of the medium are operative. It is tempting to speculate that low-titer congenitins will increase the sticking, and that this is the explanation for the better effect with NBCS (16), as well the relative ineffectiveness of gamma globulin-free serum. The moderate effect of DEAE-dextran may be explained along the same lines.

The molecular basis of the lytic phenomenon demonstrated in our system is largely unknown. The presence of complement did not alter the reaction significantly in FCS or mouse serum. The morphological observation may indicate that some sort of osmotic shock takes place. The results obtained with media of various osmolarities and serum concentrations support this idea. It is also in keeping with preliminary time-lapse recordings. By this method we have found that normal biconcave erythrocytes in the course of 5-6 min after contact with macrophages become spherocytic and crack, finally ballooning again to become ghosts.

The efficiency of the cytotoxic effect caused by activated macrophages should be noted. In our experiments about  $10^6$  macrophages were able to cause complete chromium release from  $10^7$  target erythrocytes in 5 h. This is an efficiency of at least 200 times that exerted by sensitized lymphocytes on target cells in vitro (17, 18). It is thus clear that even small admixtures of macrophages to lymphocyte suspensions in an in vitro system will greatly influence the experi-

mental results under conditions where macrophages are cytotoxic against the tested target cells. It is clear that an in vitro system based on the lysis of erythrocytes is widely different from cytotoxic effects on nucleated cells in vivo. Still we consider contact-mediated erythrocyte lysis as a special example of cytotoxic activity. This view is in keeping with other recent publications (4). As a matter of fact we have preliminary data to indicate that L cells are affected in a similar way as erythrocytes under proper experimental conditions.

## SUMMARY

A cytotoxic effect of mouse peritoneal macrophages against syngeneic and allogeneic erythrocytes was demonstrated by isotope release and release of hemoglobin. The cytotoxic effect was dependent on the contact between viable, activated macrophages and target cells. Activation was accomplished by prolonged cultivation of macrophages and by the presence of  $Zn^{++}$  and Con-A. Immunization did not prove necessary. Morphological observations as well as experiments with various salt concentrations indicate that the cytotoxic reaction may involve some kind of osmotic effect upon the target cells.

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