ROLE OF PHAGOCYTOSIS
IN THE ACTIVATION OF MACROPHAGES

By JÖRG SCHNYDER and MARCO BAGGIOLINI

From the Research Institute Wander, a Sandoz Research Unit, Wander Limited, Berne, Switzerland

Macrophages are found in large numbers at sites of chronic inflammation. They enter the lesions as monocytes (1) and develop in situ into large cells which synthesize and release an impressive variety of enzymes such as lysosomal hydrolases (2, 3), plasminogen activator (4), elastase (5), and collagenase (6). These enzymes are most likely involved in the extensive destruction of cellular and extracellular tissue elements which accompanies chronic inflammation.

Since macrophages are highly phagocytic, we have investigated the role of phagocytosis in their activation. We have studied the activation process in cultures of mouse peritoneal macrophages which were exposed to different types of particles. We have followed macrophage activation by assaying, in the cells as well as in the culture media, a number of enzyme activities which are known to change in the course of this process. In this paper, we present experiments which show that macrophages obtained from untreated mice become activated in culture after phagocytosis of zymosan or formaldehyde-treated sheep erythrocytes but do not become activated after phagocytosis of latex beads.

Materials and Methods

Macrophage Cultures. Macrophages were obtained from male OF1 mice (Sandoz Ltd., Basel, Switzerland) weighing 20–24 g. The mice were either untreated, or treated 4 days before cell harvesting with Brewer's thioglycollate broth or a suspension of streptococcus A cell wall fragments (3). The cell culture techniques and the biochemical assay methods used were described in an earlier paper (3).

Phagocytosis. Particles were prepared as follows. Zymosan (Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire, England) was boiled in phosphate-buffered saline (PBS)[1] for 1 h, washed three times by centrifugation, resuspended in PBS (50 mg/ml), and autoclaved at 120°C (7). Latex beads, 1.01 μm in diameter (Serva GmbH & Co., Heidelberg, W. Germany), were suspended in PBS containing 0.01% Tween 80, washed three times by centrifugation, resuspended in PBS/Tween 80 and irradiated with ultraviolet light before use (8). Sheep erythrocytes were washed five times in PBS by centrifugation, resuspended in PBS containing 3% formaldehyde for 2 days at 4°C, and then washed five more times in PBS (9).

Phagocytosis experiments were performed on cells which were allowed to adhere to the culture dishes at 37°C during 3 h after harvest. Particles were added in a small volume of PBS to the culture dishes (60 mm) containing 4–6 × 10^6 adherent cells and 3 ml of medium (3). The particle-to-cell ratios were between 7 and 30:1 for zymosan (see figures for details), 5:1 for formaldehyde-treated sheep erythrocytes, and 40:1 for the latex beads. Phagocytosis was stopped after 1 h by draining the medium and eliminating noningested particles by washing three times with PBS. Culturing in particle-free medium was then continued for up to 13 days under standard conditions (3). The medium was changed at different intervals but at least every 3rd day.

---

[1] *Abbreviations used in this paper:* LDH, lactate dehydrogenase; PBS, phosphate-buffered saline.
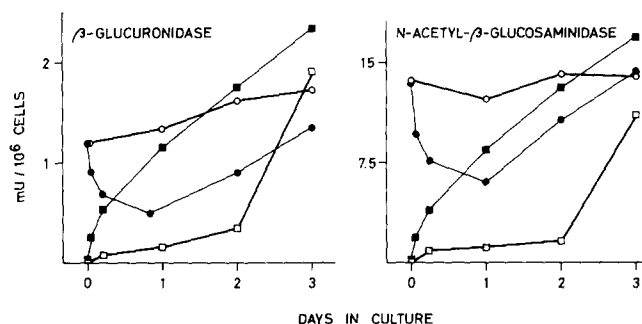
Fig. 1.   Release of lysosomal enzymes from noneliciteu macrophages. Effects of zymosan phago-cytosis. Short-term experiment showing the difference in the release kinetics of phagocytosis-stimulated (■) and of resting macrophages (□). 20 particles per cell were added at the beginning of the experiment and phagocytosis was stopped after 1 h by eliminating noningested particles through washing. Each value is the average from triplicate cultures. Squares represent enzyme levels in the media and circles represent intracellular levels.

Hexose Monophosphate Shunt.   1-[^{14}C]Glucose (1 μCi per dish containing 3 ml of medium) was added to the cultures together with the particles or with particle-free control medium. After 1 h, 2 ml of cell-free medium was transferred into the main well of Warburg-type reaction flasks, each containing a strip of filter paper wetted with 100 μl of 10% potassium hydroxide in the center well. The flasks were closed and 200 μl of 1 N sulfuric acid was added to the medium from a side arm to liberate the $^{14}CO_2$. After 15 min, the filter paper strip and two 250-μl portions of water used for rinsing the center well were transferred to scintillation counting vials containing 10 ml of Tritosol (10) and counted. Quenching was corrected by the channel ratio method. Control experiments, using 6-[^{14}C]glucose were performed exactly as described above.

Chemicals.   D[1-^{14}C]glucose was obtained from New England Nuclear, Boston, Mass. and D[6-^{14}C]glucose from The Radiochemical Centre, Amersham, England. The sources of the other reagents used is given in our previous paper (3).

Results

Short-Term Experiments.   Exposure of nonelicited macrophages to zymosan, followed, 1 h later, by the removal of noningested particles triggered a rapid and abundant release of lysosomal glycosidases into the medium (Fig. 1), which was paralleled by a corresponding drop in the intracellular contents of these enzymes. While release continued for at least 3 days at nearly constant rate, the intracellular enzyme levels returned to the initial values. As illustrated in Fig. 1, nonelicited macrophages which were not exposed to particles released only minor amounts of acid glycosidases during the first 2 days of culture but subsequently began to secrete actively (3), often releasing similar amounts of lysosomal enzymes as macrophages which had phagocytosed.

Long-Term Experiments.   If the experiments were continued for 9–10 days (Fig. 2), further biochemical changes apparently linked to the phagocytic stimulus were observed. Around day 4, the macrophages which had phagocytosed started to secrete considerable amounts of plasminogen activator. From day 4 to the termination of the experiments on day 10, the rate of plasminogen activator secretion remained constant. These macrophages also showed an increasing content in lactate dehydrogenase (LDH). 4 days after phagocytosis their LDH levels were two to three times higher than in control cells.

There was no significant difference in lysozyme secretion between resting and phagocytosis-stimulated macrophages (not shown). An increased release of LDH was observed during and immediately after phagocytosis. Subsequently, however, the loss
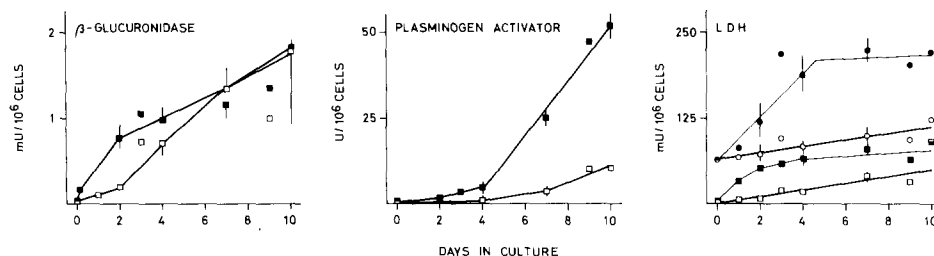
FIG. 2. Biochemical effects of zymosan phagocytosis in nonelicited macrophages. Early induction of β-glucuronidase release, delayed secretion of plasminogen activator, and increase of intracellular LDH. Zymosan-treated cultures, 7–14 particles per cell (black symbols), are compared with control cultures (white symbols). Squares represent enzyme levels in the media and circles represent intracellular levels. The day 3 and day 9 values are from a single experiment, all the others are mean values from four to five experiments. In these cases, the SEM is given as a vertical bar when larger than the symbol's height.

of LDH in phagocytosis-stimulated cultures was as low as in the control cultures (Fig. 2), suggesting that phagocytosis did not affect cell viability.

Effects of Different Particles. The triggering of lysosomal enzyme release and the induction of plasminogen activator secretion observed suggested that macrophages become activated as a consequence of zymosan phagocytosis. We therefore compared zymosan with two other particles, namely formaldehyde-treated sheep erythrocytes, which unlike zymosan are digested in phagocytic vacuoles (11), and latex beads which we expected to be biologically inert. Morphological controls under the inverted microscope using intact and Giemsa-stained cultures as well as electron microscopy showed that all three test particles were readily engulfed by the macrophages.

As shown in Fig. 3, phagocytosis of sheep erythrocytes induced a rapid release of β-glucuronidase and a progressive rise in intracellular LDH. Both changes were similar to those induced by zymosan. In contrast, phagocytosis of latex beads failed to affect these two putative parameters of cell activation; the cells showed a delayed secretion of β-glucuronidase and no change in intracellular LDH. Similarly, phagocytosis of sheep erythrocytes or zymosan induced the secretion of plasminogen activator, a well-established biochemical marker of macrophage activation (4, 12), while phagocytosis of latex was without measurable effect.

Hexose Monophosphate Shunt Activation. To obtain more direct evidence for the interaction between the macrophage and the particles, we then determined the activity of the hexose monophosphate shunt in the cultures after addition of the particles. The results are presented in Table I. Phagocytosis of zymosan and sheep erythrocytes was accompanied by an 11- to 14-fold increase in $^{14}CO_2$ formation from C_1-labeled glucose, i.e., by a strong increase in glucose metabolism via the hexose monophosphate pathway. In contrast, no shunt activation was observed when macrophages were exposed to latex beads. Thus, after latex phagocytosis macrophages appear to remain biochemically resting with respect to both secretion and energy metabolism. Phagocytosis did not affect significantly $^{14}CO_2$ formation from C_6-labeled glucose.

Effects of Serum and Protein Synthesis Inhibition. Using zymosan, we studied the influence of certain culture conditions on phagocytosis-induced cell activation. Fig. 4 shows a marked response to zymosan phagocytosis even when serum is omitted from the culture medium. This suggests that serum components are not involved in the
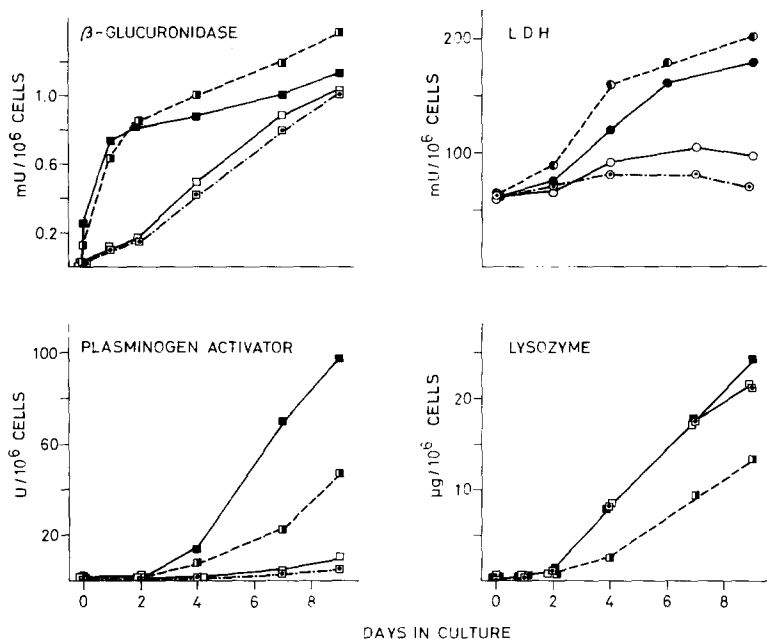
Fig. 3. Biochemical effects of phagocytosis of different types of particles in nonelicited macrophages. Enzymes were determined in the media (square symbols) or in the cells (round symbols). The particles were: formaldehyde-treated sheep erythrocytes, 5 particles per cell (black symbols), zymosan, 7 particles per cell (half-black), latex, 40 particles per cell (dotted). Control cultures (white symbols) were not exposed to particles. Each value is the average from triplicate cultures.

TABLE I

Effects of Different Particles on Hexose Monophosphate Shunt

Particle	$^{14}CO_2$ Liberation from $[1-^{14}C]$Glucose (dpm, mean values ± SEM)	Number of determinations	Relative change with respect to control
None (control)	426 ± 30	14	1
Zymosan	5,978 ± 212	11	14
Sheep erythrocytes	4,809 ± 45	3	11
Latex	438 ± 23	8	1

The particle-to-cell ratio was 7:1 for zymosan, 5:1 for sheep erythrocytes, and 40:1 for latex.

activation process. Inhibition of protein synthesis by the addition of 0.25 μg/ml cycloheximide to the culture medium suppressed the rise in intracellular LDH and plasminogen activator secretion, but inhibited only slightly the release of β-glucuronidase (Fig. 5). This may indicate that β-glucuronidase is secreted mainly from the pre-existing lysosomal pool while plasminogen activator secretion is fully dependent on de novo enzyme synthesis.

Elicited Macrophages. All the above results were obtained with nonelicited macrophages. In a last series of experiments, we studied the effects of phagocytosis on macrophages which had been elicited by treating the mice either with a suspension of streptococcus A cell walls or with thioglycollate medium. As we have shown earlier, both treatments enhance the macrophage yield, but only thioglycollate appears to activate the cells (3). The consequences of zymosan phagocytosis in elicited macro-
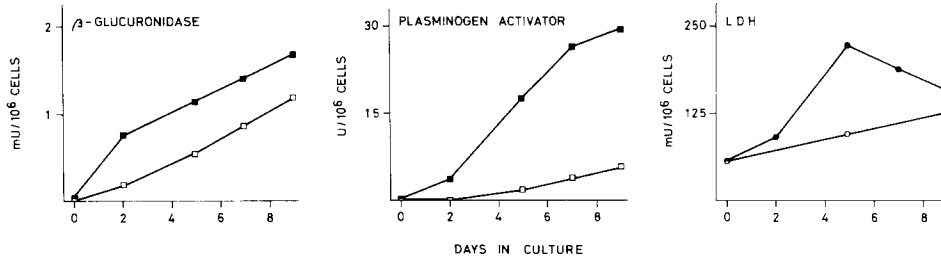
Fig. 4. Biochemical effects of zymosan phagocytosis in serum-free cultures of nonelicited macrophages. Zymosan-treated cultures, seven particles per cell (black symbols), are compared with control cultures (white symbols). Squares represent enzyme levels in the media and circles represent intracellular levels. Each value is the average from triplicate cultures.
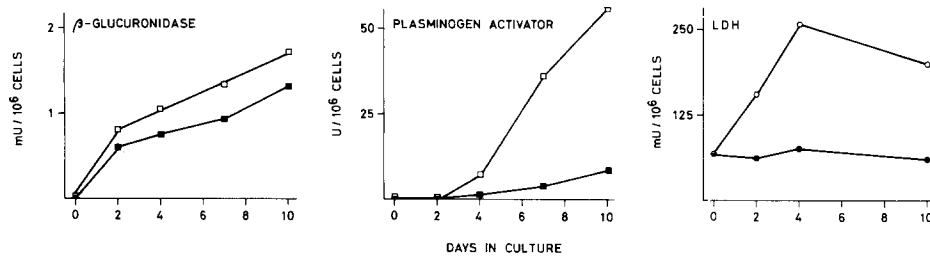

Fig. 5. Effect of cycloheximide at the concentration of 0.25 μg/ml (black symbols) on phagocytosis-induced synthesis and release of enzymes in nonelicited macrophages. Zymosan, seven particles per cell, added 15 min after cycloheximide, was used as test particle. Squares represent enzyme levels in the media and circles represent intracellular levels. In control cultures (white symbols) cycloheximide was omitted. Each value is the average from triplicate cultures.
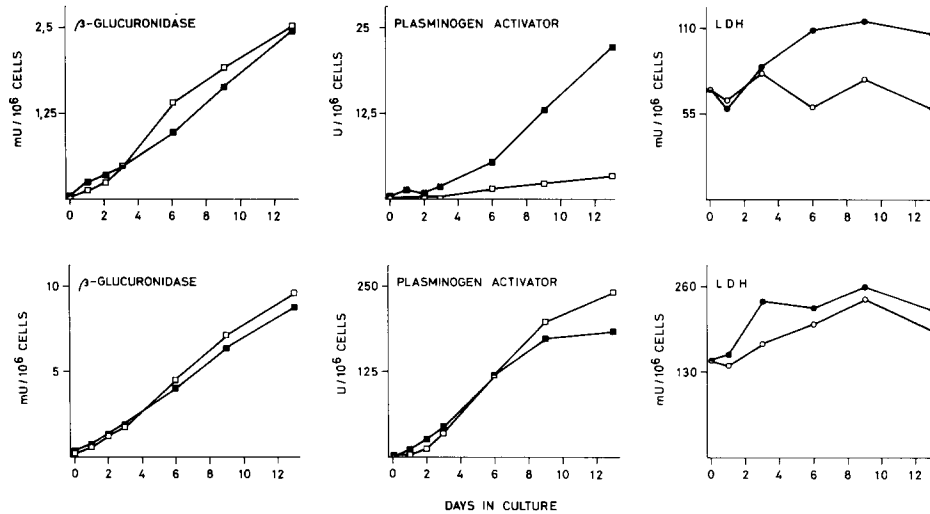

Fig. 6. Biochemical effects of zymosan phagocytosis in macrophages obtained from mice treated intraperitoneally with a streptococcus A cell wall suspension (upper graphs) or with thioglycollate broth (lower graphs). Zymosan-treated cultures, 15 and 30 particles per cell, respectively, (black symbols) are compared with control cultures (white symbols). Squares represent enzyme levels in the media and circles represent intracellular levels. Each value is the average from triplicate cultures.

phages are shown in Fig. 6. Streptococcus A-elicited cells did not show a change in β-glucuronidase secretion after phagocytosis, but responded with secretion of plasminogen activator and with a rise in intracellular LDH as did nonelicited macro-

phages. By contrast, phagocytosis did not affect these parameters in macrophages which were elicited with thioglycollate and were therefore already fully activated.

Discussion

We show in this paper that macrophages become activated in vitro, after phagocytosis of certain particles. Immediately upon particle uptake, the cells start releasing large amounts of lysosomal hydrolases. This is followed by a progressive increase in cellular LDH and, about 4 days after phagocytosis, by the production and secretion of plasminogen activator. By using different types of particles we found that the activating stimulus is not the phagocytic uptake per se. Sheep erythrocytes act like zymosan but latex beads, which are phagocytosed equally well, have no effect. Searching for an explanation, we found that zymosan and sheep erythrocytes both strongly stimulate glucose metabolism via the hexose monophosphate shunt while latex beads are phagocytosed without altering the activity of the shunt. In other respects, however, the two macrophage-activating particles differ considerably; unlike zymosan, sheep erythrocytes are digested after uptake (11) and are not able to activate complement by the alternative pathway (P. Dukor, Ciba-Geigy Ltd., Basel, Switzerland, unpublished data). This suggests that the stimulation of the hexose monophosphate shunt may be a triggering event in macrophage activation. On the other hand, phagocytosis-induced macrophage activation does not appear to depend on the intracellular fate of the ingested material or to occur only with particles which have the ability to activate complement, as suggested by Schorlemmer et al. (13).

The results obtained with elicited macrophages are in agreement with the above conclusions. Zymosan stimulates the hexose monophosphate shunt also in elicited cells (data not shown) and has the capacity to activate them unless they are already strongly activated by the eliciting procedure. In fact, zymosan phagocytosis induces plasminogen activator secretion in macrophages elicited with streptococcus A cell walls but does not increase the secretion rate of thioglycollate-elicited macrophages.

Release of lysosomal glycosidases as a consequence of phagocytosis has been reported earlier by Davies et al. (2) who also observed a rise in the cellular levels of LDH and leucine aminopeptidase. These authors suggested that the mechanism of release was selective exocytosis rather than regurgitation. Our results support this interpretation since acid hydrolase release continues for several days after removal of the particles. As already indicated, we think that phagocytosis and the consequent stimulation of the hexose monophosphate shunt are initiating events. In nonactivated macrophages, they trigger off lysosomal enzyme secretion, a process which is characteristic for the macrophage and which eventually occurs in culture independently of phagocytosis (3). In cells such as thioglycollate-elicited macrophages, which already secrete lysosomal hydrolases, phagocytosis does not stimulate the secretory process further.

Axline and Cohn (11) found that the phagocytosis of digestible particles, e.g. sheep erythrocytes or aggregated gamma globulin, induced the production of acid phosphatase, and to a lesser extent, of cathepsin D and β-glucuronidase in mouse peritoneal macrophages. No effect was observed when polyvinyl toluene, polystyrene, or starch particles were used. This is in accord with the activating effect of sheep erythrocytes and the lack of activation by latex beads reported here. The lack of effect of latex particles, however, cannot be explained by the fact that they are not digested in the

vacuoles, since other nondigestible particles like streptococcus A cell walls (2) or zymosan as shown in this paper are very powerful inducers of lysosomal enzyme production and release. In our experiments, phagocytosis of zymosan and sheep erythrocytes had similar effects on the release of β-glucuronidase, suggesting that this response does not depend on the digestibility of the particles. Weissmann et al. (7) found release of β-glucuronidase by mouse peritoneal macrophages during phagocytosis of zymosan but not of sheep erythrocytes. However, since the test period was only 2 h these data may reflect regurgitation rather than selective release.

Our findings that macrophages are activated by phagocytosis of zymosan but not of latex beads are in agreement with data on the effects of these two particles on prostaglandin synthesis and release. As reported by several laboratories (14–16), nonelicited mouse macrophages in culture respond with the release of prostaglandins when they are challenged with zymosan but show no release when exposed to latex beads.

There is little information on the influence of phagocytosis on induction of plasminogen activator secretion. Gordon et al. (12) found only minimal secretion of plasminogen activator by unstimulated macrophages during a culture period of 4 days after phagocytosis of latex or sheep erythrocytes. These results are not incompatible with those presented here since our macrophages begin to secrete significant amounts of plasminogen activator approximately 4 days after the phagocytic stimulus. A further observation along these lines was made by Hamilton et al. (17) who found that exposure to asbestos induced a delayed increase in fibrinolytic activity in nonstimulated macrophages. Our results show that between the 4th and 10th day after a phagocytic stimulus nonelicited macrophages secrete high amounts of plasminogen activator at nearly constant rate and that secretion does not depend on the intracellular persistence of the ingested particles. The fact that more plasminogen activator is secreted by macrophages which have phagocytosed sheep erythrocytes than by those which have ingested zymosan shows again that digestible particles can be very powerful stimuli.

In summary, the results presented in this paper, together with evidence provided by other studies (2, 12, 17), show that macrophages may become activated as a consequence of phagocytosis. Our data suggest that biochemical reactions associated with the burst of hexose monophosphate shunt activity, which accompanies the phagocytosis of different types of particles, could be an initiating event in the long and complex process of macrophage differentiation and activation.

Summary

Macrophages were obtained by peritoneal lavage from untreated mice or from mice which had received either Brewer's thioglycollate broth or a suspension of streptococcus A cell walls intraperitoneally 4 days before. 3 h after harvesting, adherent cells from untreated mice were allowed to phagocytose zymosan, formaldehyde-treated sheep erythrocytes, or latex beads. Phagocytosis was stopped after 1 h and culture was continued for up to 10 days. Phagocytosis of zymosan or sheep erythrocytes triggered the immediate release of lysosomal glycosidases, stimulated the synthesis of cellular lactate dehydrogenase, and induced the delayed production and secretion of plasminogen activator. No such changes were observed upon phagocytosis of latex. Although

all three particles used were phagocytosed, only zymosan and sheep erythrocytes stimulated glucose oxidation via the hexose monophosphate shunt.

Similar findings were obtained in macrophages elicited with streptococcus A cell walls after zymosan phagocytosis. Thioglycollate-elicited macrophages, however, which were already secreting lysosomal hydrolases and plasminogen activator, could not be activated further by zymosan.

The results of this study show that macrophages become activated after phagocytosis of particles that stimulate the activity of their hexose monophosphate shunt. The triggering event appears to be the burst of shunt activity itself or shunt-related biochemical reactions rather than phagocytic uptake per se or particle-dependent complement activation by the alternative pathway. Once initiated, macrophage activation proceeds independently of the intracellular fate of the ingested material.

References

1. Van Furth, R., and Z. A. Cohn. 1968. The origin and kinetics of mononuclear phagocytes. *J. Exp. Med.* **128:**415.
2. Davies, P., R. C. Page, and A. C. Allison. 1974. Changes in cellular enzyme levels and extracellular release of lysosomal acid hydrolases in macrophages exposed to group A streptococcal cell wall substance. *J. Exp. Med.* **139:**1262.
3. Schnyder, J., and M. Baggiolini. 1978. Secretion of lysosomal hydrolases by stimulated and non-stimulated macrophages. *J. Exp. Med.* **148:**435.
4. Unkeless, J. C., S. Gordon, and E. Reich. 1974. Secretion of plasminogen activator by stimulated macrophages. *J. Exp. Med.* **139:**834.
5. Werb, Z., and S. Gordon. 1975. Elastase secretion by stimulated macrophages. Characterization and regulation. *J. Exp. Med.* **142:**361.
6. Werb, Z., and S. Gordon. 1975. Secretion of a specific collagenase by stimulated macrophages. *J. Exp. Med.* **142:**346.
7. Weissmann, G., P. Dukor, and R. B. Zurier. 1971. Effect of cyclic AMP on release of lysosomal enzymes from phagocytes. *Nat. New Biol.* **231:**131.
8. Ulrich, F., and D. B. Zilversmit. 1970. Release from alveolar macrophages of an inhibitor of phagocytosis. *Am. J. Physiol.* **218:**1118.
9. Rabinovitch, M. 1967. Attachment of modified erythrocytes to phagocytic cells in absence of serum. *Proc. Soc. Exp. Biol. Med.* **124:**396.
10. Fricke, U. 1975. Tritosol: a new scintillation cocktail based on Triton X-100. *Anal. Biochem.* **63:**555.
11. Axline, S. G., and Z. A. Cohn. 1970. In vitro induction of lysosomal enzymes by phagocytosis. *J. Exp. Med.* **131:**1239.
12. Gordon, S., J. C. Unkeless, and Z. A. Cohn. 1974. Induction of macrophage plasminogen activator by endotoxin stimulation and phagocytosis. *J. Exp. Med.* **140:**995.
13. Schorlemmer, H. U., D. Bitter-Suermann, and A. C. Allison. 1977. Complement activation by the alternative pathway and macrophages enzyme secretion in the pathogenesis of chronic inflammation. *Immunology.* **32:**929.
14. Humes, J. L., R. J. Bonney, L. Pelus, M. E. Dahlgren, S. J. Sadowski, F. A. Kuehl, Jr., and P. Davies. 1977. Macrophages synthesise and release prostaglandins in response to inflammatory stimuli. *Nature (Lond.).* **269:**149.

15. Glatt, M., H. Kälin, K. Wagner, and K. Brune. 1977. Prostaglandin release from macrophages: an assay system for anti-inflammatory drugs in vitro. *Agents Actions.* 7:321.

16. Gemsa, D., M. Seitz, W. Kramer, G. Till, and K. Resch. 1978. The effects of phagocytosis, dextran sulfate, and cell damage on PGE_1 sensitivity and PGE_1 production of macrophages. *J. Immunol.* 120:1187.

17. Hamilton, J., J.-D. Vassalli, and E. Reich. 1976. Macrophage plasminogen activator: induction by asbestos is blocked by anti-inflammatory steroids. *J. Exp. Med.* 144:1689.