THE PINOCYTIC RATE OF ACTIVATED MACROPHAGES*

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Animals systemically infected with various intracellular parasites can show an increased ability to resist subsequent challenge with the same, or certain other, microorganisms. This resistant state is associated with a striking enhancement in the antimicrobial capacity of the fixed macrophages in the liver and spleen, as well as a family of physiologic and morphologic changes in the peritoneal macrophage population, which has been called "activation" (11). These "activated" cells are larger, spread more rapidly on glass, have a larger complement of intracellular hydrolases (5), and display more plasma membrane activity than the resident peritoneal cells (2). A phenomenologically similar sort of "activation" has been accomplished by the intraperitoneal injection of endotoxin, thioglycollate medium, or proteose-peptone broth, among other materials.

Since Lewis' morphologic observations (8), and subsequent observations by Cohn (4), it has been clear that the resident peritoneal macrophage has a striking and characteristic pinocytic capacity. With the development of a sensitive and reliable technique for measuring the pinocytic rate of cultured cells (13), it was possible to carry these analyses further, and to compare the activity of the macrophage with other cell types (14). This method depends upon including, in the culture medium, the soluble enzyme horseradish peroxidase (HRP),\(^1\) and then measuring the rate of accumulation of that enzyme within the cells. Previous work (6, 13) has shown that HRP is taken up by cells in the bulk phase, with little, if any, adsorption to the plasma membrane, and is included solely in membrane-bounded cytoplasmic vesicles by a temperature-sensitive, and energy-dependent process. Once interiorized, these HRP-containing pinosomes fuse with lysosomes, and the HRP is degraded with a half-life time of 8–10 h. Thus, over brief periods of time, up to 2 h, there is a linear accumulation of HRP in the cells which can be taken as an accurate measure of the volume of fluid pinocytosed during that time.

We have studied the pinocytic activity of mouse peritoneal macrophages obtained after the intraperitoneal injection of several stimulating materials, in an effort to identify objective, quantitative differences between such cells and the resident macrophage population. Our studies show that pinocytic activity is

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\(^1\) Abbreviations used in this paper: Con A, concanavalin A; 199-20% FCS, tissue culture medium 199 supplemented with 20% heat-inactivated, filtered fetal calf serum; HRP, horseradish peroxidase.
such a distinguishing characteristic, and further, that it is possible to discriminate not only between resident and stimulated cells, but also among peritoneal cells stimulated with various agents.

Materials and Methods

**Macrophages.** Cells were washed from the peritoneal cavities of NCS mice of either sex, weighing from 25-30 g, as previously described (6). In some cases, the mice had been previously injected intraperitoneally with one of several "stimulating" agents (see below). Recovered cells were resuspended in tissue culture medium 199 supplemented with 20% fetal calf serum which had previously been heated to 56°C for 30 minutes (199-20% FCS), plated on plastic Petri dishes or glass cover slips, washed to remove nonadherent cells, and incubated at 37°C until use.

"Stimulating" Agents. Lyophilized endotoxin (Escherichia coli 0111:B4; Difco Laboratories, Detroit, Mich.) was suspended in sterile normal saline and stored frozen at a concentration of 100 µg/ml. Mice were injected with 30 µg of endotoxin 4 days before collecting the peritoneal exudate.

Brewer's thioglycollate medium (thioglycollate), proteose-peptone powder, and agar were purchased from Difco Laboratories. Thioglycollate medium was prepared exactly as recommended by the vendor, and stored in the dark until use. Mice were injected with 1.0 ml of the reconstituted medium and cells were generally harvested 4-days later. Proteose-peptone (1% and 10% solutions) and agar (0.5 mg/ml) were prepared with sterile distilled water and autoclaved. Mice received 1.0 ml of these fluids intraperitoneally 4 days before the exudate was collected. Latex spheres (polyvinyltoluene particles, 1.09 Am diameter, Dow Diagnostics Corp., Midland, Mich.) were also suspended in sterile distilled water and approximately 10^6 particles were injected intraperitoneally into each mouse.

Measurement of Pinocytic Rate. The pinocytic rates of monolayer cultures were measured as previously described (6), using the method described by Steinman and Cohn (13). HRP (Type II, Sigma Chemical Co., St. Louis, Mo.) was dissolved in 199-20% FCS and added to cells at a concentration of 1 mg/ml. Cell lysates, prepared in 0.05% Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.) were assayed for peroxidase activity using o-dianisidine (Sigma Chemical Co.) as substrate, in the presence of 0.3% H_2O_2, and the rate of change of absorbance of the reaction mixture, at 460 nm, was measured on a Gilford spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) equipped with an automatic chart recorder.

Light Microscopy. Macrophages cultivated on glass coverslips were exposed to HRP (1 mg/ml) dissolved in 199-20% FCS at 37°C in a CO_2 incubator. The cells were then rinsed with medium, fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 5 min at room temperature, and then stained for peroxidase activity as outlined previously (6), using a 10 min exposure to diaminobenzidine in Tris buffer. The stained cover slips were mounted on glass microscope slides and examined and photographed with a Zeiss Ultraphot microscope equipped with both bright-field and phase-contrast optics (Carl Zeiss, Inc., New York). Cells were photographed on Kodak Ektapan sheet film (Eastman Kodak Co., Rochester, N. Y.).

Electron Microscopy. Cells were stained for peroxidase activity, fixed and processed for electron microscopy as previously described (6). Cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4) for 10 min at room temperature, and exposed to diaminobenzidine (30 mg%) in 0.05 Tris buffer (pH 7.6) and 0.01% hydrogen peroxide for 10 min. The cells were then postfixed in a 1:2 mixture of 2.5% glutaraldehyde and 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4), for 15 min on ice, and then were exposed to 0.5% magnesium uranyl acetate in normal saline (pH 5.0) at room temperature for an additional 15 min before being pelleted in agar for dehydration in graded alcohols and embedding in Epon (Shell Chemical Co., New York).

Miscellaneous. Differential cell counts were performed on air-dried, methanol-fixed smears stained with Giemsa stain (Gradwohl Laboratories, St. Louis, Mo.). Protein was assayed by the method of Lowry et al. (10), using lysozyme as the standard.

Results

Pinocytic Rate of Thioglycollate- and Endotoxin-Stimulated Macrophages. Resident peritoneal macrophages, maintained for 24 h in culture in
20% FCS interiorize HRP at a linear rate of 56 ng HRP/h/100 μg cell protein, during a 2 h exposure to the marker enzyme. Thioglycollate-stimulated macrophages, maintained in 20% FCS for 24 h, pinocytize HRP at a rate approximately three times greater than the rate of resident peritoneal macrophages (Fig. 1). Endotoxin-stimulated cells, similarly cultivated, show a pinocytic rate somewhat greater than control cells, but considerably lower than that of thioglycollate-stimulated cells. The accumulation of cell-associated HRP is linear with time, over 2 h, for each of the three cell types, and is reduced to background levels when the cells are kept on ice (Table I). Binding of HRP to the dish itself is negligible under these conditions.

Endogenous Peroxidatic Activity in Thioglycollate Macrophages. Although it is firmly established that resident mouse peritoneal macrophages, unlike their precursor cells, have no demonstrable peroxidase (18), it was necessary to examine the possibility that some endogenous activity was present in stimulated cells. Thioglycollate cells, maintained in culture for 48 h, were therefore lysed in Triton X-100, and the lysate was assayed for peroxidatic activity using o-dianisidine as the substrate, in the presence of hydrogen peroxide. The equivalent of 1.8 ng of HRP activity could be detected per 100 μg of cell lysate. No effort was made to determine the dependence of this activity on the presence of hydrogen peroxide, or to characterize it any further.

Cytochemistry of Macrophages Exposed to HRP. Cultivated macrophages, exposed to HRP for 5 min to 1 h, were examined by light and electron microscopy, after being reacted with diaminobenzidine in order to locate the peroxidase which had become associated with the cells. This method yields semiquantitative data on the number and staining intensity of the organelles in the vacuolar apparatus. As shown in Fig. 2, both unstimulated and thioglycollate-stimulated cells were well spread with extensive ruffled membranes and considerable numbers of phase-dense cytoplasmic granules, presumably lysosomes. The most striking difference between the two cell varieties is the presence of conspicuous phase-lucent lakes in the cytoplasm of the thioglycollate-stimulated cells. Thioglycollate cells are also somewhat larger than the unstimulated cells, with thin membrane ruffles more generally distributed over the entire cell periphery, rather than being accumulated in only a few locations, as they are on unstimulated cells.

Under bright-field examination of thioglycollate-stimulated and unstimulated cells, each exposed for 20 min to 1 mg/ml HRP in 199-20% FCS, there is considerably more reaction product in the thioglycollate cells. The thioglycollate cells are more reactive both as a population, and on an individual cell basis. Nearly all the thioglycollate cells stained positively for HRP, while only about half of the unstimulated cells gave a visible reaction with diaminobenzidine. Both populations showed a wide range of individual cell reactivities, but the most strongly reactive cells in the thioglycollate population were considerably more positive than any of the unstimulated cells. As shown in Table II, less than 10% of the unstimulated cells were moderately stained with diaminobenzidine and about 1% stained intensely. The vast majority of cells stained only weakly, if at all, after a brief exposure to HRP. However, nearly every thioglycollate cell could be readily stained under these conditions, and about 20% of the cells were intensely stained with reaction product.
Examination of thin sections of cells exposed to HRP and then stained with diaminobenzidine (Fig. 3), showed that the reaction product was confined to membrane-bound cytoplasmic vesicles and granules. The cell shown has a large membrane-bound cytoplasmic inclusion containing moderately electron-dense amorphous material. This inclusion may correspond to the phase-lucent lakes seen by light microscopy in thioglycollate cells, and may contain agar or other poorly digestible particulate material present in the thioglycollate medium.

Effect of In Vitro Cultivation on Pinocytic Rate. Thioglycollate-stimulated, endotoxin-stimulated, and unstimulated peritoneal macrophages were cultivated in 20% FCS-supplemented medium for up to 72 h. As shown in Fig. 4, the qualitative differences among the three groups persisted in culture. Although there was some increase in endocytic rate with cultivation, at least for the thioglycollate-stimulated cells, the effect is not major, nor does it affect the qualitative differences among the three cell groups.

Components of Thioglycollate Medium Responsible for Pinocytic Stimulation. As Brewer's medium is a mixture of several ingredients (3) we injected some of its components separately into the peritoneal cavities of mice and assayed the pinocytic activity of the resulting exudate cells 4 days later. The assays were carried out after the cells had been kept in culture for 48 h.

As summarized in Table III, proteose-peptone, in the concentration used in Brewer's medium (1%), agar, or a combination of the two materials, caused only a modest rise in the yield of peritoneal cells. Proteose-peptone in higher concentration (10%) improved the yield somewhat. Endotoxin alone or in combination with latex also caused little if any increase in peritoneal cell yield. Latex alone was slightly more effective. None of the agents tested approached the effectiveness of complete thioglycollate medium.

Proteose-peptone at either concentration, agar, agar plus proteose-peptone, or
latex all gave cells with pinocytic rates comparable to or slightly above that of endotoxin-stimulated cells, which was considerably lower than the endocytic rate of thioglycollate-stimulated cells. However, the endocytic rate of cells stimulated simultaneously with endotoxin and latex was considerably elevated, although still lower than thioglycollate cells.

Temporal Development of Pinocytic Stimulation after the Intraperitoneal Injection of Thioglycollate. As Table IV summarizes, the initial response to thioglycollate is primarily an increase in the number of neutrophils in the peritoneal cavity. This response, however, is limited to the first 72 h after injection, and no neutrophils were recovered on the 4th day. There is a moderate increase in the number of peritoneal monocytes and macrophages on the 2nd and 3rd days, but the main mononuclear response occurs on the 4th day, when the total number of cells recovered is tripled, and the number of macrophages increases nine times. It is also interesting that a small number of eosinophils were seen on the 4th day after injection.

The pinocytic rate generally mirrored the pace of mononuclear accumulation (Table V). It was unchanged, as compared with control values, on the 1st day, but showed some increase on the 2nd day, to levels similar to those seen after the injection of endotoxin. The high pinocytic rate characteristic of thioglycollate-stimulated cells developed on the 3rd day and persisted for the next 24 h.

Effect of Concanavalin A (Con A) on the Pinocytic Rate of Thioglycollate-Stimulated Macrophages. Con A, a protein isolated from the jack bean (*Canavalia ensiformis*), and able to bind glucopyranoside and mannopyranoside residues, can stimulate the pinocytic rate of resident peritoneal macrophages about threefold (6). We therefore exposed thioglycollate-stimulated macrophages to Con A (100 μg/ml) in 199-20% FCS for periods of up to 2 h. As Fig. 5 shows, the pinocytic rate of these cells, while they were exposed to Con A, was increased about 3-fold as compared with untreated cells, and nearly 10-fold in comparison

| Condition | Pinocytic rate (ng HRP/100 μg cell protein/h) |
|-----------|-----------------------------------------------|
| Control macrophages | 37 56 |
| Endotoxin-stimulated macrophages | 37 85 0 3.7 |
| Thioglycollate-stimulated macrophages | 37 190 0 11.3 |
| No cells (dish control) | 37 4.2* |
| Thioglycollate-stimulated macrophages, no added HRP (endogenous activity) | 1.8 |

* ng HRP accumulated after 120 min.
FIG. 2. Light micrographs of macrophages exposed to HRP (1 mg/ml) for 20 min, and then stained with diaminobenzidine. Each set pictures identical microscope fields, viewed by phase-contrast (left) or bright-field (right) optics. (a and b) Thioglycollate macrophages, x 400. (c and d) Unstimulated cells, x 400. (e and f) Thioglycollate macrophages, x 1,000. (g and h) Unstimulated cells, x 1,000.
Table II

Distribution of Reactivity to Diaminobenzidine of Unstimulated and Thioglycollate-Stimulated Cells Exposed to HRP (1 mg/ml) for 20 min

|                         | Total cells | Intensely stained | Moderately stained | Weakly stained |
|-------------------------|-------------|-------------------|-------------------|---------------|
| Unstimulated            | 160         | 2 (1.3%)          | 10 (6.3%)         | 148 (92.5%)   |
| Thioglycollate-stimulated | 125         | 23 (18.4%)        | 99 (79.2%)        | 3 (2.4%)      |

with the rate of resident peritoneal cells. This stimulated rate was reduced to background levels when the cells were incubated on ice, instead of 37°C. The levels of enzyme activity at 0°C were somewhat higher in the presence of Con A (104 μg after 2 h) than the comparable value without Con A (11.3 μg), but in neither case do these values represent a significant fraction of the HRP actually endocytized by the cells under the two conditions.

Calculation of Pinocytic Rates per Cell. All results were normalized for the variable dish contents of cell protein. As stimulated macrophages may have more protein per cell than unstimulated cells (2), it seemed useful to recalculate our results on a per cell basis. In order to do this, we estimated the protein content of a known number of purified macrophages of various types.

Unstimulated cells, or cells obtained after endotoxin or thioglycollate injection were cultivated for 24 h in 20% FCS. We then thoroughly washed the dishes to remove nonadherent cells, and scraped the cells from the dishes into a known volume of buffered saline. We counted the suspended cells in a hemocytometer, and assayed the protein content of a second portion of the same suspension. As shown in Table VI, unstimulated mouse macrophages have a protein content of 80 μg per million cells. Endotoxin cells show an increase to about 106 μg per million cells, and thioglycollate cells are highest with a protein content of 130 μg per million cells.

Discussion

This report establishes that thioglycollate-stimulated macrophages display a pinocytic rate of 190 ng HRP/100 μg cell protein/h, a rate several times greater than that of resident peritoneal cells (which is 56 ng HRP/100 μg cell protein/h). This stimulated rate appears 3 days after the intraperitoneal injection of thioglycollate medium, and is increased somewhat by cultivating the cells in tissue culture medium supplemented with 20% FCS. Other agents injected intraperitoneally, including proteose-peptone, agar, or endotoxin, produce cells with a pinocytic rate higher than that of unstimulated cells, but considerably lower than the rate of thioglycollate-stimulated cells. However, the addition of latex to endotoxin leads to a considerable increase in the pinocytic rate, although it is still lower than the thioglycollate rate.

HRP has been previously validated for use in measuring the pinocytic rate of resident peritoneal cells (13). It is taken up by these cells in small electronlucent vesicles by a process which is temperature and time dependent. There is
FIG. 3. Electron micrograph of a thioglycollate-stimulated macrophage exposed to HRP (1 mg/ml) for 20 min, and stained with diaminobenzidine. Note black reaction product enclosed in membrane-bounded vesicles or smaller dense granules. There is a large membrane-bounded cytoplasmic vesicle (V) containing amorphous material, which may correspond to the phase-lucent cytoplasmic lakes evident in Figs. 2 a and e. Unstained section, original magnification 11,050.

no endogenous peroxidatic background in these cells (18), and insignificant amounts of the enzyme remain extracellular, or are interiorized after adsorption to the plasma membrane. Finally, the intracellular half-life of the enzyme, 8-10 h, is long enough to make the total accumulated enzyme, over 1-2 h, a good approximation for the total amount of enzyme interiorized over the same period.

HRP may also be used to measure the pinocytic rate of stimulated macrophages. There is little, if any, endogenous peroxidatic activity in these cells, and the HRP is taken up by a temperature-sensitive process with a constant rate of accumulation for up to 2 h. Extracellular accumulation of enzyme is negligible and the enzyme is distributed in the cells in membrane-bounded cytoplasmic
vesicles, consistent with its interiorization by pinocytosis. We have not studied the rate of degradation of HRP in stimulated cells. If HRP is metabolised more rapidly by stimulated than by unstimulated macrophages, we are underestimating the pinocytic rate of the stimulated cells. Alternately, if the degradative rate were reduced to zero in stimulated cells, it could not account for the increase in pinocytic rate. Consequently, possible changes in rate of degradation of HRP cannot explain the qualitative differences between unstimulated and stimulated macrophages.

Thioglycollate-stimulated cells are able to further increase their pinocytic rate in response to Con A, like unstimulated cells, and, in fact, show about a threefold increase in their pinocytic rate during exposure to Con A (100 µg/ml). This rate persists over a 2 h exposure to the lectin, but rapidly reverts to unstimulated levels when the lectin is removed from the incubation medium (P. J. Edelson, unpublished observations) or cleared from the cell surface.

The measured pinocytic rate of thioglycollate-stimulated cells allows us to

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**Table III**

*Effect on Macrophage Pinocytic Rate of Several Agents Injected Intraperitoneally*

| Stimulating agent                  | Average yield of peritoneal cells per mouse (× 10^4) | Pinocytic rate (ng HRP/100 µg cell protein/h) |
|-----------------------------------|---------------------------------------------------|---------------------------------------------|
| Proteose-peptone, 10%            | 10.6                                               | 116.5                                       |
| Proteose-peptone, 1%             | 8.6                                               | 87.1                                        |
| Agar (0.5 mg/ml)                 | 7.6                                               | 99.7                                        |
| Proteose-peptone 1% + agar       | 7.3                                               | 81.9                                        |
| Endotoxin                        | 6.4                                               | 81.1                                        |
| Endotoxin + latex                | 6.8                                               | 143.2                                       |
| Latex                            | 8.8                                               | 107.9                                       |
| Thioglycollate medium            | 18.3                                              | 214.2                                       |
| Unstimulated                     | 5.7                                               | 59.3                                        |

* Averages of four mice.
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**Table IV**

*Total Cell Counts, and Absolute Differential Counts, for Peritoneal Exudates Accumulating after the Intraperitoneal Injection of Thioglycollate Medium*

| Days after intraperitoneal thioglycollate injection | Total cells* per mouse (average)* | Monocytes/macrophages* | Polymorphonuclear leukocytes* | Large lymphocytes* | Small lymphocytes* |
|-------------------------------------------------|-----------------------------------|------------------------|-------------------------------|-------------------|------------------|
| 0                                               | 10.1                              | 2.3                    | 0.7                           | 6.8               | 0.3              |
| 1                                               | 11.1                              | 1.9                    | 6.1                           | 1.9               | 1.2              |
| 2                                               | 15.5                              | 4.4                    | 3.6                           | 6.7               | 0.8              |
| 3                                               | 23.8                              | 15.5                   | 7.1                           | 4.8               | 2.9              |
| 4                                               | 29.0§                             | 17.5                   | 0                             | 4.4               | 5.5              |

* In millions of cells.
† Averages of four mice.
§ Total count for day 4 includes $1.5 \times 10^8$ eosinophils not listed in table. Eosinophils were not seen on days 0–3.

**Table V**

*Pinocytic Rate of Exudate Macrophages Harvested up to 4 days after the Intraperitoneal Injection of Thioglycollate Medium, and Cultivated for 24 h*

| Days after intraperitoneal thioglycollate injection | Rate of HRP uptake (ng/100 μg/h) |
|---------------------------------------------------|---------------------------------|
| 0                                                 | 59*                             |
| 1                                                 | 57                              |
| 2                                                 | 112                             |
| 3                                                 | 201                             |
| 4                                                 | 214*                            |

* Rate measured in experiment described in Table II.

estimate the fraction of cell surface area which must be interiorized by these cells per minute. We have found that $10^6$ thioglycollate-stimulated cells interiorize 247 ng HRP/h, when incubated in a 1 mg/ml solution of HRP. Thus, these cells are interiorizing 247 nl of fluid/h. In order to make a reasonable estimate of the fraction of plasma membrane required to include this volume, we consider the idealized situation where the entire endocytic volume is being interiorized in uniform pinosomes 0.1 μm in radius. Such pinosomes would each include a vol of $4.19 \times 10^{-3}$ μm³. As the vol interiorized per cell, per minute, is 4.12 μm³, we would require 982 such pinosomes per minute. The surface area enclosing each pinosome is 0.126 μm², for a total surface area of 123.7 μm² interiorized per cell per minute. The surface area of an unstimulated cell has recently been estimated to be 935 μm² by morphometric analysis. Steinman, R. M., and Z. A. Cohn. Manuscript in preparation.
exchangeable cholesterol pool is a good reflection of the quantity of cell plasma membrane, and therefore of cell surface area, then the surface area of thioglycollate cells is about 1.8 times that of unstimulated cells, or about 1,680 μm². If 123.7 μm² is internalized per cell per minute, this would represent 7.4% of the total cell surface area, so that under these assumptions an area equivalent to 100% of the cell membrane would be internalized every 13.5 min.

In a similar way, we may calculate the fraction of cell volume internalized per minute. For this calculation, we begin with the estimate of the vol of a normal macrophage of 435 μm³, also based on morphometric measurements.² If we assume that the increase in cell protein of thioglycollate cells is an approximate measure of the increase in cell volume, we find that the vol of a thioglycollate cell is 707 μm³. The minute vol internalized is 4.12 μm³ per cell, or about 0.58% of the cell volume per minute.

The fact that it takes several days after thioglycollate stimulation to recover a population of cells with increased pinocytic activity may be an important clue to understanding the mechanisms by which such cells develop. Any or all the components of the induced inflammation may be necessary for the final development of pinocytically stimulated cells, including a variety of locally generated inflammatory factors, such as complement components, vasoactive peptides, or components of the coagulation system, systemically circulating inflammatory agents, products of lymphoid cells, and components or products of the neutrophils which precede the mononuclear exudate in the peritoneal cavity. There may also be a direct effect of the injected material on macrophages or their
precursors in the cavity. Whatever explanations are offered for the development of the final activated population it is clear that they will have to account for the considerable delay in the detection of such a population.

In addition to the question of what mechanisms are responsible for pinocytic stimulation, there is also the issue of the source of the active cells. There are two alternatives. They may arise from cells already present in the peritoneal cavity before the introduction of the inflammatory stimulus, or the active cells may be predominantly derived from circulating precursors which enter the peritoneal cavity after, and perhaps as a result of, the inflammatory stimulus. These cells are then driven to become activated macrophages either directly, or through their progeny. Work by van Furth and Cohn (17) and by Volkman and Gowans (19) indicate on the basis of thymidine-labeling experiments that the bulk of the macrophages recovered in an inflammatory exudate originate from circulating monocytes which enter the focus after the inflammatory stimulus has been introduced. North (12), however, has argued that resident peritoneal phagocytes are the origin of the majority of the exudate cells. This work relied on his ability to prelabel resident peritoneal cells with latex beads, and then identify activated cells with tritiated thymidine autoradiography. Since the large majority of labeled cells contained latex particles, North concluded that the inflammatory macrophages are primarily derived from a pre-existing resident peritoneal population. This experiment, however, is complicated by the fact that latex itself is an inflammatory stimulus so that it is not possible to assume that the only cells available to ingest the latex originally were resident peritoneal cells. Thus, the results do not necessarily conflict with the view that macrophages in an inflammatory exudate are generally not derived from the pre-existing resident peritoneal population.

It is also relevant to this question of the origin of the activated macrophages that Lin and Stewart (9) have recently identified a cell in thioglycollate-induced peritoneal exudates which can proliferate in culture and seems committed to the production of mononuclear phagocytes. Such cells may be the precursors of the activated macrophages present in peritoneal exudates, and it would be important to define the state of activation of the in vitro progeny of these cells.

It is important in all these considerations to be aware of the obvious heterogeneity of the exudate cells. Even if we focus only on the macrophages, it is clear, for example, from the cytochemical observation of these cells, that not all of them accumulate HRP at the same rate at all times. In resident populations,
about 1% of the cells at any given time seem to be considerably more active
pinocytically than the majority, while even among thioglycollate cells some cells
seem considerably more active than others. We do not know whether each cell
has its own characteristic pinocytic rate which it maintains continuously, or
whether we are seeing a population effect, with individual cells varying their
rates over time, but the fraction of cells at any given rate remaining relatively
constant. It is clear that the pinocytic rate characteristic of the population as a
whole is rather constant in culture, with little evidence that the overall rate
may mature with time.

A further issue is the question of what determines the intrinsic pinocytic rate
of a cell. This is really a matter of asking what characteristic of the thioglyco-
lolate cell is being reflected in its increased pinocytic rate. Although its surface
area seems to be somewhat larger than that of unstimulated cells, our estimates
suggest that the pinocytic rate is increased out of proportion to the increased
surface, and probably to the increased volume as well. Pinocytic rate could
reflect an increase in some other cell characteristic, for example, levels of ATP
or metabolic rate, or could be the result of a physical or chemical change in the
plasma membrane itself.

The activated macrophage was initially defined by Mackaness as a cell
capable, after specific immunologic stimulation, of developing a global antibacte-
rial capacity not characteristic of the unstimulated cell (11). Blanden (2) subse-
quently summarized other properties characteristic of the activated peritoneal
macrophage, including an accelerated rate of spreading on glass, and an in-
creased complement of mitochondria and phase-dense vesicles, presumably
lysosomes. In addition, he noted an increased number of phase-lucent cyto-
plasmic vesicles in these cells, which he interpreted to indicate an increased
pinocytic rate. Stubbs et al. (15) have added to this list by examining some
metabolic characteristics of macrophages from mice systemically infected with
Listeria. These cells were found to oxidize glucose labeled with $^{14}$C in either the
1, 3, 4, or 6 positions, or uniformly labeled at essentially the same rate as
unstimulated cells. However, after an exposure to polystyrene beads, the in-
crease in glucose oxidation, either via the hexose monophosphate shunt, or the
TCA cycle was considerably greater than that of the normal cells. This does not
necessarily imply that the activated cells are intrinsically more labile metabol-
ically than their normal counterparts, but could, at least in part, be due to an
increase in the number of beads phagocytized by the activated cells as compared
to the control cells. These workers did, in fact, find that the activated cells
showed a greater rate and extent of phagocytosis of $^{14}$C-labeled mycobacteria,
but detected no difference in their capacity for or rate of ingestion of labeled
starch granules. It should be mentioned that in that report, no difference was
detected between the protein contents of normal and activated cells. While this
may be true for cells from Listeria-infected animals, it is not true of thioglycol-
late-or endotoxin-stimulated cells, and it is difficult to come to a firm conclusion
on the issue as the results seem to represent measurements on mixed peritoneal
cell populations, rather than estimates of the protein content of more purified
macrophage populations.

Recently several new observations have been made which illustrate the
differences between resident peritoneal macrophages and stimulated cells, and among cells stimulated by various agents. Unkeless et al. (16) have reported that thioglycollate cells, but not resident cells, synthesize and secrete a neutral serine protease which can convert plasminogen to plasmin, while Bianco et al. (1) have reported that thioglycollate cells, but not normals, can ingest particles bound solely to the macrophage receptor for a fragment of the third component of complement (C3b). In each of these cases endotoxin-stimulated cells display the characteristic at an intermediate level. In the case of the plasminogen activator, this level can be increased by exposing the cells to a phagocytic stimulus (7). Our experiments in which endotoxin and latex were injected simultaneously suggest that the pinocytic rate may also respond synergistically to the two stimuli. This raises the possibility that such a synergistic effect may also be the basis for the special effectiveness of complete thioglycollate medium.

It is clear that a catalogue of distinctive objective markers of macrophage activation is being accumulated. Such a collection is essential to a formal examination of the mechanisms of macrophage activation, and to a complete physiologic and biochemical description of the activated cell.

Summary

Peritoneal macrophages from mice injected 4 days previously with Brewer's thioglycollate medium have a pinocytic rate, in culture, of 190 ng horseradish peroxidase (HRP)/100 µg cell protein/h, compared to the rate of resident peritoneal cells of 56 ng HRP/100 µg cell protein/h. Mice injected with endotoxin or with only certain of the components of the Brewer's medium show an intermediate level of stimulation. The rate of unstimulated, endotoxin-stimulated, or thioglycollate-stimulated cells shows little change over several days in culture. The pinocytic rate of thioglycollate-stimulated cells can, however, be further increased by exposure to concanavalin A. Although cells may show transient increases in their pinocytic rate in many situations, a sustained increase in pinocytic rate is a sign of the "activated" state of macrophages.

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