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Listericidal activity of non-stimulated and stimulated human macrophages *in vitro*

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SUMMARY

An *in vitro* biological assay system has been described to quantify the listericidal activity of human macrophages grown from peripheral blood monocytes. Within 15 min after *in vitro* infection, the activated macrophages showed a faster and higher degree in killing capacity as compared to the nonactivated ones; the killing process started early and was of short duration.

INTRODUCTION

The increase in anti-microbial activity of macrophages which occurs in animals infected with living microorganism is frequently non-specific (Mackaness, 1971). When peritoneal exudate cells (PEC, containing predominantly macrophages and lymphocytes) from guinea-pigs immunized against Bacillus Calmette-Guérin (BCG) are incubated *in vitro* with the appropriate antigen, macrophages in the culture acquire an enhanced microbicidal activity (Simon & Sheagren, 1971), a process generally referred to as *activation*. Macrophage activation is an immunological phenomenon, presumably depending on the interaction of sensitized lymphocytes with antigen, and the release by these cells of macrophage activating lymphokines (David, 1974; Lane & Unanue, 1972; Mackaness, 1971). *Listeria monocytogenes* has been largely used as target microorganism in the demonstration of such an enhanced bacterial killing (Blanden, 1974).

In the present report, the process of macrophage activation *in vitro* has been studied, using human macrophages derived from cultured blood monocytes. The various parameters of the system are analysed, including the role of antibiotics in the culture fluid.

MATERIALS AND METHODS

Blood was obtained hrough the Swiss Red Cross blood transfusion centre from clinically healthy donors of either sex, with a positive Mantoux test due to previous BCG vaccination, or from donors having negative Mantoux test who had not been BCG vaccinated. Volunteers were Mantoux tested and individuals with skin reactions 15 × 25 mm or larger (read after 48 hr) were chosen as positive test subjects. The experiments were performed 2–15 weeks after the skin test. Volunteers with negative Mantoux test were used as controls.

Purified protein derivative (PPD). The same batch of PPD RT 23 (Statens Seruminstitut, Copenhagen) was used for skin testing and *in vitro* experiments. The Mantoux test was performed as recommended by the manufacturer; for the *in vitro* tests, preservative-free PPD was used at a final concentration of 500 u (10 μg) per ml.

Peripheral blood leucocytes (PBL) separation. Fifty millilitres of fresh venous blood were transferred to a 100 ml Erlemeyer bottle containing 15–20 glass beads and rotated for 15 min at room temperature. A sample was diluted in Turk's (E. Merck, Darmstadt) solution, and leucocytes were counted. The defibrinated blood was distributed to two 35-ml glass centrifuge tubes. After centrifugation at 500 *g* for 10 min at 4°C, the serum was collected and kept in ice until use. RPMI-1640 medium (Microbiological Associate Inc., Bethesda, Maryland) containing 20 u of Heparin (Vitrum, Sweden) per ml was added to the packed cells up to the original defibrinated blood volume. The resuspended cells were layered on to the 10 ml Ficoll–Isopaque (9·6 g Ficoll, Pharmacia, Sweden; 130 ml distilled water; 20 ml Ronpacon 440. Cilag Chemie AG, Switzerland) in each of

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two 35-ml centrifuge tubes. After 20 min centrifugation at 400 *g* at 20°C, cells at the medium–Ficoll interface were collected in one centrifuge tube. The pooled cells were then washed three times with RPMI-1640 medium without heparin by centrifugation at 350 *g* for 5 min at 20°C each time. After the last wash, 3 ml RPMI-1640 medium supplemented with 10% of nutrient mixture were added to the pellet. The nutrient mixture contained: 1·0 g L(+)-glutamine puriss, Fluka AG, Buchs SG, Switzerland; 1·5 g Lactalbumin–Hydrolysat, enzymat. pulv., Fluka Ag, Buchs SG, Switzerland; 1·5 g Casein-Hydrolysat, enzymat., Fluka Ag, Buchs SG, Switzerland; 10·0 ml TC amino acids non-essential Minimal Eagle 100 times, Difco, Detroit, Michigan; 1 ml phosphate-buffered saline (PBS) ten times, PH 7·2; 4·0 g D(+)-Glucose, anhydrous for bacteriology, Fluka AG, Buchs SG, Switzerland; 77·0 ml RPMI-1640 medium, further called modified RPMI-1640 (RPMI-E). The cells were resuspended and counted using Turk's solution. The total yields of PBL after Ficoll-Isopaque separation ranged from 10–31% with a mean of 19% in twenty-one experiments. More than 98% of these cells were viable as determined by the Trypan blue (0·05%) exclusion test.

Distribution of PBL suspension. Macrophage culture (glass-adherent cells). A part of PBL suspension was adjusted to $3{\cdot}4\times10^6$ cells/ml, suspended in RPMI-E medium supplemented with 40% fresh autologous serum (AS). The use of fresh autologous serum is necessary to get optimum conditions to grow macrophages for the first 3 days (own observation). For experiments involving the quantitation of intracellular *Listeria*, 0·3 ml volumes of the cell suspension were distributed in $60\times11{\cdot}5$-mm flat bottomed glass tubes (Auer and Co AG, Zurich); for the microscopic observation of Giemsa-stained macrophages, 3-ml volumes of suspension were introduced to 35-ml petri dishes (Falcon plastic 3001) containing four round coverslips (12 mm diameter). Cells in the small tubes or in petri dishes were incubated in an atmosphere of 5% CO_2 at 37°C for 3 days, and subsequently washed twice to obtain adherent cells. Such a cell population will subsequently be referred to as 'macrophage culture'.

Lymphocyte culture. The other part of PBL suspension was adjusted to 1×10^6 cells/ml with RPMI-E medium supplemented with 10% decomplemented (56°C, 30 min) pooled AB serum (AB)/ml, and aliquots placed into two 13×100-ml culture glass tubes. Ten microlitres PPD was added to one tube and 10 microlitres to the other one. Both tubes were incubated at 37°C in an atmosphere of 5% CO_2 for 3 days, and subsequently the non-adherent cells were collected. Such non-adherent cells will be referred to as 'lymphocyte culture'.

In some experiments, thymidine (thymidine(methyl-H_3), the Radiochemical Centre, Amersham, incorporation of PPD-stimulated and control cultures was determined. Cells were kept in the incubator for 5 days, thymidine was then added to a final concentration of 2 μCi/ml and counted the following day. The mean of the counts of the PPD-treated lymphocytes was 7·6 times higher than the controls (nine experiments).

Activation of macrophage culture with PPD-stimulated lymphocyte suspension. After 3 days of incubation, macrophage cultures were overlaid with 0·2 ml (small flat-bottomed tubes) or 2 ml (petri dishes) of autologous lymphocyte cultures in the presence (Ly+PPD) or in the absence of PPD (Ly-PPD) (see above). Each culture also received 0·1 ml (tubes) or 1 ml (dishes) of AB serum. Both experimental and control cell mixtures were incubated for another 3 days to allow for activation of the macrophages. At day 6, the mixtures in the tubes were washed to remove non-adherent cells, and the adherent cells assessed for quantitative bactericidal activity; coverslips were removed from the dishes, stained with Giemsa and examined under the microscope.

Activation of macrophage culture with PPD. PBL suspended in RPMI-E medium containing 40% AS serum were incubated for 3 days, then the non-adherent cells were removed. RPMI-E medium containing 40% AB serum and PPD was added to the tubes where required. Tubes were incubated for another 3 days. Afterwards, the macrophage cultures were assessed for their listericidal capacity.

Culture of Listeria monocytogenes. A stock culture, pathogenic for mice, was a kind gift from Dr G. B. Mackaness, supplied in lyophilized form. To minimize heterogeneity in the *Listeria* population, a subculture was prepared from one colony after passage through human macrophages. *Listeria* were grown in RPMI-1640 medium overnight at 37°C, distributed in small plastic tubes (Falcon plastic 2003) and frozen at −70°C. This procedure reduced the original viable bacterial numbers by 20–30%; they then remained stable for at least 10 months. Before adding to the macrophages, frozen bacteria were thawed in water at room temperature. The tube was then left to warm at 37°C for 10 min.

Bactericidal assay. To determine the number of viable *Listeria* ingested by macrophages, 0·1 ml of a suspension containing 70×10^6 viable bacteria/ml in RPMI-E medium supplemented with 40% AB serum was added to each macrophage culture tube (or 1 ml of the same suspension was added to each dish). The initial PBL to *Listeria* ratio was 1:7.

Quantification of viable bacteria in macrophages. The term *infection* is used to indicate that when *Listeria* suspension is overlaid on macrophage culture for a certain period of time, a certain number of bacteria remain associated with the macrophages after extensive washing; presumably they are phagocytosed.

To remove extracellular *Listeria*, the infected cultures were washed four times with warm (37°C) Hanks's balanced salt solution (HBSS, Difco); warm HBSS was used to minimize cell detachment. Three-tenth millilitre RPMI-1640 medium containing 10% heated (56°C, 30 min) pooled human serum (PHS) was then added, followed immediately with 0·1 ml of 0·25% sodium dodecyl sulphate (SDS) in PBS to rupture the macrophages (Mauel, Behin & Biroum-Noerjasin, 1973). Triplicate tubes were shaken on a Vortex mixer (speed control, 5) for 50 sec and 0·5 ml of PHS was immediately added. The tubes were shaken again for a few seconds and left in ice. Volumes of 0·05 ml of the suspensions were spread on brain heart infusion (BHI)-agar (Difco, Detroit, Michigan) and the colonies were counted the next day under a dissecting microscope. In control experiments, the growth of *Listeria* was found not to be affected by treatment with SDS or 10% PHS serum.

Statistics. The mean values are given ± standard error of the mean. Student's *t*-test was used to estimate statistical significance.

RESULTS

I. *Studies of non-activated macrophages*

Population of adherent cells. In order to study the composition of the macrophage cultures, the following experiment was performed. Ten million PBL suspended in 3 ml RPMI-E medium supplemented with 40% fresh autologous serum were cultured in a petri dish containing four coverslips. After 3 days of incubation, the non-adherent cells were removed, and the macrophage culture was re-incubated for another 3 days in the presence of RPMI-E medium supplemented with 40% AB serum. After 6 days of incubation (total) the non-adherent cells were removed for the second time, washed three times, and live *Listeria* suspension was added. After 40 min of incubation, coverslips were harvested and stained with Giemsa. In ten experiments, they were found on morphological grounds to be composed of: macrophages, 90–97%; lymphocytes, 1–7%; multinucleated giant cells, 1–3%; granulocytes, 0%. The macrophages and giant-cells contained several bacteria per cell.

Phagocytic activity. To assess the phagocytic capacity of macrophages, 6 day old macrophages on coverslips were overlaid with *Listeria* suspension. Coverslips were harvested and stained at 10-min intervals; then the phagocytic cells were counted under the microscope. As shown in Fig. 1a, the percentage of cells with intracellular bacteria was maximal after 20 min exposure to bacteria; in addition, individual macrophages were found to contain more bacteria with increasing time of exposure.

To determine the number of viable intracellular *Listeria* with increasing times of infection, 6-day-old tube cultures of macrophages were overlaid with bacteria suspension. Triplicate tubes were removed from the incubator at 10-min intervals, and the infected cells were immediately processed for colony counts of viable intracellular bacteria. The number of ingested bacteria was found to increase almost linearly with increased time of exposure to *Listeria* (Fig. 1b).

Quantification of intracellular killing and multiplication of bacteria. To follow the fate of the bacteria after infection, macrophages were overlaid with a *Listeria* suspension for 10 min. All tube cultures were then washed to remove extracellular bacteria, then, 0·3 ml RPMI-E medium containing 40% AB serum was added to each tube and incubation was resumed; except for triplicate tubes which were directly

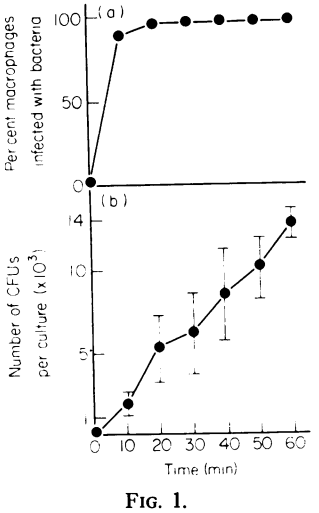

FIG. 1.

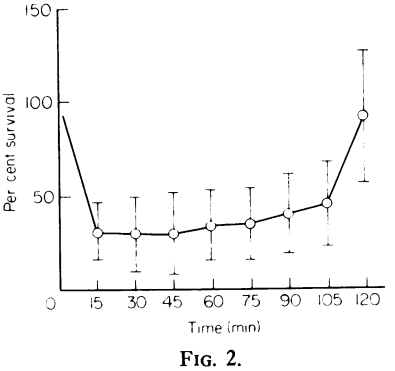

FIG. 2.

FIG. 1. (a) Per cent of macrophages by time which contained bacteria. (b). Viable bacteria phagocytosed by non-stimulated human macrophages during 60 min of incubation. Points represent mean and bars represent s.e. of triplicate cultures of three experiments.

FIG. 2. Fate of intracellular bacteria in non-stimulated human macrophages. Points represent mean and bars represent s.e. of triplicate cultures (four experiments).

processed for the zero time bacterial counts. The re-incubated tube cultures were then processed for colony counts at 15-min intervals. As shown in Fig. 2, the number of viable intracellular *Listeria* decreased within the first 15 min of incubation to 16–52% (mean 30%) of the original ingested viable *Listeria* (zero time counts), indicating a marked bacteriolytic effect of non-stimulated cells (four experiments). Colony counts then remained stable for at least 45 min, indicating a bacteriostatic phase. Beyond 105 min of harvest, the number of viable intracellular bacteria increased rapidly, indicating the existence of a multiplication phase.

II. *The role of antibiotics in the listericidal activity of macrophages*

To determine whether antibiotics might influence the fate of intracellular *Listeria*, tube cultures of normal macrophages were infected with *Listeria* for 10 min, then washed and re-incubated in the presence or in the absence of penicillin (50 u/ml, Benzylpenicillin-Natrium, Specia) in the culture fluid. Colony counts of intracellular bacteria were performed 3 and 5 hr later. Tubes with and without antibiotics were thoroughly washed before cell disruption to avoid carrying over antibiotics with the *Listeria*

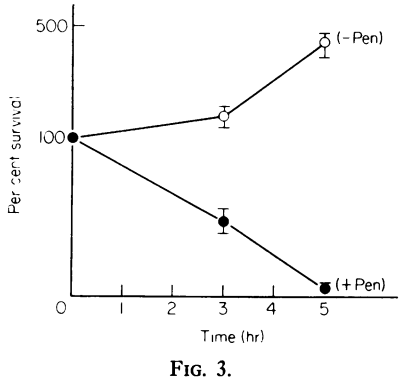

FIG. 3.

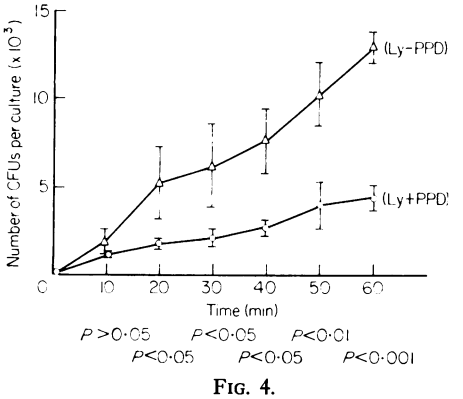

FIG. 4.

FIG. 3. Killing of *Listeria monocytogenes* by non-stimulated human macrophage culture during a 5-hr period of incubation, re-incubated in the presence of 50 u penicillin/ml medium (●), killing continued up to 5 hr. The control culture (without antibiotics) (○) showed an increase in the number of viable bacteria. Points represent the mean and bars represent s.e. of triplicate cultures (one experiment).

FIG. 4. Viable *Listeria monocytogenes* phagocytosed by non-stimulated (△) and stimulated (○) human macrophage cultures during 60 min of incubation. During the period of phagocytosis, the non-stimulated macrophages contained more *viable* bacteria as compared to the stimulated macrophages. After 10 min phagocytosis the difference in the bacterial counts was not significant ($P > 0{\cdot}05$), but became significant ($P < 0{\cdot}05$) after 20 min, and more so after 60 min ($P < 0{\cdot}001$) of incubation. Points represent mean and bars represent s.e. of triplicate cultures. The number of *viable* bacteria phagocytosed by both experimental and control cultures increased with time, but significantly higher by non-stimulated macrophages (three experiments).

suspension to the BHI-agar dishes. A marked difference was observed between the fate of viable intracellular *Listeria* in cultures incubated with medium containing antibiotics as compared with similar cultures without antibiotics. Colony counts on *Listeria* harvested from antibiotic treated macrophages were found to decrease continuously with time. In marked contrast, the 3- and 5-hr harvests of the cultures incubated without antibiotics showed rapid intracellular growth (Fig. 3). In view of the potent effect of extracellular penicilline on the fate of intracellular *Listeria*, antibiotic free culture media were used for all experiments.

III. *Studies of activated macrophages*

Quantification of ingestion, killing and multiplication of Listeria. To compare the phagocytic activity of activated and non-activated macrophages, dish cultures were washed free of non-adherent cells, infected with *Listeria* for 10 min, then coverslips were harvested, stained and examined microscopically. No difference was found in the percentage of phagocytic cells in both types of cultures (90% in the activated vs. 89% in the non-activated macrophages).

To determine the number of phagocytosed viable *Listeria*, tube cultures of activated and non-activated macrophages were overlaid with *Listeria* suspension as described in materials and methods. Triplicate tubes were removed from the incubator at 10-min intervals, and the infected cells were immediately processed for colony counts of intracellular micro-organisms. As shown in Fig. 4, there was no significant difference ($P>0{\cdot}05$) between the number of viable *Listeria* from activated and non-activated macrophage cultures infected for 10 min. At longer times of exposure, however, a significant difference was observed. The difference became highly significant ($P<0{\cdot}001$) after 60 min of exposure. Based on these three experiments, a standard infection period of 10 min was chosen to minimize the difference at the starting point in subsequent experiments aimed to follow the fate of *Listeria* ingested by activated macrophages.

To determine whether activated macrophages have an increased listericidal activity as compared to control cells, tube cultures of activated and non-activated macrophages were infected for 10 min. All tube cultures were then thoroughly washed, and the incubation resumed. Triplicate tubes of activated and control tubes were harvested for the zero time counts, and the remaining tubes were harvested at 10 min intervals for colony counts of intracellular bacteria. Under these conditions, the number of viable *Listeria* was found to decrease faster and to a greater degree in the activated cells (Fig. 5). In addition, whereas the number of viable bacteria began to increase in non-activated macrophages between 50 and 60 min after infection, no such an increase was observed in the activated cultures during this time period. Based on these three experiments, tube cultures in the following experiments were harvested 1 hr after infection.

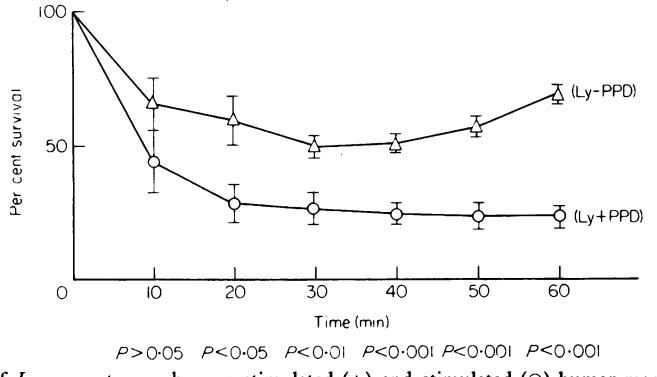

FIG. 5. Killing of *L. monocytogenes* by non-stimulated (△) and stimulated (○) human macrophage cultures during 60 min of incubation. The bactericidal capacity of the stimulated macrophages is higher than the non-stimulated macrophages. At 10 min harvest the difference in bactericidal capacity is not significant ($P>0{\cdot}05$), but becomes significant ($P<0{\cdot}05$) at 20 min harvest, and more so at 60 min harvest ($P<0{\cdot}001$). Points represent mean and bars represent s.e. of triplicate cultures (three experiments).

IV. *Studies of macrophages obtained from Mantoux-positive and Mantoux-negative individuals*

Quantification of killing of *Listeria* by macrophages obtained from six Mantoux-positive individuals showed that, after 1 hr infection, the PPD-treated cultures (via lymphocytes) showed 11–31% (mean 20%) bacterial survival and the macrophages without PPD showed 39–51% (mean 44%); the mean of the colony counts of non-activated macrophages was 2·2 times higher than the activated ones. This difference is highly significant ($P<0{\cdot}001$, six experiments), Fig. 6.

Attempts were made to activate macrophage cultures directly with PPD, in the absence of added autologous lymphocytes. Tube cultures were left in the incubator for 3 days and the adherent cells were washed extensively, and incubated for another 3 days in the presence of 500 u/ml of PPD in RPMI-E medium containing 40% of AB serum, then tested for listericidal activity. When tubes were processed 1 hr after infection, the PPD added macrophage cultures showed 15–23% (mean 19%) of bacterial survival and the control macrophage cultures showed 38–44% (mean 41%). The mean of the colony counts of control cultures was 2·1 times higher as compared to the PPD treated macrophage cultures (two experiments). The cells from Mantoux-negative individuals, when incubated with PPD, did not differ in their listericidal activity from control preparations without added PPD.

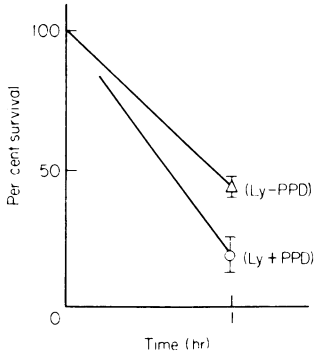

FIG. 6. Studies of six different Mantoux-positive individuals. Lymphocytes with or without PPD were transferred to the autologous macrophage cultures. Killing of *L. monocytogenes* by non-stimulated (△) and stimulated (○) macrophage cultures. At 1-hr harvest, the stimulated macrophages showed higher degree of bactericidal capacity ($P < 0{\cdot}001$) as compared to the non-stimulated macrophages. Points represent mean and bars represent s.e. of triplicate cultures (six experiments).

Results of the studies of six Mantoux-negative individuals showed that, 1 hr after infection, macrophage cultures co-cultivated with lymphocytes plus PPD showed 17–33% (mean 30%) of bacterial survival and the control cultures showed 15–31% (mean 28%). The mean of colony counts of non-PPD added cultures was 0·97 times lower than the PPD treated cultures. The difference is not significant ($P > 0{\cdot}05$, six experiments).

DISCUSSION

Assessment of the fate of intracellular bacteria can be complicated by extracellular multiplication and continuous phagocytosis of these bacteria by macrophages. To overcome this difficulty, investigators have resorted to the addition of antibiotics to the culture fluid after phagocytosis (Armstrong & Sword, 1964; Cline, 1970; Steigbigel, Lambert & Remington, 1974). However, as discussed by Wilder & Edberg (1973) and by Hart (1974), the bactericidal capacity of macrophages cultured in the presence of antibiotics may be altered by the capacity of these cells to concentrate these drugs from the surrounding fluid. Our data show that the number of viable bacteria recovered from non-activated human macrophages incubated in the presence of 50 u of penicillin/ml decreases continuously with time; a similar finding was reported by Cline (1970). However, macrophages incubated in medium without antibiotics show an increase in the number of viable intracellular *Listeria* with time (Fig. 3). To avoid possible errors in the interpretation of the data, the fate of intracellular *Listeria* was studied in this investigation using media without antibiotics. To minimize the role of continuous phagocytosis of multiplying extracellular bacteria, the fate of intracellular *Listeria* was assessed (in most experiments) 1 hr after infection, that is within the lag-phase of extracellular bacterial growth, which as determined by Cline (1970) is at least of 120 min. The possibility that multiplying extracellular *Listeria* were re-phagocytosed is difficult to exclude when the fate of intracellular bacteria was assessed beyond 2 hr of incubation. The need for an early quantitation of intracellular bacteria was also suggested by Hart (1974).

Using different assay conditions, Bast *et al.* (1974) have reported that activated guinea-pig macrophages can produce soluble factors which have bactericidal effect on *Listeria*. The system I have described here measures only the capacity of macrophages to kill *Listeria* which have already been phagocytosed, and the results are not influenced by possible extracellular killing due to such factors.

The initial PBL: *Listeria* ratio of 1:7, and a phagocytosis period of 10 min, were chosen for the following reasons; under these conditions: (1) 90% of the 6-day-old macrophages are found to have ingested bacteria; (2) the number of viable *Listeria* phagocytosed is sufficient to demonstrate killing activity within a short time; (3) convenient numbers of colonies can be counted in the BHI-agar dishes; (4) for the comparison of the fate of *Listeria* in activated and non-activated macrophages, the viable bacterial counts after 10 min of infection (zero time) show no ($P > 0{\cdot}05$) statistical difference (Fig. 4).

To observe the growth curve of the intracellular *Listeria*, non-stimulated macrophages were infected for 10 min, washed thoroughly and incubation was resumed. Fig. 2 shows that those macrophages displayed remarkable killing activity for the first 15 min post-infection. This capacity appears to be of very short duration as no further decrease in the number of viable bacteria was observed beyond 15 min. This could be explained by an exhaustion *in vitro* of the cellular bactericidal mechanisms, or by the existence of a susceptible sub-population of macrophages as reported by Blanden (1968) and discussed by North (1974b), or by the existence of a bacterial sub-population resisting the listericidal activity of the cells. The bactericidal phase was followed by a bacteriostatic phase which lasted for 45 min. This can be interpreted as reflecting either a lag-phase in the growth of the remaining intracellular bacteria, or equal rates of bacterial killing and multiplication. Finally, starting from 105 min after infection, the number of viable intracellular bacteria began to increase. A similar, biphasic bacterial growth curve was described by Steigbigel *et al.* (1974) in their study of the bactericidal activity of human monocytes in suspension. Whereas these authors reported an initial decrease, followed after 2 hr by an increase in intracellular bacterial counts, it appears from the present data that a 'bacteriostatic' period of relatively long duration separates these two phases. However, this can be detected only when harvest of intracellular bacteria are performed at close intervals.

As described in a variety of systems, macrophages activated by antigen- or mitogen-stimulated lymphocytes display an enhanced killing capacity towards *Listeria monocytogenes* and other intracellular micro-organisms. Similarly, the experiments reported here show that human macrophages co-cultivated with autologous lymphocytes plus PPD destroyed *Listeria* faster and to a higher degree than control macrophage populations. The difference in bacterial counts between activated and control cultures was significant 20 min after infection (Fig. 5), and more so with longer incubation times. It was interesting to note that the initial period of rapid destruction of the bacteria did not last longer in activated cells than in normal controls; the arrest phase, on the other hand, appeared to be considerably prolonged. Such observations suggest that activated macrophages differ quantitatively rather than qualitatively from normal ones, in being capable of carrying to a higher degree the normal tasks of these cells.

The results presented here also indicate that bacterial killing starts very early after phagocytosis. After 10 min of infection, activated and control macrophages were similar in both their percentage of phagocytic cells as judged by microscopic observation of stained preparation, and in the number of viable intracellular *Listeria* (Fig. 5). However, macrophage inhibition factor (MIF) stimulated macrophages have been shown to display enhanced rates of phagocytosis of dead Mycobacteria (Klun & Youmans, 1973). The fact that an increased recovery of viable bacteria was observed in activated preparations after 10 min of infection suggests that activated macrophages are endowed at the same time with increased rates of both phagocytic and bactericidal activities, and that bacterial destruction is a rapid event. Blanden (1968) has similarly reported killing of *Salmonella typhimurium* by macrophages of immune mice within minutes of phagocytosis. As discussed by others (Anderson & Remington, 1974; Blanden, 1968; North, 1974b), it is not known whether such behaviour reflects a general enhancement of the bactericidal properties of all the macrophages in the culture, or an increase in the percentage of cells capable of killing the micro-organisms. The mechanisms of macrophage activation are still controversial. Although interaction of lymphoid cells of T-lineage with antigen has been shown to be necessary to obtain protection against virulent micro-organisms *in vivo* (Lane & Unanue, 1972; Mackaness, 1971; North, 1974a), whether the same cells are required for macrophage activation *in vitro* is a matter of debate. Normal macrophages can be activated to kill intracellular micro-organisms when incubated with lymphocytes stimulated with antigen (Behin *et al.*, 1975; Simon & Sheagren, 1972), plant lectins (Klun & Youmans, 1973), or allogeneic cells (Mauel, Biroum-Noerjasin & Behin, 1974), implying a possible role for soluble factors in mediating this phenomenon (David, 1974; Fowles *et al.*, 1973; Nathan, Karnovsky & David, 1971). On the other hand, Simon & Sheagren (1972) reported macrophage activation on exposure to antigens in cultures of PEC from BCG immunized guinea-pigs, even after extensive washing to remove contaminating lymphocytes. Similarly in the experiments reported here, activation could apparently be obtained by incubation of macrophage cultures from Mantoux-positive subjects with PPD, even after removal of the non-adherent cells. Whether this represents a direct effect of antigen on the macrophages

armed *in vivo*, a phenomenon described by Evans & Alexander (1972), or activation via the remaining adherent lymphocytes, is not known.

The system described in the present report may be a useful test of human monocyte function. Particularly, alterations in monocyte behaviour of certain patients with recurrent infections can easily be observed by determining the listericidal capacity of these cells under carefully controlled conditions (Biroum-Noerjasin & Cruchaud, in preparation).

In a recent extension of these studies, we have found that macrophages activated either by PPD or PHA can act as potent cytotoxic effector cells against human tumour cells (Biroum-Noerjasin & Carrel, manuscript in preparation). Thus, the cytotoxic activity of macrophages may not be restricted to an antibacterial role *in vivo*.

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REFERENCES

ANDERSON, S.E. & REMINGTON, J.S. (1974) Effect of normal and activated macrophages on *Toxoplasma gondii*. *J. exp. Med.* **139**, 1154.

ARMSTRONG, A.S. & SWORD, C.P. (1964) Cellular resistance in listeriosis. *J. infect. Dis.* **114**, 2586.

BAST JR, R.C., CLEVELAND, R.P., LITTMAN, B.H., ZBAR, B. & RAPP, H.J. (1974) Acquired cellular immunity: extracellular killing of *Listeria monocytogenes* by a product of immunologically activated Macrophages. *Cell. Immunol.* **10**, 248.

BEHIN, R., MAUEL, J., BIROUM-NOERJASIN & ROWE, D.S. (1975) Mechanisms of protective immunity in experimental cutaneous leishmaniasis of the guinea-pig. II. Selective destruction of different *Leishmania* species in activated guinea-pig and mouse macrophages. *Clin. exp. Immunol.* **20**, 351.

BLANDEN, R.V. (1968) Modification of macrophage function. *J. Reticuloend. Soc.* **5**, 3, 179.

BLANDEN, R.V. (1974) T-cell response to viral and bacterial infection, (ed. by Goran Moller), pp. 56. The Immune Response to Infectious Diseases. *Transplantation review.* **19**, Munksgaard, Copenhagen.

CLINE, M.J. (1970) Bactericidal activity of human macrophages analysis of factors influencing the killing of *Listeria monocytogenes*. *Infect. Immunol.* **2**, 2, 156.

DAVID, J R (1974) Macrophage activation induced by lymphocyte mediators. p. 1 Karolinska Symposia on research methods in reproductive endocrinology; 7th Symposium. *Immunological approaches to fertility control.* Forum Printer 15, Copenhagen, Denmark.

EVANS, R. & ALEXANDER, P. (1972) Mechanism of immunologically specific killing of tumour cells by macrophages. *Nature (Lond.)*, **236**, 168.

FOWLES, R.E., FAJARDO, I.M., LEIBOWITCH, J.L. & DAVID, J.R. (1973) The enhancement of macrophage bacteriostatis by products of activated lymphocytes. *J. exp. Med.* **138**, 952.

HART, P. D'ARCY (1974) Critical approach to the technique of assessment of antibacterial effects of activated mouse peritoneal macrophages. pp. 131. (ed. by W.-H. Wagner and H. Hahn) Activation of Macrophages: Workshop conferences Hoechst. Volume 2. American Elsevier Publishing Corp., New York.

KLUN, C.L. & YOUMANS, G.P. (1973) The induction by *Listeria monocytogenes* and plant mitogens of lymphocyte supernatant fluids which inhibit the growth of *Mycobacterium tuberculosis* within macrophages *in vitro*. *J. Reticuloend. Soc.*, **13**, 3, 275.

LANE, F.C. & UNANUE, E.R. (1972) Requirement of thymus T lymphocytes for resistance in listeriosis. *J. exp. Med.* **135**, 1104.

MACKANESS, G.B. (1971) Delayed hypersensitivity and the mechanisms of cellular resistance to infection. pp. 413. (ed. by B. Amos), *Progress in immunology: First International Congress of Immunology.* Academic Press, New York.

MAUEL, J., BEHIN, R. & BIROUM-NOERJASIN (1973) Quantitative release of live microorganisms from infected macrophages by sodium dodecyl sulphate. *Nature, New Biology:* 244, 93.

MAUEL, J., BIROUM-MOERJASIN & BEHIN, R. (1974) Killing of intracellular parasites as a measure of macrophage activation. pp. 260. (ed. by W.-H. Wagner and H. Hahn) *Activation of macrophages: workshop conferences Hoechst.* volume 2. American Elsevier Publishing Corp. Inc. New York.

NATHAN, C.F., KARNOVSKY, M.L. & DAVID, J.R. (1971) Alterations of macrophage functions by mediators from lymphocytes. *J. exp. Med.* **133**, 1356.

NORTH, R.J. (1974a) T-cell-dependent macrophage activation in cell-mediated anti-*Listeria* immunity. pp. 210. (ed. by W.-H. Wagner and H. Hahn) *Activation of macrophages: workshop conferences Hoechst.* volume 2. American Elsevier Publishing Corp. Inc., New York.

NORTH, R.J. (1974b) Cell mediated immunity and the response to infection. pp. 185. (ed. by R.T. McCluskey and S. Cohen) *Mechanisms of cell-mediated immunity*, John Wiley and Sons, Inc., New York.

SIMON, H.B. & SHEAGREN, J.N. (1971) Cellular immunity *in vitro*. I. Immunology mediated enhancement of macrophages bacteridical capacity. *J. exp. Med.* **133**, 1377.

SIMON, H.B. & SHEAGREN, J.N. (1972) Enhancement of macrophage bactericidal capacity by antigenically stimulated lymphocytes. *Cell. Immunol.* **4**, 163.

STEIGBIGEL, R.T., LAMBERT, JR, L.H. & REMINGTON, J.S. (1974) Phagocytic and bactericidal properties of normal human monocytes. *J. clin. Invest.* **53**, 131.

WILDER, M.S. & EDBERG, J.C. (1973) Interaction of virulent and avirulent *Listeria monocytogenes* with cultured mouse peritoneal macrophages. *Infect. Immunol.* **7**, 3, 409.