

METABOLIC AND FUNCTIONAL STUDIES ON ACTIVATED MOUSE MACROPHAGES*

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(Received for publication 8 November 1972)

The macrophage responds to some infections with an adaptive increase in its defense capacities. Mackaness (1) has shown that the enhancement of macrophage function during infection has an immunologic basis and involves the lymphocyte. The "activated" macrophages obtained from animals during a certain stage of infection spread out on glass more extensively, appear more heavily endowed with mitochondria and lysosomes, are more phagocytic, and are better able to kill bacteria than their normal counterparts. In attempts to elucidate the mechanism of interaction between macrophages and lymphocytes that might underlie these phenomena, Nathan et al. (2) found that a factor produced by antigen-stimulated lymphocytes altered the function of normal macrophages. When these cells were exposed to this preparation in vitro,¹ they exhibited increases in cell adherence, ruffled membrane activity, phagocytosis, and oxidation of glucose carbon-1 to CO₂. The experiments described below were designed to determine whether macrophages, activated in vivo by *Listeria monocytogenes* (3), also showed enhanced glucose oxidation and the pattern of enhanced phagocytic capabilities that were noted to result from the factor(s) described by Nathan et al. (2). Such comparisons might assist in elucidating the mechanism of activation of macrophages in vivo.

Materials and Methods

Animals.—Cox Swiss mice (males, 4-5-wk old), obtained from Charles River Laboratories, Wilmington, Mass., were used in these experiments.

Organisms.—The strain of *Listeria monocytogenes* used was obtained from Dr. G. B. Mackaness. Suspensions of the bacteria were prepared from an 18 h culture of *Listeria* grown in Trypticase soy broth (Difco Laboratories, Inc., Detroit, Mich.). The organisms were washed twice and resuspended in sterile saline to give the approximate number of bacteria per milliliter as determined by the optical density. The suspensions (or dilutions of the suspension) were plated and bacterial colonies counted to determine the specific number of *Listeria* in the original suspension. The LD₅₀ for this strain when injected intravenously was found to be 5×10^5 microorganisms. A sublethal dose of *Listeria*, 1.5×10^4 , was injected intravenously into the mice.

* This work was supported by U. S. Public Health Service grants A1-03260 and A1-07685 from the National Institute of Allergy and Infectious Diseases.

¹ Fractions rich in migration-inhibitory factor (MIF) obtained by Sephadex chromatography of supernatant fluids from antigen-stimulated lymphocytes (2).

Collection of Mouse Peritoneal Exudates.—7 days after infection, the mice were exsanguinated and peritoneal exudates collected, using no irritant, in Hanks' balanced salt solution (Grand Island Biological Co., Grand Island, N. Y.). They were washed three times in Krebs-Ringer phosphate solution (KRP). The peritoneal exudates from normal mice (controls) were also collected. A direct cell count was made and the cell suspensions were adjusted with KRP to give 1×10^7 macrophages/ml. Peritoneal macrophages were determined to be activated if they spread on a glass slide when incubated at 37°C for 15 min according to the method of Mackaness (3).

Chemical Determinations.—DNA was determined using the method of Kissane and Robins (4). Protein was measured according to Lowry et al. (5).

Oxidation of Specifically Labeled Glucose to $^{14}\text{CO}_2$.—The incubations were carried out in 10-ml flasks fitted and sealed with rubber vaccine ports carrying a hanging plastic center well with 0.2 ml of 20% KOH for collection of evolved $^{14}\text{CO}_2$ (Kontes Glass Co., Vineland, N.J.). The flasks contained approx. 1×10^7 macrophages, 7.5 mM glucose (with 0.5 μCi of $[\text{U-}^{14}\text{C}]$, $[3,4\text{-}^{14}\text{C}]$, or $[1\text{-}^{14}\text{C}]$ or 2.5 μCi $[6\text{-}^{14}\text{C}]$ glucose), and Krebs-Ringer phosphate solution to a final vol of 1 ml. Polystyrene spherules (0.1 ml; 2.5 mg) were added where phagocytosis was to be studied. After 60 min incubation at 37°C with shaking in a water bath, 0.2 ml of 20% H_2SO_4 was injected through the port to release any bound $^{14}\text{CO}_2$. After 30 min at room temperature the contents of the center wells were transferred to vials containing 15 ml of Buhler's solution (6), and the vials counted on an Ansitron II Liquid-Scintillation Counter (Picker Nuclear Corp., Newton, Mass.). The specific activity of each labeled glucose was determined, so that radioactivity measurements could be converted into nanomoles of glucose oxidized per hour per milligram of cell protein.

Measurement of Phagocytosis.—Phagocytic activity was determined by the method of Michell et al. (7) using ^{14}C acetyl starch or heat-killed ^{14}C -labeled *Mycobacterium tuberculosis hominis* H37Rv as the particles. Preparation of these particles has been described elsewhere (7-9), and their specific activity, based on mass or number of particles, was determined. Particles were added to monolayers for precisely timed periods, and uptake of particles per milligram of cell protein was assessed.

RESULTS

DNA and Protein Content of Cells.—Table I gives the values for DNA and protein of normal and activated macrophages. In early experiments the activated cells appeared higher with respect to the latter measurement than normal cells; for all experiments the increase was 13%, which was not statistically different from the control value.

Differential counts showed that in both the normal and activated cell suspensions approximately 90-95% were mononuclear cells, of which two-thirds

TABLE I
Protein and DNA Content of Normal and Activated Macrophages

	Normal	Activated	P
	$\mu\text{g}/10^6$ cells		
DNA	13.70 \pm 1.8 (5)	13.84 \pm 1.6 (5)	>0.1
Protein	65.6 \pm 7.7 (12)	73.9 \pm 4.99 (12)	>0.1

Results expressed as mean \pm standard error with number of observations in parentheses.

were macrophages and one-third were lymphocytes. The remaining cells included polymorphonuclear leukocytes (ca. 4%) and mast cells (ca. 1%).

Glucose Oxidation.—Oxidation of glucose by the activated cells was greater than that in the normal cells both at rest and during phagocytosis. This indicates increased metabolic activity (see Fig. 1). Oxidation of specifically labeled glucose gave indications of bigger increases in the conversion to $^{14}\text{CO}_2$ of 3,4- ^{14}C -, and 6- ^{14}C -labeled glucose in the resting activated cells compared with [U- ^{14}C] or [1- ^{14}C]glucose. During phagocytosis there was a marked elevation of $^{14}\text{CO}_2$

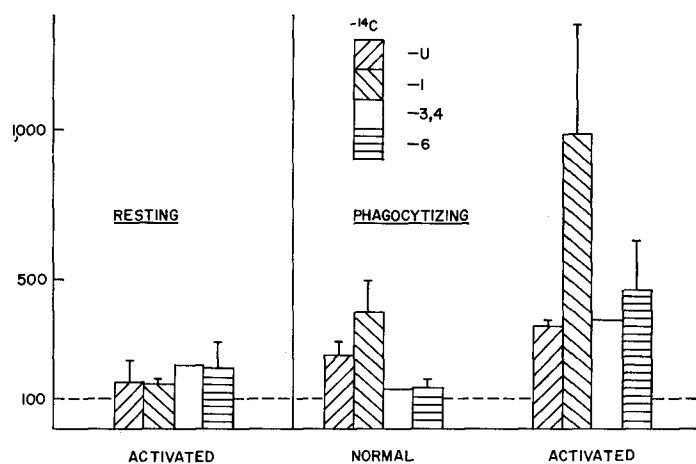


FIG. 1. Oxidation of specifically ^{14}C -labeled glucose by activated and normal macrophages at rest and during phagocytosis. Oxidation of [^{14}C]glucose by normal macrophages at rest is taken to be 100%. The absolute values are given below, as nanomoles of $^{14}\text{CO}_2$ produced per hour and milligram of cell protein, based on the specific activity of the substrate expressed per labeled glucose carbon atom. The mean values for two experiments (each performed in duplicate or triplicate) are given, except in the case of [3,4- ^{14}C]glucose, with which only one experiment was performed. Glucose: [1- ^{14}C], 11.7; [3,4- ^{14}C], 11.6; [6- ^{14}C], 1.6; [U- ^{14}C], 36.9.

formation from all the labeled glucose species, with [U- ^{14}C]glucose yielding the smallest relative increase in $^{14}\text{CO}_2$. Oxidation of glucose carbon-1 to $^{14}\text{CO}_2$ is an approximate indicator of hexose monophosphate shunt activity and this increase during phagocytosis is well documented (Oren et al. [10], Karnovsky et al. [11]), especially in the case of neutrophilic granulocytes. Macrophages also show a significant increase in conversion of [1- ^{14}C]glucose to $^{14}\text{CO}_2$ during phagocytosis, except for alveolar macrophages (Oren et al. [10]).

Phagocytosis.—The time-course of phagocytosis by normal and activated macrophages showed differences dependent on the type of particle offered for ingestion. When ^{14}C -labeled starch was offered, there was no detectable increase in uptake by the activated cells (see Fig. 2). When ^{14}C -labeled tubercle bacilli were offered, at least a twofold increase was seen throughout the period of ob-

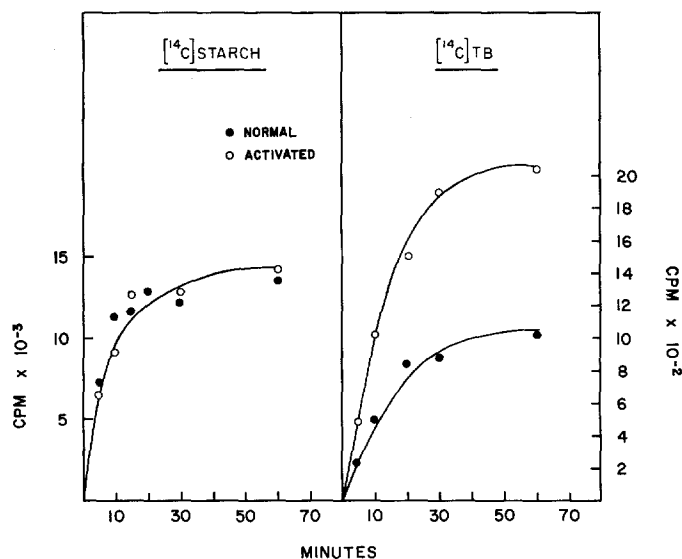


FIG. 2. Time-course of phagocytosis by normal and activated macrophages. Results are expressed as counts per minute per milligram of protein. Specific activity of starch: 2,200 cpm/mg of starch. Specific activity of tubercle bacilli: 1,385 cpm/mg of tubercle bacilli (TB). Note: data shown are from one representative experiment that was confirmed.

servation. Thus both the rate of uptake and capacity for dead tubercle bacilli were increased in the activated cells compared with normal cells.

DISCUSSION

Although the activated macrophages have increased metabolic and functional activity, there was no statistically valid increase in protein or DNA content indicating that the enhanced "spreading" of these cells seen microscopically is not due to an increase in cell mass.

Macrophages activated *in vivo* show increases in metabolic activity (as indicated by increases in glucose oxidation) and of phagocytic activity as did cells activated *in vitro* (2). However, with respect to resting cells especially, a dissimilarity was noted, i.e., the oxidation of [1- ^{14}C]glucose to $^{14}\text{CO}_2$ was increased only about 50% in activated mouse macrophages, while there was a fourfold increase in the case of guinea pig macrophages activated *in vitro*. Further, mouse macrophages appeared to oxidize glucose carbon-6 to $^{14}\text{CO}_2$ more effectively than guinea pig macrophages.

After these experiments were completed, a paper by Ratzan et al. (12) appeared in which findings were reported on the oxidation of carbon-1 of glucose, increased resistance to infection, and inhibition of bacterial growth in macrophages activated *in vivo*. The activated macrophages used by these investiga-

tors produced about 60% more $^{14}\text{CO}_2$ at rest from $[1\text{-}^{14}\text{C}]$ glucose than did normal macrophages and two to five times more $^{14}\text{CO}_2$ during phagocytosis. Our data with this labeled sugar are similar. In addition our studies showed that activation of mouse macrophages caused notable (about threefold) increases in the oxidation of both $[3,4\text{-}^{14}\text{C}]$ - and $[6\text{-}^{14}\text{C}]$ glucose during phagocytosis, as compared with normal cells. This is true also of the increment with $[\text{U-}^{14}\text{C}]$ glucose, when one considers the absolute magnitude of $^{14}\text{CO}_2$ production in that case.

In general, it would seem that during phagocytosis the cells activated in vivo exhibited a much greater stimulation of the tricarboxylic acid cycle during phagocytosis than their controls, as well as a greater stimulation of the hexose monophosphate shunt pathway.

It is of interest that the activated macrophages phagocytized the tubercle bacilli at a greater rate compared with normal cells, while this was not so with the starch particles. Ratzan et al. (12) determined the percentage of mouse macrophages that ingested three or more live *Listeria* in vitro. They observed that macrophages activated in vivo with *Listeria* or BCG were about three times more effective than controls. The method for determining phagocytosis was different from that used here and detected the proportion of cells able to phagocytize under the conditions used rather than determining rates and capacities (7).

Nathan et al. (2) found that the rate and extent of phagocytosis of dead tubercle bacilli particles were greatly enhanced in macrophages activated in vitro by an MIF-rich fraction. The rate and extent of phagocytosis of starch were only slightly increased (ca. 20 and 10%, respectively), but this was statistically significant (2). Our observations with macrophages activated in vivo are indeed rather similar. It should be noted that whereas the MIF system in vitro employed guinea pig macrophages elicited with caseinate, in the current study we used mouse peritoneal macrophages obtained without elicitation. In addition to the species difference, account should probably also be taken of the maturity of the cells.

In our experiments comparisons were made between cells obtained from mice injected with live *Listeria monocytogenes* and cells obtained from normal untreated mice. Control experiments were also done in which mice were injected with 1.5×10^4 heat-killed *L. monocytogenes* (the same number as used in the experiments with living bacteria) and 5×10^7 dead organisms (the number to which it is estimated the *Listeria* would increase during the first few days of infection). In both sets of animals the macrophages obtained after 7 days were found to have phagocytic and metabolic activity similar to, or slightly lower than, the normal macrophages from untreated mice. Thus, *Listeria* must be living in order to bring about the enhancement of activity we have observed, consistent with the requirement stipulated for activation in terms of cellular immunity (3).

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