

**AN AUTOMATED SYSTEM FOR MEASUREMENT OF LEUKOCYTE METABOLISM**

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***The hexose monophosphate shunt by which 1-<sup>14</sup>C-glucose is converted to <sup>14</sup>CO<sub>2</sub> is stimulated by phagocytosis. We have developed and evaluated an automated system which permits quantification of this phenomenon in 25 samples sequentially. Polymorphonuclear leukocytes were suspended in a balanced salt solution containing 1-<sup>14</sup>C-glucose and placed in a sterile sampling vial. Incubation at 37°C was terminated by citric acid addition at 90 min. Sampling was performed automatically in an instrument which aspirates the atmosphere of each vial into an ion chamber for measurement. Siliconization of the sample vials greatly reduced the basal metabolism of the white cell suspensions. Terminating the incubation with citric acid increased <sup>14</sup>CO<sub>2</sub> production by about 50%. Agitation during incubation was found to be of no value. There was a direct relationship between <sup>14</sup>CO<sub>2</sub> production and both the number of leukocytes and the log of the antigen concentration. During phagocytosis, there was a peak in <sup>14</sup>CO<sub>2</sub> production about 90 min after the addition of particles to the white cell suspension.***

In 1969, we proposed a system for the automated detection of bacterial growth based on measurement of the conversion of <sup>14</sup>C-labeled substrates to <sup>14</sup>CO<sub>2</sub> (1). This principle can also be used to measure the metabolism of polymorphonuclear leukocytes. Phagocytosis of solid particles by polymorphonuclear leukocytes (PMNs) is accompanied by stimulation of oxygen consumption, H<sub>2</sub>O<sub>2</sub> production, and production of <sup>14</sup>CO<sub>2</sub> from 1-<sup>14</sup>C-glucose via the hexose monophosphate shunt (HMP) (2). These metabolic events associated with polymorphonuclear leukocyte metabolism have been found to be abnormal in certain immune disorders. In Chediak-Higashi disease, in which phagocytosis occurs but subsequent death of the phagocytized organism does not follow, <sup>14</sup>CO<sub>2</sub>

production from <sup>14</sup>C-glucose is increased when the leukocytes are observed in the absence of particles and rises to greater than normal levels after phagocytosis. In chronic granulomatous disease of children, phagocytosis occurs but subsequent <sup>14</sup>CO<sub>2</sub> release is impaired greatly.

**MATERIALS AND METHODS**

**Preparation of leukocytes.** Sixty to 100 ml of dog's blood was obtained by venesection and allowed to sediment 1 hr in 50 ml of 0.1 M EDTA and 6% Dextran in saline. The white cell-rich plasma was then pipetted into a plastic centrifuge tube and centrifuged at 1,500 rpm for 8 min at 4°C. After decanting the supernatant, the remaining red cells were lysed by adding 2 ml of distilled water, followed in 20 sec by 2 ml double-strength Hank's balanced salt solution (HBSS), followed immediately by 10 ml HBSS modified by 10 mg glucose/100 ml (MHBSS). This mixture was then recentrifuged at 800 rpm for 10 min at 4°C. After decanting the supernatant, the cells were resuspended in 27 ml MHBSS. Each milliliter of the resulting solution contained from  $4.3 \times 10^6$  to  $30.0 \times 10^6$  WBC. The differential counts ranged from 83 PMNs and 17 lymphs to 54 PMNs, 35 lymphs, and 11 other granulocytes. By the trypan blue exclusion test, more than 85% of the leukocytes were alive after 6 hr at room temperature under unstimulated conditions.

**Particulate antigens and antibody.** Phenol-killed salmonella O types A, C, and E (BioQuest of Becton-Dickinson) and 1-micron diam polystyrene latex particles from Dow Diagnostics were used as antigens. Various concentrations from 10<sup>5</sup> to 10<sup>8</sup> bacteria/ml were obtained by dilution in MHBSS. Rabbit antisera hyperimmune to the specific salmonella antigens were obtained commercially from BioQuest

Received Oct. 10, 1973; original accepted Nov. 21, 1973.  
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and diluted to various concentrations in MHBSS from 1:10,000 to 1:10.

**Incubation bottles.** Glass incubation bottles (50-ml size) were rinsed with distilled water, heat dried at about 200°C for 2 hr, and siliconized by a 10-sec rinse with a 1% solution of Siliclad in distilled water, followed by repeated rinsing with distilled water.

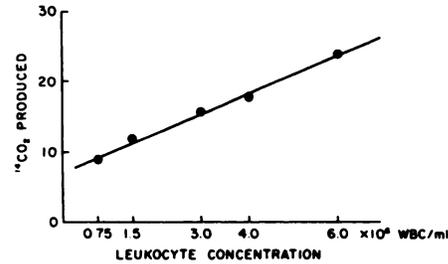
**Incubation and detection of phagocytosis.** Each incubation bottle contained 5 ml of total mixture: 1 ml of WBC suspension, 1 ml of approximate Ag suspension, 1 ml of appropriate Ab solution, 1.8 ml of MHBSS, and 1.0 mCi of  $1\text{-}^{14}\text{C}$ -glucose supplied by Amersham/Searle (57 mCi/mM.) After incubation at 37°C for 1.5 hr, the incubation was stopped by the addition of 1 ml of 62.5% citric acid which killed the white cells and lowered the media pH to about 2, thus liberating virtually all the metabolized  $^{14}\text{CO}_2$  from the buffered media into the atmospheres in the bottles. After an equilibration period of 30 min, the  $^{14}\text{CO}_2$  in the bottles was determined using the automated system (Bactec 225, Johnston Laboratories, Cockeysville, Md.) which samples up to 25 bottles sequentially. The details of the operation of this system have been published previously (3).

**Microscope slides.** Incubation mixtures prepared separately in plastic test tubes but of the same ratio of components as the incubation bottles were observed for clumping of Ag by Ab and for evidence of phagocytosis using Wright-stained smears. A phagocytic index was calculated in units of bacteria per cell by counting the number of bacteria seen to be superimposed upon the white cells totally within the borders of the cell membrane. Twenty-five consecutive cells were counted in each slide.

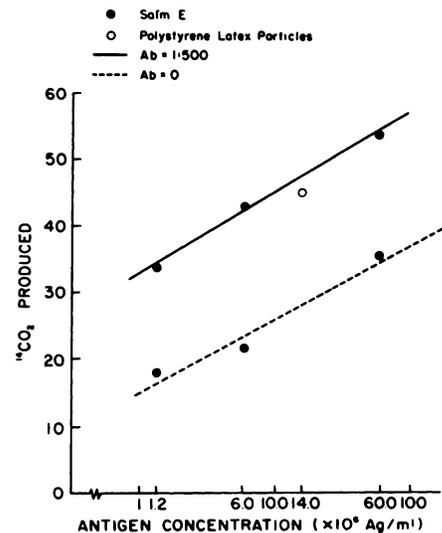
## RESULTS

**Leukocyte number.** With a constant number of salmonella organisms ( $6 \times 10^7$ ) and a constant salmonella antisera dilution (1:500), there was a direct linear relationship between the number of leukocytes and the amount of  $^{14}\text{CO}_2$  liberated, over the range of  $0.75 \times 10^6$  WBC/ml to  $6.0 \times 10^6$  WBC/ml (Fig. 1).

**Antigen concentration.** There was a nearly linear relationship between the LOG of the antigen concentration and amount of  $^{14}\text{CO}_2$  liberated (Fig. 2). It can be seen that the presence of antibody at a dilution of 1:500 increased the quantity of  $^{14}\text{CO}_2$  produced, but the relationship to antigen concentration remained linear. The minimal antigen concentration easily detectable was  $1.2 \times 10^6$  Ag/ml; for salmonella A and C, detectable effects were noted with  $2.5 \times 10^6$  Ag/ml and  $0.6 \times 10^6$  Ag/ml, respec-



**FIG. 1.** Effect of leukocyte concentration on amount of  $^{14}\text{CO}_2$  produced at constant volume. Leukocytes incubated for 1 hr with Salmonella O type C antigen =  $6.0 \times 10^7$ /ml and antibody = 1:500.



**FIG. 2.** Effect of antigen concentration on leukocyte metabolism. Leukocytes incubated with salmonella E or polystyrene latex particles, with antibody = 1:500 or 0.

tively. Addition of polystyrene latex particles (PSP) at a concentration of  $14.0 \times 10^6$  PSP/ml resulted in a release of  $^{14}\text{CO}_2$  comparable to that released by the corresponding concentration of opsonized salmonella E particles.

**Incubation time.** For all antigens tested,  $^{14}\text{CO}_2$  release was first detected after 1 hr of incubation ( $\frac{1}{2}$  hr samples were equal to controls) and peaked sharply in the second hour while declining after 3 hr (Fig. 3). Figure 3B shows the earlier time course for the interiorization and disappearance of the particles as observed microscopically.

**Effect of siliconization.** In Fig. 4 it can be seen that siliconization of the incubation glassware resulted in lower values of  $^{14}\text{CO}_2$  production by resting leukocytes. Without siliconization, PMNs respired if stimulated even though the media contained MHBSS but no particles.

**Termination with citric acid.** One milliliter of 62.5% citric acid was used to terminate phagocytosis and release  $^{14}\text{CO}_2$  from the media. In general, it was

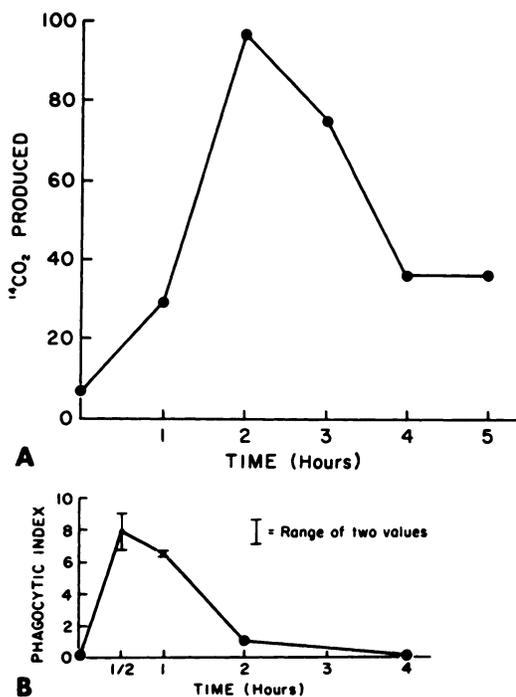
found that this amount of citric acid liberated two to three times as much  $^{14}\text{CO}_2$  from matched incubation samples with all three types of salmonella (A, C, and E). There was a tenfold increase in sensitivity to Ab concentration noted at low Ab concentrations when citric acid was used. Without citric acid, Ab diluted to 1:1,000 was the lowest concentration distinguishable from controls. With citric acid, Ab diluted to 1:10,000 could clearly be distinguished from controls.

The amount of citric acid added (0.5–5.0 ml) made little difference (12%) in the amount of  $^{14}\text{CO}_2$  produced. The time allowed for equilibration after the addition of citric acid (5–60 min) affected the amount of  $^{14}\text{CO}_2$  released. The longer times yielded 50–100% higher values than the 5 min readings.

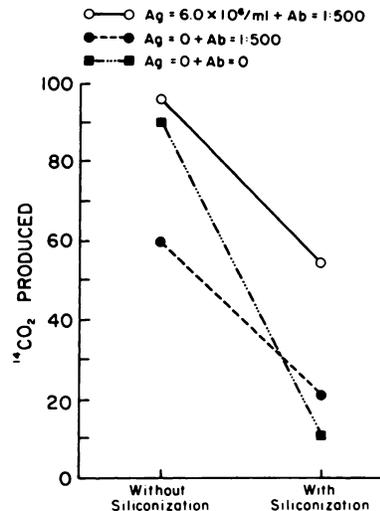
**Agitation.** Samples incubated with occasional stirring (two to three times) at 30°C before the addition of citric acid released as much or slightly more  $^{14}\text{CO}_2$  than the samples incubated with constant agitation at about 100 cycles/min in a water bath at 30°C.

DISCUSSION

The relationship between leukocyte concentration and  $^{14}\text{CO}_2$  produced corroborates previous studies by Skeel (4). However, we observed a linear relationship between the LOG of the Ag concentration



**FIG. 3.** (A) Time course of phagocytosis-associated metabolism. Leukocytes incubated with Salmonella E antigen =  $6.0 \times 10^7/\text{ml}$  and antibody = 1:200. (B) Time course of interiorization seen by microscope. Leukocytes incubated with Salmonella E antigen =  $6.0 \times 10^7/\text{ml}$  and antibody = 1:200.



**FIG. 4.** Effect of siliconization of incubation bottles on leukocyte metabolism. Leukocytes incubated with Salmonella E antigen and antibody for 2 hr.

and  $^{14}\text{CO}_2$  released rather than a simple linear relationship (4). Polystyrene particles stimulated  $^{14}\text{CO}_2$  release to the same degree as the opsonized salmonella E bacteria. The minimum number of particles that produced a detectable effect was  $1.2 \times 10^6$ .

Most particles were engulfed by leukocytes 15–30 min after exposure while the associated HMPS activity occurred later, starting at 1 hr and peaking sharply in the next hour. These observations are similar to those of Malawista (5). Metabolic observation must be performed after 1–3 hr of incubation.

The data in Fig. 4 clearly indicate a need to siliconize all incubation glassware. Without siliconization, there is little difference between a resting cell and a stimulated cell. Both are producing near maximal levels of  $^{14}\text{CO}_2$ . With siliconization, the cells stimulated by particulate Ag continue to metabolize via the HMPS whereas control cells do not. PMNs stick to bare glass but not to siliconized glass. It seems likely that the act of sticking to glass invokes the same cell membrane phenomenon responsible for triggering the HMPS burst in phagocytosis. Rossi (6) has shown that ruffling of the PMN cell membrane by antileukocyte antibody alone in the absence of phagocytosis stimulates the HMPS. Without siliconization, we observed that the presence of Ab alone reduced basal metabolism. It seems possible that the protein coated the bare glass and inhibited sticking. In any event, it is clear that to obtain accurate data, the leukocytes must be protected from all active surfaces.

The experiments on the citric acid support the conclusion that terminating incubation with 1 ml of 62.5% citric acid is worthwhile. In this and other

tests, citric acid treatment doubled to tripled the  $^{14}\text{CO}_2$  values and resolved the areas of minimal stimulation from controls so that sensitivity was roughly ten times greater for detecting Ab concentrations.

Previous investigators have reported that agitation or even tumbling of the leukocytes in test media is necessary for the act of phagocytosis (5,7,8). Our data show that in the automated system occasional stirring (two to three times) at 37°C before the addition of citric acid is better than constant agitation at 100 oscillations/min at 37°C.

#### ACKNOWLEDGMENTS

We want to thank Marianne Chen and Michele Melville for their technical assistance during the course of these studies.

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