THE FATE OF BACTERIA WITHIN PHAGOCYTIC CELLS

III. Destruction of an Escherichia coli Agglutinogen within Polymorphonuclear Leucocytes and Macrophages*

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The injection of particulate bacterial antigens into a vertebrate host results in the formation of specific globulins directed against various immunogenic components of the organism. One of the first stages of this process is thought to be the capture of the particle by phagocytes of the blood and reticuloendothelial system. These cells which presumably do not produce antibody may degrade the immunogen, possibly releasing units which then interact with other lymphoid cells. Although the ability of phagocytic cells to kill intracellular bacteria is widely recognized, little information is available concerning their ability to alter immunogens of these organisms. Since the phagocytes constitute a wide array of cell types which differ morphologically and biochemically it seemed possible that they might also differ in degradative potential.

This report will deal with the differential destruction of an *Escherichia coli* agglutionogen by three distinct populations of phagocytic cells. A two stage system has been employed in which relatively homogeneous suspensions of rabbit polymorphonuclear (PMN) leucocytes, peritoneal macrophages, and BCG-induced alveolar macrophages were interacted with bacteria *in vitro*. After various periods of intracellular residence, the residual immunogen was assayed by its ability to stimulate agglutinin formation in mice.

A preliminary report of these studies has appeared elsewhere (1).

Materials and Methods

Bacteria.—The majority of experiments were performed with a non-motile, smooth strain of *Escherichia coli* K-12. This organism was maintained on penassay agar slants and passed at intervals of 3 weeks. At the time of an experiment bacteria were grown in penassay broth for 16 hours, collected by centrifugation, washed twice with pyrogen-free saline, and resuspended in the same medium to the appropriate concentration. When heat-killed organisms were required, aliquots of the viable washed suspension were heated in a water bath (60–100°C) and washed twice with saline. A limited number of experiments were performed with laboratory strains of *Bacillus subtilis*, *Staphylococcus albus*, and *Salmonella typhimurium*-RIA

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under the same cultural conditions. The inoculum employed for subsequent experiments was standardized by viable plate counts and nephelometry.

Animals.—Rabbits, weighing 3 to 4 kg, were employed as the source of phagocytic cells.

Mice were from the NCS Swiss strain maintained at The Rockefeller Institute. These animals weighing 25 gm contained few coliform organisms as part of their intestinal flora and were free from other specific pathogens and ectoparasites. These mice gave more uniform antibody titers when compared to other laboratory strains and were never found to contain agglutinating antibody to *E. coli* K-12 under the conditions employed.

Phagocytic Cells.—

Polymorphonuclear leucocytes: These were obtained from glycogen-induced peritoneal exudates (2) and contained less than 2 per cent of other cell types.

Peritoneal macrophages: Mineral oil–induced peritoneal macrophages were obtained by a previously described method (3).

BCG-induced alveolar macrophages: These cells were obtained in large numbers by a modification of the technique of Myrvik *et al.* (4) as described previously (3). Rabbits were injected intravenously with 20 mg of dead BCG on two successive days and their lung macrophages harvested 3.5 to 4.5 weeks after injection. The preparations used for this study contained on the average 85 per cent large macrophages, 5 to 15 per cent smaller mononuclear cells, and 2 to 10 per cent polymorphonuclear leucocytes. The phagocytic activity and biochemical properties of these cells have been reported (3).

Sera.—

Rabbit immune sera: Adult New Zealand rabbits were immunized three times per week for a total of 5 injections. *E. coli* K-12 antigen (60°C/30 minutes) was suspended to an OD 650 mμ of 0.3 and 0.5 ml given in the marginal ear vein. Seven days after the last injection the animals were bled by cardiac puncture.

Mouse sera: Mice which had been injected with *E. coli* alone or *E. coli*–phagocyte suspensions were bled as a routine on the 10th day. Following light ether anesthesia cardiac blood was removed by means of a Pasteur pipette, allowed to clot, and stored at 4°C for clot retraction. Serum separated under these conditions was then stored at −20°C until assay. In most instances single sera were obtained whereas pools of 2 to 4 animals were occasionally collected.

Preparation of Cells and Bacteria for in Vitro Interactions.—The three types of rabbit phagocytes were collected by centrifugation at 500 RPM (International, type 1) for 5 minutes, washed once with modified Hanks' solution at ph 7.5, counted in a hemocytometer, and resuspended to a concentration of 35 to 40×10^6/ml in a medium composed of Hanks' solution, 100 mg per cent glucose, and 15 per cent fresh rabbit serum. The cells were then dispensed to 13 × 100 mm glass tubes. *E. coli* K-12, suspended in saline, was then added to give a final stock dilution of 1/10. The total volume of the incubation tubes was 5.0 to 6.0 ml, 9 parts leucocyte suspension, 1 part *E. coli*, and the final concentration of cells and serum had been reduced by 10 per cent. The tubes were closed with white rubber stoppers and incubated at 37°C on an end over end rotator at 8 RPM (New Brunswick Scientific Company, New Brunswick, New Jersey, model ST). At intervals from T_0 (immediately after addition of *E. coli*) to T_{300} minutes, aliquots were removed, placed in an ice bath, and 1.0 ml injected intraperitoneally into mice. On occasion, the leucocytes were disrupted either by six cycles of freezing and thawing in a dry ice–alcohol bath or by homogenization with a motor driven teflon pestle prior to mouse inoculation. This procedure did not alter subsequent agglutinin titers. Injection of leucocytes alone never resulted in detectable antibody to *E. coli*.

Mouse Assay for Agglutinogen.—Aliquots of either leucocytes, *E. coli* alone, or *E. coli*–leucocyte suspensions, in a volume of 1.0 ml, were injected intraperitoneally into 25-gm NCS mice. Neither the bacteria, either viable or heat-killed, nor the leucocytes produced any gross toxic manifestations in the animals. Sera obtained by cardiac puncture were obtained

as a routine 10 days after intraperitoneal antigen although antibody production curves have been performed from the 5th to the 20th day.

Agglutination tests were performed on individual sera according to the following procedure: twofold dilutions of sera were prepared in saline in perspex depression trays. *E. coli* antigen was prepared by heating a washed 18 hour culture to 70–85°C/40 minutes. This was washed twice with saline and resuspended to an OD 450 mμ of 0.3 in a Junior Coleman spectrophotometer. Antigen was employed for no more than 3 days after harvesting and was always washed and calibrated before use. The antigen, in 0.1 ml aliquots, was then added to serial dilutions of serum contained in a similar volume. After thorough mixing the trays were covered with plastic, incubated at 40°C for 2 hours, and then overnight at 4°C. The last dilution demonstrating macroscopic agglutination was then determined.

Determination of in Vitro and in Vivo Ingestion Kinetics.—The procedures employed for the *in vitro* assay of the kinetics of particle ingestion and intracellular bactericidal activity have been reported in previous publications (2). Viable suspensions of *E. coli* K-12 were mixed with the three types of phagocytes and subsequently sampled for both total and extracellular bacterial populations up to 120 minutes of incubation. The concentration of phagocytes and serum was identical with that previously described for *in vitro* interactions. The number of bacteria was maintained to give the same multiplicity as in the immunogenicity experiments.

The *in vivo* fate of *E. coli* K-12 in the mouse peritoneal cavity as well as the cellular response to this form of challenge were studied by techniques previously employed for staphylococci (5). Data were obtained on the total and extracellular bacterial population as well as on the total white blood cell and total PMN counts recoverable from the peritoneum for 5 hours after the inoculation of 10^8 *E. coli* K-12.

RESULTS

General Consideration.—Prior to an examination of the intraphagocytic fate of a bacterial immunogen a system was required which had the following prerequisites: (*a*) a dose-response relationship with an adequate slope, (*b*) efficient *in vitro* phagocytosis in the presence of normal serum, (*c*) potent immunogenicity, (*d*) low virulence, and (*e*) the availability of large numbers of relatively homogeneous phagocytes. Because of the possible presence of antibody-producing cells in the phagocyte populations a heterologous recipient, *i.e.* the mouse, was selected so that rapid rejection of such cells would occur.

Initial studies employing strains of *E. coli*, *S. typhimurium*, *B. subtilis*, and *S. albus* were conducted with the previous requirements in mind. Each of these organisms had been employed previously in the study of the degradation of bacterial macromolecules (6). None of the Gram-positive bacteria were of sufficient immunogenicity to be employed for the study. On the average, 10 to 100 times more bacteria were required to give a detectable agglutinin response than with Gram-negative bacilli, and this number was too large to use for efficient *in vitro* phagocytosis. Of the two Gram-negative bacilli, *E. coli* was selected because its immunogenicity could be studied over a wider range of doses. All subsequent experiments to be presented were conducted with this organism.

Fig. 1 illustrates the dose-response titration of *E. coli* K-12 after intraperitoneal injection. Each point represents the mean of 18 to 22 individual sera. In

all subsequent experiments 10^8 *E. coli* per ml of phagocytic cells was employed. This represented a multiplicity of 2 to 3 bacteria/leucocyte. The use of heat-inactivated bacteria (70°C/30 minutes) resulted in identical agglutinin titers.

The in Vitro and in Vivo Fate of E. coli.—Before comparing the influence of the three phagocyte populations on the *E. coli* agglutinogen it was neccesary to

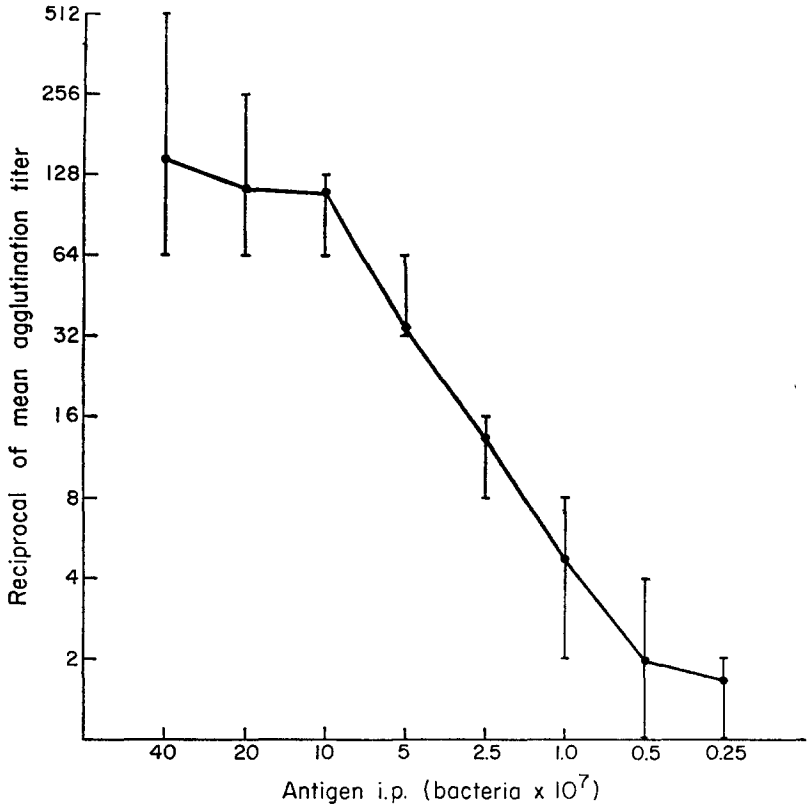

FIG. 1. Dose-response relationship between intraperitoneally administered immunogen and agglutination titer.

evaluate the rate and efficiency of phagocytosis in the *in vitro* system. Fig. 2 illustrates the results of one such experiment. Rapid phagocytosis and intracellular inactivation occurred with each cell type and within 30 minutes 95 per cent or more of the inoculum had been ingested and rendered non-viable. Therefore, any significant difference in the temporal fate of the agglutinogen was not likely to be related to the kinetics of the *in vitro* ingestion process.

The fate of the same organism was also studied within the mouse peritoneal cavity. Fig. 3 shows that *E. coli* was again rapidly ingested and killed under these circumstances. During the 1st hour when the vast majority of organisms

was ingested, the bacilli were exposed to an almost homogeneous population of mononuclear phagocytes. Only at later stages did singificant numbers of granulocytes enter the cavity. Examination of the spleen, liver, and kidneys of these animals never indicated bacterial multiplication. This finding as well as the similar agglutinin titer obtained with dead bacteria indicated that the injection

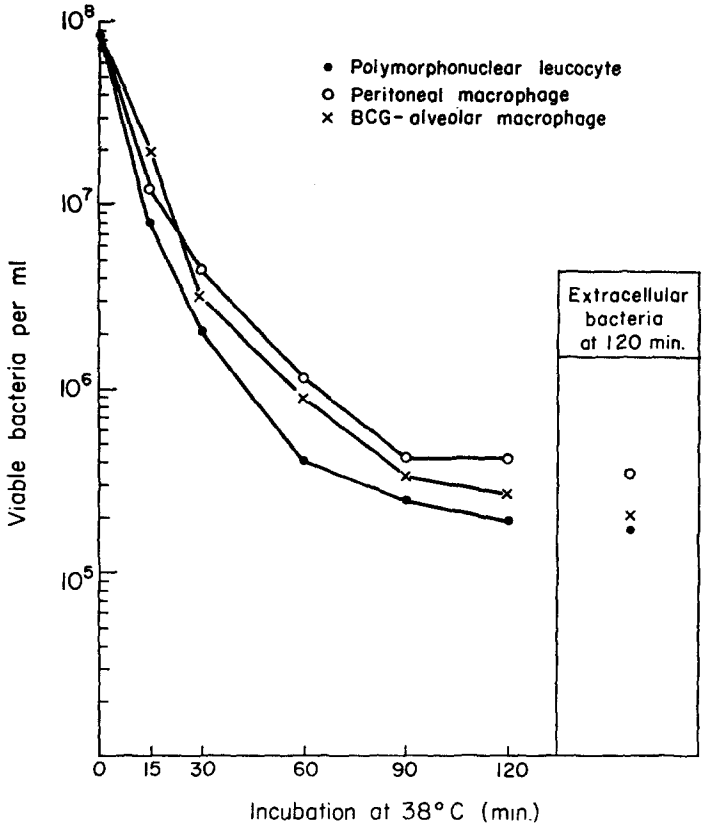

FIG. 2. The *in vitro* interactions between *E. coli* K-12 and three types ra ofbbit phagocytes.

of live bacteria was not associated with a significant increase in immunogenic mass.

Nature of Agglutinogen.—A number of experiments were performed to characterize the gross nature of the agglutinogen under study.

Rabbit and mouse antisera were prepared against (*a*) viable *E. coli*, (*b*) bacteria heated to 80°C/40 minutes, and (*c*) organisms heated to 100°C/120 minutes. Each of these antisera was then tested against the following antigens for agglutination in block titrations: (*a*) viable washed bacteria, (*b*) bacteria heated to 60° and 80°C for 40 minutes, and (*c*) bacteria boiled for 2 hours. After heating the organisms they were washed twice with saline.

Each of the rabbit and mouse antisera demonstrated the same agglutination end point when tested with either viable or heated bacteria. This indicated that the agglutinogen was heat-stable and a somatic component.

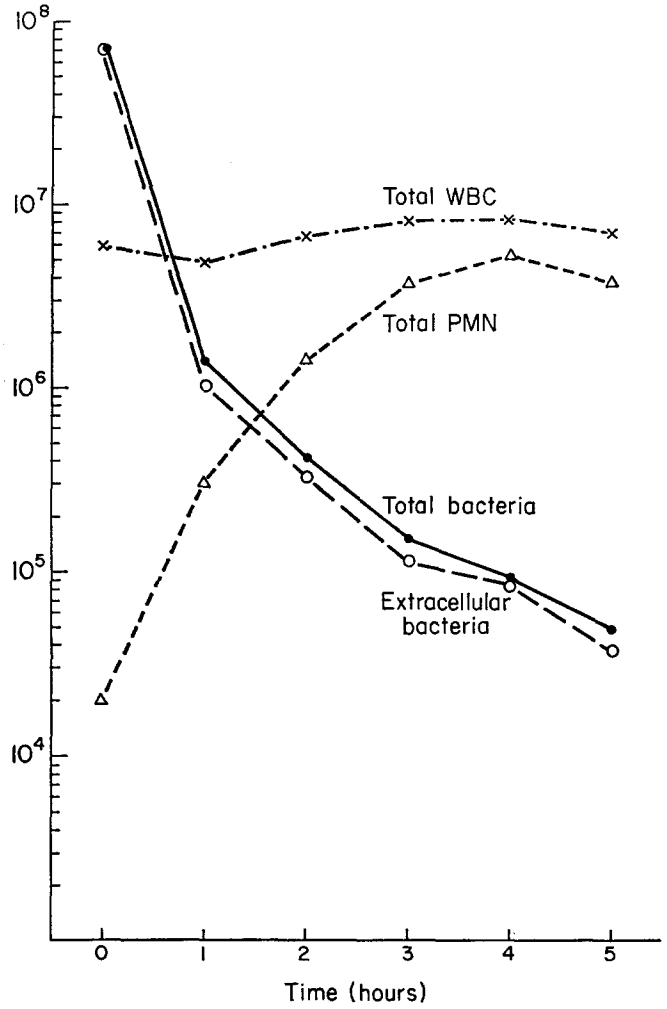

FIG. 3. The *in vivo* fate and cellular response of *E. coli* K-12 in the mouse peritoneum.

The Fate of Agglutinogen within Three Types of Rabbit Phagocytes.—Fig. 4 represents the composite results with three distinct phagocyte populations from the rabbit. Each point on the graph is the mean titer of 15 to 20 individual sera. These sera were obtained from mice which had been injected 10 to 11 days previously with *E. coli*–phagocyte suspensions. The results with PMN leucocytes are shown on the left. At zero time, prior to phagocytosis but im-

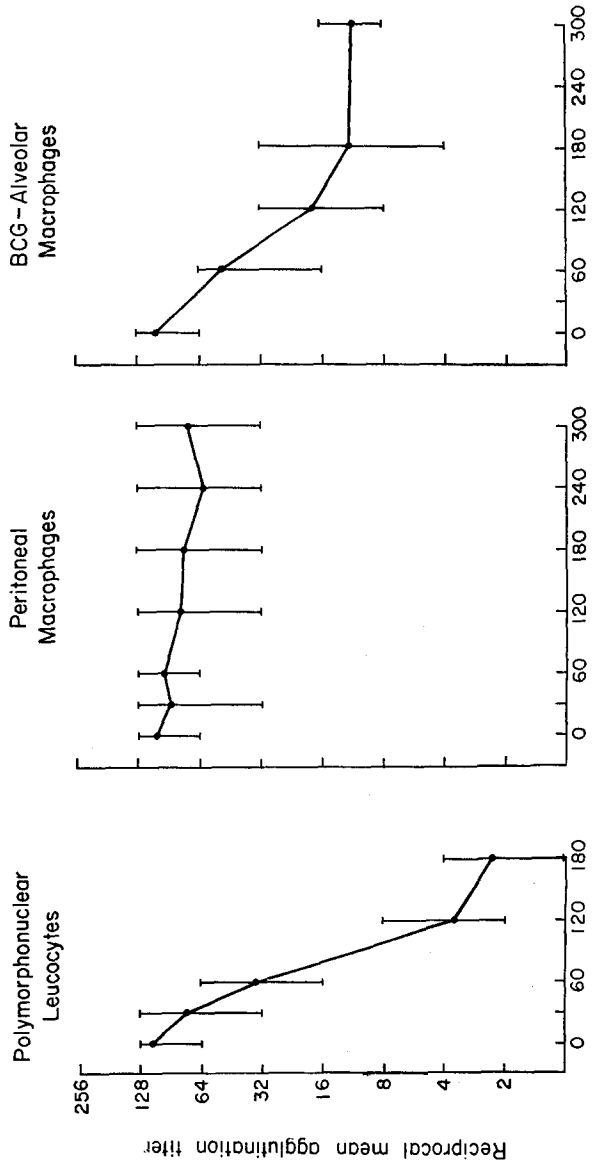

FIG. 4. The fate of an *E. coli* agglutinogen within rabbit polymorphonuclear leucocytes, oil-induced peritoneal macrophages, and BCG-induced alveolar macrophages after *in vitro* incubation.

mediately after mixing *E. coli* with the cells, the resulting agglutinin titer was the same as that obtained after the injection of *E. coli* alone. Following phagocytosis and the subsequent intracellular events there was a marked reduction in the immunogenicity of the suspension. During the first 30 minutes there was little change in the agglutinogen. Thereafter a progressive loss took place so that at 120 minutes the agglutinin titer of mouse serum was less than 1:4. This corresponded to a loss in immunogenic mass of more than 90 per cent when compared to the dose-response relationship in Fig. 1.

In contrast, the events which occurred in peritoneal macrophage suspensions were quite different. Even though phagocytosis and killing took place at the

TABLE I

The Influence of Immune Serum on the Fate of an E. coli Agglutinogen within PMN Leucocyte and Peritoneal Macrophages

Phagocyte	Rabbit serum	Immunogen	Incubation time	Reciprocal of agglutination titer*
			min.	
—	Normal	*E. coli*, viable	120	115
—	Immune	" "	120	92
PMN	Normal	" "	120	6
"	Immune	" "	120	2
Peritoneal macrophage	Normal	" "	120	96
" "	Immune	" "	120	84
" "	Normal	*E. coli*, 80°C/30 min.	120	92
" "	Immune	" "	120	76

* Mean of 10 to 16 individually assayed mouse sera.

same rate, little change occurred in the immunogenicity of the bacteria. There was, however, a slight reduction in mean titer after 5 hours of intracellular residence.

At this point in the investigation it seems clear that there were major differences in the disposition of the immunogen within the two types of phagocytes. Whether this effect would apply to other types of macrophages was not known. To examine this point additional experiments were conducted using the BCG-induced alveolar macrophage. As shown on the right side of Fig. 4 these cells inactivated the agglutinogen to a much greater extent than the peritoneal macrophage but less efficiently than the PMN leucocyte. After 180 minutes of intracellular residence approximately 75 per cent of the immunogen had been destroyed and no further reduction occurred during the subsequent 2 hours.

In view of the relative inability of peritoneal macrophages to alter the *E. coli* agglutinogen it was of interest to examine the interaction in the presence of specific immune serum and with heat-killed bacilli. Table I presents such a study with both PMN leucocytes and peritoneal macrophages. When granu-

locytes were used, there appeared to be a slightly greater destruction of immunogen in the presence of immune serum. This, however, may be related to the slight effect of immune serum alone when incubated with bacteria for 2 hours *in vitro*. It should be pointed out that this strain of *E. coli* is ingested as readily in normal rabbit serum as in immune serum (7). The effect of immune serum in the presence of macrophages was essentially the same. This was the case when either viable or heat-killed bacteria were employed.

The Formation and Nature of Mouse Agglutinins to E. coli.—Although not previously discussed in detail, it seemed possible that one of the effects of residence within the phagocytes might relate to a delayed agglutinin response in the mouse. This necessitated a study of the temporal sequence of agglutinin formation after the inoculation of *E. coli* alone and in the presence of leucocytes. Such an experiment is illustrated in Fig. 5. Following the injection of immunogen alone, peak titers were reached at approximately 10 days (untreated serum) and fell off slightly at 17 days. A similar result was obtained with the residual immunogen, remaining after 60 minutes of residence within the PMN leucocytes. The shapes of both antibody production curves were comparable although the absolute titer was reduced after leucocytic action. The same result was obtained with the alveolar macrophage but is not presented.

Another factor which could conceivably be altered after intraleucocytic residence was the type of antibody formed in the mouse. The open circles show the titers of the same sera after treatment with 2-mercaptoethanol (2-M.E.) (8). This agent presumably inactivated the high molecular weight antibody present in the samples. It is apparent that considerable 2-M.E.-sensitive antibody is initially present in both cases. The relative concentration of sensitive and insensitive antibody is then reversed and at 14 days the majority is insensitive and presumably 7S in character.

From these results it appeared that intraleucocytic residence did not alter either the temporal sequence of antibody formation in the mouse or the nature of the antibody produced.

Intracellular Localization of the Residual E. coli Agglutinogen.—It was next of interest to examine the subcellular fractions from phagocyting leucocytes in order to determine the localization and possible solubilization of the agglutinogen.

Two experiments employing PMN leucocytes and alveolar macrophages are presented in Fig. 6.

Viable *E. coli* was mixed with the phagocytes in the usual reaction mixture containing 10 per cent fresh normal rabbit serum. Immediately after addition, and after 60 and 120 minutes' intracellular residence, aliquots were chilled in an ice bath, centrifuged at 800 RPM, and washed once with 0.25 M sucrose. All subsequent steps were performed at 0–4°C. The cells were then suspended in sucrose, dispersed to chilled tubes, and homogenized with a motor-driven teflon pestle until 85 to 95 per cent of the cells were disrupted. Aliquots of the total homogenate (*H*) were retained and the remainder of the homogenate fractionated by

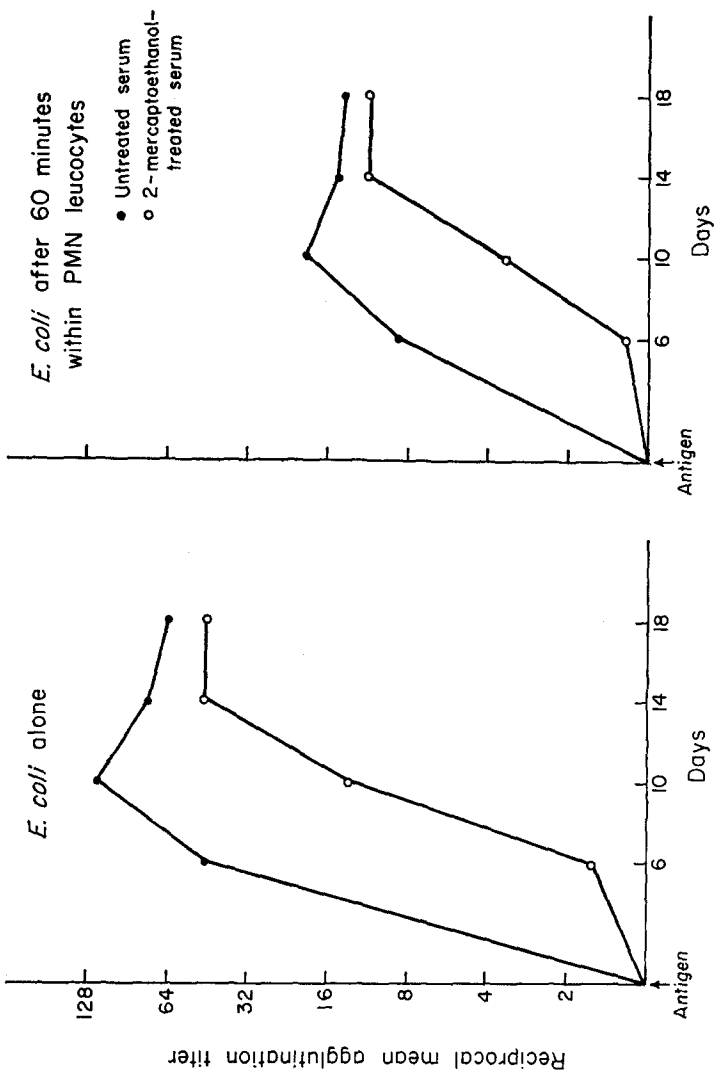

FIG. 5. The influence of *in vitro* intraphagocytic residence on the temporal sequence and mercaptoethanol sensitivity of mouse agglutinins.

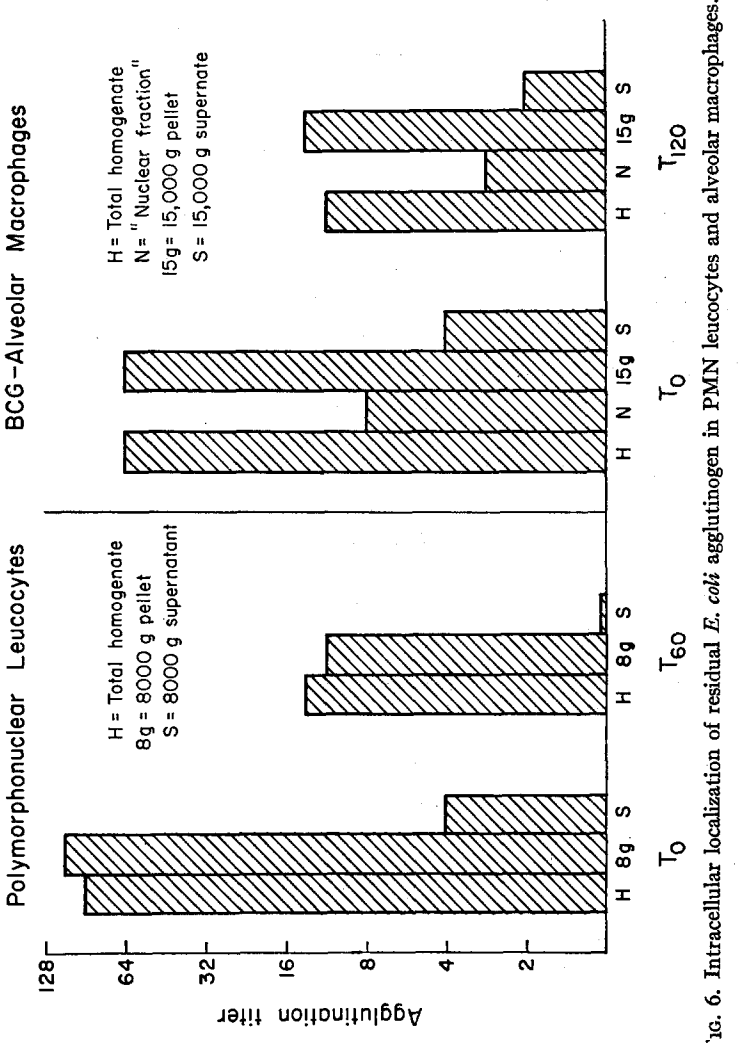

FIG. 6. Intracellular localization of residual *E. coli* agglutinogen in PMN leucocytes and alveolar macrophages.

differential centrifugation according to previously described procedures (9). All fractions were taken up in isotonic sucrose to comparable volumes and 1.0 ml aliquots were injected intraperitoneally into mice. Animals were bled at 10 days and individual sera tested for agglutinins.

Control experiments were performed in which subcellular fractions were obtained from non-phagocyting cells, then mixed with a standard dose of *E. coli* and injected into mice. These revealed that none of the fractions had either a potentiating or depressing effect on antibody formation and resulted in identical titers when compared to *E. coli* alone.

The values obtained at zero time were internal controls, prior to phagocytosis, and measured the centrifugal distribution of *E. coli* in the presence of the leucocyte homogenate. The major portion of the activity sedimented in the 8000 *g* and 15,000 *g* fractions from PMN leucocytes and alveolar macrophages respectively, and equalled the immunogenicity of the total homogenate. This localization would be expected from merely sedimenting the organism in 0.25 M sucrose. After 60 minutes within the PMN leucocyte the immunogenicity of the total homogenate had been reduced and essentially all the activity resided in the high-speed pellet. The same was true for the alveolar macrophage fractions. In both cases little or no activity was present in the soluble phase of the cell cytoplasm.

Since it was possible that a solubilized immunogen was in some way enclosed within the phagocytic vacuole and therefore sedimented with moderate centrifugal speeds, additional experiments were performed.

The 8 *g* and 15 *g* fractions from phagocyting cells were frozen and thawed for 8 cycles in a dry ice–alcohol bath. This resulted in the disruption of both cytoplasmic granules and phagocytic vacuoles as evidenced by the solubilization of acid hydrolases. This material was then centrifuged at 15,000 *g* for 15 minutes and the pellet resuspended to volume with 0.25 M sucrose. Both the pellet and supernatant fluid were then assayed for immunogenicity.

Under these conditions all of the immunogen was recovered in the 15,000 *g* pellet from the frozen and thawed fractions, although about 90 per cent of acid phosphatase cathepsin and β-glucuronidase was present in the soluble phase. This strongly suggested that the immunogen was associated with a relatively large particle, *e.g.* bacterial cell wall, and that little was present in a "soluble" form. Study on the extracellular medium from such incubation mixtures revealed no detectable agglutinogen and was therefore evidence against extraleucocytic release.

A single experiment performed with oil-induced peritoneal macrophages showed the same type of results and indicated that at 3 hours of intracellular residence all detectable agglutinogen was associated with the 8000 *g* pellet. In this case, however, little change occurred in total immunogenicity.

DISCUSSION

The results of these experiments are most readily interpreted in terms of the intracellular breakdown of the *E. coli* agglutinogen. Unfortunately, the chemical composition of this substance is unknown at this time so that a more exact description of its degradation is not possible. Irrespective of composition, the immunogen was handled differently within the phagocytes employed. The polymorphonuclear leucocyte and BCG-induced alveolar macrophage were able to inactivate the immunogen within their cytoplasm during a relatively short period of time. In contrast, the oil-induced peritoneal macrophage, although ingesting, inactivating, and degrading certain components of the bacilli (6) was not capable of significantly altering immunogenicity. The latter finding is analogous to the earlier results of Walsh and Smith who employed *S. typhi* (10). These results may not, however, be applicable to all types of antigens since guinea pig peritoneal macrophages rapidly degrade the proteins of antigen-antibody complexes to acid-soluble products (11).

Many differences exist in the morphology and biochemistry of the three phagocyte populations. In terms of the present experiments, the content of granule-associated hydrolytic enzymes is of obvious importance. These lysosomal hydrolases are released into the phagocytic vacuole (12, 13) and presumably aid in the digestion of the engulfed particle. Although many qualitative and quantitative enzymatic differences exist in the three populations (3, 9) it is uncertain whether these are related to the present results. In this context both the alveolar macrophage and PMN leucocyte are rich in lysozyme whereas the peritoneal macrophage has low levels of this polysaccharidase (3). Further speculation remains unwarranted until the nature of the immunogen is determined.

Although these experiments were conducted in an *in vitro* environment they pertain to events within the intact animal. The results suggest first that the fate of certain antigens may depend upon the type of cell to which they are exposed and thereby influence the production of antibody. Secondly, antigen may persist for longer periods within certain cell lines and the previous work of McMaster (14) suggests localization in mononuclear phagocytes. These cells which are probably long lived could then serve as depots of antigenic stimulation. No evidence for a more active role of the macrophage in antibody formation (15) is suggested by these studies.

SUMMARY

The fate of a heat-stable *Escherichia coli* agglutinogen within three types of rabbit phagocytic cells was examined. A system is described whereby quantitative ingestion of viable *E. coli* by suspensions of PMN leucocytes, BCG-induced

alveolar macrophages, and oil-induced peritoneal macrophages took place *in vitro*. After various periods of intracellular residence aliquots were injected intraperitoneally into NCS mice and the resulting agglutinins assayed. The loss of immunogenicity within phagocytes was estimated by comparison with a dose-response titration prepared with bacteria alone. Under these conditions no increase in immunogenic mass occurred *in vivo* or *in vitro* when viable organisms were were employed.

PMN leucocytes and alveolar macrophages destroyed the majority of the immunogen within 2 hours of intracellular residence. In contrast, the immunogenicity of *E. coli* was maintained within peritoneal macrophages for periods up to 5 hours. The use of heat-killed bacilli or specific immune serum did not significantly influence the intracellular fate of the immunogen. Residual immunogenicity was associated with a particle having the same centrifugal properties as the intact organism and essentially none was released in a soluble form. Intracellular residence within phagocytic cells did not influence the resulting temporal sequence of antibody formation nor the proportions of mercaptoethanol-sensitive and resistant immune globulins.

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