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Phagocytosis and Killing of *Salmonella typhimurium* by Peritoneal Exudate Cells

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Normal peritoneal cells from conventional, germfree, or *nu/nu* mice readily killed opsonized salmonellae, an observation that suggests that this activity in the normal peritoneal cavity may not be dependent on either environmental antigenic stimulation or T-cell mediation. In contrast, peritoneal cells elicited 4 days after injection with thioglycolate medium failed to kill opsonized salmonellae but appeared to be highly phagocytic. Peritoneal cells from thioglycolate-treated mice could be induced to kill opsonized salmonellae by giving the mice a primary footpad injection and a secondary intraperitoneal injection of *Corynebacterium parvum*. This activation by *C. parvum* appeared to be thymus dependent, since it did not occur in *nu/nu* mice.

Data from several sources suggest (6, 20, 23, 31; J. W. Jutila and K. W. Lee, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, B42, p. 22), but do not prove (11), that T-dependent macrophage activation is important for the survival of mice infected with *Listeria monocytogenes* and *Salmonella typhimurium*. Studies of the ability of macrophages to be activated to kill or to arrest the growth of microbes have generally been done with pathogens such as mycobacteria, toxoplasmas, and trypanosomes, which grow in peritoneal macrophages of normal mice but which either fail to grow or are killed within highly activated macrophages (18, 24, 29). Studies on the effects of macrophage activation on the killing of salmonellae or listeriae are complicated by two factors: (i) most, but not all, peritoneal macrophages are already capable of killing salmonellae or listeriae without further activation (22); and (ii) for reasons as yet unknown, in vitro phagocytosis and eventual killing of salmonellae by activated macrophages occur with very low efficiencies (12, 19). Our observation that, unlike normal peritoneal exudate cells (PEC), thioglycolate (TG)-elicited PEC are unable to kill salmonellae (D. E. Briles and J. E. Lehmeyer, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, E122, p. 71) provided us with a good starting cell population for developing a T-independent PEC activation assay. We found that, if mice that had been given an intraperitoneal (i.p.) injection with TG were also given primary footpad and secondary i.p. injections of *Corynebacterium parvum*, their PEC became highly bactericidal. This activation was T dependent and required both *C. parvum* injections. The basic in vivo assay methodology for this procedure was adapted from the earlier studies of Jenkin and Rowley, in which opsonized live salmonellae were injected into the peritoneal cavities of mice, and the peritoneal contents were plated within 60 min (13). By injecting radioactive salmonellae, Jenkin and Rowley could show that salmonellae were being killed and were not simply leaving the peritoneal cavity (13). We based our immunization procedure on one developed previously by Tuttle and North, who injected small doses of *C. parvum* into *C. parvum*-sensitized mice to stimulate a local, T-dependent cell-mediated immune response to tumor cells (35).

MATERIALS AND METHODS

Mice. CBA/J female mice (6 to 10 weeks old) were obtained from Jackson Laboratory, Bar Harbor, Maine. Germfree Swiss, conventional Swiss, *nu/nu* Swiss, and *nu/+* littermates (all 6 to 10 weeks old) were obtained from S. Michalek and J. McGee at the University of Alabama in Birmingham, Birmingham.

Hybridoma anti-*Salmonella* antibody. CBA/J mice were given five weekly intravenous injections of 10^9 acetone-killed (1) *S. typhimurium* cells. At 4 days after the last injection, myeloma cells (P3x63-Ag8.653; 15) and spleen cells were fused (17). Hybrids were cloned by limiting dilution on feeder layers of PEC in 96-well tissue culture plates. Antibody-secreting clones were assayed for anti-*S. typhimurium* activity by a modified enzyme-linked immunosorbent assay (9). Briefly, 100 μl of 5×10^8 heat-killed *S. typhimurium* cells were dried on polyvinyl microtiter plates at 60°C. The plates were fixed with methanol for 10 min and air dried before use. The anti-*S. typhimurium*-secreting clones were then identified with class-specific re-

agents prepared as previously described (14). By this procedure, clone ST-1 (μ, κ) was isolated. ST-1 was grown as an ascites tumor in (DBA/2 × CBA/N)F_1 mice. The ascitic fluid contained about 6×10^3 μg of antibody per ml as determined by radioimmunoassay (4a).

Salmonellae. *S. typhimurium* SR-11 (33) was obtained from L. J. Berry, University of Texas at Austin, and maintained virulent by intravenous passage through mice. Cultures were stored at −70°C and grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) containing 0.5% yeast extract (Difco) for 4 to 6 h before use. Bacteria were harvested by centrifugation at 4,000 × *g* for 7 min and washed by centrifugation three times in 4°C sterile Ringer lactate (Travenol Laboratories, Deerfield, Ill.). The washed bacteria were suspended in Ringer lactate, and the absorbance at 420 nm was determined. The number of colony-forming units (CFU) per milliliter was estimated, assuming that an absorbance of 1 equaled 5×10^8 CFU/ml. The actual numbers of CFU were determined by plating on bismuth sulfite agar (Difco). For use as a carrier during centrifugation, live salmonellae were washed in Ringer lactate, killed at 56°C, washed, and suspended in 1% bovine serum albumin (BSA).

Tritium-labeled salmonellae. Salmonellae were labeled with ^{3}H-amino acids by growing them in modified RPMI 1640 prepared with a Select-Amine kit (GIBCO Diagnostics, Madison, Wis.) reconstituted to have 1/20 the normal amount of each amino acid and four times the normal amount of glucose. Salmonellae were grown for 10 to 16 h in 0.5 ml of the medium described above plus 0.3 ml of ^{3}H-labeled protein hydrolysate (Schwarz/Mann, Orangeburg, N.Y.) containing a total of 300 μCi of ^{3}H. The labeled bacteria were collected on a 0.45-μm membrane (Millipore Corp., Bedford, Mass.), washed with sterile Ringer lactate, and rinsed from the membrane in Ringer lactate containing 0.1% BSA.

Injections. Some mice were injected i.p. with 1 ml of Brewer TG medium (Difco) 4 days before sacrifice. Killed *C. parvum* was obtained courtesy of R. L. Tuttle (Wellcome Reagents Ltd., Research Triangle Park, N.C.) as a 7-mg/ml suspension preserved with 0.01% thiomersal. Mice were injected in both rear footpads with a total of 100 μg of *C. parvum* in 0.1 ml of Ringer lactate 7 days before sacrifice. Mice were injected i.p. with 15 μg of *C. parvum* in 0.1 ml of Ringer lactate (generally 18 to 20 h) before sacrifice.

In vivo salmonella killing assay. Unless indicated otherwise, mice were injected with TG and *C. parvum* as described above. ^{3}H-labeled salmonellae (strain SR-11) were opsonized by suspending them at 10^7 CFU/ml in Ringer lactate containing 20 μg of the immunoglobulin M anti-*Salmonella* antibody produced by hybridoma ST-1 (a 1/300 dilution of ST-1 ascites fluid) per ml. After 30 min of incubation at 37°C, the salmonellae were injected into mice that had received i.p. injections of *C. parvum* 18 to 20 h earlier. After 30 to 60 min, each mouse was injected i.p. with 3 ml of Ringer lactate containing 15 U of heparin per ml. The mice were immediately killed by cervical dislocation, and the fluid in the peritoneal cavity was removed. A 0.5-ml amount of the peritoneal fluid was added to a scintillation vial containing 1 ml of Protosol (New England Nuclear Corp., Bedford, Mass.) and ethanol (1:2), and 0.1 ml of peritoneal fluid was added at 0°C to a tube containing 4 ml of 0.01% BSA in water and 1 optical density unit (420 nm) of heat-killed SR-11 to act as a carrier during centrifugation. The BSA-water was used to lyse the phagocytes (10). Lysis was apparently complete since subsequent sonication did not increase the numbers of SR-11 CFU. The tube was mixed and centrifuged at 4,000 × *g* for 7 min, and the pellet was suspended in 0.7 ml of BSA-water. Three serial 1/7 dilutions were made by transferring 0.1 ml to 0.6 ml of BSA-water at 0°C. To the cold tubes, 7.5 ml of 50°C bismuth sulfite agar was added by mixing with a Vortex mixer, and the contents of the tubes were immediately poured into 100-mm plastic petri dishes. Colonies were counted after 16 to 20 h of incubation at 37°C. Since a selective medium was used, the plating procedures were not carried out aseptically. To determine the CFU for the inoculum, a sample was centrifuged and plated in parallel with the samples of exudate fluid. The scintillation vials were capped tightly, incubated at 60°C for 1 h with shaking, and then cooled to room temperature. A 0.5-ml amount of 30% H_2O_2 was added dropwise, and the vials were capped loosely and incubated for 30 min at 60°C. The vials were then cooled again, and 15 ml of Scintiverse (Fisher Scientific Co., Pittsburgh, Pa.) and 0.5 ml of 0.5 N HCl were added. ^{3}H counts were measured in a liquid scintillation counter. This procedure decolorized the contents of the vials, thus eliminating artifacts resulting from occasional contamination of peritoneal fluid with blood. The fraction of CFU alive was expressed as: (CFU in peritoneal fluid/counts per minute in peritoneal fluid)/(CFU in inoculum/counts per minute in inoculum). This fraction has been referred to as relative CFU/cpm.

Identification of macrophages and neutrophils. Cytocentrifuge slides of the PEC were prepared and stained with Wright stain and for nonspecific esterase (36) for differential counts of 300 cells. The term "polymorphonuclear leukocytes" (PMNs) includes neutrophils, eosinophils, and basophils.

In vivo-in vitro opsonization and killing. The in vivo-in vitro opsonization and killing procedure is a modification of that of Blanden et al. (4). Injection schedules and opsonization methods for these experiments were the same as those described above, except the peritoneal cavities were washed out after only 3 min. A sample of the recovered fluid was processed as described above to determine total CFU. In addition, 1 ml of peritoneal fluid was added to 2 ml of 4°C Ringer lactate containing 0.01% BSA and 15 μg of heparin per ml, layered over 3 ml of fetal calf serum, also at 4°C and containing 15 μg of heparin per ml, and centrifuged for 5 min at 250 × *g*. The supernatant was aspirated off, and a sample was plated on bismuth sulfite agar to determine "free CFU." The pellet was suspended in 5 ml of 4°C Ringer lactate and centrifuged at 250 × *g* for 4 min. This final pellet was suspended in 2 ml of Hanks balanced salt solution (GIBCO) containing 5% fetal calf serum. One half of this mixture was centrifuged at 4,000 × *g*, and the pellet was lysed with BSA-water, serially diluted, and

plated out to determine "cell-associated CFU." The remaining half was incubated in a 37°C water bath for 40 min, centrifuged, lysed, and plated as described above to determine "surviving CFU." Since the sum of the cell-associated CFU and the free CFU always equaled "total CFU," we have not presented the data for free CFU.

RESULTS

In vivo killing. When opsonized, ^{3}H-labeled, live salmonellae were injected into the peritoneal cavities of untreated mice and mice previously given primary footpad and secondary i.p. injections of *C. parvum*, most of the CFU were eliminated within 45 min (Fig. 1). In contrast, when opsonized salmonellae were injected into the peritoneal cavities of mice pretreated with an i.p. injection of TG 4 days earlier, little or no killing occurred.

Although very few CFU remained in the peritoneal cavities of the normal or *C. parvum*-treated mice, much of the radioactivity was recoverable even after 60 min (Fig. 1). Thus, it is apparent that, in normal and *C. parvum*-treated animals, the eliminated CFU were actually being killed. By comparing the CFU/cpm ratio of each individual mouse with the same ratio for the injected inoculum, we were able to determine accurately the percentage of salmonellae surviving in each case. By performing the calculation in this way, we could compensate both for any inadequate mixing of the peritoneal contents during sampling and for the fact that a portion of the inoculum might accidentally be injected into the peritoneal tissues or organs.

Activation of TG-elicited PEC with *C. parvum*. Further investigation indicated that if TG-treated mice were also given the primary footpad and secondary i.p. *C. parvum* injections, their PEC were induced to kill salmonellae (Fig. 2). Both of the *C. parvum* injections were required to induce optimal salmonella killing by the PEC of TG-treated mice, and the activation of TG-elicited cells could be accomplished over a broad range of primary (Fig. 2, experiment 2) and secondary (Fig. 2., experiment 1) *C. parvum* doses. Opsonization with antibody was also required for optimal killing. By using relative CFU/cpm as an index of killing, we observed that, after opsonization with as little as 6 μg of ST-1, at least 90% of the salmonellae were killed over a period of 60 min in vivo. When unopsonized salmonellae or salmonellae incubated with heat-inactivated normal mouse serum were injected, fewer than 25% were killed over the same period. We also observed that salmonellae could be opsonized with sera from CBA/J mice hyperimmunized with an acetone-killed SR-11 vaccine (data not shown).

Kinetics of appearance of bactericidal activity. In mice treated with both TG and *C. parvum*, we found that *C. parvum*-induced killing did not occur until about 14 h after the boost (Fig. 3). We also examined the numbers of macrophages and PMNs recoverable from the peritoneal cavity as a function of time postboost (Fig. 4). After the *C. parvum* boost, we observed a decrease in the number of recoverable macrophages, which approached the lowest level at about 16 h, i.e., during the period of maximal bactericidal activity. This reduction to one-fourth of the normal number of recoverable macrophages probably represents the "macrophage

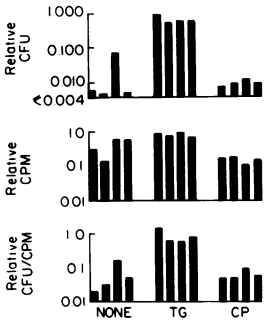

FIG. 1. *Recovery of ^{3}H-labeled S. typhimurium SR-11 from the peritoneal cavities of normal and TG- or C. parvum (CP)-treated CBA/J mice. Mice were injected i.p. with 10^6 opsonized CFU of ^{3}H-labeled salmonellae (20,000 cpm). The mice were killed, and their peritoneal cavities were washed out after 45 min. Each bar represents the data for an individual mouse.*

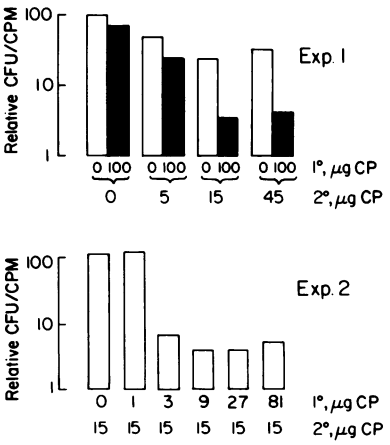

FIG. 2. *Activation of TG-elicited PEC with primary footpad and secondary i.p. injections of C. parvum (CP). Mice were killed 60 min after injection with opsonized ^{3}H-labeled salmonellae. Each bar represents the average value for two or three CBA/J mice.*

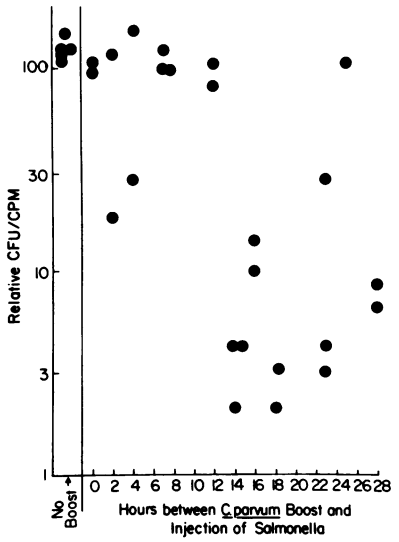

FIG. 3. *Kinetics of C. parvum-induced killing of opsonized salmonellae. Each point represents the value for a single CBA/J mouse. Mice were killed 60 min after injection with ^{3}H-labeled salmonellae.*

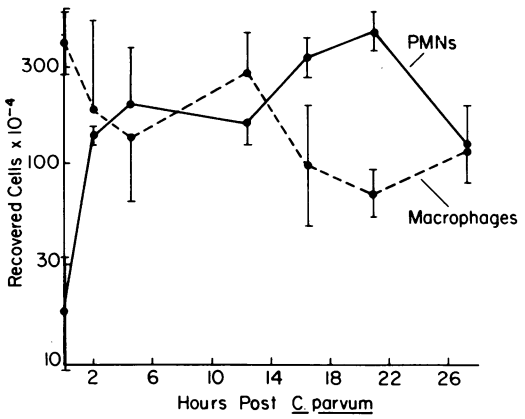

FIG. 4. *Changes in the numbers of macrophages and PMNs in the peritoneal cavities of TG-treated mice that were also given primary and secondary injections of C. parvum. Each datum point represents the average and standard error of three to six CBA/J mice. The number of nonmacrophage, non-PMN peritoneal cells was about 1.2 × 10^6 and did not change significantly during the experiment.*

disappearance" phenomenon that Nelson and North have described previously in guinea pigs and have shown to be associated with delayed-type hypersensitivity (26). In contrast, the number of PMNs showed a dramatic rise within the first 2 h after the *C. parvum* boost. There was a further increase in the numbers of PMNs during the period of maximum bactericidal activity (Fig. 4). Although PMNs may account for some of the bacterial killing, their increase in numbers alone cannot account for the *C. parvum*-induced killing of salmonellae. Between 2 and 12 h post-boost, the number of PMNs was 50% of maximal, yet no increase in killing was observed until after 12 h.

In vivo-in vitro opsonization and killing. It seemed likely that *C. parvum*-induced killing was due to either activation or removal of cells, presumably macrophages, that phagocytize but do not kill. Such cells would protect any engulfed salmonellae from destruction by PMNs or activated macrophages. To examine this hypothesis, we studied the ability of PEC from mice to ingest and kill salmonellae. The mice for this experiment were treated in the same manner as those used in the in vivo killing studies, except the peritoneal cavities were washed out after 3 min rather than after 30 to 60 min. The recovered fluid was assayed for total SR-11 CFU, percentage of cell-associated CFU, and the fraction of cell-associated CFU that could be killed by incubating the PEC for 40 min at 37°C.

After the 3-min in vivo incubation, we were able to recover at least half of the bacteria from the mice in each of the three groups. The lower recoveries in the normal mice and the TG-*C. parvum*-treated mice were probably the result of bacterial killing during the 3 min in vivo (Fig. 5). After centrifugation of the normal and TG-elicited PEC through calf serum, most of the bacteria were associated with the cell pellet, whereas the PEC from mice treated with both TG and *C. parvum* phagocytized almost none of the bacteria (Fig. 5). When the recovered PEC and their associated bacteria were incubated for 40 min at 37°C, the normal and *C. parvum*-activated PEC killed most of their associated bacteria. Incubation of the TG-elicited PEC, on the other hand, caused no net decrease in the numbers of associated CFU (Fig. 5). Thus, it appears that TG-induced cells are able to phagocytize salmonellae very rapidly but are unable to kill them. PEC elicited with both TG and *C. parvum*, on the other hand, may phagocytize more slowly but kill very rapidly. In this study, salmonellae were opsonized with anti-*Salmonella* hybridoma antibody ST-1. As shown in Table 1, the ability of the TG-elicited PEC to ingest salmonellae was dependent on opsonization with antibody, since incubation with heat-inactivated normal mouse serum (no antibody) did not result in any association of bacteria with cells.

Kinetics of opsonization and killing. The small fraction of bacteria associated with the TG-*C. parvum*-elicited PEC after 3 min in vivo may be due in part to the fact that these cells

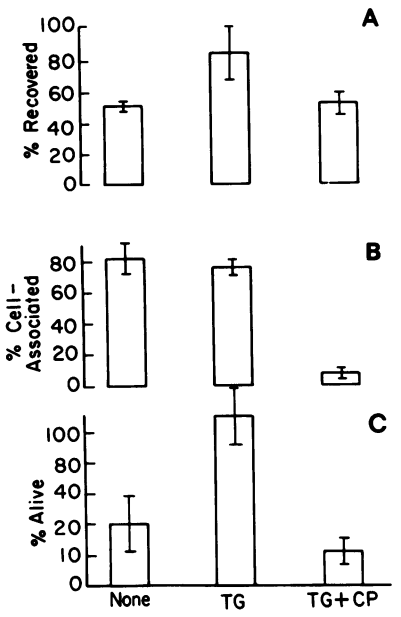

FIG. 5. *In vivo phagocytosis and in vitro killing of opsonized salmonellae. Data are shown for three treatment groups of CBA/J mice, each consisting of three mice. Mice were treated with either TG or TG and C. parvum (CP), as described in the text, or left untreated. At 20 h after the last C. parvum injection, the mice were injected with 10^6 opsonized live salmonellae. (A) Percentage of injected salmonellae that were recovered alive after 3 min; (B) percentage of the recovered salmonellae that were cell associated; (C) percentage of the cell-associated salmonellae that were still alive after incubation at 37°C for 40 min.*

TABLE 1. *Effect of opsonization on bacterial association with PEC*[a]

Bacteria incubated with:	% Recovered	% In pellet
1/20 NMS	96	12
20 μg of ST-1 per ml	81	84
20 μg of ST-1 per ml (no PEC)[b]		<1

[a] Thioglycolate-treated CBA/J mice were injected with 10^5 SR-11 cells that had been incubated with anti-*Salmonella* antibody, as a 1/300 dilution of ascites fluid, or diluted heat-inactivated normal serum (NMS) for 30 min at 37°C. After 3 min, the peritoneal contents were washed out and a sample was plated to determine the percentage of the inoculum recovered. Another sample was centrifuged through calf serum (5 min at 250 × *g*) to determine the percentage of recovered salmonellae that were associated with the PEC. The bacteria incubated with NMS served as a "no-antibody control."

[b] As a control for bacterial pelleting due to agglutination, 10^5 opsonized SR-11 cells were centrifuged through calf serum. Less than 1% was recovered in the fluid pellet.

are less phagocytic, but this is probably also due to the rapid destruction of any salmonellae that are phagocytized by these cells. To investigate this possibility in more detail, we studied the kinetics of phagocytosis and killing as a function of the length of time after injection of the opsonized salmonellae into TG-*C. parvum*-treated mice. The number of cell-associated CFU rose between the 2- and 5-min time points and then decreased (Fig. 6). However, the numbers of cell-associated CFU were never more than 5% of the injected CFU. The number of total CFU, on the other hand, decreased rapidly even by the first time point. Since bacterial killing in the peritoneal cavities of TG-*C. parvum*-treated mice begins immediately after salmonella injection, we would not expect to see an accumulation of salmonellae associated with TG-*C. parvum*-elicited cells. In contrast, there can be an accumulation of bacteria associated with the TG-elicited PEC since these cells do not kill salmonellae. Even when the assumption is made that all killing requires phagocytosis, it still appears that phagocytosis occurs more rapidly in the peritoneal cavities of TG-treated mice than in those of TG-*C. parvum*-treated mice, since within 3 min virtually all salmonellae in TG-treated mice are cell associated, whereas in TG-*C. parvum*-treated mice at 3 min, about 50% of the injected

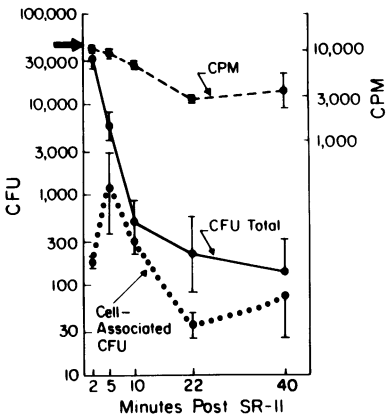

FIG. 6. *Kinetics of the elimination of ^{3}H-labeled opsonized salmonellae from the peritoneal cavities of mice treated with TG and both primary and secondary injections of C. parvum. Mice were injected with live ^{3}H-labeled S. typhimurium SR-11 20 h after the secondary injection of C. parvum. This figure depicts the numbers of total SR-11 CFU and cpm recovered from the peritoneal cavity. Also depicted are the numbers of cell-associated CFU recovered. The arrow on the vertical axis indicates the numbers of injected CFU and cpm. Each time point represents the geometric mean and standard error for three CBA/J mice.*

bacteria are neither killed nor cell associated (Fig. 5).

T-dependence of activation. The delayed kinetics of *C. parvum*-dependent activation and the fact that maximal killing was associated with macrophage disappearance suggest that the killing may be the result of a T-cell-dependent activation of macrophages. To test this hypothesis directly, we performed in vivo killing experiments with athymic (30) *nu/nu* mice. The results (Fig. 7) show that *C. parvum* cannot activate the TG-elicited PEC of these mice to kill salmonellae. We also examined the ability of normal *nu/nu* mice to kill salmonellae and found that it was unaltered (Fig. 7). Thus, it appears that the ability of resident peritoneal macrophages to kill salmonellae is a T-independent process. This contrasts with the activation of TG-elicited macrophages by *C. parvum*, which is T dependent.

Antigen independence of normal PEC activity. To determine whether the bactericidal activity of normal PEC was the result of either subclinical infections or antigen stimulation from gut bacteria, we compared the ability of PEC in conventional and germfree mice to kill salmonellae in vivo. Much to our surprise, the germfree mice showed slightly better killing than did the conventional controls (Fig. 8). Thus, it appears that, not only is the activation of normal PEC T independent, but it may also be antigen independent.

DISCUSSION

Comparison of bacterial killing and phagocytosis in PEC elicited by TG or TG-*C. parvum*. We have shown that resident and

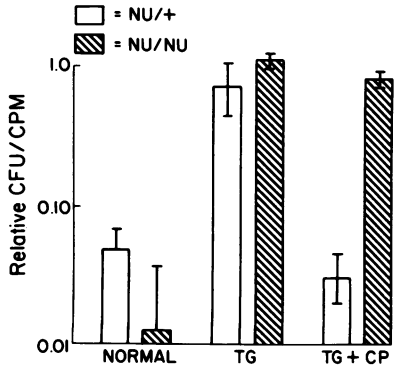

FIG. 7. *Killing of opsonized salmonellae in the peritoneal cavities of nu/nu and nu/+ mice. Each group contained four to eight Swiss mice. Geometric means and standard errors are shown. Injections of TG and C. parvum (CP) are described in the text. NORMAL, No TG or C. parvum treatment.*

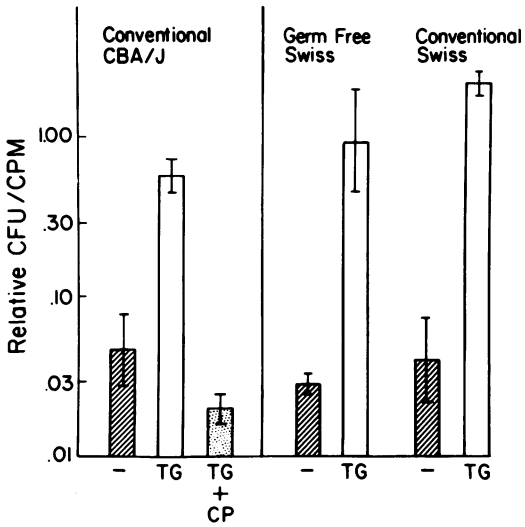

FIG. 8. *Killing of opsonized salmonellae in the peritoneal cavities of germfree and conventional Swiss mice. Conventional CBA/J mice were included as controls. Each group contained four to eight mice. Geometric means and standard errors are shown. Injections of TG and C. parvum (CP) are as described in the text. —, Neither TG nor C. parvum treatment given.*

C. parvum-activated PEC readily killed salmonellae, whereas TG-elicited PEC failed to kill salmonellae. When we examined these same cell populations for their ability to phagocytize in vivo, we found an inverse relationship, with the maximum phagocytosis occurring in mice treated with TG. Although the difference in phagocytosis may be caused in part by the higher numbers of macrophages in the peritoneal cavity of TG-treated mice, it is probably also a reflection of the intrinsic phagocytic activity of the PEC obtained with the different treatments. Bianco et al. have shown that TG-elicited peritoneal exudate macrophages are more phagocytic than normal macrophages (3). Nathan and Terry have shown that macrophages elicited with bacillus Calmette-Guérin are much less phagocytic than normal macrophages or macrophages elicited by TG (25). Our observation that TG-elicited macrophages fail to kill salmonellae is in agreement with similar results in studies of antitrypanosome immunity (24) and with studies in which the injection of mice with TG before i.p. infection with listeriae caused increased susceptibility to infection (2).

In recent years, it has become evident that activated macrophages do not represent a single state of differentiation and that not all of the properties that have been associated with activated macrophages are present in all activated

or elicited cells at any one time (28). Our studies confirm and extend some of these earlier findings. For example, we observed that TG-elicited PEC are the least able to kill salmonellae and thus, by this criterion, are less activated than resident PEC. However, in other studies, it has been clearly shown that TG-elicited, but not resident, PEC can phagocytize via their C3b receptors and thus, by this criterion, are considered to be more activated than normal PEC (3).

Thymus, antigen, and antibody dependence of salmonella killing by TG-*C. parvum*-elicited PEC. It seems likely that the *C. parvum*-induced bacterial killing that we have observed with TG-elicited PEC is dependent both on T-cell activation of macrophages and on antibody to facilitate phagocytosis. The first injection probably primes T cells in the draining lymph nodes, and some of these cells probably circulate to the peritoneal cavity and are restimulated by the second injection. Although the supposition that the salmonellae are being killed by activated macrophages is not proven in this paper, it is based on many previous studies of similar models (6, 20, 24, 29, 31). The evidence for T-cell involvement, on the other hand, is quite strong, especially since *C. parvum* fails to activate TG-elicited PEC in athymic *nu/nu* mice. This conclusion is supported by several of our other observations. The requirement for both *C. parvum* injections makes it unlikely that the *C. parvum* in the second injection is simply stimulating the macrophages directly. The fact that maximal killing did not occur until 14 to 16 h after the i.p. injection is consistent with a cell-mediated, rather than an antibody-mediated, immune response (6). Finally, the fact that macrophage disappearance was observed during the period of maximal bactericidal activity also indicates that a cellular immune response was at least concurrently in progress. Nelson and North have shown that macrophage disappearance is associated with cell-mediated immunity and is the result of activated macrophages becoming sticky and adhering to the peritoneal membranes (26).

If some of our activated macrophages are adhering to the peritoneal membranes, we might expect that part of the loss of salmonellae from the peritoneal cavity of normal and TG-*C. parvum*-treated mice might be due to a sequestering of salmonellae in adherent macrophages. This probably occurs to some extent since, based on ^{3}H counts, the percentages of ^{3}H-labeled salmonellae recovered from TG-*C. parvum*-treated mice and untreated mice were lower than those from mice injected with TG (Fig. 1). Even so, it is clear that killing of salmonellae is occurring in the peritoneal cavities of normal and TG-*C. parvum*-treated mice since the ratio of CFU to cpm recovered from the peritoneal cavities of these mice is less than 1/10 of the CFU/cpm ratio for the inoculum.

In this assay system, we have also observed that there appears to be a T-dependent accumulation of PMNs in the peritoneal cavity 16 to 20 h after the second *C. parvum* injection. Similar observations have been reported previously in mouse delayed-type hypersensitivity reactions (7, 8, 16). Although the PMNs that enter the peritoneal cavity may kill some of the injected salmonellae, their influx cannot account for the *C. parvum*-mediated increase in bacterial killing. If they did, we would expect to see more killing between 2 and 12 h postboost, when the number of neutrophils in the peritoneal cavity is 50% of the number that is observed at the time of maximal killing. We feel that the *C. parvum*-induced killing is due either to removal or to activation of the TG-induced PEC, mainly macrophages, that phagocytize the salmonellae and protect them from PMNs or more activated macrophages.

T-cell and antigen independence of bacterial killing by resident PEC. One major outcome of these studies was the observation that, whereas our procedure for activating TG-elicited PEC was both antigen *C. parvum* dependent and T dependent, the killing ability of PEC in untreated mice appeared to be neither T dependent nor antigen dependent. These findings reemphasize the studies of others which indicate that macrophages may be activated by many different routes (5, 21, 32, 34).

Possible application of the TG-*C. parvum* treatment to the study of antibacterial immunity. Our observation that TG-elicited macrophages can be activated by *C. parvum* in a T-dependent manner to kill salmonellae in vivo may be useful for a number of different studies of antibacterial or antitumor immunity and should be particularly applicable to studies of anti-*Salmonella* immunity.

The fact that our assay is in vivo may give rise to several advantages. It has not been possible to devise in vitro conditions that allow more than a small percentage of the added salmonellae to be killed (12, 19). Even when phagocytosis is allowed to proceed in vivo and killing is allowed to proceed in vitro, only a small fraction of bacteria are phagocytized at any one time (reference 4 and Fig. 6). We feel that this is because, whereas phagocytosis with activated macrophages occurs slowly, killing occurs rapidly. As a result, among the cell-associated CFU recovered from the in vivo opsonization, there

would be a disproportionate number of CFU in those peritoneal macrophages that are not activated, since most bacteria that enter activated macrophages would be killed before the in vitro assay. An in vivo assay may have an additional advantage since macrophages have been shown to change their state of activation in culture (24, 27). Thus, even if a completely in vitro assay could be made to work, it might not accurately reflect the in vivo situation.

For certain studies in which the genetic susceptibility to salmonella infection is investigated, it may be advantageous to use heat-killed *C. parvum* rather than salmonellae as a macrophage-activating agent. Collins and Mackaness have shown that cell-mediated protection against salmonellae is much more effective with a live than with a killed salmonella vaccine (6). In any experiment designed to compare the ability of salmonella-susceptible and salmonella-resistant mouse strains to activate macrophages, a live vaccine would be unacceptable since it would grow more in the susceptible than in the resistant strains and thus provide unequal immunization. By using heat-killed *C. parvum* as an activating agent, it should be possible to avoid this problem and still be able to screen salmonella-susceptible mouse strains for those that might have a general defect in T-dependent macrophage activation.

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