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Role of Transferrin, Transferrin Receptors, and Iron in Macrophage Listericidal Activity

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Summary

It is not yet known what properties distinguish macrophages which can kill facultative intracellular bacteria, such as *Listeria monocytogenes*, from those which cannot. Listeria is an organism which requires iron for growth, yet macrophage listericidal mechanisms are also likely to be iron dependent. We show here that resident peritoneal macrophages and thioglycollate-elicited macrophages cannot kill listeria, but proteose peptone-elicited and FCS-elicited macrophages can. All these cell populations phagocytose listeria. Transferrin receptor expression is low on resident cells, intermediate on peptone- and FCS-elicited cells, and high on thioglycollate-elicited cells. Transferrin transports iron into cells via the transferrin receptor: thus, iron content of resident cells is low, of peptone- and FCS-elicited cells is intermediate, and of thioglycollate-elicited cells is high. Moreover, antibody to transferrin, which prevents it binding its receptor, inhibits listericidal macrophages from killing this bacterium. Finally, nonlistericidal cells with high transferrin receptor expression and high intracellular iron become listericidal if they are incubated with apotransferrin, an iron-free ligand which prevents iron uptake by cells. These data suggest that macrophages must have enough available intracellular iron to support listericidal mechanisms, but too much iron favors growth of the bacterium, which no longer can be killed by the macrophage.

Acquired resistance to infection with facultative intracellular bacteria, such as *Listeria monocytogenes*, has been shown to depend on cell-mediated immunity (1, 2). This cell-mediated immune response requires the specific sensitization of T lymphocytes to listeria antigens. These T cells subsequently cause macrophages with enhanced microbicidal capabilities to accumulate at the site of infection (3–8). Despite the widespread acceptance of the concept that immunologically-activated macrophages are potent killers of these facultative intracellular bacteria, some populations of macrophages, which appear to be activated by some criteria, are capable of killing listeria, while others are not (9–13). It seems probable that before the complex mechanisms by which one acquires resistance to facultative intracellular pathogens can be understood, we must understand the mechanisms by which macrophages kill these microbes.

Many mechanisms have been proposed to explain how macrophages kill facultative intracellular bacteria. Among these are mechanisms dependent on iron, such as the generation of bactericidal products of oxygen metabolism (14). Paradoxically, however, evidence suggests that hosts with iron overload may be at higher risk for developing infection caused by *L. monocytogenes* (15–18). For instance, patients with chronic iron overload have increased incidence of infection with *L. monocytogenes* (15, 16). In addition, mice homozygous for a genetic defect which causes murine β-thalassemia show increased susceptibility to listeria and to *Salmonella typhimurium*, another facultative intracellular bacterium (18).

Other experimental evidence suggests that levels of intracellular iron are important regulators of macrophage listericidal mechanisms. The primary mechanism by which most cells obtain iron is believed to be via the transferrin receptor (19), and macrophages have been shown to possess both intracellular and cell surface receptors for transferrin (20). Most cells regulate their intracellular iron levels by iron-dependent modulation of the expression of the transferrin receptor (21–27). Macrophages at different levels of activation vary transferrin receptor expression (20), raising the possibility that cellular iron status also changes with activation. Moreover, resident peritoneal macrophages and macrophages induced by chronic infection of mice with bacillus Calmette-Guérin (BCG)[1], which have low numbers of transferrin receptors (20) and presumably low intracellular iron levels, are not bactericidal for *L. monocytogenes* (10, 11). Listericidal activity of FCS-elicited macrophages, which have a 6–7-fold higher level

[1] *Abbreviations used in this paper:* BCG, bacillus Calmette-Guerin; NMS, normal mouse serum; TPB, tryptose phosphate broth.

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of transferrin receptor expression than resident peritoneal macrophages (20), and presumably a concomitant increase in intracellular iron levels, has not been studied. However, thioglycollate-elicited macrophages, which have 10–15-fold higher levels of transferrin receptors than resident macrophages (20) are not able to kill listeria (9). Interestingly, alveolar macrophages from normal non-smokers express the transferrin receptor and are capable of killing listeria (28–30) while alveolar macrophages from smokers, which have 4-fold higher iron levels, cannot kill listeria (29, 31).

Taken together, these data suggested to us the hypothesis that there is a minimum level of intracellular iron necessary for macrophages to express listericidal activity, but if the macrophage iron levels become excessive the cell is no longer able to kill this bacterium. To test this hypothesis, we have examined the role of transferrin, the transferrin receptor and iron in killing of listeria by macrophages. The data demonstrate that cells with low levels and high levels of transferrin receptor and intracellular iron are not listericidal, while those with intermediate numbers of surface transferrin receptors, and intermediate iron content are listericidal. Moreover, antibody to transferrin, which prevents it binding its receptor, inhibits listericidal cells from killing this bacterium.

Materials and Methods

Preparation of Peritoneal Macrophages. BDF_1 (C57BL/6 × DBA/2)F_1 mice 10 to 20 wk-of-age were obtained from Jackson Laboratories (Bar Harbor, ME) and used for the preparation of all macrophage populations. Mice were housed either in the National Jewish Center for Immunology and Respiratory Medicine Animal Care Facility or the University of Colorado Health Sciences Center Animal Facility. All animals were maintained on food and water *ad libitum*. Mice of a single age and sex were used in each experiment. To obtain inflammatory peritoneal macrophages mice were injected i.p. with either 1.0 ml of sterile 10% proteose peptone (Difco Laboratories, Detroit, MI), 1.0 ml FCS (HyClone Labs, Inc., Logan, UT), or 1.0 ml of sterile 4% Brewer thioglycollate medium (Difco Laboratories). Mice were killed by CO_2 narcosis 48, 48, or 72 h later, respectively, and peritoneal exudate cells were harvested by peritoneal lavage with 10 ml of balanced salt solution (BSS, reference 32). Resident macrophages were harvested from normal mice by peritoneal lavage as above. Cells were centrifuged, resuspended in BSS, counted, and used.

Bacteria. *L. monocytogenes*, strain EGD, was maintained by periodic passage through BDF_1 mice. Bacteria were grown to log phase in tryptose phosphate broth (TPB, Difco Laboratories), divided into portions, and stored at −70°C. For each experiment, organisms were grown overnight in TPB, recovered, and diluted as needed.

Iron Assay. The iron content of a known number of peritoneal exudate cells was determined by a modification of Carter's method (33) using the buffer recommended by McGowan (34). To do this, cells were suspended in deionized water and an equal volume of 6N HNO_3 (Fisher Scientific, Fairlawn, NJ) was added. The samples were heated at 80°C for 18–24 h to release protein-associated iron. All iron was reduced to Fe^{++}, and ferrous iron concentration was quantitated spectrophotometrically by its characteristic absorbance at 562 nm after being complexed with ferrozine (Sigma Chemical Co., St. Louis, MO). The concentration of iron, presented as ng/10^6 cells, was calculated by comparing the absorbance of the samples with a standard curve in which absorbance was plotted against known concentration of iron.

Reagents and Antibodies. A FITC-labelled mouse anti-human transferrin receptor mAb, clone L01.1, was purchased from Becton Dickinson (Mountain View, CA). For immunofluorescent labelling, the hybridoma TIB 219, which secretes a rat IgG_{2a} mAb against the murine transferrin receptor (28), was purchased from ATCC (Rockville, MD). The cells were cultured in Dulbecco's modified Eagle's medium with 4.5 g/liter glucose (Gibco Laboratories, Grand Island, NY), 2×10^{-5} M 2-ME, and 2% FCS (Hyclone Labs) and gentamicin at 50 μg/ml (Quality Biologicals, Gaithersburg, MD). Individual cultures were allowed to grow until the hybridomas died, at which time the tissue culture supernatants were collected. The supernatants were centrifuged at 1000 × *g* for 10 minutes and then filtered to remove the debris. The antibody was then purified by passage over a protein G sepharose column (Pharmacia LKB Biotechnology AB Uppsala, Sweden). For indirect immunofluorescence, a FITC-labeled goat anti-rat Ig antibody (Jackson Immunochemicals, West Grove, PA) was used. Iron-saturated transferrin, apotransferrin (obtained as transferrin, substantially iron-free), and a fractionated monospecific goat anti-human transferrin antibody were purchased from Sigma Chemical Co., St. Louis, MO. In some experiments, normal goat IgG (Sigma Chemical Co.) was used as a control.

Assay for Transferrin Receptor Expression on Macrophages Using Flow Cytometry. Cells were aliquoted to round-bottom floppy microtiter wells (1×10^6 cells/well) containing 100 μl staining medium (phosphate buffered saline + 2% FCS + 0.2% sodium azide). The plate was centrifuged at 250 × *g*. Cells were then incubated either with staining medium to assess autofluorescence, with FITC-labeled goat anti-rat antibody to assess non-specific binding of the secondary antibody, with rat anti-mouse transferrin receptor mAb followed by incubation with FITC conjugated goat anti-rat Ig antibody, or with FITC-labelled mouse anti-human transferrin receptor mAb. Cells were incubated with the primary antibody or staining medium in the dark for 45 min at 4°C, then washed three times with staining medium. Cells were then incubated with the secondary antibody in the dark for 30 min at 4°C, washed three times with staining medium, and resuspended in 1 ml staining medium. Flow cytometric analysis of the cells was then performed on a flow cytometer (Epics C; Coulter Electronics Inc., Hialeah, FL) equipped with an argon laser tuned to 488 nm and run at 200 mW. The flow cytometer was gated on forward light scatter and right angle scatter so as to select macrophages and exclude lymphocytes, cell debris, and nonviable cells. The log fluorescence intensity of 5,000 macrophages from each sample was displayed as a frequency distribution histogram.

Phagocytosis and Bactericidal Assays. Phagocytosis was determined as described earlier (10). Briefly, 2.5×10^6 cells and 2.5×10^7 bacteria were incubated, rotating end-over-end, in 1 ml BSS containing 5% normal mouse serum (NMS). After 30 minutes, cells were harvested, washed three times, counted, cytocentrifuged, and stained with Diff-Quik (Dade Diagnostics, Aguada, PR). The phagocytic index was determined as percent positive macrophages (those containing more than one bacterium) times mean number of bacteria per positive macrophage.

Bactericidal activity was assayed as described earlier (10). Briefly, 2.5×10^6 cells, 2.5×10^6 bacteria, and 5% NMS were mixed in a total volume of 1 ml BSS in polypropylene tubes (Sarstedt, Newton, NC). Tubes were rotated in a 37°C incubator for 2 h as above. At the beginning of the incubation 0.1 ml samples were removed from control tubes, diluted in sterile distilled water to

lyse the cells, and plated on tryptic soy agar plates (Remel, Lenexa, KS). After the tubes were incubated for an additional 2 h with end-over-end rotation, samples were removed as before, diluted, and plated. After 24 to 48 h, the number of bacterial colonies was determined and the mean and SEM were calculated. Log_{10} killing was calculated as (mean log_{10} bacteria after 2 hr incubation with cells as indicated) − (mean log_{10} bacteria at 0 time).

Pretreatment of Mouse Peritoneal Macrophages. In some experiments cells were washed with BSS, incubated in polypropylene tubes at 5×10^6 cells per ml in complete medium (RPMI 1640; Irvine Scientific, Santa Ana, CA; 10% FCS; 5×10^{-5} M 2-ME; 2 mM glutamine) alone or containing 10 mg/ml human apotransferrin or iron-saturated human transferrin (Sigma Chemical Co.). Cells were then washed with BSS before being used in the assays.

In other experiments, peritoneal exudate cells elicited with proteose peptone or thioglycollate were either incubated for 1 h in complete medium or medium containing goat anti-human transferrin antibody or goat IgG as a control. Cells were then washed with BSS before being used in the assays.

Statistics. Results were analyzed for significance between groups by one-tailed Student's *t* test using the Wormstat program on a Macintosh SE computer. Groups were considered statistically different if $p < 0.01$.

Results

Listericidal and Phagocytic Activity of Different Macrophage Populations. The ability of four different macrophage populations to kill listeria was compared and the data from 7–10 similar experiments are presented in Fig. 1 *A*. Macrophages elicited with either proteose peptone or FCS were capable of killing listeria, whereas resident macrophages and macrophages elicited with thioglycollate medium were unable to do so. This finding could not be correlated with the size of the exudate as FCS elicited only twice as many peritoneal exudate cells as were harvested from resident mice, and thioglycollate elicited more cells than either FCS or proteose peptone (Table 1). Similarly, the percent macrophages in these four cell populations has ranged from 77–95 over the past 5 yr. This suggests that differences in anti-listericidal activity of these peritoneal cell populations probably is not due to differences in the number of macrophages.

One possible explanation for the differences in listericidal activity of the various macrophage populations is that these cells do not phagocytose listeria equally, since phagocytosis is necessary for listericidal activity (10). Thus, the phagocytic ability of the different macrophage populations was measured (Fig. 1 *B*). Resident macrophages were significantly less phagocytic than any of the inflammatory macrophages, which might account for their decreased ability to kill listeria. However, non-listericidal thioglycollate-elicited macrophages were more phagocytic than either the FCS-elicited or the proteose peptone-elicited macrophages. Thus, differences in phagocytosis alone could not account for the fact that thioglycollate-elicited macrophages exhibited no listericidal activity.

Transferrin Receptor Expression on Different Macrophage Populations. Hamilton et al. (20) reported that transferrin receptor expression on macrophages varied with the state of activation of the macrophage. In order to determine if transferrin receptor expression is associated with macrophage listericidal activity, the relative expression of the transferrin receptor was examined in each of the four macrophage populations. Representative flow cytometric analyses for each macrophage popu-

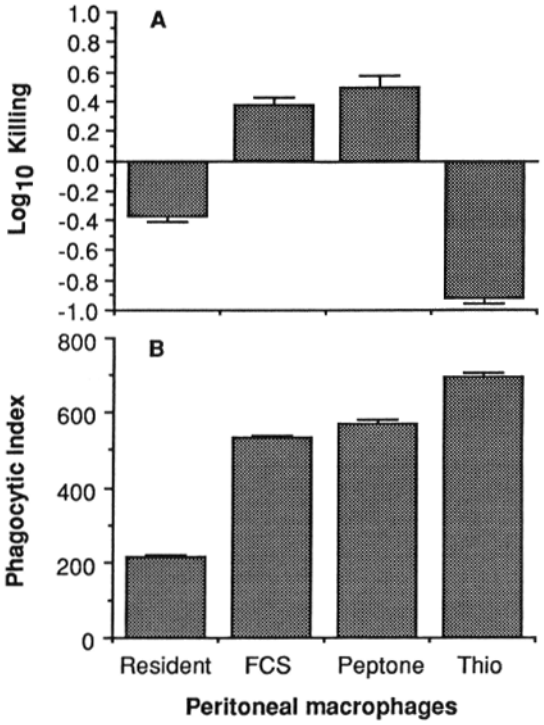

Figure 1. Listericidal (Fig. 1 *A*) and phagocytic (Fig. 1 *B*) activities of resident peritoneal macrophages, or of peritoneal exudate macrophages elicited with FCS, proteose peptone (Peptone), or thioglycollate (Thio). In Fig. 1 *A*, the line indicates the number of listeria at the beginning of the incubation, and the bars indicate listeria colonies 2 h after incubation with cells as indicated. Positive values reflect killing of bacteria, and negative values indicate bacterial growth. The data are mean log_{10} killing ± SEM of 7–10 experiments. Fig. 1 *B* depicts mean phagocytic index (% phagocytic macrophages × mean listeria per positive macrophage) ± SEM of 6–9 experiments.

Table 1. *Peritoneal Exudates Elicited by Different Inflammatory Stimuli*

Peritoneal cells*	No. of experiments	Mean PEC per mouse ± SEM‡
		× 10^6
Resident‡	7	2.67 ± 0.09
FCS-elicited	8	5.07 ± 0.25
Peptone-elicited	10	9.74 ± 0.59
Thioglycollate-elicited	7	16.33 ± 0.10

* Mice were not injected (resident), or injected with inflammatory agents, as indicated, and peritoneal cells harvested 48 or 72 h later from 3 to 10 mice per group, and counted.

‡ Data indicate mean number of peritoneal exudate cells (PEC) ± SEM from the number of experiments indicated. Differences between resident cells and all other cells, and between thioglycollate-elicited cells and all other cells, were statistically significant.

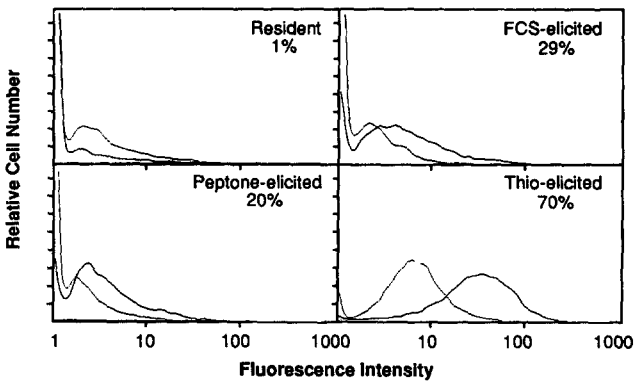

Figure 2. Transferrin receptor expression on resident peritoneal macrophages, or on peritoneal exudate cells elicited with FCS, proteose peptone (Peptone), or thioglycollate (Thio). Fluorescence intensity of cells stained with rat anti-transferrin receptor antibody (TIB 219) + FITC-labeled goat anti-rat antibody (dark lines) or with FITC-labeled antibody alone (*light lines*) are shown. The numbers represent percent positive cells.

lation are shown in Fig. 2. Percent cells expressing transferrin receptors were determined by gating on cells stained with the secondary antibody alone, and subtracting these secondary antibody controls from profiles obtained when cells were stained with anti-transferrin antibody and secondary antibody. Mean fluorescence intensity, reflecting relative number of transferrin receptors per cell, was determined to be 1.36 arbitrary units for resident cells, 2.51 for peptone-elicited cells, 6.15 for FCS-elicited cells, and 10.86 for thioglycollate-elicited cells. These data are pooled from 3, 4, 2, and 4 similar experiments, respectively. Data obtained when cells were stained directly with FITC-labeled mouse anti-human transferrin receptor mAb were similar, and are not presented here.

These experiments show that resident macrophages, which do not kill listeria, expressed a low level of transferrin receptors. Proteose peptone-elicited and FCS-elicited macrophages expressed a slightly higher level of this receptor, while thioglycollate-elicited macrophages expressed high levels of this receptor. These differences are detected both in the number of transferrin receptor positive cells and in relative receptor expression per cell. Transferrin receptor expression per cell is similar to results obtained by others (20), who used Scatchard analysis to show that thioglycollate-elicited cells have 5–6-fold more transferrin receptors than do resident cells. Byrd and Horwitz (35), demonstrated that transferrin receptor expression on human monocytes measured by flow cytometry was similar to receptor expression detected using Scatchard analysis. The data presented here show that an increase in transferrin receptor expression over that displayed by resident macrophages correlates with the ability of macrophages to express listericidal activity but that cells with high levels of transferrin receptors are unable to kill the bacterium.

Iron Content of Different Macrophage Populations. The primary function of transferrin is believed to be the delivery of iron into the cell. To determine if transferrin receptor expression correlated with cellular iron content, the iron content of the four different macrophage populations was measured and is presented in Table 2. The iron content of resident macrophages was 1.52 ng/10^6 cells, while the iron content of macrophages which kill listeria is increased 6-fold. The iron content of thioglycollate-elicited macrophages, on the other hand, is increased 18-fold over resident macrophages, and 3-fold over FCS- and proteose peptone-elicited macrophages. Thus cellular iron content correlated with transferrin receptor expression.

Table 2. *Iron Content of Different Populations of Peritoneal Macrophages*

Peritoneal cells*	No. of experiments	Iron
		ng/10^6 cells‡
Resident	3	1.52 ± 0.13
FCS-elicited	3	8.36 ± 0.21
Peptone-elicited	3	9.25 ± 0.21
Thioglycollate-elicited	3	27.83 ± 0.39

* Mice were not injected (resident) or were injected in inflammatory agents, as indicated, and peritoneal cells harvested 48 or 72 hr later from 3 to 10 mice per group.

‡ Iron content was measured and is presented as ng per 10^6 cells ± SEM. Differences between all cells, except between FCS- and peptone-elicited cells, was statistically significant.

Inhibition of Binding of Transferrin to its Receptor Decreases Macrophage Listericidal Activity. To test the hypothesis that cellular iron is necessary for expression of macrophage listericidal activity, macrophages which kill listeria were treated with substances that would decrease the ability of mouse serum transferrin to provide iron to the cell. The ability of transferrin to deliver iron to the cell requires binding of the iron-transferrin complex to the cellular transferrin receptor (34, 36, 37). Proteose peptone-elicited macrophages were pretreated for 1 h with a polyclonal goat antibody to human transferrin

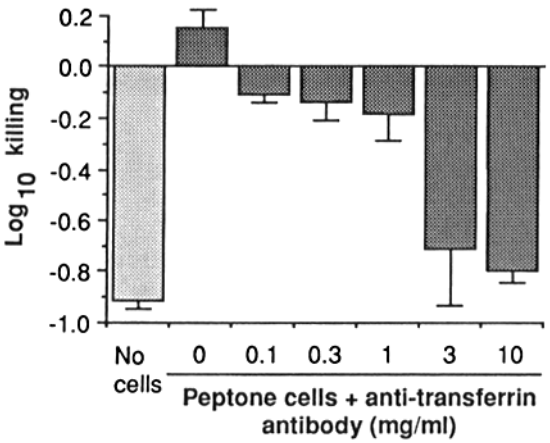

Figure 3. Effect of anti-transferrin antibody on listericidal activity of peptone-elicited macrophages (*dark shading*). Cells were incubated with or without anti-transferrin antibody for 1 h, washed, and bactericidal activity assayed. The light bar (*no cells*) indicates bacterial growth in the absence of cells. The data are $\log_{10}$ killing ± SEM in one of three experiments with similar results.

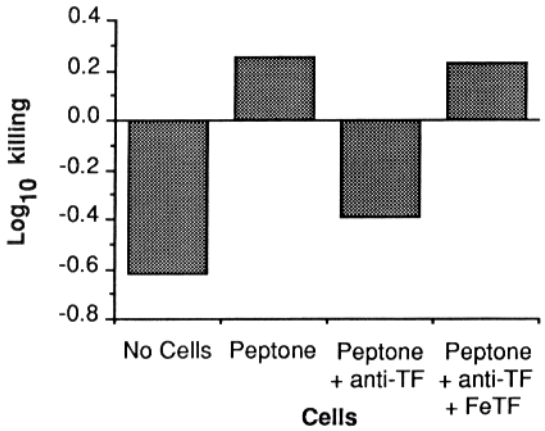

Figure 4. Transferrin reverses inhibition of listericidal activity by anti-transferrin antibody. Cells were preincubated with or without anti-transferrin antibody (1 mg/ml) for 1 h. Bactericidal activity was then assayed in the presence of anti-transferrin antibody ± iron-saturated transferrin (FeTF, 10 mg/ml). Data indicate mean $\log_{10}$ killing of two identical experiments.

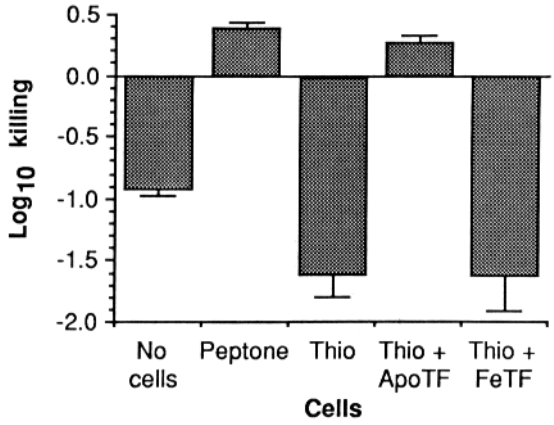

Figure 6. Apotransferrin, but not iron-saturated transferrin, induces thioglycollate-elicited macrophages to kill listeria. Thioglycollate-elicited cells (Thio) were incubated for 20 h with apotransferrin (ApoTF, 1 mg/ml) or iron-saturated transferrin (FeTF, 1 mg/ml), and bactericidal activity assayed. Bactericidal activity of peptone-elicited cells (Peptone) is included as a positive control. Data show mean $\log_{10}$ killing ± SEM in one of two similar experiments.

to prevent the binding of transferrin to its cell surface receptor, and bactericidal activity determined. Anti-transferrin antibody inhibited macrophage listericidal activity in a dose-dependent manner (Fig. 3). In a separate experiment, anti-transferrin antibody at the same concentration decreased intracellular iron content of peptone-elicited cells by 30%. The antibody had no direct effect on the growth of listeria (data not shown). Inhibition could be completely reversed by the addition of iron-saturated human transferrin to the assay system (Fig. 4), documenting the specificity of the antibody effect. In order to rule out the possibility that inhibition was due to non-specific effects of goat Ig, proteose peptone-elicited macrophages were treated with pooled goat Ig (Fig. 5). Goat Ig at the same concentration as the anti-transferrin antibody (3 mg/ml) did not inhibit macrophage listericidal activity,

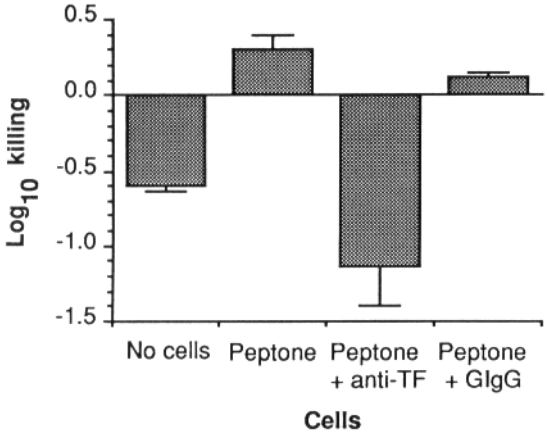

Figure 5. Ability of goat anti-transferrin antibody (anti-TF) to inhibit listericidal activity of peptone-elicited macrophages is not due to nonspecific effects of goat Ig. Bacteria were incubated for 2 h with no cells or with peptone-elicited cells ± goat Ig (GIgG, 1 mg/ml) or anti-TF (1 mg/ml). Data show mean $\log_{10}$ killing ± SEM in one of three similar experiments.

suggesting that the inhibition was specific for the anti-transferrin antibody.

The inhibition seen with the antitransferrin antibody was not due simply to an effect on phagocytosis, as the phagocytic index of peptone-elicited cells treated with this antibody was 532 ± 6.2, which is not significantly different from untreated peptone-elicited cells, which had a phagocytic index of 569 ± 11.5.

Excess Cellular Iron Decreases the Ability of Macrophages to Kill Listeria. Since the lack of listericidal activity in thioglycollate-elicited macrophages could not be accounted for by decreased phagocytic ability, we tested whether the decreased listericidal activity was due to an increase in cellular iron content as a result of the increased expression of the transferrin receptor. This hypothesis predicts that lowering intracellular iron by incubating cells with apotransferrin, a form of transferrin which has little or no iron bound to it, should induce them to become listericidal. Thus, thioglycollate-elicited macrophages were incubated with apotransferrin overnight in polypropylene tubes, rotating end-over-end. Cells incubated with apotransferrin do not internalize iron and should show a net decrease in intracellular iron since they are utilizing, but no longer importing, iron. Treatment of nonlistericidal thioglycollate-elicited cells with apotransferrin, but not with iron-saturated transferrin, induced them to express listericidal activity (Fig. 6). Moreover, in a representative separate experiment, intracellular iron content of thioglycollate-elicited cells was 36.8 ng/10^6 cells before incubation, 10.4 ng/10^6 cells after 22 hr. incubation in RPMI + 1% BSA containing 10 mg/ml apotransferrin, and 36.2 ng/10^6 cells after incubation in medium containing 10 mg/ml iron-saturated transferrin. This suggests that apotransferrin increased bactericidal activity of thioglycollate-elicited macrophages because it decreased intracellular levels of iron. The increase in listericidal ability was not due to differences in phagocytosis as the phagocytic index was similar for un-

treated cells, cells treated with iron-saturated transferrin, and cells treated with apotransferrin (data not shown).

Discussion

The mechanisms by which macrophages kill the facultative intracellular bacterium, *Listeria monocytogenes*, are unknown. We show here that certain populations of macrophages, notably peritoneal exudate cells elicited with proteose peptone or FCS, are listericidal, while other populations, such as thioglycollate-elicited or resident peritoneal macrophages, are not. Non-listericidal cells may nevertheless phagocytose this organism normally. Experiments presented here show that expression of intermediate amounts of the transferrin receptor is required for macrophages to express listericidal activity. Moreover, listericidal cells have intermediate amounts of cellular iron. If binding of transferrin to its receptor is prevented by anti-transferrin receptor antibody, listericidal macrophages lose their ability to kill, but not to phagocytose this bacterium. Similarly, macrophages which have high transferrin receptor expression, high iron content, and are not listericidal become listericidal following incubation with apotransferrin, which decreases import of iron into the cell.

Iron-saturated transferrin contains two molecules of ferric iron. At neutral pH, the transferrin receptor on a cell binds iron-saturated transferrin, and this receptor-ligand complex is endocytosed, thus transporting iron into the cell (28, 36). Once inside the cell, the endocytic vacuole is acidified, and transferrin releases ferric ions. This internalized iron enters the available iron pool, where it can be utilized by the cell or by organisms such as listeria (37). The transferrin receptor-apotransferrin complex is relocated to the cell surface, where apotransferrin dissociates from its receptor, which is now able to bind iron-saturated transferrin and reinitiate the cycle (38). In this manner, macrophages and other cells acquire intracellular iron, and so do iron-dependent bacteria which can grow intracellularly.

L. monocytogenes requires iron for growth (17). Data presented here demonstrate that listeria grow in thioglycollate-elicited macrophages, which supply high amounts of iron, but are killed by FCS- and proteose peptone-elicited macrophages, which supply less iron. Others have demonstrated that *Legionella pneumophila*, another facultative intracellular bacterium, also requires appropriate amounts of iron for growth inside human monocytes (35). However, FCS- and peptone-elicited macrophages do not merely restrict growth of listeria by denying the organism adequate iron to support its growth, they kill this bacterium, suggesting that these cells also have active listericidal mechanisms. These listericidal mechanisms may require iron, perhaps for generation of toxic metabolites as in the Haber-Weiss reaction, which requires ferrous iron to generate hydroxyl radicals (39). It is also possible that iron is required to generate toxic nitric oxides, molecules recently demonstrated to be important in killing of certain fungi and obligate intracellular parasites (40, 41). Resident macrophages, which have low amounts of intracellular iron, also do not kill listeria. This may be because there is too little iron in these cells to support their listericidal mechanisms, or because listeria are phagocytosed poorly or into the wrong compartment by these cells. Alternatively, iron in resident macrophages may be compartmentalized in a way that prevents it from being used by cellular listericidal mechanisms.

Evidence supporting transport of iron into cells as an essential component of listericidal activity is provided by experiments describing effects of anti-transferrin receptor antibody. In these experiments, anti-transferrin antibody inhibited the ability of peptone-elicited macrophages to kill listeria. This was not due to non-specific effects of the goat anti-human transferrin antibody since purified goat IgG did not affect listericidal activity of these cells. The specificity of the antibody effect was confirmed by experiments demonstrating that transferrin restored listericidal activity to cells incubated with the anti-transferrin antibody. These experiments suggest that for a macrophage to express listericidal activity, it must be able to transport iron into the cell.

The concept that too much iron inside a cell favors growth of listeria, and effectively neutralizes listericidal mechanisms, is supported by studies using thioglycollate-elicited macrophages. As shown here, thioglycollate-elicited macrophages phagocytose listeria very well, but cannot kill this organism. They have high numbers of transferrin receptors on their surfaces (20, Fig. 2), and contain high amounts of iron (Table 2). Moreover, when thioglycollate-elicited cells were incubated with apotransferrin to decrease import of iron, they became listericidal. These data suggest that thioglycollate-elicited macrophages may allow growth of listeria either because the high iron content favors the organism, or because the iron is recompartmentalized within the cell in a manner which decreases its availability for listericidal activity. It is also possible that in a high iron intracellular environment, listeria more easily escape from the hostile environment of the phagolysosome into the cytoplasm, where bacterial growth is not impeded by normal listericidal mechanisms.

Finally, experiments here demonstrate that macrophages which phagocytose listeria are not necessarily able to kill this organism. Thioglycollate-elicited macrophages and peptone-elicited macrophages treated with anti-transferrin antibody are both highly phagocytic, but neither can kill listeria. Peritoneal macrophages elicited by BCG injection 2–7 weeks earlier are highly phagocytic, but are not listericidal (11 and PAC, unpublished observation). This suggests that phagocytosis is necessary for listeria to be killed intracellularly, but it is not sufficient. Experiments examining phagocytic and listericidal capabilities of human bronchoalveolar lavage macrophages demonstrate that under certain circumstances these cells, too, can phagocytose but not kill *L. monocytogenes* (29, 30).

In summary, data presented here suggest that for a macrophage to be listericidal, it must have sufficient surface transferrin receptor expression to allow binding and internalization of amounts of iron adequate to support listericidal mechanisms. If a cell has too few surface transferrin receptors, and thus too little internal iron, it cannot kill listeria. Moreover, if a macrophage has high levels of transferrin receptor ex-

pression and high iron content, then growth of this facultative intracellular bacterium is favored, and the cell is not able to express listericidal activity. Listericidal activity of macrophages is inhibited if binding of transferrin to its receptor is prevented.

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