Gamma Interferon Enhances Internalization and Early Nonoxidative Killing of *Salmonella enterica* Serovar Typhimurium by Human Macrophages and Modifies Cytokine Responses

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Received 1 October 2004/Returned for modification 29 November 2004/Accepted 24 January 2005

Gamma interferon (IFN- γ) is a critical cytokine in host defense against salmonella infections, but its role in phagocytic killing of intracellular *Salmonella* spp. has been investigated mainly in animal rather than human cells. We measured the effect of recombinant IFN- γ (rIFN- γ) priming on bacterial internalization, intracellular killing, oxidative burst, and cytokine release during phagocytosis of *Salmonella enterica* serovar Typhimurium by human monocyte-derived macrophages (MDM). Eleven-day-old MDM, primed for 72 h with rIFN- γ (100 ng/ml) exhibited an increased proportion of cells with associated bacteria (31% versus 26%, $P = 0.036$) and a 67% increase in internalized bacteria per cell compared to unprimed cells ($P = 0.025$). Retrieval of viable bacteria following internalization was reduced 3.6-fold in 72-h primed versus unprimed MDM (interquartile range, 3.1 to 6.4) at 0.5 h due to enhanced early intracellular killing, and this difference was maintained up to 24 h. In contrast, cells primed for only 24 h exhibited no increase in early killing. MDM were competent to produce an early oxidative burst when stimulated with phorbol myristate acetate, which was fully abrogated by the respiratory burst inhibitor diphenyleneiodonium chloride (DPI), but infection of MDM with *S. enterica* serovar Typhimurium did not cause an increase in the early respiratory burst under unprimed or primed conditions, and DPI had no effect on the early killing of bacteria by primed or unprimed MDM. During 24 h following infection, rIFN- γ -primed MDM released more interleukin-12 (IL-12) and less IL-10 relative to unprimed cells. We conclude that 72-h priming with rIFN- γ increases the efficiency of internalization and nonoxidative early intracellular killing of *S. enterica* serovar Typhimurium by human macrophages and modifies subsequent cytokine release.

Salmonella enterica serovar Typhimurium is an important pathogen of animals and humans. It causes a diarrheal illness that is usually mild and self-limiting in immunocompetent adults, but infections may be severe, invasive, persistent, or recurrent in patients with human immunodeficiency virus (HIV) infection, hemoglobinopathies, chronic granulomatous disease, or severe malarial anemia. Failure to clear intracellular bacteria probably plays an important role in the recurrence of human salmonella infections, particularly in HIV-infected patients (10).

Intracellular survival in cells of monocyte/macrophage lineage is an important component of the pathogenesis of salmonellosis (8), and tissue culture studies have demonstrated that gamma interferon (IFN- γ) primes murine macrophages for efficient killing of *Salmonella* (9, 16, 32). Different models have been used to demonstrate the role of IFN- γ in enhancing both earlier oxidative killing (9) and later nitrosative killing and control of *Salmonella* (32). Murine macrophages control internalized *Salmonella* by the rapid initial generation of reactive oxygen species by the respiratory burst phagocyte oxidase (phox) over the first 6 to 12 h, causing rapid bacterial killing.

This is followed by production of nitric oxide products, leading to sustained later bacteriostasis at 24 to 48 h (31). Vazquez-Torres et al. (32) have used phox^{-/-} and iNOS^{-/-} mouse strains to show that overnight priming of primary murine cells with IFN- γ and lipopolysaccharide (LPS) mainly enhances later, nitrogen-mediated suppression of wild-type *S. enterica* serovar Typhimurium in mouse macrophages, with no measurable effect on early oxidative killing in the first hours after internalization. In contrast to data from murine models, there is limited information about the effect of IFN- γ during infection of *Salmonella* spp. in primary human cells. Stevanin et al. have used a *Salmonella hmp* mutant to demonstrate a role for nitrosative killing of salmonella in primary human cells, which is detectable in the early stages of infection (28), but the effect of IFN- γ on this killing pathway is unknown. Janssen et al. have found that recombinant IFN- γ (rIFN- γ) promotes intracellular killing of *S. enterica* serovar Typhimurium in human monocyte-derived macrophages (MDM) from healthy volunteers and that this response is defective in MDM from patients with IFN- γ receptor deficiency (15).

Type 1 cytokines perform key roles in the host response to salmonella infection; interleukin-12 (IL-12) is produced by cells of monocyte-macrophage lineage in response to infection and primes lymphocytes for high-output production of IFN- γ , which in turn activates macrophages for enhanced killing of intracellular pathogens. IL-10 and IL-4 form part of a counter-

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regulatory mechanism (6). IFN- γ and IL-12 are essential for successful host defense against salmonellosis in the mouse, and IFN- γ has been shown using different models to play a central role in both the early limitation of replication and later clearance of *Salmonella* (11, 19, 20, 22, 25). Neutralization of IL-12 results in impaired survival (17) and reduced tissue levels of IFN- γ , while exogenous IFN- γ can counter the deleterious effects of IL-12 neutralization (18).

Human genetic studies have demonstrated that type 1 cytokines also play an essential role in the control of salmonellosis in humans. Adults and children with genetic deficiencies of the IL-12 receptor (2), IL-12 p40 subunit (3), or the IFN- γ receptor (24) have increased susceptibility to serious infections with *Salmonella* spp. and atypical *Mycobacteria*. The therapeutic use of IFN- γ has been described in patients with other intracellular infections, including leishmaniasis and some *Mycobacteria* spp. infections (13), and a phase 1 trial of IL-12 in HIV infection has also been reported (14). Type 1 cytokines may have clinical utility as adjunctive therapy in patients who are susceptible to severe and recurrent salmonellosis.

In this study we measured the effect of priming with rIFN- γ on the ability of human MDM to internalize and kill *S. enterica* serovar Typhimurium and to release type 1 cytokines in response to the infection.

MATERIALS AND METHODS

Preparation of MDM and priming of cells with IFN- γ . Volunteers for blood donation were recruited among healthy hospital staff and students (age range, 21 to 46 years). Peripheral blood mononuclear cells were separated from donated sterile venous blood (CPDA blood collection system; Baxter, United Kingdom) by centrifugation on a Ficoll-Paque Plus density gradient (Amersham Biosciences, Uppsala, Sweden) and then washed and resuspended at 10^6 cells/ml in RPMI 1640 (Life Technologies, Renfrewshire, United Kingdom) supplemented with 10% heat-inactivated newborn calf serum (Life Technologies), and 1-ml aliquots were cultured at 37°C in 5% CO₂ in 24-well tissue culture trays (Nunc, United Kingdom). Cells to be used for fluorescence microscopy were prepared in wells containing sterile glass coverslips (BDH, United Kingdom). Medium was exchanged after 24 h, and the adherent population of MDM thereby selected was subsequently cultured in RPMI medium supplemented with 10% heat-inactivated fetal calf serum (HI-FCS) (Life Technologies). Day 8 MDM were primed using medium containing 100 ng/ml (1,000 IU/ml) recombinant human IFN- γ (PharMingen, San Diego, Calif.) in RPMI medium with 10% HI-FCS for a period of 72 h (days 8 to 11) prior to infection with *S. enterica* serovar Typhimurium. Control MDM were mock-primed with medium alone.

Infection of MDM with *S. enterica* serovar Typhimurium. On the day of infection of MDM (day 11), stationary phase *S. enterica* serovar Typhimurium (NCTC 12023) from an overnight stationary-phase brain heart infusion broth (Oxoid Ltd., Basingstoke, United Kingdom) was inoculated into fresh brain heart infusion broth, cultured at 37°C to mid-exponential phase (2 h), washed, and resuspended in RPMI medium with 10% HI-FCS. The viable count of the inoculum was confirmed for each experiment using a dilution plating method. Triplicate sets of primed and unprimed MDM were inoculated with 5×10^7 bacteria/well. Control MDM were mock-infected in the same manner. Additional MDM that had been fixed with 2% paraformaldehyde and washed (three times) with phosphate-buffered saline (PBS) were also infected as internalization controls. Bacterial suspensions were coincubated with MDM without centrifugation for 15 min at 37°C in 5% CO₂. Additional primed and unprimed MDM were infected as above but maintained at a temperature of 4°C to permit binding of bacteria to MDM without internalization in order to determine whether IFN- γ priming altered bacterial binding to the cell surface independently of internalization. The end of the 15-min period of the capture/binding/internalization of bacteria by macrophages was denoted time zero. Triplicate sets of coverslip-adherent MDM which had been infected at 4°C and at 37°C were washed and fixed in 2% paraformaldehyde at the end of internalization (time zero) for fluorescence microscopy.

Measurement of intracellular survival of *S. enterica* serovar Typhimurium. At time zero excess bacteria were removed by washing with PBS, and cells were

incubated at 37°C in prewarmed medium containing gentamicin (200 μ g/ml) (Roussel Labs Ltd., Uxbridge, United Kingdom) for 30 min to kill remaining extracellular *Salmonella* and prevent ongoing replication and internalization. At 0.5 h, cells were washed and then lysed by treatment with PBS containing 1% saponin for 12 min. The yield of CFU released from lysed cells in each well was determined using a dilution plating method. In the remaining wells, medium was replaced with medium containing gentamicin at a lower concentration (20 μ g/ml) to suppress bacterial replication for the remainder of the experimental period of 24 h. At 4 h and 24 h, conditioned medium was harvested from experimental and control MDM wells; the cells were washed and then lysed, and the yield of CFU from each well was determined as above.

Two controls were used at all time points to ensure that there was adequate external killing of excess bacteria prior to lysis. One was plating of the final (third) wash supernatant from live infected cells prior to lysis, and the second was the yield from fixed internalization control cells after saponin treatment. The number of bacteria in both controls was required to be at least 1 log below the number subsequently released by lysis of live infected cells. Experiments that did not meet these criteria were rejected. Gentamicin at 200 μ g/ml was determined in development experiments to be the minimum dose that could reliably achieve these criteria.

In order to confirm that external gentamicin did not reduce the yield of internalized bacteria, development experiments were conducted with increasing concentrations of gentamicin from 50 to 400 μ g/ml. These established that while increasing concentrations of gentamicin resulted in increasingly efficient killing of residual external bacteria, the yield of internalized bacteria released from cell lysis was constant, regardless of the concentration of gentamicin used.

Measurement and inhibition of oxidative burst. To inhibit the macrophage oxidative burst, we added diphenyleneiodonium chloride (DPI) to the cell medium at a concentration of 25 mM for 30 min prior to challenge of cells. Cells were then washed and incubated with *S. enterica* serovar Typhimurium for 15 min as described above. A gentamicin exclusion assay was performed as above, with viable counts determined at 1 h, to determine the effect of DPI on killing of *S. enterica* serovar Typhimurium in primed and unprimed cells.

As a positive control for respiratory burst, phorbol 12-myristate 13-acetate (PMA; 100 ng/ml) was added to separate wells for the same period (15 min). MDM that had been challenged with PMA or with *S. enterica* serovar Typhimurium (as above) were then washed and placed in medium containing 200 μ g/ml gentamicin and 25 μ M lucigenin (Sigma, Poole, United Kingdom). The resultant chemiluminescence was measured over 60 min with a Lumistar Galaxy (BMG Labtechnologies, Offenbach, Germany) instrument.

Measurement of MDM cell density and viability. At 4 h and 24 h, experimental and control MDM were stained with trypan blue, and standardized cell density and viability counts were performed in each well quadrant by a single investigator (M.E.L.), who was blinded to experimental conditions, using an inverted microscope.

Fluorescence microscopy. Each fixed coverslip was washed and stained for 12 min with 1:20 rabbit anti-*Salmonella* poly(A) antibody (Difco, Detroit, Mich.) and then washed and stained for 12 min with 1:20 fluorescein-isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (Sigma, Poole, United Kingdom). Finally, coverslips were washed and stained with 4'6'-diamidino-2-phenylindole (DAPI) (Molecular Probes, Eugene, Oreg.) to counterstain bacterial DNA and cell nuclei and mounted on slides in an antifade agent (Vectashield; Vecta, Burlingame, CA). All slides were coded to disguise experimental conditions and counted by a single investigator (M.A.G.) using a Leica DMRB fluorescence microscope (Leica, Wetzlar, Germany). Cell density counts were determined on triplicate coverslips for each condition at time zero, counting 10 grids in each quadrant (approximately 300 to 400 cells per coverslip). Cell-associated bacteria were defined as bacteria that lay within the limits of the cell cytoplasm. Total cell-associated bacteria per cell and the proportion of cells with any cell-associated bacteria were counted at time zero for each coverslip. Cell-associated bacteria were further defined either as externally bound or internalized bacteria. DAPI-counterstained bacteria that were internalized were distinguished from externally bound bacteria by the absence of a ring of fluorescein-isothiocyanate-stained anti-immunoglobulin G on internalized bacteria.

Cytokine measurement. The concentrations of IL-12 (p40/p70) and IL-10 in triplicate 24-h conditioned medium samples, which were harvested and stored at -80°C, were determined using enzyme-linked immunosorbent assay kits (R&D Systems, Abingdon, United Kingdom) following the manufacturer's instructions.

Statistical analysis. Data were analyzed using Stata 8 (Statacorp) software. All parameters were tested for normality of distribution using the Wilks-Shapiro test and summarized. Comparisons between experimental conditions were made using a two-tailed paired *t* test for data with a normally distributed difference and a Wilcoxon's matched pairs signed-rank test for parametric data. Differences in

FIG. 1. The effect of priming MDM with IFN- γ for 72 h on cell association and internalization of *S. enterica* serovar Typhimurium over a 15-min incubation at 4°C or 37°C measured by fluorescence microscopy. White columns represent total cell-associated bacteria, and gray columns represent cell-associated bacteria that were internalized (90% of cell-associated bacteria in warm primed and unprimed cells). Figures are derived from triplicate data for eight different subjects for each condition. *, $P = 0.035$; **, $P = 0.025$.

chemiluminescence were determined by analysis of variance and a t test with a Bonferroni correction for multiple comparisons.

RESULTS

Priming with IFN- γ for 72 h increases internalization of *S. enterica* serovar Typhimurium by human macrophages. By fluorescence microscopy, MDM that were infected for 15 min at 4°C exhibited a low proportion of MDM with cell-associated bacteria (mean, 5% of cells) and low numbers of cell-associated bacteria per cell. No bacteria had been internalized, so all cell-associated bacteria were externally bound (Fig. 1). There were no differences in these parameters between primed and unprimed MDM.

By contrast, MDM that were infected for 15 min at 37°C exhibited an increased overall proportion of MDM with cell-associated bacteria and increased numbers of cell-associated bacteria per cell. Under these conditions, the effect of priming MDM with IFN- γ for 72 h was to modestly increase the proportion of MDM with cell-associated bacteria (31% primed versus 26% unprimed cells, $P = 0.036$) and to increase both the total number of cell-associated bacteria per cell and the number of internalized bacteria per cell by 67% (Fig. 1). Under both conditions, 90% of all cell-associated bacteria were internalized by 15 min, leaving only 10% of bacteria externally bound. There was no effect of rIFN- γ on the proportion of cell-associated bacteria that were internalized.

Intracellular killing of *S. enterica* serovar Typhimurium by human macrophages is enhanced by priming with IFN- γ . The yields of surviving bacteria from 72-h primed MDM are shown in Fig. 2. The number of bacteria detected in the final control wash before lysis was more than 10-fold below the yield following lysis in all cases. There was no difference in the numbers of bacteria removed in the control washes prior to lysis (mean at third wash, 1.6×10^2 primed versus 1.9×10^2 unprimed cells; $P = 0.12$). The bacterial yield from paraformaldehyde-fixed control MDM was less than 1% of that from experimental wells in every case, and there was no difference between

FIG. 2. The effect of priming MDM with IFN for 72 h on the absolute yield of CFU from lysed MDM over 24 h after phagocytosis of *S. enterica* serovar Typhimurium. The graph shows medians (lines), IQRs (boxes), and 5 to 95% ranges (whiskers). Figures are derived from triplicate data for eight different subjects for each condition. t, time; *, $P = 0.0001$; **, $P = 0.004$.

primed and unprimed cells (data not shown), confirming that gentamicin killed all noninternalized *Salmonella* bacteria equally in both groups.

Despite modestly enhanced internalization of bacteria among primed cells, there was a highly significant reduction in absolute yield of CFU at all time points from MDM that were primed for 72 h compared to unprimed cells ($P = 0.001$) (Fig. 2).

The proportional reduction in yield following IFN priming was quantified as the recovery ratio, calculated as the ratio of the yield of CFU from unprimed cells to the yield of CFU from primed cells; a ratio of >1 therefore implies a proportional reduction in the yield of CFU from primed cells, due to intracellular killing of bacteria. MDM primed for 72 h yielded 3.6-fold fewer bacteria (median) than unprimed cells by 0.5 h (interquartile range [IQR], 3.1 to 6.4) and this enhanced killing was sustained to 4 h and 24 h (median recovery ratio of 3.4-fold; IQR, 2.7 to 4.6). Priming with rIFN- γ therefore enhanced intracellular killing of *S. enterica* serovar Typhimurium as early as 0.5 h, and the effect was sustained at the same magnitude up to 24 h (Fig. 3, top).

MDM that had been primed with the same concentration of IFN for only 24 h ($n = 5$) (Table 1) exhibited no significant difference in yields or recovery ratio between primed and unprimed cells at 0.5 h (median recovery ratio of 0.83; IQR, 0.6 to 1.9), suggesting that prolonged priming with IFN- γ is necessary to achieve optimal early killing in human MDM under the conditions used in this study.

Oxidative mechanisms are not responsible for the enhanced early killing observed in IFN- γ -primed cells. To determine whether IFN- γ -enhanced killing that was apparent within 30 min of internalization is due to the phagocytic oxidative burst, we pretreated primed and unprimed MDM with 25 mM DPI prior to infection with *S. enterica* serovar Typhimurium (Fig. 3, bottom). Using unprimed MDM, DPI treatment did not sig-

FIG. 3. The proportional yield of *S. enterica* serovar Typhimurium from infected MDM under different conditions, calculated as the ratio of the yield of CFU from untreated MDM to the yield of CFU from treated MDM in gentamicin protection assays. A ratio greater than 1 implies a proportional reduction in the yield of *S. enterica* serovar Typhimurium from primed MDM compared to untreated MDM, due to intracellular killing. The graphs show medians (lines), interquartile ranges (boxes), and 5 to 95% ranges (whiskers); they are derived from triplicate data for eight different subjects for each condition. The top graph shows the effect of priming with IFN- γ for 72 h on the yield of *S. enterica* serovar Typhimurium after 0.5 h, 4 h, and 24 h. The bottom graph shows the effect of DPI, an inhibitor of the oxidative burst, on early (1 h) yield of *S. enterica* serovar Typhimurium from IFN-primed and unprimed MDM. t, time.

nificantly alter the baseline yield of CFU at 1 h compared to cells not treated with DPI (for CFU for cells without IFN and without DPI versus CFU without IFN and with DPI treatment, $P = 0.91$) (Table 1) or the recovery ratio relative to untreated cells (median recovery ratio with DPI treatment, 1.1; IQR, 0.7 to 1.7) (Fig. 3, bottom).

Using 72-h primed cells, we again demonstrated an in-

creased recovery ratio resulting from priming with IFN- γ compared to unprimed cells ($P = 0.018$) (Fig. 3, bottom), but pretreatment with DPI failed to abrogate the reduction in yield of CFU that was seen in primed versus unprimed MDM in the gentamicin exclusion assays (for values from cells with IFN and without DPI versus cells with IFN and without DPI, $P = 0.436$) (Table 1), and the recovery ratio was not significantly abrogated in primed cells versus primed cells treated with DPI (with IFN, a median killing ratio of 3.0 and an IQR of 2.5 to 6.4; with IFN and with DPI, a median killing ratio of 2.1 and an IQR of 1.1 to 6.5; $P = 0.24$) (Fig. 3, bottom).

To ensure that the model was robust, we measured chemiluminescence of lucigenin in unprimed and 72-h primed MDM in response to *S. enterica* serovar Typhimurium and PMA, a positive control (see Fig. 4). Control MDM (Fig. 4) were shown to be competent to produce a rapid and significant oxidative burst following stimulation with PMA ($P = 0.001$; $n = 8$), and preincubation of MDM with 25 mM DPI for 30 min prior to stimulation was shown to be sufficient to fully abrogate the oxidative burst produced by PMA at a saturating concentration in unprimed control cells ($P = 0.013$; $n = 8$).

We did not detect lucigenin-enhanced chemiluminescence significantly above an unchallenged control background from either primed or unprimed macrophages when challenged with *S. enterica* serovar Typhimurium, and DPI pretreatment had no significant effect on lucigenin-enhanced chemiluminescence in MDM infected with *S. enterica* serovar Typhimurium (Fig. 4). These data are all consistent with an early nonoxidative mechanism of killing by human MDM, which is enhanced by priming with IFN- γ .

The effect of priming with IFN- γ on cell viability. To determine whether any experimental treatments resulted in loss or death of cells and to exclude this as a confounding variable in the gentamicin protection assay, cell density counts were made and were not significantly different between experimental conditions at 0, 4, or 24 h (Table 1, data shown at 24 h). The percentage of cells taking up trypan blue was not different between conditions at 4 h, being below 5% for all conditions, but was marginally higher in 72-h primed infected cells compared to unprimed infected cells at 24 h (6.3% versus 1.7%; $P = 0.08$) (Table 1, data shown at 24 h).

The 72-h priming with IFN- γ modulates IL-10 and IL-12 release by human macrophages following phagocytosis of *S. enterica* serovar Typhimurium. Uninfected control MDM did not produce any detectable IL-10 or IL-12, regardless of whether they were primed with IFN- γ or not. IL-10 was decreased in 24-h conditioned medium collected from infected 72-h primed MDM (48.5 pg/ml; 95% confidence interval [CI], 14 to 163) compared to infected unprimed MDM (80.1 pg/ml; 95% CI, 27 to 237; $P = 0.019$; $n = 8$). By contrast IL-12 (p40/p70) was modestly but significantly increased in 24-h conditioned medium from primed MDM (43.2 pg/ml; 95% CI, 12.3 to 152) compared to unprimed MDM (29.3 pg/ml; 95% CI, 10.5 to 82.2; $P = 0.018$; $n = 8$).

DISCUSSION

The data presented here show that priming with IFN- γ for 72 h has several important effects on the interaction between *S. enterica* serovar Typhimurium and human MDM. First,

TABLE 1. The effect of 24- or 72-h priming with rIFN- γ and of pretreatment with DPI on bacterial yield, MDM cytokine secretion, cell density, and cell death after infection of human MDM with *S. enterica* serovar Typhimurium^a

Parameter and condition	Treatment	n	Mean	95% CI	P
Log ₁₀ yield of CFU at <i>t</i> = 0.5 h ($\pm$ 72-h priming)	No IFN	8	3.95	3.46–4.25	0.0001
	72-h IFN	8	3.17	2.72–3.62	
Log ₁₀ yield of CFU at <i>t</i> = 24 h ($\pm$ 72-h priming)	No IFN	8	3.67	3.26–4.09	0.0001
	72-h IFN	8	3.15	2.61–3.68	
Log ₁₀ yield of CFU at <i>t</i> = 0.5 h ($\pm$ 24-h priming)	No IFN	5	3.8	2.9–4.7	0.88
	24-h IFN	5	3.7	3.4–4.1	
Log ₁₀ yield of CFU at <i>t</i> = 24 h ($\pm$ 24-h priming)	No IFN	5	4.3	3.6–4.9	0.022
	24-h IFN	5	3.9	3.5–4.4	
Log ₁₀ yield of CFU at <i>t</i> = 1 h ($\pm$ DPI)	No IFN	8	3.9	3.1–4.8	0.91
	No IFN, +DPI	8	3.9	2.3–4.7	
Log ₁₀ yield of CFU at <i>t</i> = 1 h ($\pm$ DPI)	72-h IFN	8	3.4	2.7–4.2	0.44
	72-h IFN, +DPI	8	3.6	3.0–4.3	
Cells/quadrant at 24 h, uninfected cells	No IFN	8	106	63–147	0.18
	72-h IFN	8	98	70–128	
Cells/quadrant at 24 h, infected cells	No IFN	8	111	64–150	0.23
	72-h IFN	8	104	65–142	
% Cells dead at 24 h, uninfected cells	No IFN	8	0.8	0.6–1.0	0.077
	72-h IFN	8	3.5	0.3–7.0	
% Cells dead at 24 h, infected cells	No IFN	8	1.7	0.6–2.9	0.008
	72-h IFN	8	6.3	0.4–8.5	

^a All data are derived from triplicate observations for each experimental condition and are expressed as means and 95% confidence intervals (arithmetic for normal data, geometric for nonnormal data). *P* values are derived from a paired *t* test (normal data) or a Wilcoxon's signed-rank (nonnormal data) test. *n*, number of subjects. Data for the percentages of dead cells are based on trypan blue staining.

priming with IFN- γ caused an increase in the numbers of internalized *Salmonella* bacteria. This occurred by both a modest increase in the proportion of MDM that were able to bind and internalize *Salmonella* and a 67% increase in the number of *Salmonella* bacteria that were bound to and internalized by each cell. Efficiency of internalization of bound bacteria appeared to be the same in primed and unprimed cells (90%). To

our knowledge, this effect of IFN- γ on bacterial internalization has not been previously described.

The gut mucosa is relatively deficient in complement, and in order to mimic macrophage phagocytosis in this environment we have studied nonopsonized bacteria. The cellular receptors which engage nonopsonized *Salmonella* are undetermined, but the organism is capable of entering many eukaryotic cells

FIG. 4. The oxidative burst of MDM, which were unprimed or primed for 72 h with IFN- γ . Cells were stimulated with either PMA (as a positive control) or by infection with *S. enterica* serovar Typhimurium. Data for each condition are from MDM derived from eight volunteers.

quickly and efficiently, using a bacterial type III protein secretory apparatus encoded by the virulence locus *spiI*, which allows direct introduction of bacterial invasion proteins through the cell membrane and rapid phagocytosis of the organism. The high proportion of cell-associated bacteria that were internalized (90%) might be explained by such rapid and efficient internalization mechanisms or else by a relatively weak initial surface binding of salmonella to the cell surface, such that noninternalized bacteria might have been washed off before staining.

The mechanisms by which IFN- γ priming of MDM could increase the number of internalized *Salmonella* bacteria have not been studied, but our data suggest that IFN- γ increased the opportunity for bacterial-macrophage interaction, perhaps by an increase in the number or density of surface receptors or binding sites or by an increase in the size or mobility of the cell. Such morphological changes have previously been described following priming of human MDM with IFN- γ (21). We assessed 72-h IFN- γ -treated MDM compared to controls by analysis of forward scatter by cytometry, and image analysis of microscopy and found there was no difference between primed and unprimed cells (data not shown). Our experience cannot, therefore, corroborate the changes previously described and makes it less likely that morphological changes account for the differences we saw in internalization. Modification of the receptor repertoire of MDM by IFN- γ is a likely alternative mechanism, and a large number of candidate receptors could potentially be involved. This is an area requiring further work.

Second, despite the increased number of bacteria internalized by IFN- γ -primed cells, there was decreased bacterial survival in 72-h primed cells over the 24-h chase, with the yield of bacteria decreased 3.6-fold as early as 0.5 h and 3.4-fold at 24 h. Ingestion of *Salmonella* spp. is known to induce cell death in macrophages, and we were concerned that the observed loss of viable bacteria could be due to cell death or detachment during the experiment, particularly if SPI-1 were induced, as it may have been under our experimental conditions. The cell density count data, however, were unaffected by experimental conditions up to 24 h after experimental challenge. The small increase in the number of adherent nonviable cells at 24 h (1.8% versus 6.3%) would not explain the large differences noted in bacterial killing, particularly since cell death increased with time, only reaching significance at 24 h, while the difference in bacterial survival was greatest at 30 min.

It therefore seems likely that priming with IFN- γ increased the efficiency with which the primed cells killed *S. enterica* serovar Typhimurium in the period immediately following internalization. The experimental methodology used here to measure differences in intracellular viability relies upon the relatively poor penetration of cells by gentamicin. This antibiotic does penetrate cells by pinocytosis, albeit poorly (4), especially at the initial concentration used as a pulse to kill extracellular bacteria if used for more than 30 min. This effect has been shown to be relevant with regard to *Listeria* (1), which is an intracytoplasmic rather than a compartmentalized pathogen. There are no direct grounds for assuming that these findings would extend to the intracellular compartment in which *Salmonella* bacteria reside, and we demonstrated in development experiments that increasing doses of gentamicin up to 400 μ g/ml improved the efficiency of extracellular killing but

did not affect the yield of internalized bacteria. These are reasonable grounds for reassurance that the intracellular killing we observed was not an artifact of gentamicin penetration.

As the oxidative burst is thought to be an early mediator of killing, at least in murine macrophages, we investigated the mechanism of this IFN-enhanced early killing in human cells using DPI, a flavoenzyme inhibitor. DPI failed to abrogate the increased killing by IFN- γ -primed cells. We went on to investigate the lucigenin-enhanced chemiluminescence resulting from the oxidative burst in primed and unprimed MDM challenged with *S. enterica* serovar Typhimurium or PMA. We found that while MDM were competent to produce an immediate oxidative burst in response to PMA, which was fully abrogated by the DPI, MDM from the same individuals did not produce any measurable oxidative burst upon infection with *S. enterica* serovar Typhimurium. These data suggest that the very early killing we have observed following priming with IFN- γ is independent of oxidative mechanisms.

Vazquez-Torres et al. (32) showed that IFN- γ mainly enhances late (>24 h after phagocytosis), nitrogen-mediated suppression of wild-type *Salmonella* by mouse macrophages but not early oxidative killing. Recently, however, Foster et al. (9) used the J774 murine cell line to demonstrate that priming with increasing doses of IFN- γ alone produced an earlier sustained oxidative burst at 7 to 12 h (but not at 2 h), which was abrogated by a reactive oxygen intermediate (ROI) inhibitor. Priming with IFN- γ was required for control of infection with wild-type *S. enterica* serovar Typhimurium compared to avirulent mutants, and control coincided with the enhanced respiratory burst and was abolished by an ROI inhibitor (9).

Kagaya et al. (16) primed susceptible mouse peritoneal macrophages for 12 h and 48 h with IFN- γ and LPS and found that both durations of priming caused an increase in killing of *S. enterica* serovar Typhimurium from 8 h (but not as early as 2 h) but that a longer duration and higher dosage of IFN- γ were required to increase H₂O₂ production than were required to enhance killing of *Salmonella*. In addition, ROI scavengers failed to have any effect upon intracellular survival of *Salmonella*. They concluded that the increase in early killing induced by IFN- γ priming was not mediated by ROIs and attributed it to the observed increased rate of phagosome-lysosome fusion in primed cells, followed by an oxygen-independent early killing mechanism.

Some of these studies are apparently in mutual conflict, but this may be explained by the use of different murine cell types and strains, different doses or duration of priming, or the concurrent use of LPS during priming, which has discrete mechanisms to augment cell activation (12).

With regard to one previous study with human cells, our work is consistent with that of Janssen et al. (15), who showed in MDM from healthy controls that a similarly high dose of IFN (1,000 U) was required to achieve increased control of intracellular *Salmonella* at 18 h following experimental inoculation. The duration of priming (18 h) was, however, shorter than in our study, and they did not investigate the early component of killing.

No previous study in murine or human cells has reported an effect of priming with IFN- γ on killing of intracellular *Salmonella* as early as 30 min after internalization; peritoneal macrophages derived from parenterally IFN-treated mice (30) and

MDM derived from human subjects who had been pretreated with parenteral IFN- γ injections (23) failed to show an increase in killing of salmonella at early time points. The effects of IFN- γ on oxidative killing have generally been reported at 6 to 12 h. This may be because of methodological differences; some studies use the initial earliest yield of CFU following infection as a baseline denominator, reporting later yields at 6 to 12 h as a fraction or percentage of this figure. By prepriming with IFN and by making a careful simultaneous microscopic assessment of the rate of internalization after 15 min, we have been able to report the earliest available yield following gentamicin treatment in its own right and have shown a substantial reduction in yield of CFU from primed cells compared to unprimed cells even at this early time point, despite a modest increase in microscopic internalization in primed cells. Prolonged priming for 72 h was necessary to exhibit this enhanced early killing in human cells, and it was not seen after only 24 h of priming.

What could be the mechanism of this enhanced early microbial killing? Macrophages kill *Salmonella* bacteria following internalization by phagolysosome formation and maturation, by the effects of oxidative and nitrosative stress, and by antimicrobial cationic peptides and enzymes. We have noted a killing effect that requires relatively prolonged priming in order to be established, perhaps requiring substantial cellular synthesis or structural rearrangement, and yet that is present in immediately active form upon internalization. Interferon is known to have important effects on early phagosome-lysosome fusion (16) as well as later maturation (33) in intracellular infection. While the role of killing by reactive oxygen species themselves has been well accepted, there is increasing recognition of the role of proteases activated by ion influxes in the phagocytic vacuole as mediators in their own right (1), and these may prove to be important effectors in relation to other killing pathways, in addition to the oxidative burst. Finally, our own laboratory has established that there is a modest and very early contribution of nitrosative killing to salmonella by human MDM (28). Although the ability of IFN- γ to prime this is unknown, IFN- γ is known to prime mouse cells for enhanced nitrosative killing. The possible contribution of these various mechanisms to the very early component of killing in human cells and the role of IFN- γ in this process warrant further study.

Third, we have demonstrated that priming with IFN- γ modifies IL-10 and IL-12 production over 24 h by human MDM in response to *S. enterica* serovar Typhimurium infection. In our experiments, IL-10 production was decreased in primed MDM, and IL-12 levels were increased in primed compared to unprimed cells. These data are in keeping with the findings from animal models of *Salmonella* infection (29).

What are the likely molecular mechanisms of the effect of IFN- γ on bacterial killing and cellular cytokine secretion? Ligation of the IFN- γ receptor subunits by IFN- γ causes activation of several transduction pathways, most notably that mediated by the JAK kinases and STAT1, and binding of transcription factors to the gamma-activated sequences of a large number of IFN- γ -regulated genes. This causes alteration of early and late expression of mRNA for a host of regulatory, transcription, and growth factors and secreted chemokines (26). Rosenberger et al. found that a notable effect of IFN- γ

priming is to increase the steady-state expression of mRNA encoding several families of transcription factors (27). A human genetic mutation of the STAT1 gene causing loss of gamma activating factor (5) has been found in a patient with atypical mycobacterial infections, suggesting that this pathway, at least, is important in human defense against intracellular infections.

In summary, we have demonstrated that priming of human macrophages with IFN- γ has modest but significant effects on bacterial internalization and early killing and on type 1 cytokine responses in response to intracellular infection with *Salmonella* serovar Typhimurium. The mechanisms of these effects on internalization and killing are not clear and require further investigation, but the phagocyte oxidative burst does not contribute to the increase in very early killing observed in this model using IFN- γ -primed human MDM. The undoubted role of the oxidative burst in control of human salmonella infections is well demonstrated by patients with chronic granulomatous disease who suffer frequent serious salmonella infections, and these patients partially respond to exogenous IFN (7); but early nonoxidative IFN-dependent mechanisms may also be of additional clinical importance in human disease. Our findings may inform studies of the mechanism of invasive or recurrent *S. enterica* serovar Typhimurium in other conditions, including HIV/AIDS, and support possible future therapeutic use of IFN- γ in patients susceptible to recurrent or persistent salmonellosis.

ACKNOWLEDGMENTS

M.A.G. is a Wellcome Trust (United Kingdom) Training Fellow in Clinical Tropical Medicine. R.C.R. receives support from the Wellcome Trust and the BBSRC for related work. D.L.J. is supported by a grant from the Meningitis Research Foundation, and D.H.D. is a Wellcome Trust Advanced Fellow.

The authors thank S. B. Gordon for helpful discussions and comments.

The South Sheffield Research Ethics Committee approved this study and the protocol was adhered to throughout the study (protocol no. SSR 001206). All subjects gave informed consent.

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Editor: F. C. Fang