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Tumor Necrosis Factor Alpha Binding to Bacteria: Evidence for a High-Affinity Receptor and Alteration of Bacterial Virulence Properties

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Human and murine receptors for tumor necrosis factor alpha (TNF-α) are present on most somatic cells and have been characterized and cloned. In contrast, very little is currently known about whether TNF-α can bind to pathogens and whether such binding results in important biological consequences for the infected host. We now report that a number of gram-negative bacteria have receptors for TNF-α. Using ^{125}I-labeled TNF-α, we show that *Shigella flexneri* has 276 receptors for TNF-α, with a K_d of 2.5 nM. The binding of labeled TNF-α to these bacterial receptors can be inhibited by cold TNF-α but not by cold TNF-β. Binding of ^{125}I-TNF-α to *S. flexneri* was inhibited by trypsin treatment of bacterial cells or incubation at 52°C for 3 min. Monoclonal antibody to either the 55-kDa or the 75-kDa TNF-α receptor, which are present on different eukaryotic cells, had no effect on ^{125}I-TNF-α binding to bacteria. A number of gram-negative bacteria were capable of binding ^{125}I-TNF-α. Gram-positive bacteria bound significantly less ^{125}I-TNF-α than gram-negative bacteria. Pretreatment of *S. flexneri* with TNF-α resulted in enhanced bacterial invasion of HeLa cells and enhanced uptake by human and murine macrophages. Pretreatment of HeLa cells with antibody to the 55-kDa TNF-α receptor abrogated enhanced invasion of HeLa cells by TNF-α–bacterium complexes. These results suggest that TNF-α–bacterium complexes can interact with TNF-α receptors present on eukaryotic cells. This report shows that gram-negative bacteria have receptors for TNF-α and that a virulence property of a bacterium is altered as a consequence of cytokine binding.

Tumor necrosis factor alpha (TNF-α) is produced by monocytes/macrophages in response to different stimuli, including endotoxin and gram-negative bacteria (3, 20). TNF-α is a proinflammatory cytokine which has numerous biological activities (15, 26) believed to play important roles not only in host defense (11, 15, 18) but also in some of the pathological sequelae associated with different bacterial infections (7, 13, 30). The receptor(s) for TNFα has recently been characterized and cloned and is found on most somatic cells (1, 21, 25, 27, 29). While much is known about the biological effects of TNF-α and other proinflammatory cytokines and about their binding to eukaryotic cells, it has only recently been demonstrated that some cytokines can bind to different pathogens. Porat et al. (23) have shown that virulent strains of *Escherichia coli* have receptors for interleukin 1 (IL-1) and that IL-1 enhances the growth of these bacteria. The growth of virulent strains of *E. coli* also has been shown to be enhanced by IL-2 and granulocyte-macrophage colony-stimulating factor (5). Recently, Treseler et al. (31) have shown that *Candida albicans* can bind IL-2 and that these complexes can induce the proliferation of an IL-2-dependent cell. In contrast, little is currently known about whether TNF-α can bind to different pathogens and whether such binding alters the biological properties of these pathogens. We report here that bacteria can bind TNF-α, that *Shigella flexneri* has high-affinity receptors for TNF-α, and that an important virulence property of *S. flexneri* is enhanced as a consequence of TNF-α binding.

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MATERIALS AND METHODS

Reagents. ^{125}I-labeled recombinant human TNF-α (200 to 800 Ci/mmol) was obtained from commercial sources (Amersham Corp., Arlington Heights, Ill., or DuPont Co., Wilmington, Del.) or was produced by the iodogen method (8, 11) with recombinant human TNF-α (2×10^8 U/mg) obtained from UBI Inc., Lake Placid, N.Y. Unlabeled recombinant human TNF-α (2×10^7 U/mg) and TNF-β (3×10^7 U/mg) were also purchased from Genzyme, Boston, Mass. Rabbit antibody to TNF-α was obtained from Genzyme. Monoclonal antibody (immunoglobulin G1 [IgG1]) specific for TNF-α was purchased from UBI (200 ng of antibody per ml reported to give 50% neutralization of 20 ng of TNF-α per ml). An IgG1 control antibody was purchased from Biodesign International, Kennebunkport, Maine. Monoclonal antibodies to the 55- and 75-kDa TNF-α receptors were a kind gift from Manfred Brockhaus, Department of Protein Biochemistry, Hoffmann-La Roche, Basel, Switzerland. Utr-1 (anti-p75) and htr-9 (anti-p55) have been previously described (4, 6, 9). Soybean trypsin inhibitor and trypsin were obtained from Sigma, St. Louis, Mo.

Bacteria and cells. HeLa, U937, and RAW264.7 cells were obtained from the American Type Culture Collection, Rockville, Md. *S. flexneri* serotype 2a, SA100, a noninvasive isogenic variant (SA100NI) of SA100, and *Salmonella typhimurium* TML have been previously described (8). *Listeria monocytogenes*, *E. coli*, *S. aureus*, and *Streptococcus mitis* were all obtained from Clinical Microbiology Services of John Sealy Hospital at University of Texas Medical Branch, Galveston, Tex.

Bacterium–^{125}I-TNF-α binding. Overnight cultures of bac-

teria were grown to mid-logarithmic phase and assessed for their ability to bind ^{125}I-TNF-α at either 37 or 4°C. For binding at 37°C, bacteria were incubated 10 min at 37°C with 0.01% azide in RPMI 1640 medium and then washed twice. Bacteria were not treated with azide when binding was assessed at 4°C. Bacteria (2×10^9 CFU) were then incubated in 250 μl of phosphate-buffered saline (PBS) containing 1% heat-inactivated fetal calf serum or 1% bovine serum albumin (BSA) plus various concentrations of labeled or unlabeled TNF-α or TNF-β. After appropriate incubation at 37 or 4°C with mixing every 10 to 20 min, bacterium-TNF mixtures were transferred to a Swinnex syringe-filter fitted with a 0.45-μm-pore-size nitrocellulose filter. Tubes (BSA-coated microcentrifuge tubes) which contained bacterium-TNF mixtures were washed with 250 μl of RPMI medium, and this volume was added to the syringe-filter. Bacteria were then collected by filtration, and filters containing bacteria were washed with 1 ml of RPMI medium. Filters were then assessed for the amount of bound ^{125}I-TNF-α. Filters used in these experiments were pretreated with fetal calf serum and then washed with RPMI medium. ^{125}I-TNF-α binding to filters in the absence of bacteria was ≤6% of the total counts per minute added to the binding mixture. Nonspecific binding to bacteria was assessed by using a ≥100-fold excess of unlabeled TNF-α. This value was subtracted from the number of counts per minute obtained from the collected ^{125}I-TNF–bacterium complexes, and the resultant value was defined as specific binding. Nonspecific binding was always ≤10% of total bound counts per minute. Scatchard analysis was performed as described by Stuart (28).

Trypsin treatment of bacteria was achieved by incubating 4×10^9 CFU of *S. flexneri* SA100 in 10 ml of PBS with trypsin (100 μg/ml; Sigma) for 30 min at 37°C. Soybean trypsin inhibitor (100 μg/ml; Sigma) was then added, and after 15 min at 37°C, the bacteria were pelleted and washed. Trypsin-treated and control-treated bacteria were then assessed for their ability to bind ^{125}I-TNF-α. In some experiments *S. flexneri* was pretreated with htr-9, Utr-1, or control antibody (5 μg/ml) and assessed for ^{125}I-TNF-α binding as described above.

^{125}I-TNF-α binding to *S. flexneri* varied between different commercial lots of ^{125}I-TNF-α. This appeared to correlate with the level of biological activity retained by the ^{125}I-labeled TNF-α. Little binding was detected when ^{125}I-TNF-α had <20% biological activity as measured by the L-929 bioassay.

Phagocytic uptake and HeLa cell invasion by TNF-α-pretreated *S. flexneri*. *S. flexneri* (5×10^4 to 10^5 CFU) were incubated in 1 ml of RPMI 1640 with or without various concentrations of TNF-α (specific activity = 2×10^7 to 2×10^8 U/mg). After 4 h at 4°C with mixing every 20 min, bacteria were diluted fivefold, pelleted by centrifugation (1,500 × *g*), and then washed with 4 ml of medium. Bacterial cells in 250 μl were then added to monolayers of HeLa cells or a mouse macrophage cell line (RAW264.7) or to suspension cultures of a human monocytic cell line (U937). After 1 h of incubation at 37°C, cells were washed three times with medium and lysed with 0.1% Triton X-100. Quantitation of cell-associated bacteria was determined by spreading aliquots of serially diluted cell lysates on Congo red agar. In some experiments, TNF-α-pretreated bacteria were assessed for their ability to invade HeLa cells. HeLa cell invasion was assessed as previously described (19). Briefly, HeLa cell monolayers (2×10^5 per well) were established in 24-well plates. HeLa cell monolayers were exposed to *S. flexneri* for 90 min. Noninternalized *S. flexneri* were counterselected with 50 μg of kanamycin per ml for 2 h. *Shigella*-infected cells were overlaid with 300 μl of 0.5% agarose in distilled water followed by 300 μl of 2× L broth which contained 0.5% agar. Bacterial colonies detected after 24 h of incubation at 37°C represented single or adjacently infected HeLa cells. To assess the role of TNF receptors in invasion by TNF-α–bacterium complexes, HeLa cells were pretreated with htr-9 or Utr-1 (10 μg/ml) for 1 h at 37°C and then challenged with *S. flexneri* complexes.

RESULTS AND DISCUSSION

TNF-α binding to bacteria was investigated by using ^{125}I-labeled human recombinant TNF-α with bacterium–^{125}I-TNF-α complexes quantitated by filtration. As illustrated in Fig. 1, *S. flexneri*, a gram-negative bacterium, bound significant levels of ^{125}I-labeled TNF-α. This binding was saturable, with maximal binding being attained at 40 min when binding was measured at 37°C (Fig. 1A and B). When binding was measured at 4°C, maximal binding was observed at 4 h (data not presented). When binding was assessed at 37°C, bacteria were pretreated with azide to inhibit bacterial replication. ^{125}I-TNF-α binding to azide-treated bacteria was identical to binding to untreated bacteria during the first 20 min of incubation at 37°C. However, since non-azide-treated bacteria had a doubling time of approximately 30 to 40 min in the binding medium, non-azide-treated bacteria consistently bound more TNF-α at time periods longer than 20 min. The binding of ^{125}I-TNF-α to *S. flexneri* was inhibited by various concentrations of unlabeled TNF-α; Scatchard analysis indicated a K_d of 2.5 nM, with 276 binding sites for TNF-α per bacterium (Fig. 1C). Interestingly, unlabeled TNF-β was ineffective at competing with ^{125}I-TNF-α for binding to *S. flexneri* (Fig. 1C). In contrast, TNF-α receptors on eukaryotic cells can be occupied by both TNF-α and TNF-β (1, 27). Thus, the bacterial receptors for TNF-α appear to differ from TNF-α receptors on eukaryotic cells with regard to binding specificity for TNF-α versus TNF-β. In this regard, using monoclonal antibodies to the 75- and 55-kDa TNF receptors present on eukaryotic cells, we found no cross-reactive epitopes present on bacteria by Western blotting (immunoblotting) or enzyme-linked immunosorbent assay techniques (data not presented). In addition, we could not block binding of TNF-α to bacteria with these antibodies (Fig. 2). However, pretreatment of ^{125}I-TNF-α with a monoclonal antibody to TNF-α completely inhibited TNF-α binding to bacteria (Fig. 2).

TNF-α binding to bacteria appeared to utilize a receptor which has a protein component. Heating (52°C for 3 min), formalin fixation, and trypsin treatment of bacteria each resulted in complete to partial reduction of TNF-α binding (Table 1). These results suggest that TNF-α binding involves a protein component. However, the possibility that carbohydrate and/or another structure(s) could contribute toward TNF-α binding also exists. IL-2 binding to *C. albicans* was shown to be inhibited by mannose and to be mediated by low-affinity receptors (31). Lectin-like binding has also been proposed to explain the binding of IL-1 to *E. coli* (23).

The ability to bind TNF-α was not unique to *S. flexneri*. An avirulent *E. coli* strain (DH5α) and a virulent *Salmonella typhimurium* strain (TML) both bound significant levels of ^{125}I-TNF-α (Table 2). Further, both a virulent *S. flexneri* strain (SA100) and an isogenic nonvirulent *S. flexneri* strain (SA100NI) (8) appeared to bind comparable levels of ^{125}I-TNF-α (Table 2). In addition, no difference between levels of

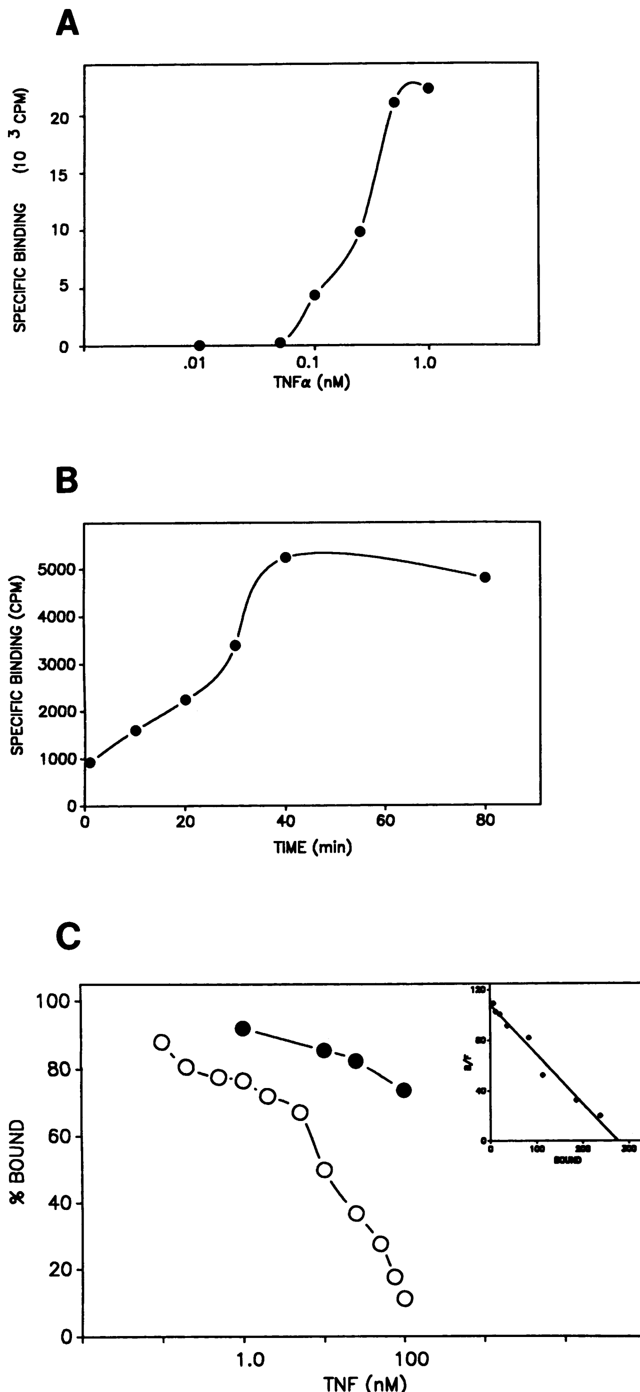

FIG. 1. TNF-α binding to *S. flexneri*. (A) Saturation binding curve of ^{125}I-TNF-α after 40 min at 37°C with *S. flexneri*. (B) Time course of ^{125}I-TNF-α binding at 37°C with 0.1 nM ^{125}I-TNF-α (3×10^4 to 4×10^4 cpm). (C) Competition of TNF-α and TNF-β with ^{125}I-TNF-α for binding to *S. flexneri*. ^{125}I-TNF-α (0.1 nM) was incubated (37°C, 40 min) with *S. flexneri*, along with various concentrations of unlabeled TNF-α (○) or TNF-β (●). The results are plotted as specific binding relative to results of an assay with no added competitor. (Inset) Scatchard plot of data. All data are means of duplicate determinations and are representative of at least two separate experiments.

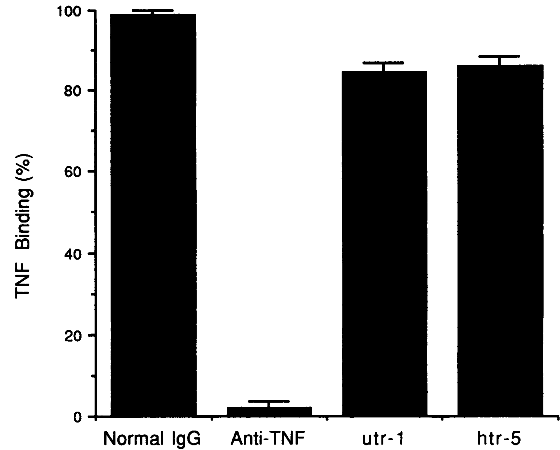

FIG. 2. Antibody to TNF-α inhibits ^{125}I-TNF-α binding to *S. flexneri*. ^{125}I-TNF-α binding was assessed as described in the legend to Fig. 1. ^{125}I-TNF-α (1 nM) was incubated in medium alone or with 25 ng of monoclonal antibody to TNF-α per ml or control IgG1. After 45 min at room temperature, the ^{125}I-TNF-α mixtures were assessed for binding to *S. flexneri* at 4°C. In a separate experiment, *S. flexneri* (2×10^8 CFU) were pretreated with either medium, control IgG1 (4 μg/ml, mouse anti-human IgG), htr-9 (4 μg/ml), or Utr-1 (4 μg/ml) and assessed for ^{125}I-TNF-α binding. The amount of bound ^{125}I-TNF-α is expressed as a percentage of the control value, obtained with bacteria or ^{125}I-TNF-α incubated in medium only. Data are means ± standard deviations of duplicate determinations and are representative of at least two separate experiments.

TNF-α bound by rough strains and levels bound by smooth strains of *Salmonella* was found (data not presented). These data may indicate that bacterium–TNF-α binding may be a common property of gram-negative bacteria. In contrast, significantly lower levels of ^{125}I-TNF-α binding occurred when a number of gram-positive bacteria were assessed (Table 2). Whether high- or low-affinity TNF receptors are present on these bacteria is unknown and currently under investigation.

We next investigated the biological consequences that might follow TNF-α binding to a bacterium. In this regard, an important host defense against invasive bacterial pathogens is the phagocytic uptake and antimicrobial activities of polymorphonuclear leukocytes and macrophages. TNF-α has been shown to act on both of these cell populations by increasing phagocytic functions and antimicrobial activities (12, 14, 17). Therefore, we investigated whether pretreat-

TABLE 1. Characteristics of TNF-α–bacterium binding

S. flexneri treatment[a]	Specific binding (cpm) of TNF-α at[b]:	
	4°C	37°C
None	7,065 ± 48	5,226 ± 83
Formalin	5,818 ± 33	4,176 ± 66
Heat	3,199 ± 91	3,056 ± 8
None	3,588 ± 99	
Trypsin	327 ± 97	

[a] *S. flexneri* was assessed for TNF-α binding following heat treatment (52°C for 3 min), fixation by 1% formaldehyde, or trypsin treatment.

[b] Data are means ± standard deviations of duplicate determinations per condition and are from one experiment whose data were representative of data obtained from three experiments. TNF-α was used at 0.1 nM (30,000 cpm).

TABLE 2. TNF-α binds to gram-positive and gram-negative bacteria

Bacterium[a]	Specific binding (cpm) of TNF-α at[b]:	
	4°C	37°C
Gram negative		
S. typhimurium TML	6,591 ± 124	5,994 ± 404
E. coli DHSα	5,801 ± 58	6,150 ± 150
S. flexneri SA100	4,951 ± 160	5,850 ± 300
S. flexneri SA100NI	5,213 ± 65	5,414 ± 215
Gram positive		
L. monocytogenes	1,815 ± 16	
S. aureus	1,905 ± 111	
S. mitis	2,101 ± 40	

[a] ^{125}I-TNF-α binding to different bacteria was assessed as described in Materials and Methods and in the legend to Fig. 1. Bacteria were incubated with 0.1 nM ^{125}I-TNFα (3×10^4 to 4×10^4 cpm) for 40 min at 37°C or for 4 h at 4°C.

[b] Data are means ± standard deviations of duplicate determinations per experimental condition and are from one experiment whose data were representative of data obtained from six experiments.

ment of *S. flexneri* SA100 with TNF-α had any effect on the phagocytic uptake of these complexes. *S. flexneri* SA100 (5×10^4 CFU) was pretreated with saturating levels of TNF-α for 4 h at 4°C. TNF-α-treated bacteria were then washed and compared with control-treated bacteria for their ability to be phagocytized by human and mouse macrophage cell lines. A two- to fivefold increase in phagocytic uptake and/or cell-associated bacteria was consistently observed with TNF-bacterium complexes compared with control-treated bacteria. Specifically, uptake (in CFU per 5×10^5 macrophages) was as follows: for U937 cells, 19 ± 2 (control-treated *S. flexneri*) versus 106 ± 4 (TNF-α–*Shigella* complex); for RAW264.7 cells, 293 ± 39 (control) versus 900 ± 103 (TNF-α–*Shigella* complex). (Data are means ± standard deviations of triplicate determinations of one representative experiment.) These results suggest that TNF-α–bacterium complexes are more susceptible than bacteria alone to phagocytic uptake. The ability of TNF-α–gram-positive-bacterium complexes to be taken up more efficiently by macrophages and the ability of TNF-α-bound bacteria to survive within macrophages are currently under investigation.

An important virulence determinant of *S. flexneri* is its ability to penetrate and replicate within epithelial cells of the intestinal mucosa, which then results in tissue damage (24). Thus, we investigated whether pretreatment of *S. flexneri* SA100 with TNF-α had any effect on its ability to invade HeLa cells. *S. flexneri* SA100 (10^5 CFU), pretreated with saturating levels of TNF-α for 4 h at 4°C and then washed, had a 20-fold enhancement in cellular invasion (Fig. 3). Binding of TNF-α to bacteria was a prerequisite for enhanced bacterial uptake, since adding TNF-α to bacterium-HeLa cell cultures at the initiation of invasion resulted in no enhancement of bacterial uptake by HeLa cells (data not presented). Enhanced cellular uptake by TNF-α-pretreated bacteria was dependent upon a bacterial virulence factor(s). Noninvasive *S. flexneri* (SA100NI), which bound equivalent levels of ^{125}I-TNF-α (Table 1), could not be converted to an invasive phenotype by TNF-α binding (Fig. 3). These results show that bacterial invasion of nonprofessional phagocytic cells (HeLa cells) is greatly enhanced subsequent to TNF-α binding to the surface of the bacteria.

Recently, Baird et al. (2) have shown that herpes simplex

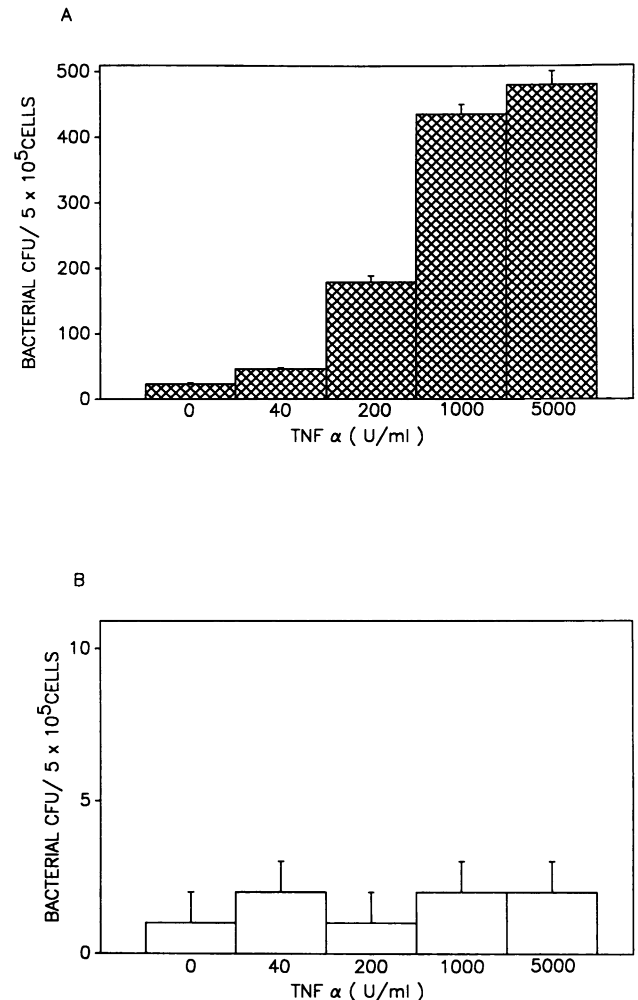

FIG. 3. Enhancement of cellular invasion by TNF-α-pretreated *S. flexneri*. (A) HeLa cell invasion by TNF-α-pretreated versus untreated *S. flexneri* SA100; (B) HeLa cell invasion by TNF-α-pretreated versus untreated *S. flexneri* SA100NI. *S. flexneri* (SA100 or SA100NI) was incubated in 1 ml of RPMI 1640 with or without various concentrations of TNF-α (specific activity = 2×10^8 to 2×10^7 U/mg). After 4 h at 4°C, bacteria (10^5 CFU/ml) were washed and treated as described in Materials and Methods. Bacteria (pretreated with medium or TNF-α) were then assessed for their ability to invade HeLa cells. HeLa cell invasion was assessed as previously described (20). Data are from triplicate determinations and are representative of eight experiments.

virus type 1 can bind basic fibroblast growth factor and that this association appears to be necessary for virus penetration into target cells. We therefore investigated whether enhanced HeLa cell invasion by TNF-*Shigella* complexes was being mediated by TNF-*Shigella* interactions with the TNF-α receptors present on HeLa cells. HeLa cells have been previously shown to express only the p55 form of the TNF-α receptor (4). Monoclonal antibodies to the p55 TNF-α receptor (htr-9) have been shown to completely block ^{125}I-TNF-α binding to HeLa cells. In contrast, antibody to the p75 TNF-α receptor (Utr-1) was shown to have no effect on ^{125}I-TNF-α binding to HeLa cells (4). To investigate the role of TNF-α receptors present on HeLa cells in enhanced invasion by TNF-α–*Shigella* complexes, HeLa cells were pretreated with either Utr-1 (anti-p75) or

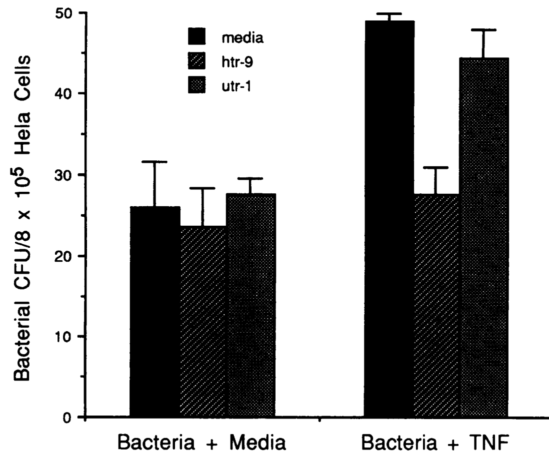

FIG. 4. Enhanced invasion of HeLa cells by TNF-α–*Shigella* complexes is inhibited by pretreatment of HeLa cells with antibody to the p55 TNF-α receptors. HeLa cell monolayers were pretreated for 1 h at 37°C with medium only, Utr-1 (10 μg/ml), or htr-9 (10 μg/ml). Treated cells were then exposed to *S. flexneri* alone or TNF-α–*Shigella* complexes as described in the legend to Fig. 3. Data are from triplicate determinations and are from one experiment whose data were representative of data obtained from three experiments.

htr-9 (anti-p55) (10 μg/ml; both are IgG1) for 1 h at 37°C and then assessed for susceptibility to enhanced invasion by *Shigella* organisms alone versus TNF-α–*Shigella* complexes. As seen in Fig. 4, pretreatment of HeLa cells with htr-9, but not Utr-1, completely inhibited enhanced invasion by TNF-α–*Shigella* complexes. HeLa cell invasion by untreated *S. flexneri* was not affected by either antibody. These results suggest that TNF-α–bacterium complexes can interact with TNF-α receptors present on HeLa cells and that this interaction results in enhanced binding and invasion.

A number of pathogenic bacteria have receptors for different plasma proteins (22, 32) and for different extracellular matrix proteins (10, 16, 33). Bacterial binding of collagen, fibronectin, and laminin has been proposed to be an important mechanism for bacterial adherence to target tissues (33), such as the small intestine. The possibility that bacteria could also have receptors for cytokines has only recently been explored. IL-1, IL-2, and granulocyte-macrophage colony-stimulating factors have all been shown to enhance the growth of virulent strains of *E. coli* (5, 23). In both of those studies, TNF-α had no effect on *E. coli* growth. However, the ability of TNF to bind to *E. coli* or to alter bacterial virulence was not addressed. Our results document that bacteria can bind TNF-α and that this binding results in important biological effects. The implication of TNF-α binding to a bacterium with regard to host defense versus pathology is currently unknown. Enhanced invasion of epithelial cells by TNF-coated bacteria could help bacteria evade antibody and phagocytic cells and could result in alterations in epithelial cell metabolism such that intracellular bacterial growth is enhanced. Alternatively, invasion of epithelial cells by TNF-bacterium complexes could result in early epithelial cell destruction and inhibition of intracellular growth of the bacteria. We have previously shown that fibroblasts and epithelial cells infected with gram-negative bacteria (*Salmonella* and *Shigella* spp.) have enhanced susceptibility to the cytotoxic action of TNF (13). The consequences of enhanced phagocytic uptake of TNF-bacterium complexes could also affect host defense and pathology. In preliminary studies (data not presented), we have found that TNF-coated bacteria have impaired growth in macrophages compared with control-treated bacteria. These preliminary results suggest that TNF-coated bacteria can enhance bactericidal and bacteriostatic functions of macrophages. In conclusion, the results presented in this study pose new questions regarding how interactions between bacteria and cytokines might contribute to host defense and/or the pathology of invasive bacterial infections.

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