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A Systemic Downregulation of Gamma Interferon Production Is Associated with Acute Shigellosis

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Production of cytokines by peripheral blood mononuclear cells from *Shigella*-infected patients was assessed. The frequencies of tumor necrosis factor alpha (TNF-α), TNF-β, and transforming growth factor β mRNA-expressing cells were persistently upregulated during the course of shigellosis in comparison to those of healthy controls. In contrast, the frequency of gamma interferon (IFN-γ) mRNA-expressing cells was significantly reduced during the acute stage compared to that during the convalescent stage and to that of healthy Bangladeshi controls ($P < 0.01$). Constitutive IFN-γ production in Bangladeshi controls was significantly upregulated compared to that in Swedish controls.

Natural infection with a *Shigella* species induces the production of a broad spectrum of cytokines in the rectal tissues which persists for up to 1 month after the onset of the disease, maintaining an acute inflammatory reaction in the rectal mucosa (12). Severe inflammation was associated with increased production of proinflammatory cytokines in the rectum compared to that during mild inflammation. Increased concentrations of proinflammatory cytokines were detected in stool and plasma specimens from patients with acute *Shigella* infections, and increased levels correlated with the clinical severity of the disease (15). However, a significant downregulation of gamma interferon (IFN-γ) was observed at the local site as well as in stool and plasma specimens from all patients during the acute stage of the disease, and it gradually increased during the convalescent phase (12, 14, 15). To investigate whether this reduced cytokine response was due to local or systemic immune system impairment, we examined constitutive cytokine mRNA expression in the peripheral blood of *Shigella dysenteriae* type 1- and *Shigella flexneri*-infected patients during the acute and the convalescent stages of the disease. The results from these patients were compared to those of healthy adults. We aimed to investigate whether the lack of an IFN-γ response was due to either a transcriptional or a translational inhibition of IFN-γ. To elucidate this, we have used (i) in situ hybridization to quantify cytokine mRNA-expressing cells, (ii) an enzyme-linked immunospot (ELISPOT) assay to determine the frequencies of cytokine-secreting cells, and (iii) an enzyme-linked immunosorbent assay (ELISA) to determine the concentrations of secreted cytokines.

Adult patients with complaints of bloody, mucoid diarrhea and severe abdominal cramps who presented at the treatment center of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDRB), in Dhaka were selected for the study. Stool samples were examined by direct microscopy for cysts or parasites and cultured for the following: *Salmonella*, *Shigella*, and *Aeromonas* species; *Vibrio cholerae* O1 and O139 strains; and *Campylobacter jejuni*. For the study, nine patients with *S. dysenteriae* type 1 infections and nine with *S. flexneri* infections (age range, 18 to 45 years) were selected. Informed consent was obtained from each patient. The study was approved by the ethical review committee of the ICDDRB. A clinical evaluation of each patient was carried out. Patients were discharged from the hospital within a week of when diarrhea subsided and were requested to return for a follow-up visit 30 days later. Blood samples were obtained from each patient twice, at the time of admission and 1 month after admission. All patients received antimicrobial treatment according to the antibiotic susceptibility pattern of the infecting strain.

Six healthy age-matched adult males living in an area in which shigellae are endemic were recruited as Bangladeshi controls (BC). Six healthy individuals residing in an area in which shigellae are not endemic were recruited as Swedish controls (SC). Physical examinations and clinical and microbiological investigations were carried out for controls as for patients to exclude those with a history of infection or fever within the last 5 months. Blood was collected once from all control individuals.

Shiga toxin (ShT) was prepared from *S. dysenteriae* type 1 strain 114SD, an auxotrophic mutant due to a transposon Tn*10*-inactivated *aroD* gene. The toxin was prepared by affinity chromatography as described by Ryd et al. (16). The protein content of the toxin preparation was measured by the Lowry method (8). The endotoxin content of the ShT preparation, as determined by a chromogenic *Limulus* amoebocyte lysate assay (M. A. Bioproducts, Walkersville, Md.), was 18 pg/ml (cutoff level, 7.5 pg/ml). Lipopolysaccharide (LPS) preparations from *S. dysenteriae* 1 and *S. flexneri* Y were obtained by the hot phenol-water extraction method (9). Antigens were diluted in RPMI 1640 with 10% fetal calf serum (Gibco Ltd., Paisley, Scotland) for the stimulation studies.

Mononuclear cells (MNC) were separated from freshly collected heparinized blood of either patients or controls by centrifugation through Ficoll-Hypaque (Histopaque; Sigma Chemical Co., St. Louis, Mo.). MNC were counted and kept in liquid nitrogen until used. The MNC were thawed at 37°C and then washed and suspended in RPMI 1640 medium containing 0.29 mg of glutamine per ml and supplemented with 0.11 mg of sodium pyruvate per ml, 100 U of penicillin G per ml, 100 μg of streptomycin per ml, and 10% fetal calf serum (Gibco). The fetal calf serum, RPMI medium, and Ficoll-Hypaque, when

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TABLE 1. Frequency of spontaneous expression of various cytokine mRNAs by MNC from patients with *S. dysenteriae* type 1 or *S. flexneri* infections during the acute (2 to 6 days after onset) and the convalescent (30 to 35 days postonset) stages of the disease and from healthy BC and SC

Cytokine	Constitutively expressed cytokine mRNA/10^5 MNC in[a]:						*P* value[b]
	Healthy controls		Patients in acute stage of infection with:		Patients in convalescent stage after infection with:		
	BC	SC	*S. dysenteriae* type 1	*S. flexneri*	*S. dysenteriae* type 1	*S. flexneri*	
IFN-γ	125 ± 50	10 ± 8[c]	26.5 ± 11[c]	50 ± 25[c]	100 ± 60	150 ± 60	0.001
TNF-β	35 ± 15	6 ± 6[c]	63 ± 30	55 ± 25	116 ± 30[c]	65 ± 25	0.03[d]
IL-4	40 ± 25	10 ± 4[c]	85 ± 90	65 ± 25	75 ± 55	75 ± 35	NS[e]
IL-6	30 ± 20	4.5 ± 3[c]	50 ± 20	60 ± 25	45 ± 15	50 ± 20	NS
TNF-α	45 ± 10	9 ± 8[c]	125 ± 75[c]	95 ± 25	155 ± 85[c]	105 ± 45[c]	NS
TGF-β	35 ± 20	ND[f]	72.5 ± 40	45 ± 35	115 ± 60	100 ± 40[c]	0.01[g]

[a] Data are expressed as means ± standard errors.
[b] Probability values were determined by Wilcoxon/Kruskal-Walis test, comparing the data between BC and SC, between patients in the acute stage and BC, between convalescent-stage patients and BC, and between patients in the acute and convalescent stages.
[c] Value is statistically significantly different from that of BC ($P < 0.05$).
[d] *P* value is for *S. dysenteriae* type 1-infected patients only.
[e] NS, not significant.
[f] ND, not determined.
[g] *P* value is for *S. flexneri*-infected patients only.

tested for endotoxin levels by the *Limulus* amoebocyte assay, showed <0.01, 0.05, and 0.02 ng of endotoxin per ml, respectively (1 endotoxin unit/ml = 0.1 ng/ml). For the ELISPOT assay, MNC were suspended at a concentration of 10^5/ml. For in situ hybridization studies, MNC were aliquoted into 50-ml polypropylene conical tubes (Falcon; Becton Dickinson, Lincoln Park, N.J.) at 5×10^6/ml.

Synthetic oligonucleotide probes (Scandinavian Gene Synthesis, Köping, Sweden) for various cytokines (IFN-γ [6], tumor necrosis factor beta [TNF-β] [5], interleukin-4 [IL-4] [18], IL-6 [17], TNF-α [11], and transforming growth factor beta [TGF-β] [3]) were labelled with [^{35}S]deoxyadenosine 5′-α-(thio)-triphosphate by using terminal deoxynucleotidyl transferase (Amersham, Little Chalfont, United Kingdom). The oligonucleotide sequence was obtained from GenBank through the use of the Mac Vector system. Probes complementary to the antisense strands of the studied cytokine probes (exons 4) were used as control probes in parallel slides to ensure the specificity of the hybridization signals.

In situ hybridization was performed by a previously described method for tissue sections that was modified for use with MNC (2). A control probe used in parallel with the cytokine probes on the fixed cells produced a uniformly weak background signal without revealing any positive cells. The variability of the hybridization procedure was ±15% between duplicates. For stimulation studies, MNC were stimulated with either ShT (100 ng/ml) or LPS (100 ng/ml) or (for controls) incubated in RPMI at 37°C in a humidified atmosphere containing 7% CO_2 for 48 h. After culture, supernatants were collected for measurement of secreted cytokines and cells were harvested, washed in fresh RPMI, and used for in situ hybridization. After in situ hybridization, labelled cells (positive cells containing >20 grains/cell) were counted at a magnification of ×400 and expressed as the number of positive cells per 10^5 cells. Slides were counted by two persons, and the variability of the counting procedure was ±15%.

A modified ELISPOT assay for detection of secretion of IFN-γ and IL-4 by MNC was performed as described previously (4). Spots corresponding to cells that secreted IFN-γ or IL-4 were counted under a dissection microscope (magnification, ×40). Results were expressed as the number of cytokine-secreting cells (spots) per 10^5 MNC. Concentrations of cytokines in culture supernatants were measured by ELISAs that detected both receptor-bound and unbound IFN-γ (Endogen, Woburn, Mass.) or IL-4 (Quantakine; R&D Systems, Minneapolis, Minn.) according to the manufacturer's instruction. The minimum detectable concentration of IFN-γ was 2 pg/ml, and that of IL-4 was 3 pg/ml. Interassay variation was 7 to 10% for repeated measurements.

The data were processed by using Microsoft Excel 5.0 (Microsoft Corporation, Redmond, Wash.) and JMP 3.1 (SAS Institute, Cary, N.C.). Statistical analyses were performed by the use of the Wilcoxon/Kruskal-Walis test. Differences were regarded as significant if they reached a 95% level of confidence ($P < 0.05$).

Frequencies of MNC constitutively expressing mRNA for TNF-α, TNF-β, IL-4, IL-6, and TGF-β in patients with acute shigellosis were comparable to those in BC (Table 1). The only exception was TNF-α mRNA-expressing cells, which were significantly higher in number in patients during the acute stage of *S. dysenteriae* type 1 infection than in BC ($P < 0.05$). In contrast, a marked downregulation of IFN-γ mRNA-expressing cells was noticed in both *S. dysenteriae* type 1- and *S. flexneri*-infected patients ($P < 0.005$) during the acute stage compared to BC (Table 1). In comparison to BC, patients in the convalescent phase exhibited a significant upregulation of cells expressing mRNA for TNF-α, TNF-β, and TGF-β in cases of *S. dysenteriae* type 1 infection and for TNF-α and TGF-β in cases of *S. flexneri* infection ($P \leq 0.05$). Upregulation of TNF-β mRNA-expressing cells in *S. dysenteriae* type 1-infected patients and of TGF-β mRNA-expressing cells in *S. flexneri*-infected patients was evident in the convalescent stage when compared to the acute stage ($P < 0.05$). Both BC and patients exhibited significantly increased numbers of cells constitutively expressing cytokine-specific mRNA in comparison to SC. In particular, there was a significantly higher incidence of IFN-γ mRNA-expressing cells in the blood of BC than in that of SC ($P < 0.005$).

To correlate the mRNA expression by MNC to the actual secretion of cytokines, an ELISPOT assay was performed to study antigen-specific IFN-γ- and IL-4-secreting cells in the peripheral circulation. Patients infected with *S. dysenteriae* type 1 or *S. flexneri* showed similar kinetic patterns of cytokine-secreting cells, with no significant differences in their numbers

TABLE 2. Comparative analysis of constitutive as well as in vitro-stimulated cytokine production in patients with *S. dysenteriae* type 1 ($n = 9$) and *S. flexneri* ($n = 9$) infections during the acute (2 to 6 days after onset) and convalescent (30 to 35 days postonset) stages of shigellosis and in controls from areas in which *Shigella* spp. are (BC; $n = 6$) or are not (SC; $n = 6$) endemic

Study group	Cytokine production by:							
	Unstimulated cells				Phytohemagglutinin-stimulated cells			
	IFN-γ[a]		IL-4[b]		IFN-γ[a]		IL-4[b]	
	No. of secreting cells	Concn in supernatant	No. of secreting cells	Concn in supernatant	No. of secreting cells	Concn in supernatant	No. of secreting cells	Concn in supernatant
Controls								
BC	7.3 ± 6	12 ± 4	0	UD[c]	73 ± 35	59 ± 39	15 ± 4	6.3 ± 2.2
SC	2 ± 1.4[d]	3.2 ± 4[d]	0	UD	144 ± 35[d]	115 ± 27[d]	22 ± 5	3.2 ± 2
Patients								
Acute stage	22 ± 7[d]	4.3 ± 5[d]	2.1 ± 1[d]	UD	187 ± 22[d]	67 ± 22	71 ± 6[d]	14.6 ± 3
Convalescent stage	52 ± 7[d]	9.8 ± 6	0.1 ± 0.1	UD	366 ± 42[d]	143 ± 48[d]	58 ± 20[d]	10.5 ± 3
P value[d]	0.05	0.04	0.05		0.02	0.03	NS[e]	NS

[a] Results are expressed as the mean number of cells secreting the cytokine per 10^5 MNC ± the standard error, as determined by ELISPOT assay.
[b] Results are expressed as the concentration (in picograms per milliliter) of the cytokine in the culture supernatant ± the standard error, as measured by quantitative sandwich enzyme immunoassay after 48 h of incubation.
[c] UD, undetectable.
[d] The value is significantly different from that of BC ($P < 0.05$).
[e] Probability values were determined by the Wilcoxon/Kruskal-Walis test in comparing BC and SC data, acute-stage and BC data, and convalescent-stage and BC data.

between the two groups of patients during the course of the disease (Table 2). Numbers of IFN-γ-secreting cells were significantly higher in patients throughout the disease course than in BC, in contrast to the findings of IFN-γ mRNA-expressing cells. The discrepancy could be due to the difference in the sensitivities of the two techniques. IFN-γ-secreting cells were significantly lower in number at the acute stage than at the convalescent stage ($P < 0.05$). To address this discrepancy, ELISAs were performed on culture supernatants to measure secreted cytokines (IFN-γ and IL-4). The concentration of IFN-γ was significantly higher in BC than in patients in the acute or convalescent stage (Table 2), similar to the findings of IFN-γ mRNA-expressing cells (Table 1). Although the frequency of IFN-γ-secreting cells was lower in BC, the capacity of individual cells to secrete IFN-γ was probably higher. Cells secreting IL-4 were not detected in the supernatant, and no spontaneous secretion into the culture supernatant was evident. Stimulation of MNC with LPS or ShT induced a significant upregulation of IFN-γ mRNA-expressing cells in comparison to the constitutive expression in patients during the acute stage (Table 3). However, specific antigen stimulation did not result in a significant increase in the number of IFN-γ-secreting cells (Table 3).

This report demonstrates a systemic activation of MNC constitutively synthesizing cytokine-specific mRNA (TNF-α, TNF-β, IL-4, IL-6, and TGF-β) in patients with shigellosis during both the acute and the convalescent stages. Production of IFN-γ was temporarily suppressed at the onset of the disease. This was compatible with our earlier findings of persistent production of cytokines in the rectal mucosa in the same group of patients during the course of shigellosis and a selective suppression of IFN-γ in the acute stage (12, 13). However, we do not know why a disparity in the induction of the cytokine response with a selective immunosuppression of the IFN-γ response is seen in patients with shigellosis. Do the bacteria themselves selectively downregulate the IFN-γ response, or is it a previous infection or exposure to antigen that causes this downregulation? Studies by Klapproth et al. showed that lysates from two pathogenic strains of *Escherichia coli* inhibited mitogen-stimulated expression of lymphokines, although production of cytokines by monocytes was not affected (7). The immunosuppressive factors produced by pathogenic bac-

TABLE 3. Frequency of MNC from patients with shigellosis at the acute stage of the disease (2 to 6 days after onset) and from healthy controls induced to produce IFN-γ after stimulation for 48 h with ShT or LPS

Antigen	Mean no. of IFN-γ-expressing cells/10^5 MNC ± SE for[a]:							
	Healthy controls				Patients infected with:			
	BC ($n = 6$)		SC ($n = 6$)		*S. dysenteriae* type 1 ($n = 9$)		*S. flexneri* ($n = 9$)	
	IFN-γ mRNA-expressing cells	IFN-γ-secreting cells	IFN-γ mRNA-expressing cells	IFN-γ-secreting cells	IFN-γ mRNA-expressing cells	IFN-γ-secreting cells	IFN-γ mRNA-expressing cells	IFN-γ-secreting cells
None	125 ± 50	7.3 ± 6	10 ± 8[b]	2 ± 1.4	26.5 ± 11	21 ± 7[b]	50 ± 25	23 ± 9[b]
LPS	243 ± 56	6.5 ± 5	48 ± 10[b]	6.7 ± 4	384 ± 41	25 ± 8[b]	347 ± 35	27 ± 12[b]
ShT	119 ± 44	6.3 ± 3	24 ± 6[b]	2.2 ± 1.3	100 ± 34	24.3 ± 10[b]	132 ± 58	26.7 ± 15[b]

[a] Probability values were determined by the Wilcoxon/Kruskal-Walis test in comparing BC and SC data as well as acute-stage and BC data while considering either IFN-γ mRNA-expressing cells or IFN-γ-secreting cells.
[b] Value is significantly different from that of BC in the same category ($P < 0.05$).

teria may be important in modifying gastrointestinal responses in enteric infections. It could also be a general phenomenon that is entirely unrelated to any antigen-specific immune response. This event may also be due to a downregulation of IL-18, which is an IFN-γ-inducing cytokine (10).

The present study indicates that selective immunosuppression of the IFN-γ response is due to a systemic inhibition of IFN-γ induction. The peripheral blood MNC did not exhibit a selective IFN-γ anergic response, since a significant upregulation of IFN-γ-secreting cells was observed after stimulation with phytohemagglutinin (Table 2). Stimulation with specific antigens such as LPS and ShT induced a significant increase in the number of IFN-γ mRNA-expressing cells compared to that in constitutive expression, yet no significant change in the frequency of IFN-γ-secreting cells was observed (Table 3). This suggested that there was a restriction at the translational level upon stimulation with *Shigella*-specific antigens. In patients with bronchial asthma due to viral infections of the lungs, $CD8^+$ T cells activated by specific viral antigens switch to a Th2 cytokine-secreting pattern in an IL-4-dependent manner (1). These cells secrete more Th2 cytokine and less IFN-γ and delay viral clearance. However, it is not clear from the present study if there was a change in the cytokine balance to the Th2 side, with decreased IFN-γ and increased IL-4 production, in acute shigellosis.

In conclusion, our data show that there was a persistent activation of cytokine genes in the peripheral circulation during the acute and the convalescent stages of shigellosis, as seen in the rectal mucosa (12, 13). Patients exhibited higher numbers of IFN-γ-producing cells following in vitro stimulation with ShT and LPS. Therefore, the downregulation of IFN-γ at the acute stage of shigellosis could be induced by other *Shigella*-specific antigens. Healthy individuals frequently exposed to diarrheal pathogens such as shigellae have much higher constitutive IFN-γ responses than those of control individuals from areas in which *Shigella* spp. are not endemic, implying that IFN-γ may be important for protection. To identify whether the selective immunosuppression of IFN-γ also occurs during primary infection with *Shigella* spp., the above findings must be investigated in children infected with shigellae.

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