Sporadic Enteric Reactive Arthritis and Undifferentiated Spondyloarthropathy: Evidence for Involvement of *Salmonella typhimurium*

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ABSTRACT. ***Objective.*** To define the candidate bacterial trigger and cytokine profile of synovial fluid mononuclear cells (SFMC) in patients with sporadic enteric reactive arthritis (ReA) and undifferentiated spondyloarthropathy (uSpA).

Methods. The study group comprised 10 patients with ReA and 23 with uSpA who fulfilled European Spondylarthropathy Study Group criteria. Ten patients with rheumatoid arthritis (RA) served as disease controls. IgG, IgA, and IgM antibodies to *Shigella flexneri*, *Salmonella typhimurium*, and *Yersinia enterocolitica* were measured in sera and SF by ELISA. Peripheral blood mononuclear cell (PBMC) and SFMC proliferation assays were done in the presence or absence of crude bacterial lysates. Bacterial antigens and DNA in synovial cells were detected by indirect immunofluorescence and polymerase chain reaction, respectively. Interferon-γ (IFN-γ), interleukin 10 (IL-10), and IL-4 were measured in 18 h SFMC culture supernatants in presence of bacterial lysate.

Results. Antibodies to *S. typhimurium* were significantly elevated in the sera of 8 of 25 patients compared to controls (0/22; $p < 0.05$). The ratio of SF:serum anti-salmonella IgA was significantly higher in patients compared to controls ($p < 0.0002$). The ratio of SF:serum IgA antibodies to *S. typhimurium* was higher than that for *S. flexneri* ($p < 0.007$) and *Y. enterocolitica* ($p < 0.05$). Out of 25 patients, 8, 2, and none had elevated antigen-specific SFMC proliferation response to *S. typhimurium, S. flexneri*, and *Y. enterocolitica*, respectively, whereas no control had elevated response. Salmonella antigens were detected in the synovial cells of 4 out of 14 patients. There was significantly higher IFN-γ production from SFMC of patients who had increased proliferative response to Salmonella (LTT+) in the presence of Salmonella antigens compared to antigen control. The mean ± SD of the ratio of IFN-γ:IL-10 in the LTT+ patients was significantly lower compared to controls.

Conclusion. *S. typhimurium* is probably one of the triggers for enteric ReA and uSpA in our cohort of patients, and the immune response is characterized by increased production of both IL-10 and IFN-γ. (J Rheumatol 2003;30:105–13)

Key Indexing Terms:
SERONEGATIVE SPONDYLOARTHROPATHY
PATHOGENESIS
IMMUNE RESPONSE
ENTERIC BACTERIA

Reactive arthritis (ReA) is a form of spondyloarthropathy (SpA) that is triggered by enteric infections caused by *Shigella flexneri*, *Salmonella typhimurium*, and *Yersinia enterocolitica* or genitourinary infection by *Chlamydia trachomatis*. Patients with undifferentiated spondyloarthropathy (uSpA) have a clinical picture similar to ReA but with no history of preceding infection, which possibly represents an incomplete form of the latter in which the inciting infection is asymptomatic. We have described significantly elevated antibodies to *S. flexneri* and *S. typhimurium* in sera of patients with uSpA suggesting preceding infection[1].

The preceding bacterial infection is indirectly reflected by elevated serum or synovial fluid (SF) antibody titers to the bacteria. Because serum antibodies to these bacteria are elevated in the general population and are cross-reactive, their significance is limited[2]. The presence of SF IgA antibody to these organisms provides a more definitive clue for the bacterial trigger[3-5]. Compared to antibodies, the antigen-specific proliferation of SF T cells suggests definitive evidence of previous exposure[6], although various antigen-independent factors influence the response[7]. A dominant local response is reflected by increased SF T cell proliferation compared to the peripheral blood[8,9]. T cell proliferation in peripheral blood is time-dependent and is rarely found very early or late in the course of arthritis[10].

Bacterial products have been described in the synovial tissues and SF leukocytes by immunofluorescence assay. Typical Chlamydia elementary bodies have been observed in

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the synovial cells[11]. Although the whole bacterium-like structure was missing, Yersinia[12], Salmonella[13], and Shigella[14] antigens have been observed in SF cells. Chlamydia DNA has been detected in SF of patients with ReA and uSpA using polymerase chain reaction (PCR)[15,16]. However, investigators failed to detect enterobacteria DNA in the synovial samples from these patients[13,17]. Recently, Yersinia DNA was identified by PCR in one patient with enteric ReA[18].

A local immune response against persisting bacteria or its products in the joint may explain synovitis in ReA/uSpA. One of the mechanisms for the bacterial persistence is cytokine imbalance. Evidence from animal models for ReA associated bacteria Yersinia[19] and Chlamydia[20] suggests that Th1 cytokines such as interleukin 12 (IL-12), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) are crucial for elimination of these bacteria. The lack of these cytokines and/or elevated production of Th2 cytokines such as IL-10 inhibit clearance of these bacteria and can lead to persistence. In patients with ReA, the skewed Th2-type response was observed by *in vitro* cytokine secretion assay[21], cytoplasmic cytokine staining[22], and cytokine mRNA quantification[23].

As ReA associated bacteria are one of the major causes of diarrhea in this region[24-26], the study was undertaken to identify the bacterial trigger in our cohort of patients with sporadic enteric ReA and uSpA by evaluating antibacterial antibodies in sera and SF and T cell proliferation responses in SF and paired blood against the whole cell lysates of *S. flexneri*, *S. typhimurium*, and *Y. enterocolitica*. We also looked for the presence of the bacterial antigens by indirect immunofluorescence and DNA by PCR. Further, we assayed IFN-γ, IL-4, and IL-10 in the synovial fluid mononuclear cell (SFMC) culture supernatants to delineate the Th1/Th2 type of immune responses.

MATERIALS AND METHODS

Thirty-three patients with SpA defined by the European Spondylarthropathy Study Group criteria[27] were included in the study. ReA (n = 10) was defined as asymmetrical lower limb oligoarthritis preceded by history of diarrhea in the previous 4 weeks. uSpA (n = 23) was defined as inflammatory backache and/or asymmetrical oligoarticular arthritis of the lower limbs along with one of the following features: enthesitis, radiological evidence of sacroiliitis, buttock pain, or a positive family history. No patient had inflammatory bowel disease or psoriasis. Clinical characteristics of patients are shown in Table 1.

Table 1. Demographic and clinical characteristics of patients with reactive arthritis (ReA) and undifferentiated spondyloarthropathy (uSpA).

Feature	ReA, n = 10	uSpA, n = 23
M:F ratio	8:2	22:1
Mean age, yrs (range)	24 (19–55)	26 (13–52)
Mean duration of disease, yrs (range)	2.3 (0.1–6)	2.8 (0.3–10)
HLA-B27	7	16
Asymmetrical oligoarthritis	10	16
Inflammatory backache	5	11
Radiological evidence of sacroiliitis	3	10
Buttock pain	2	11
Enthesitis	3	5
Positive family history	0	1

The disease control group consisted of 10 patients with rheumatoid arthritis (RA).

Bacterial antigen preparation. *S. flexneri* (2b), *S. typhimurium* (ATCC 13311), and *Y. enterocolitica* (O:3) strains were grown overnight in nutrient broth at 37°C and antigens were prepared by sonication[1].

ELISA for antibacterial antibodies. Serum and SF antibacterial antibodies were evaluated by ELISA as described[1]. OD values exceeding the mean + 2 SD of 22 healthy control sera and 8 SF samples from patients with RA were taken as positive for serum and SF antibody levels, respectively.

Cell culture and proliferation assay. Mononuclear cells were separated from SF and heparinized peripheral blood and proliferation assays were performed by standard technique[28]. In the preliminary experiments, 10 µg/ml antigens were found to be optimal. Results were expressed in stimulation indices (SI) defined as ratio of proliferation (counts per min) induced by antigen to that in medium alone, and SI > 3 was considered a positive response.

Immunofluorescence for bacterial antigens in SF cells. Cytospin smears were prepared from SF cells and immunofluorescence was performed as described[29]. Rabbit antibacterial antiserum was used as primary antibody and fluorescein conjugated $F(ab)_2$ fragments of anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO, USA) as secondary antibody. The slides were mounted with glycerol containing 20 µg/ml ethidium bromide and viewed on a fluorescence microscope (Olympus, Germany). Two independent observers read the slides and a diffuse cytoplasmic staining was considered positive.

PCR for bacterial DNA in SF cells. Chromosomal DNA of *S. typhimurium* and *Y. enterocolitica* and plasmid DNA of *S. flexneri* was isolated by alkali lysis[30]. SF cell DNA was isolated by standard method[31]. The total reaction volume was 50 µl, containing 5 µl of 10× buffer, 200 µM of each dNTP (DyNAzyme II DNA polymerase kit, Finnzymes), 1 unit Taq DNA polymerase (Boehringer Mannheim, Germany), 20 pmole of each primer, and 5 µl of template. Varying concentrations of the bacterial DNA were used separately as positive controls.

Amplification of DNA from S. typhimurium. The oligonucleotide primers ST1 (5' AGC CAA CCA TTG CTA AAT TGG CGC A 3') and ST2 (5' GGT AGA AAT TCC CAG CGG GTA CGT 3') were used for first-round amplification and ST1 and ST3 (5' TTT GCG ACT ATC AGG TTA CCG TGG 3') were used for nested PCR[13]. The PCR steps included 10 cycles each at 94°C for 1 min, 51°C for 1 min, and 72°C for 2 min, 12 cycles each at 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min, and 20 cycles each at 94°C for 1 min, 47°C for 1 min, and 72°C for 2 min. The final extension time was 10 min at 72°C. Nested PCR was performed as above using 1 µl of amplified product as template. Ten microliters of amplified product were separated by agarose gel electrophoresis (2% agarose in 0.5× TBE containing 20 µg ethidium bromide) and the products were visualized in ultraviolet transilluminator. The product sizes of first-round and nested PCR were 429 bp and 350 bp, respectively.

Amplification of DNA from S. flexneri. The oligonucleotide primers SF1 (5' TCA CTG GAC GTT GCT AAT GCG 3'), SF2 (5' CCA CTT TCG CCA CAA CCA AT 3'), and SF3 (5' ACG ATT TCG CAA CTC CCC AC 3') used were specific for *S. flexneri*. For first-round PCR SF1 and SF2 primers and for nested PCR SF1 and SF3 primers were used. The PCR steps, 94°C for 1 min, 51°C for 1 min, and 72°C for 2 min, were repeated 30 times. Nested PCR was performed by touchdown protocol using 1 µl of amplified product as template. The PCR steps, 94°C for 1 min, 51°C for 1 min, and 72°C for 2 min, were repeated 13 times. The product sizes of first-round and nested PCR were 214 bp and 177 bp, respectively. Primers were checked for cross-reactivity against *S. typhimurium*, *Y. enterocolitica*, and *E. coli*.

Amplification of DNA from Y. enterocolitica. The oligonucleotide primers YE1 (5' CGC CTG TTC GTT TGT CAG CAC ACC GGC C 3') YE2 (5' GTC GGC CTG CGC GGA AGA TGT AAC GGG 3'), and YE3 (5' GAA CGT ATC ACT TAA AAC GCT TAA CG 3') were used[17]. For first-round PCR YE1 and YE2 primers and for nested PCR YE1 and YE3 primers were used. The PCR steps, 94°C for 45 s, 63°C for 1 min, and 72°C for 2 min, were repeated 40 times. The protocol for nested PCR was otherwise the same as the

first round, but the amplification cycles were repeated 30 times using 1 µl of first-round product. The product sizes of first-round and nested PCR were 154 bp and 98 bp, respectively.

Cytokine production and assay. For the bacterial antigen stimulated cytokine production, 1×10^6 SFMC were incubated 18 h in the absence or presence of 10 µg/ml bacterial antigens in 1 ml of complete RPMI-1640 medium. IFN-γ, IL-4, and IL-10 were measured in cell-free culture supernatants by colorimetric sandwich ELISA kits or matched antibody pair following manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). The lower limit for detection of IL-4 and IL-10 was 32 pg/ml and for IFN-γ it was 14 pg/ml.

HLA-B27 typing. B27 was typed by the amplification refractory mutation system (ARMS)-PCR technique[31].

Statistical analysis. Cellular proliferation and antibody responses were analyzed by Fisher's exact test. Mann-Whitney U test was applied for analysis of difference in ratio of SF:serum in patient and control groups. Student's paired t test was used for comparison of the cytokine secretion level in the disease group. Comparison of the ratio of IFN-γ:IL-10 between patients with ReA/uSpA and those with RA was by unpaired t test.

RESULTS

Antibacteria antibody responses in serum and SF. IgG antibodies to *S. typhimurium* were raised in the sera of 6 out of 25 patients. All these sera were cross-reactive with *S. flexneri* and in 4 with *Y. enterocolitica*. IgA antibodies to *S. typhimurium*, *S. flexneri*, and *Y. enterocolitica* were elevated in 2, 4 and none, respectively (Table 2). No patient had elevated antibacterial IgM antibody response. One out of 22 healthy controls had the IgA antibody response to all 3 bacteria.

The ratio of SF:serum IgA antibody to *S. typhimurium* was estimated in 13 patients and 8 disease controls. The ratio was significantly higher in the patients compared to the controls ($p < 0.0002$). The ratio of SF:serum IgA antibodies to *S. typhimurium* was higher than that for *S. flexneri* ($p < 0.007$) and *Y. enterocolitica* ($p < 0.05$) (Figure 1).

Proliferation of SFMC and PBMC responses to bacterial antigens. Thirteen out of 25 patients (5 with ReA, 8 with uSpA) had a positive SFMC proliferation response against *S. typhimurium* compared to none of 10 patients with RA ($p < 0.05$). Among these 13 patients, 8 had antigen-specific response. Two of the patients with uSpA had antigen-specific proliferation to *S. flexneri*. Although few patients had response to *Y. enterocolitica*, none had an antigen-specific response (Table 2). Patients who showed cross-reactive responses had SI values of 3.5 to 4.5, whereas most patients having antigen-specific responses had values above 4.5 and as high as 21.

Mononuclear cell proliferation response was confined to the synovial compartment, as only 2 patients with ReA/uSpA had positive PBMC proliferation responses. One control patient each had positive PBMC proliferation response to *S. typhimurium and S. flexneri*, respectively (data not shown).

Indirect immunofluorescence for bacterial antigens in SF cells. The presence of bacterial antigens was studied in SF cells from 14 patients (7 each with ReA or uSpA) by indirect immunofluorescence assay. *S. typhimurium* antigens were detected as diffuse cytoplasm staining in 4 samples (3 with ReA, one with uSpA) (Figure 2A), whereas the rest were negative (Figure 2B). All the positive samples had a positive SFMC proliferation response. Shigella and Yersinia antigens were not detected in any of the samples.

PCR for detection of bacterial DNA in SF cells. The sensitivity of the hemi-nested Salmonella and Yersinia-specific PCR was 10 femtogram (fg) and that of *S. flexneri* was 30 fg per reaction, respectively, when purified bacterial DNA was used as template (Figure 3); 10 fg and 30 fg DNA correspond to 2–3 and 5–6 organisms, respectively. The bacterial DNA was absent in all the 33 samples from patients with ReA/uSpA and 10 samples from patients with RA.

Cytokine assay in SFMC culture supernatants. Culture supernatants of SFMC from 20 patients (7 with ReA, 13 with uSpA) had measurable quantities of IFN-γ and IL-10, but undetectable IL-4 (< 32 pg/ml). For analysis of cytokine results, patients were categorized into 2 groups, 8 patients who had a positive SFMC proliferation response (LTT+) and 12 patients who did not have a proliferation response to *S. typhimurium* (LTT–).

In the LTT+ group, IFN-γ production in the presence of antigens of *S. typhimurium* was significantly higher (5496 ± 4558 pg/ml) compared to the antigen control (without antigen; 1417 ± 2168 pg/ml) ($p < 0.05$). There was, however, no significant increase in IFN-γ production with antigens of *S. flexneri* (3197 ± 2900 pg/ml) and *Y. enterocolitica* (2082 ± 1986 pg/ml) compared to the antigen control ($p < 0.05$) (Figure 4A). There was no difference among patients with ReA and uSpA. IL-10 secretion did not increase significantly upon culture in the presence of antigens of *S. typhimurium* (8594 ± 6609 pg/ml), *S. flexneri* (9360 ± 7226 pg/ml), and *Y. enterocolitica* (8174 ± 6464 pg/ml) compared to the antigen control (4638 ± 5576 pg/ml) ($p < 0.05$; Figure 4B).

In the LTT– group, the level of IFN-γ secretion was 4065 ± 4269, 3428 ± 4112, 2834 ± 2948, and 2690 ± 3109 pg/ml in the presence of antigen of *S. flexneri*, *S. typhimurium, Y. enterocolitica*, and no antigen, respectively. IL-10 production was 5091 ± 6148, 4692 ± 6471, 4211 ± 5418, and 1941 ± 2034 pg/ml in the presence of antigen *of S. flexneri, S. typhimurium, Y. enterocolitica*, and no antigen, respectively. Thus there was no significant increase in either IFN-γ or IL-10 production over the antigen control for any of the 3 antigens ($p < 0.05$).

The mean of the ratio of IFN-γ:IL-10 in the presence of Salmonella antigens in the LTT+ patients was compared with the disease controls to look for the Th1/Th2 type of immune response. The mean ± 2 SD of the ratio in the LTT+ patients (0.67 ± 0.52) was significantly lower compared to controls (4.89 ± 1.84) ($p < 0.05$).

In controls, there was consistently increased IFN-γ secretion compared to IL-10 (9850 ± 3096 vs 3048 ± 1094 pg/ml) in the absence of any antigen. Cytokine production did not increase significantly upon culture with bacterial antigens ($p < 0.05$).

Table 2. SFMC and PBMC proliferation and corresponding serum antibody responses to various bacterial antigens in patients with ReA and uSpA. Proliferation responses are expresssed as stimulation index; a value > 3 was considered a positive response and is represented in bold. Positive serum antibody response (shown in bold) was defined as > mean + SD of healthy controls (n = 22).

Sample	Patients with ReA								
	S. flexneri			*S. typhimurium*			*Y. enterocolitica*		
	PBMC	SFMC	IgG/IgA	PBMC	SFMC	IgG/IgA	PBMC	SFMC	IgG/IgA
1	ND	1.3	ND	ND	**9.2**	ND	ND	**6.0**	ND
2	1.8	1.0	ND	1.2	**7.1**	ND	1.7	1.7	ND
3	1.3	2.4	1.59/0.16	1.9	2.5	1.80/0.22	2.0	1.3	1.37/0.17
4	1.1	1.2	ND	1.2	**5.0**	ND	1.2	1.6	ND
5	1.0	**4.6**	1.17/0.29	1.5	**4.5**	1.42/0.22	1.4	**4.0**	0.92/0.34
6	1.5	1.7	0.88/0.41	1.1	**3.4**	0.93/0.15	1.2	1.1	0.83/0.43
7	1.1	ND	0.86/0.26	5.7	ND	1.03/0.53	1.1	ND	0.52/0.26
8	1.8	ND	**2.71**/0.32	1.2	ND	**2.67**/0.43	1.4	ND	2.16/0.32
9	1.6	ND	**2.63**/0.23	1.3	ND	**2.75**/0.30	1.7	ND	2.19/0.23
10	1.2	1.8	1.77/0.31	1.3	1.9	1.70/0.39	1.1	1.3	1.32/0.33

Sample	Patients with uSpA								
	S. flexneri			*S. Typhimurium*			*Y. enterocolitica*		
	PBMC	SFMC	IgG/IgA	PBMC	SFMC	IgG/IgA	PBMC	SFMC	IgGIgA
1	ND	**4.9**	0.96/0.76	ND	**3.7**	1.00/0.35	ND	1.6	0.63/0.40
2	ND	2.3	2.30/**1.20**	ND	2.4	2.30/**0.81**	ND	1.3	**2.33**/0.34
3	1.8	**4.8**	2.20/0.30	1.1	2.3	1.76/0.37	2.1	1.6	1.69/0.23
4	2.1	1.8	2.03/0.26	1.4	**11.1**	1.85/0.65	1.7	1.5	2.03/0.26
5	1.2	< 3	ND	1.5	**11.8**	ND	1.8	1.4	ND
6	3.9	**4.7**	ND	4.9	**4.6**	ND	1.3	1.8	ND
7	1.8	< 3	2.15/0.51	1.3	**9.1**	2.28/0.53	1.1	2.1	2.03/0.39
8	1.5	< 3	**2.71/1.35**	1.7	**9.4**	**2.63**/0.59	1.4	1.9	**2.61**/0.51
9	1.7	**3.4**	1.78/0.18	1.1	**3.9**	1.72/0.16	1.5	**3.3**	1.78/0.17
10	1.4	2.4	1.25/0.25	1.5	2.3	1.58/0.43	2.1	1.6	1.11/0.29
11	ND	1.8	**2.58**/0.77	ND	1.3	**2.57**/0.61	ND	1.8	**2.52**/0.24
12	ND	2.2	2.05/0.46	ND	2.1	2.15/0.50	ND	2.1	1.91/0.35
13	1.5	1.1	1.92/0.59	1.7	1.6	1.81/0.47	1.3	1.5	1.85/0.35
14	ND	**2.1**	1.32/**1.05**	ND	2.6	1.14/0.16	ND	1.3	0.95/0.28
15	1.3	2.2	0.76/0.31	1.3	2.1	0.81/0.29	2.2	2.3	0.55/0.24
16	1.7	2.5	ND	2.1	**3.5**	ND	1.8	1.5	ND
17	2.1	1.7	ND	1.5	1.4	ND	2.1	1.7	ND
18	1.5	1.3	2.33/0.45	1.3	1.9	2.37/**1.14**	1.2	1.2	1.79/0.37
19	2.0	ND	ND	2.3	ND	ND	1.3	ND	ND
20	1.7	ND	**2.74/0.94**	1.2	ND	**2.46**/0.65	1.4	ND	**2.73**/0.64
21	1.4	ND	**2.56**/0.34	2.1	ND	**2.55**/0.29	1.5	ND	**2.73**/0.28
22	1.3	ND	1.23/0.36	1.7	ND	1.22/0.40	1.6	ND	0.88/0.31
23	1.9	ND	1.59/0.20	1.8	ND	1.28/0.31	1.3	ND	1.26/0.17

ND: Not done.

DISCUSSION

This investigation shows that the majority of patients with ReA/uSpA had humoral and cellular responses to *S. typhimurium*. In a few cases, *S. typhimurium* antigens were observed in synovial cells, whereas DNA was undetectable. In the presence of *S. typhimurium* antigens, SFMC produced elevated concentrations of IFN-γ, although the ratio of IFN-γ:IL-10 favored a skewing toward a Th2 response.

Patients with sporadic enteric ReA and uSpA had serological evidence of *S. typhimurium* infection. Serum antibacterial antibody response was not specific, as suggested[2]. Even sera IgA antibodies were seen in a minority of patients, emphasizing the limited role of preceding infection in diagnosis. As IgM antibodies are generally undetectable after 3–4 weeks of infection, we found no positive IgM antibody response.

An increased concentration of SF antibacterial antibody is strong evidence of the involvement of bacteria in the pathogenesis of synovitis. Intraarticular production of antibodies against *S. typhimurium* is suggested by a higher ratio of SF:serum IgA antibodies against *S. typhimurium* in patients with ReA/uSpA compared to the disease control. Further, the ratio was significantly higher in *S. typhimurium* compared to *S. flexneri* or *Y. enterocolitica*. This observation confirms reports of local antibacterial antibody production in patients with ReA[32,33]. A local antigen driven cellular and humoral

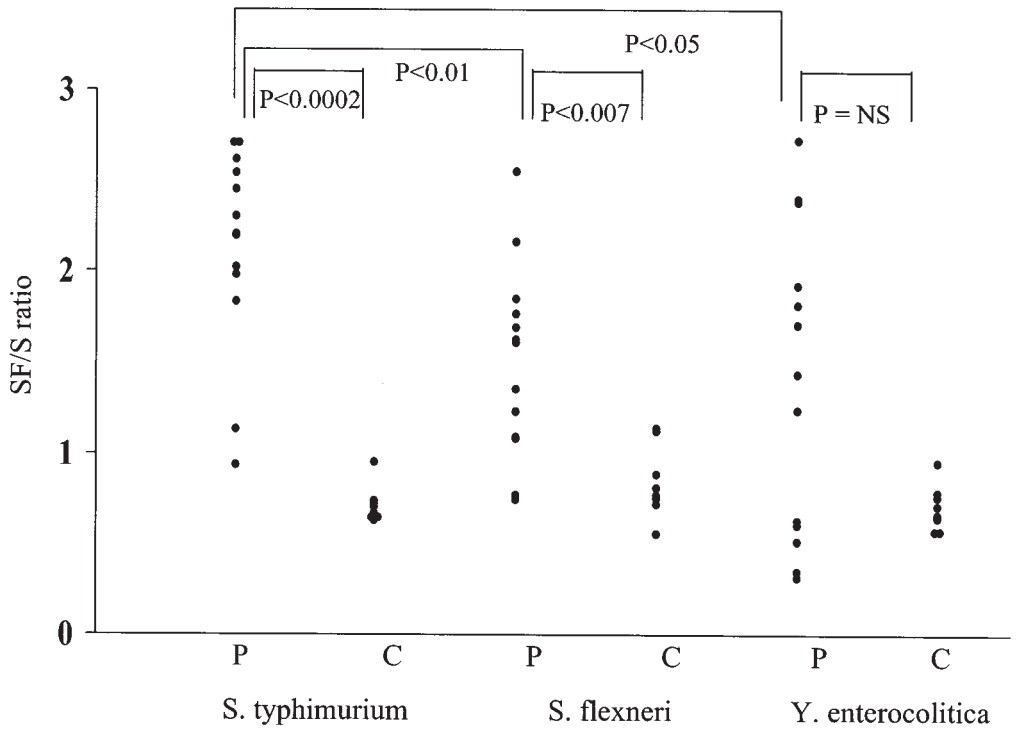

Figure 1. Ratios of SF:serum IgA antibody *to S. typhimurium*, *S. flexneri*, and *Y. enterocolitica* levels. The median ratio was significantly higher in patients compared to controls for *S. typhimurium* and *S. flexneri*. However, in patients with ReA/uSpa the ratio to *S. typhimurium* was significantly higher compared to *S. flexneri* ($p < 0.001$) or *Y. enterocolitica* ($p < 0.05$).

response is evident because the ratio of SF:serum IgA antibody was significantly higher in the patients with ReA who had positive SFMC proliferation compared to those without SFMC proliferation. Recent reports show that B cell differentiation in synovial tissue is a dynamic process and hypermutation and terminal B cell differentiation take place in synovial germinal-center-like structure[34,35].

Compared to the antibody response, the SFMC response was more striking, as almost half of them showed significant proliferation to *S. typhimurium*. Most patients having antigen-specific response to *S. typhimurium* had a very high proliferation index, suggesting increased frequency of antigen-specific T cells. A dominant SFMC proliferation response compared to the PBMC response confirmed observations in patients with Chlamydia, Yersinia, and Salmonella induced ReA and undifferentiated oligoarthritis[32,33]. The time lag between the experiment and onset of the disease[10] and/or higher antigen-specific T cell frequency in the synovium[36] could explain the differences in PBMC and SFMC responses.

The dominant local immune response observed in the synovial compartment led us to investigate the presence of bacterial products and DNA in the synovial cells. Salmonella antigens were detected in a few patients who had a positive Salmonella-specific SFMC proliferation response. The whole bacterium-like structure was missing. Failure to detect antigens in the majority of samples may be due to low concentration of the bacterial products, low sensitivity of the assay, or antigen localization to other parts of the body. Despite using highly sensitive hemi-nested PCR, we were unable to confirm the presence of bacterial DNA in the synovial cells. Unlike investigations with Chlamydia-triggered ReA[15,16], investigators have failed to identify enterobacterial DNA[13,17], except in one patient with enteric ReA where Yersinia DNA was identified by PCR using primers for pan-bacterial 16S rRNA followed by sequencing and matching of PCR product with the *Y. enterocolitica* genome[18]. The possibility of the presence of inhibitors of PCR in the sample was ruled out by successful amplification of HLA-B27 gene sequences from the same DNA preparations.

Although we did not identify the etiological agent for diarrheal illness in patients with ReA, observations that many patients with ReA or uSpA had positive SFMC proliferation response and heightened serum and SF antibody response to Salmonella and the presence of Salmonella antigens in the synovial cells suggest that these patients might have had a preceding Salmonella infection reflecting a higher incidence of gastrointestinal infection by this bacteria[25,26], and that uSpA might be forme fruste of ReA[1]. However, studies from the developed countries have shown evidence of Chlamydia and Salmonella infections as a major cause of ReA and uSpA[37].

We observed elevated secretion of Salmonella-specific IFN-γ. The source could be antigen-specific T cells in the synovium. SF T cell clones producing IFN-γ have been reported in patients with Yersinia and Chlamydia-triggered ReA[38,39].

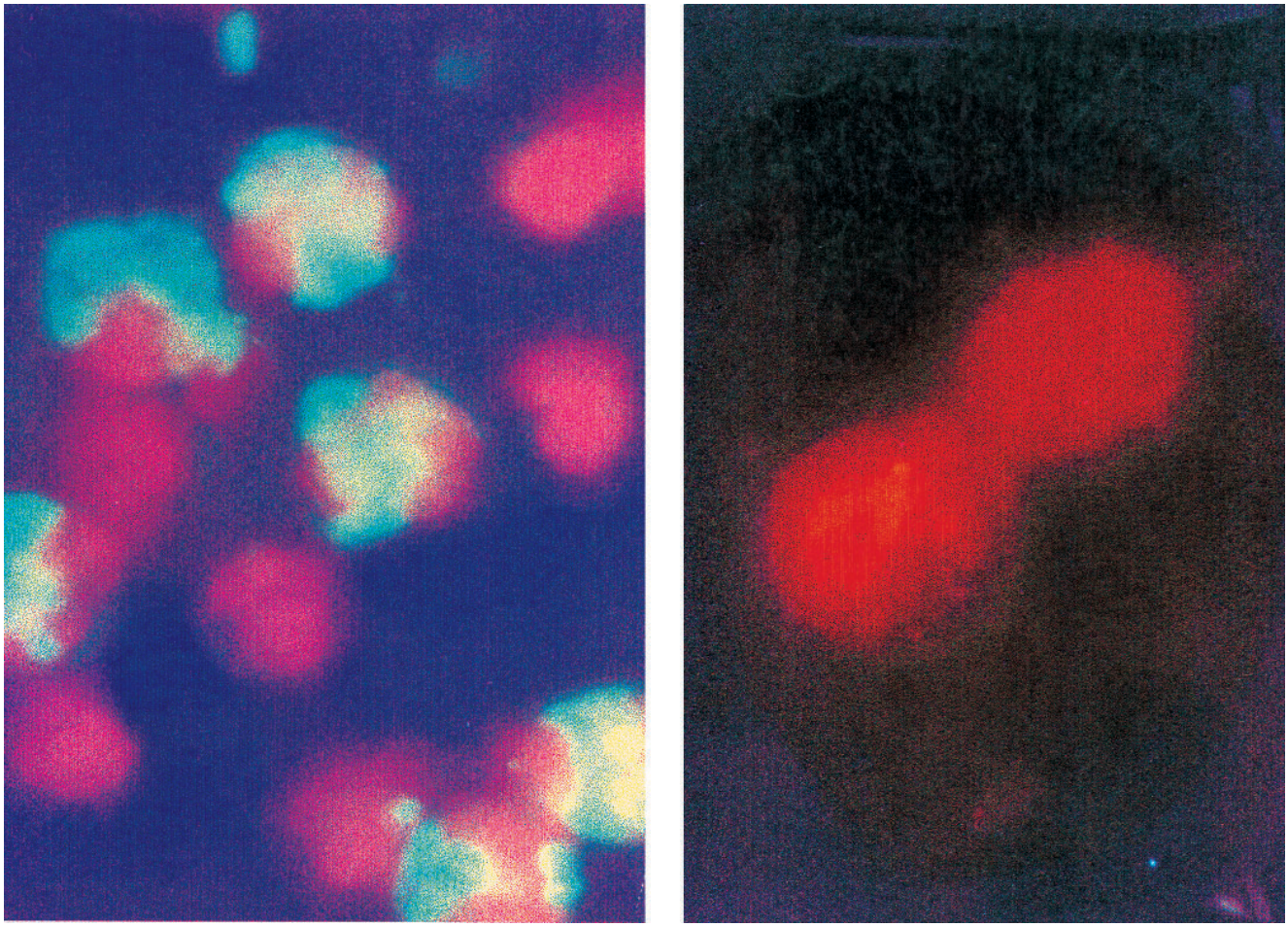

Figure 2. Cytospin smears of synovial cells from patients reacted with polyclonal anti-Salmonella antibody and developed using anti-rabbit antibody conjugate with FITC. A. Green cytoplasm staining suggests the presence of Salmonella antigen; nucleus is counterstained red with ethidium bromide. B. Negative staining suggesting absence of the antigen.

The increased secretion of IL-10 compared to IFN-γ was an antigen-independent event. The source of an antigen non-specific IL-10 production may be activated macrophages and/or lymphocytes. Partially cross-reactive antigens among these bacteria may also induce IL-10 secretion from Th2 cells, but may not induce IFN-γ production and T cell proliferation because different thresholds of activation are required to stimulate different T cell responses[40]. It is known that partially cross-reactive ligands like altered peptide ligands (APL) are able to induce cytokine secretion from Th2 cells, but cannot induce Th1-type cytokine synthesis or T cell proliferation[41].

The IFN-γ:IL-10 ratio was lower in patients with ReA compared to RA disease controls. However, IL-10 can also be produced by B cells and macrophages, and thus IL-4 is a better indicator of Th2 response. We did not detect IL-4 in the culture supernatants. In spite of a suggestion of skewed Th2-type immune response, antigen-specific secretion of IFN-γ in the patients who had Salmonella-specific SFMC proliferation indicates that the Th1/Th2 cytokine pattern is less polarized in humans than in mice, especially at the bulk culture level[42]. Although using the cytokine ratio would define the function of the cell (Th1 vs Th2), it does not take into account the quantity of cytokine produced[43]. Therefore, the presence of IFN-γ, IL-10, and many other cytokines in the local milieu might be of significant relevance in the pathogenesis of ReA and uSpA. Increased Th2 cytokines downregulate cell mediated immune responses against intracellular microbes and thus may limit the tissue-damaging effects of the Th1 response[44], or may inhibit microbial clearance[19,20]. The undetectable level of IL-4 in the culture supernatants might be because more prolonged antigen-specific stimulation is needed for its optimal secretion than for optimal secretion of IFN-γ[45].

Taken together, these data suggest that *S. typhimurium* is one of the important causes of enteric sporadic ReA and uSpA in patients in northern India.

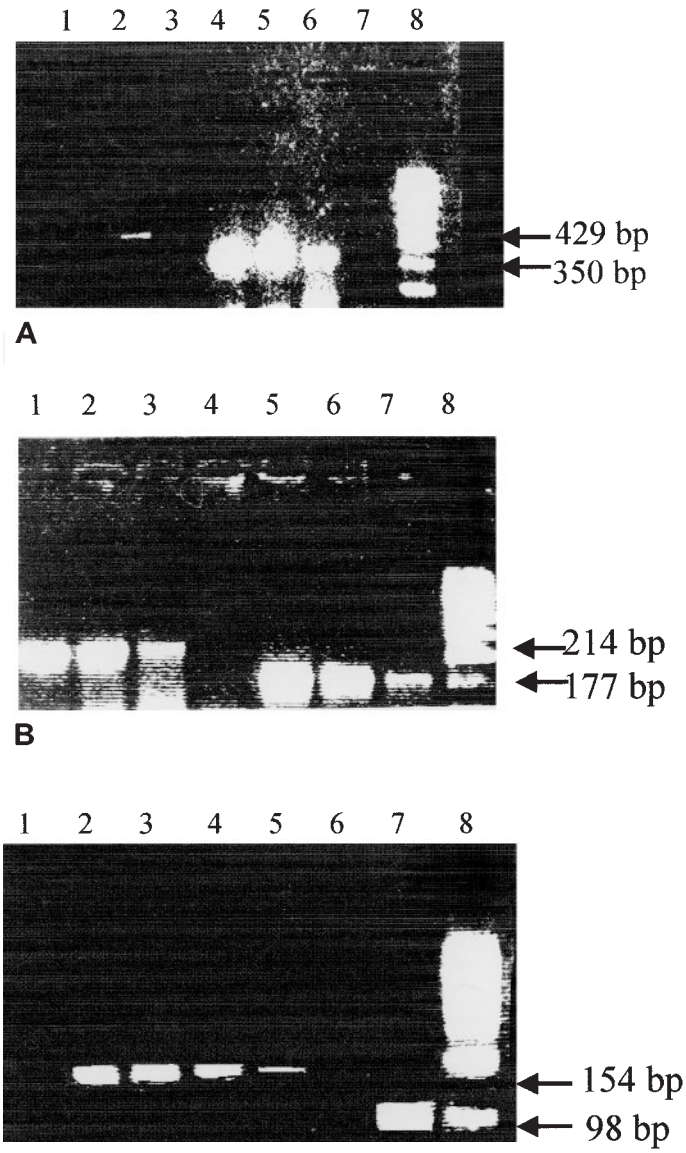

Figure 3. Agarose gel electrophoresis of PCR products showing the sensitivity of the assay using bacterial DNA as template. A. *S. typhimurium*. Lane 1: no DNA. Lanes 2 and 3 first-round PCR (429 bp product), lane 2: 1 ng, lane 3: 10 pg DNA. Lanes 4–7 nested PCR (350 bp product), lane 4: 1 pg, lane 5: 100 fg, lane 6: 10 fg, lane 7: 1 fg DNA, lane 8: 100 bp ladder. B. *S. flexneri*. Lanes 1–4 first-round PCR (214 bp product), lane 1: 1 ng, lane 2: 100 pg, lane 3: 10 pg, lane 4: 1 pg DNA. Lanes 5–7 nested PCR (177 bp product), lane 5: 1 pg, lane 6: 100 fg, lane 7: 10 fg DNA, lane 8: 100 bp ladder. C. *Y. enterocolitica*. Lanes 1–6 first-round PCR (154 bp product), lane 1: no DNA, lane 2: 1 ng, lane 3: 100 pg, lane 4: 10 pg, lane 5: 1 pg, lane 6: 10 fg DNA. Lane 7 nested PCR with 10 fg DNA (98 bp product), lane 8: 100 bp ladder.

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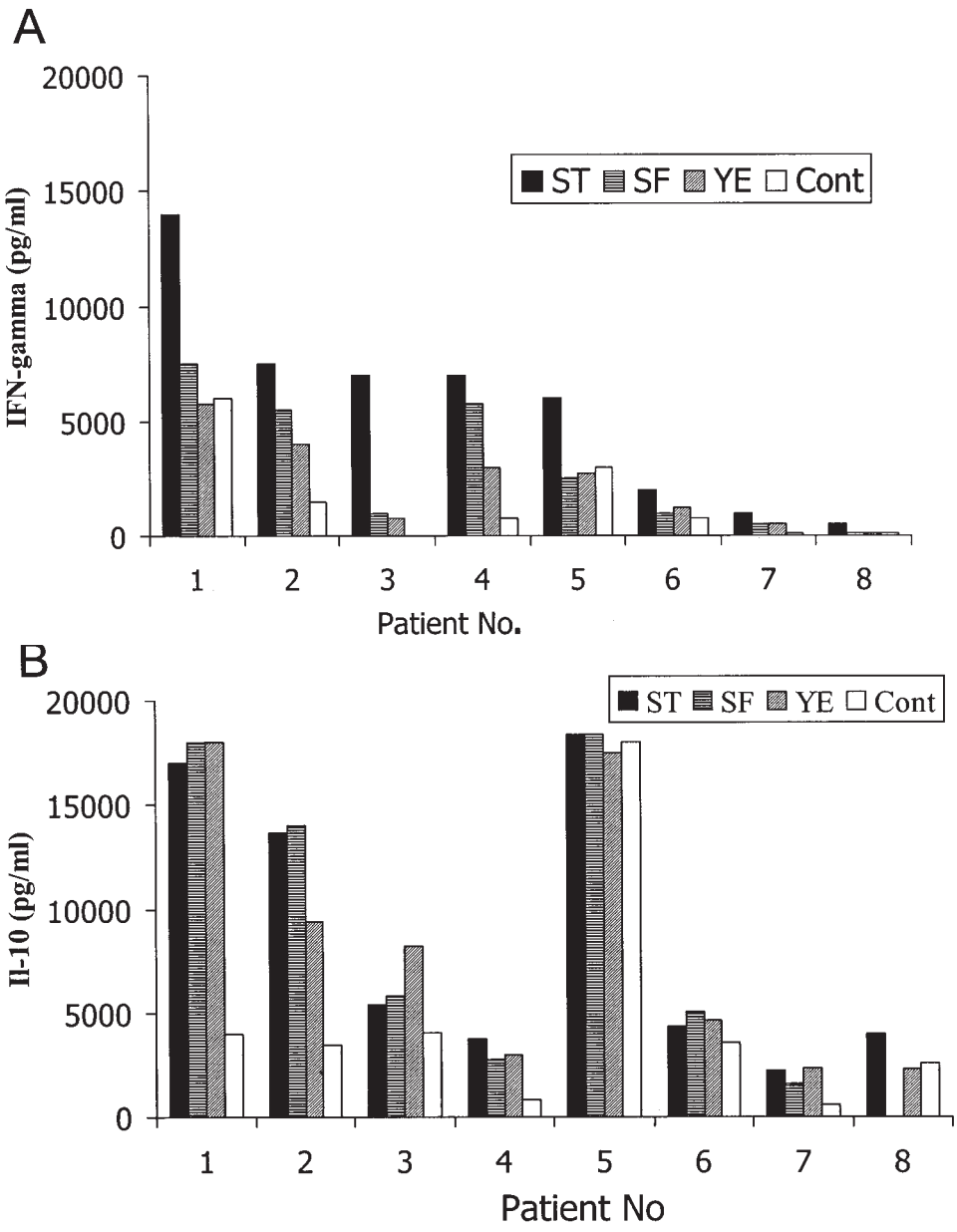

Figure 4. IFN-γ (A) and IL-10 (B) production in culture supernatants of SFMC from Patients 1–8 with positive *Salmonella*-specific SFMC proliferation response (stimulation index > 3.0). Patients 1, 2, 7, 8 had uSpA; Patients 3, 4, 5, 6 had ReA. ST: *S. typhimurium*, SF: *S. flexneri*, YE: *Y. enterocolitica*, Cont: control, no antigen.

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