Outer Membrane Protein of *Salmonella* Is the Major Antigenic Target in Patients with *Salmonella* Induced Reactive Arthritis

SHWETA SAXENA, AMITA AGGARWAL, and RAMNATH MISRA

ABSTRACT. ***Objective.*** We previously reported that *Salmonella typhimurium* was the triggering agent in one-third of our patients with sporadic enteric reactive arthritis (ReA) and undifferentiated spondyloarthropathy (uSpA). The antigens recognized by the synovial T cells in *Salmonella* induced ReA are not known. We investigated the immunodominant antigens in *Salmonella* ReA.

Methods. Synovial fluid mononuclear cells (SFMC) from 53 patients with ReA/uSpA were cultured with crude lysate of *S. typhimurium*. In 20 patients, the triggering agent was found to be *Salmonella* (stimulation index, SI, > 2.5). For cell fractionation of *S. typhimurium*, the sonicated crude lysate was separated by ultracentrifugation into a cytoplasmic supernatant (CYT) and membrane pellet (OMP). The CYT was further separated on SDS-PAGE and blotted onto nitrocellulose membrane for proliferation assays. SFMC from 20 patients with *Salmonella* ReA/uSpA were stimulated with OMP, CYT, and cytosolic fractions of *S. typhimurium*, and proliferation was measured by thymidine incorporation. Quantitation of antigen-specific cells in SF was by intracellular interferon-γ staining in 7 patients and paired peripheral blood (PB) in 5 patients after stimulation with crude *Salmonella* lysate, CYT, and OMP.

Results. Out of 20 patients with *Salmonella* ReA/uSpA, the SFMC showed a significant proliferation to OMP in 19 patients and CYT in 17 patients. The median SI of OMP (8.2, range 2.8–52.5) was significantly higher ($p < 0.0005$) than for the CYT (4.9, 2.7–18.8). Fifty percent of the patients showed proliferative response to cytosolic fractions of < 60 kDa. The mean antigen-specific T cell frequency was also higher with OMP (0.68% ± 0.59%) than CYT (0.53% ± 0.62%) but this was not statistically significant. Compared to PB, the OMP-specific cells were 7.5 times more numerous in the SF ($p < 0.05$).

Conclusion. The cellular immune response in *Salmonella* ReA/uSpA is directed predominantly against the OMP and low molecular weight proteins in the cytosolic fraction. (J Rheumatol 2005;32:86–92)

Key Indexing Terms:
IMMUNE RESPONSE ENTERIC BACTERIA
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Reactive arthritis (ReA) and undifferentiated spondyloarthropathy (uSpA) are triggered by genitourinary infection caused by *Chlamydia* or enteric infections due to *Salmonella*, *Shigella*, *Campylobacter*, and *Yersinia*[1-5]. Following epidemic of *Salmonella* infections, 0.2% to 7% of individuals develop inflammatory but sterile synovitis[6,7]. In sporadic ReA, we have recently reported that *Salmonella* accounts for one-third of cases in our community in contrast to *Yersinia* and *Chlamydia* in Western countries[8]. In *Salmonella* induced ReA, viable organisms cannot be cultured from the site of inflammation, but antigenic fragments have been found in the synovial cells[9,10]. T cell clones have been propagated from the synovial fluid (SF) of patients with *Salmonella* ReA, confirming the presence of T cells with specificity for the inciting antigen at the clonal level[11].

The antigens recognized by T cells have been studied in *Yersinia* and *Chlamydia* induced ReA. These include 19 kDa urease-ß subunit, 23 kDa ribosomal protein, heat shock protein (hsp 60), and outer membrane protein (OMP) in *Yersinia* ReA[12-14] and 60 kDa HSP and OMP of *Chlamydia*[15,16]. In typhoid fever and murine *S. typhimurium* infection, porins in the OMP are the major immunodominant antigens[17,18]. However, no such data are available in *Salmonella* ReA. We evaluated the immunodominant antigens that synovial fluid T cells recognize in ReA/uSpA.

MATERIALS AND METHODS

Selection of patients. Fifty-three patients with ReA/uSpA were studied; these were different from subjects in our previous study[8]. ReA was defined by the presence of acute onset asymmetrical oligoarthritis that was preced-

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ed by a history of diarrhea within 4 weeks, and patients with uSpA fulfilled the European Spondylarthropathy Study Group criteria[19]. Of these 53 patients, 20 had evidence for *Salmonella* induced ReA/uSpA as defined by an antigen-specific proliferative response of the synovial fluid mononuclear cells (SFMC) to the crude lysate of *S. typhimurium* (stimulation index, SI, > 2.5), but had no response to *Escherichia coli* antigen. There were 17 men and 3 women with a mean age of 24.7 years (range 13–37). Ten patients had a history of diarrhea. However, stool cultures were not done, as diarrhea had subsided when the patients reported to us. Eighteen of these were positive for HLA-B27. Demographic details of patients are given in Table 1.

ELISA for the detection of anti-Salmonella IgG and IgA in SF and serum. Ninety-six-well flat bottom plates (Nunc, Roskilde, Denmark) were coated with 100 µl of 2 µg/ml pooled lipopolysaccharide (LPS) of *S. typhimurium* and *S. enteritidis* in carbonate-bicarbonate buffer (pH 9.6) and incubated at 37°C for 1 h and then 4°C overnight. The plates were washed once with phosphate buffered saline (PBS; 0.15 M, pH 7.4) and blocked with 200 µl of 4% bovine serum albumin (BSA) in PBS for 2 h at 37°C. The plates were then washed 3 times with PBS containing 0.05% Tween 20 (PBS-T). The serum and cell-free SF were diluted 1:500 in PBS containing 1% BSA. 100 µl of diluted serum or cell-free SF were added to the wells and incubated 2 h at 37°C. The plates were washed 4 times with PBS-T and 100 µl of 1:4000 anti-human IgA-horseradish peroxidase (HRP, Dako, Berlin, Germany) and 1:6000 anti-human IgG HRP (Dako) in PBS-1% BSA were added and incubated for 2 h. The plates were washed 3 times with PBS-T and once with plain PBS. The color was developed with 100 µl of O-phenylenediamine (OPD, 4 mg in 10 ml citrate phosphate buffer, pH 5.4, and 1 µl/ml H_2O_2) and absorbance was taken at 492 nm. An optical density value exceeding the mean + 2 SD of controls was taken as positive.

Antigen preparation. Crude lysate. S. typhimurium (ATCC-13311) was grown in trypticase-soy broth overnight at 37°C. The bacteria were pelleted and washed 3 times with PBS 0.15 M, pH 7.2, and lysed using a pulse sonicator. The cells were sonicated 4 times for 30 s with 30 s rest on ice. After centrifugation at 12,000 g for 30 min at 4°C, supernatant was collected, aliquoted, and stored at –40°C. *E. coli* antigen was prepared in a similar way.

Preparation of OMP. OMP was isolated from the crude lysate according to the method described by Osborn, *et al*[20] with minor modifications. Briefly, the crude lysate of *S. typhimurium* was centrifuged at 100,000 g for 1 h to get the cytoplasmic supernatant and membrane pellet. The membrane pellet was treated with 2% sodium lauryl sarcosine for 20 min at 37°C[21]. Then it was centrifuged at 100,000 g to obtain OMP pellet. The membrane was washed with 20 mM Tris buffer (pH 7.5) 3 times at 100,000 g for 1 h at 4°C. The OMP pellet was resuspended in distilled water.

Preparation of particulate antigen. Cytosolic fractions were prepared as described[22]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of 1 mg of cytosolic supernatant (CYT) was performed in 12% linear polyacrylamide gels (Figure 1). The separated proteins were transferred from the gel onto nitrocellulose membrane (Sigma, St. Louis, MO, USA). The nitrocellulose membrane was cut into 6 fractions corresponding to molecular weights of < 20, 20–40, 40–60, 60–80, 80–100, and > 100 kDa. An additional piece of nitrocellulose membrane without antigen with the same surface area was included as negative control. Blot fractions were dried at 37°C for 1 h and solubilized in DMSO at 37°C for 1 h. Nitrocellulose particles were precipitated in equal volume of carbonate-bicarbonate buffer (0.1 M, pH 9.6) and collected by centrifugation. DMSO was removed by washing 4 times in RPMI-1640 (Sigma). Fractions were resuspended in RPMI-1640, aliquoted, and stored at –40°C; 1:20 and 1:40 dilutions of these fractions were used in proliferation assays.

Table 1. Demographic features of patients.

Features	ReA/uSpA, n = 20
M:F ratio	17:3
Median age, yrs (range)	25 (13–37)
Mean duration of disease, weeks (range)	3 (1–32)
Asymmetrical oligoarthritis, n	19
Inflammatory backache, n	17
Enthesitis, n	6
Sacroiliitis, n	3
Preceding diarrhea, n	10
Positive family history, n	4

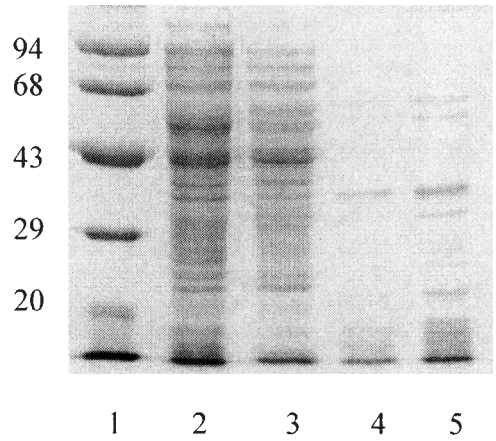

Figure 1. S. typhimurium antigens separated by 10% SDS-PAGE and stained by Coomassie blue. Lane 1, low molecular weight marker; lane 2, crude lysate; lane 3, cytosolic supernatant; lanes 4 and 5, 10 µg and 20 µg of outer membrane protein, respectively.

Lymphocyte transformation test. SFMC were isolated from SF by density gradient centrifugation over Ficoll-hypaque. The cells were adjusted to 1 × 10^6 cells/ml in complete RPMI-1640. A total of 1 × 10^5 SFMC (in 200 µl) were added to each well in 96-well plates with 10 µg/ml crude lysate of *S. typhimurium*, 10 µg/ml CYT, 10 µg/ml OMP, 1:20 and 1:40 dilutions of cytosolic fractions; 5 µg/ml PHA (Sigma) and no antigen was used as positive and negative control, respectively. Crude *E. coli* lysate was used as a control antigen. After 5 days of culture, cells were pulsed with 0.5 µCi ^{3}H-thymidine (Amersham Pharmacia Biotech, Little Chalfont, UK). After 18 h culture, the cells were harvested and radioactivity was counted in a liquid scintillation counter. ^{3}H-thymidine incorporation was measured as counts per minute and the results were expressed as stimulation index (SI); SI was calculated as counts per minute of cells with antigen divided by counts per minute of cells without antigen.

HLA-B27 typing. HLA typing was by nested polymerase chain reaction using B27-specific primers[23].

Intracellular interferon-γ (IFN-γ) staining. The SF and peripheral blood (PB) from 7 and 5 patients, respectively, with *Salmonella* induced ReA/uSpA were studied. Two hundred microliters of SF and heparinized PB were diluted 1:1 with PBS and stimulated for 6 h with 10 µg/ml of various *Salmonella* derived antigens — crude *Salmonella* lysate, CYT, and OMP in the presence of 1 µg/ml of anti-CD28 (Sigma) for costimulation; 50 ng/ml of PMA (Sigma) and 1 µg/ml of ionomycin (Sigma) were used as positive control. For the last 4 h of stimulation, the secretion inhibitor brefeldin A (Sigma) was added for the accumulation of intracellular cytokines as described[16]. For PB, lysis of red blood cells was carried out using fluorescence-activated cell sorter (FACS) lysing solution (Becton Dickinson, Heidelberg, Germany) according to the manufacturer's information. SF and PB cells were surface-stained with anti-CD4 and isotype antibody. The cells stimulated with PMA and ionomycin were surface-stained with anti-CD3. The cells were fixed and permeabilized with cytofix/cytoperm kit (Becton Dickinson) and stained with the intracellular

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cytokine, IFN-γ. All staining was performed in Perm wash buffer (Becton Dickinson).

The following antibodies were used: anti-human CD4 FITC, anti-human CD3 FITC, anti-human IFN-γ phycoerythrin (PE), isotype antibodies anti-mouse IgG1-κ PE, and anti-mouse IgG1-κ FITC (all from Becton Dickinson).

Positive cells were quantified by flow cytometry using FACS Calibur with Cell Quest software (Becton Dickinson). After gating the mononuclear cell population, 50,000 events were taken and analyzed.

Statistical analysis. The results were analyzed using the Wilcoxon signed-rank test for nonparametric data.

RESULTS

Sera samples from 17 patients with *Salmonella* ReA/uSpA, 25 patients with non-*Salmonella* ReA/uSpA, and 52 healthy controls were tested for IgG and IgA antibodies to pooled LPS of *S. typhimurium* and *S. enteritidis*. The cutoff optical density, taken as the mean + 2 SD of 52 age matched healthy controls, was 1.96 for IgG and 0.57 for IgA. There was no difference in concentration or prevalence of IgG or IgA antibodies between the 3 groups.

SF from 20 patients with *Salmonella* ReA/uSpA, 27 with non-*Salmonella* ReA/uSpA, and 19 with rheumatoid arthritis (RA) was tested. IgG antibody levels as well as prevalence were not different among the 3 groups. The mean + 2 SD of 19 disease controls for IgA antibodies to LPS of *Salmonella* was 0.296 in the SF. Eight of 20 patients (40%) with *Salmonella* induced ReA/uSpA, 8/27 (29.6%) with non-*Salmonella* ReA/uSpA, and 1/19 with RA (5.26%) were positive for IgA antibodies. The prevalence of IgA anti-*Salmonella* antibodies was higher in patients with *Salmonella* induced ReA/uSpA compared to those with RA ($p < 0.05$), but was no different from that seen in patients with non-*Salmonella* ReA/uSpA.

Response of SFMC to different S. typhimurium antigens. The SFMC from 19 out of 20 (95%) patients proliferated significantly (SI > 2.5) to OMP and induced the highest proliferative response (median SI 8.2, range 2.8–52.5) among all antigen fractions tested (Figure 2). This was significantly higher compared to crude *S. typhimurium* lysate (median SI 4.9, range 2.8–23.2; $p < 0.005$) and to CYT (median SI 4.9, range 2.7–18.8; $p < 0.0005$).

The proliferative response to CYT was found in 17 (85%) patients. However, this was not statistically significant compared with crude lysate. In 16 patients the response to the fractionated particulate cytosolic antigen was assessed. The SFMC proliferated to antigenic fractions of less than 20 kDa in 5 patients (median SI 3.74, range 2.73–6.58), to fractions of 20–40 kDa in 4 (median SI 3.55, range 2.8–7.74), and to 40–60 kDa cytosolic fractions in 4 patients (median SI 3.85, range 3.56–4.16) (Table 2).

Antigen-specific frequency of synovial T cells after stimulation with S. typhimurium derived antigens. The median numbers of IFN-γ-positive CD4+ T cells in the SF in 7 patients with *Salmonella* induced ReA/uSpA following stimulation with crude *Salmonella* lysate, CYT, and OMP were 0.4 (range 0.31–1.69), 0.36 (0.02–1.86), and 0.49 (0.15–1.93), respectively (Table 3, Figure 3). Using PMA and ionomycin as positive controls, the median IFN-γ-positive cell frequency was 16.97 (range 5.23–23.17).

Comparison of antigen-specific response in SF and PB. In 5 patients the paired PB and SF samples were also analyzed

Table 2. SFMC proliferative response to cytosolic supernatant (CYT) and various cytosolic fractions (kDa) in patients with ReA/uSpA. Proliferative responses are expressed as stimulation index (SI). SI > 2.5 was considered a positive response (shown in bold).

Patient	CYT	0–20	20–40	40–60	60–80	80–100	> 100
1	**17**	0.9	1.54	1.46	0.64	0.9	1.05
2	**6.3**	**3.16**	1.4	2.07	1.48	**2.69**	0.89
3	1.9	0.57	**2.9**	0.17	0.2	1.7	1.33
4	**4.4**	2.02	1.83	1.36	2.18	1.88	1.03
5	**5.4**	2	**2.8**	1.8	1.17	1.09	1.69
6	**2.7**	0.8	1.09	2.25	0.54	0.41	2.26
7	**13.9**	0.9	1.6	0.83	0.98	0.68	0.45
8	1.4	**6.58**	**4.2**	1.28	2.07	0.4	1.23
9	**5.8**	1.03	0.63	0.54	0.83	0.61	1.7
10	**2.56**	1.34	0.98	0.97	1.43	1.08	0.55
11	1.3	ND	ND	ND	ND	ND	ND
12	**18.82**	ND	ND	ND	ND	ND	ND
13	**3.45**	ND	ND	ND	ND	ND	ND
14	**3.89**	**3.74**	2	**3.9**	1.9	1.77	1.53
15	**10.5**	**2.73**	1.26	**4.16**	2.24	1.55	0.74
16	**2.9**	**3.76**	**7.74**	**3.56**	**3.95**	2.2	0.51
17	**17.9**	2	0.52	0.2	0.22	0.15	0.07
18	**8.2**	ND	ND	ND	ND	ND	ND
19	**14**	1.18	1.32	0.53	1.04	1.04	0.98
20	**3.07**	1.96	1.16	**3.8**	0.52	1.34	1.9

ND: not done.

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Table 3. Comparison of antigen-specific CD4+ IFN-γ+ T cells between the synovial fluid (SF) and peripheral blood (PB). Values are expressed as percentage of CD4 cells.

Patients	Crude		CYT		OMP	
	PB	SF	PB	SF	PB	SF
1	ND	1.69	ND	1.86	ND	1.9
2	0.02	0.31	0.08	0.21	0.02	0.35
3	0.21	0.36	0.17	0.17	0.04	0.49
4	0.15	0.59	0.12	0.54	0.06	0.54
5	0.38	1.14	0.27	0.36	0.14	0.48
6	ND	0.4	ND	0.53	ND	0.15
7	0.03	0.34	0.02	0.02	0.33	0.85

Crude: Crude *Salmonella* lysate, CYT: cytosolic supernatant, OMP: outer membrane protein, ND: not done.

simultaneously to look for antigen-specific frequencies. The percentage of antigen-specific T cells, as indicated by the percentage of IFN-γ-positive cells, was higher in the SF than in the PB samples. The average percentage of OMP-specific T cells was approximately 7.5 times higher in the SF than in PB samples. The antigen-specific T cell frequency was roughly 3 times higher in the SF than in the PB when stimulated with crude *S. typhimurium* lysate, and roughly 2 times higher in the SF after stimulation with CYT.

DISCUSSION

For the first time, we have demonstrated that immunodominant antigens in *Salmonella* induced ReA are present in OMP and 20–40, 40–60, and < 20 kDa cytosolic fractions. In addition, we reconfirmed our earlier observation that one-third of the sporadic ReA/uSpA in patients in our community is triggered by *S. typhimurium*.

In the absence of a culture-proven gastrointestinal infection, we have defined *Salmonella* induced ReA/uSpA based on SFMC proliferative response to bacterial antigens. Elevated antibacterial antibodies in sera have been used to define the microbial trigger[24]. However, this strategy was not useful to characterize the inciting agent due to elevated antibodies to these bacteria in healthy individuals (data not shown).

A synovial T cell response to triggering bacteria including *S. typhimurium* has been reported in patients with ReA[25]. Although bacterial antigens including LPS have been described in the synovial membrane and cells[9], the target antigens for T cell response have not been characterized to date. Based on our data, the strongest immunodominant antigen for the T cell response is the OMP of *S. typhimurium* in *Salmonella* ReA. Not only was this protein recognized by SFMC from 19 out of 20 patients, but as well the prolif-

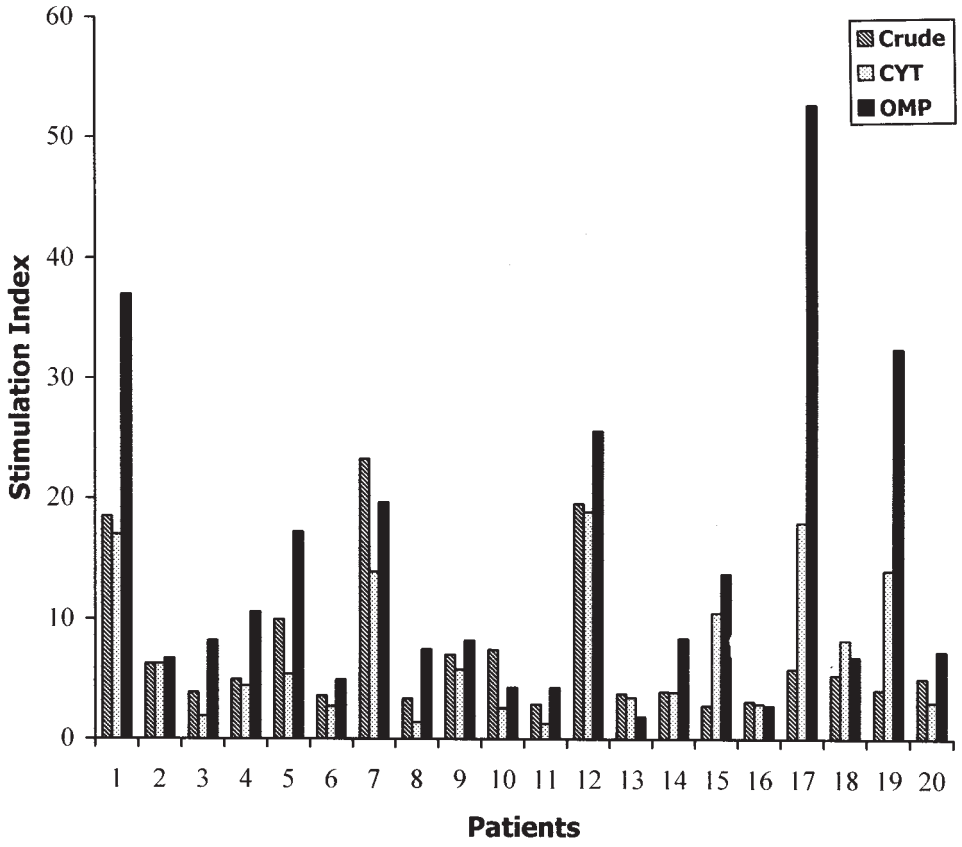

Figure 2. The proliferative response of SFMC of 20 patients with ReA/uSpA to 3 *S. typhimurium* antigens — crude lysate, cytosolic supernatant (CYT), and outer membrane protein (OMP). The highest proliferation was to OMP.

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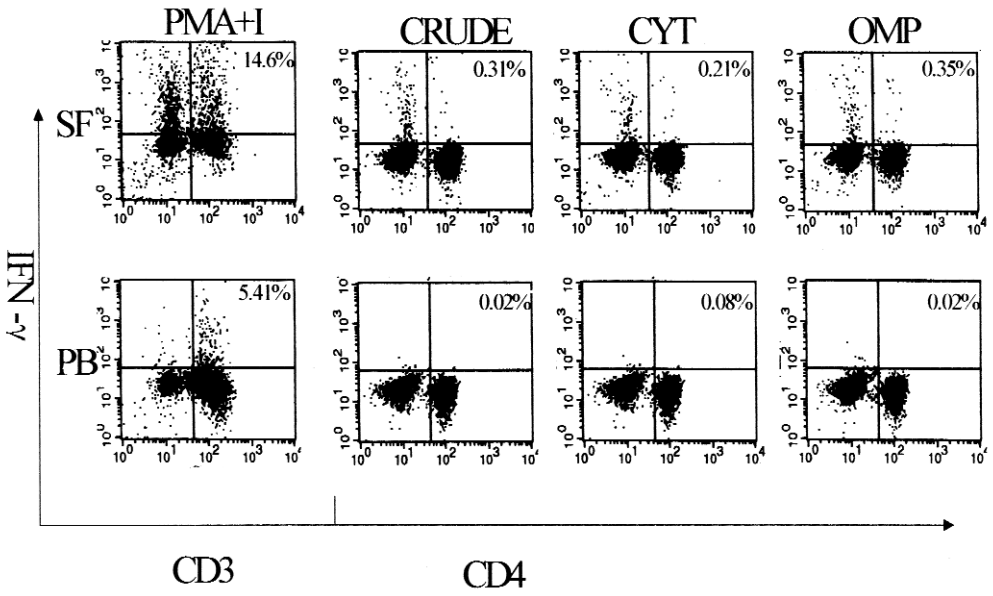

Figure 3. Dot plot of synovial fluid (SF) and peripheral blood (PB) cells of one patient with *Salmonella* induced ReA after stimulation with PMA, crude lysate, cytosolic supernatant (CYT), and outer membrane protein (OMP) of *S. typhimurium* and anti-CD28 for 6 h. After staining for the surface marker CD4, cells were fixed, permeabilized, and stained for the intracellular cytokine IFN-γ. Percentages of CD4+/CD3+ and IFN-γ-positive cells are indicated in the peripheral blood mononuclear cell gate.

erative response to this protein was much higher in magnitude than to the crude lysate of *S. typhimurium* and to the other *Salmonella* antigens. In salmonellosis, a humoral immune response to OMP has been shown to play a protective role, and a few studies even suggest it to be a target of the cellular immune response[17,18]. Further, in *Yersinia* and *Chlamydia* induced ReA, a dominant synovial T cell response against OMP has been reported[12,16]. The SDS-PAGE profile of OMP contains numerous polypeptide bands. The porins with a molecular weight of 34–36 kDa constitute the major component of the OMP and have been shown to be antigenic targets in typhoid fever[26]. In *Salmonella* ReA it remains to be seen whether the T cell response is specifically targeted against the porins or to other polypeptides of OMP.

Among the cytosolic particulate fractions, < 20, 20–40, and 40–60 kDa showed the maximum response. These fractions may contain relevant immunodominant antigens including heat shock proteins. A study described that SF T cells from 5 patients with ReA responded most strongly to a 65 kDa protein of *S. typhimurium*[27]. The 29–32 and 45–56 kDa antigenic fractions of *S. typhi* were identified as the main immunogenic fraction in typhoid fever and may play a role in postinfection immunity[28]. However, when mice were immunized with *S. typhimurium* by the oral route the immune responses of spleen cells were directed to the antigenic fractions 68–76, 50–52, and 42–45 kDa[29]. The 60 kDa HSP, one of the putative candidate antigens in inflammatory diseases[30], has been found to be the immunodominant antigen in *Yersinia* and *Chlamydia* induced ReA[12,31]. Antigenic fractions of < 20, 20–40, and 40–60 kDa need to be purified and characterized.

Our results suggest a heterogeneous immune response of the SFMC to the cytosolic fractions. Out of the 16 patients in whom the nitrocellulose-bearing cytosolic fractions were analyzed, only half showed a response to individual fractions. As well there were 2 patients who did not respond to the unfractionated cytosolic fraction who showed a proliferative response to the fractionated cytosolic antigens. Since the binding of protein to the nitrocellulose is based upon charge, the antigenicity is affected after separation. Thus, for some antigens, antigenicity is either enhanced or reduced upon fractionation. *Yersinia*-specific T cell clones failed to respond to nitrocellulose membrane-bound antigens, although there was a vigorous response to soluble antigens[32]. Even at the clonal level the immune response has been found to be heterogenous in *Yersinia* induced ReA[33].

In a conventional lymphocyte proliferation assay, antigenic specificity can be misleading due to nonspecific bystander activation of T cells. Moreover, it is not possible to quantitatively enumerate antigen-specific T cells. Antigen-specific cytometry has become an exciting new technology for the analysis of antigen-specific T cells[34]. It has been found by this method that the antigen-specific T cell frequencies parallel the frequencies estimated by tetramer technology[35]. Hence, we used intracellular IFN-γ staining to estimate the antigen-specific cells. The antigen-specific frequencies were higher to OMP than to CYT. In SF the frequency was 2–7 times higher compared to PB. Thus activated T cells in ReA are localized to the synovial com-

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partment. The intracellular data also complement the results of lymphocyte proliferation assays and suggest that OMP is the immunodominant antigen.

However, in flow cytometry it would have been better to do triple staining with CD3, CD4, and IFN-γ to confirm that these antigen-specific cells are CD4+ T cells. It would be interesting to know what population of cells is producing IFN-γ besides CD4+ cells on stimulation with *Salmonella* antigens: possibly they are CD8+ T cells or monocytes. Use of a control antigen like *E. coli* would have confirmed that these T cells are responding only to *Salmonella*.

ReA has a very strong genetic association with the HLA class 1 molecule HLA-B27. Most of our patients were also HLA-B27-positive. The exact mechanism by which HLA-B27 contributes to the pathogenesis of ReA has not been determined. One of the postulated mechanisms is molecular mimicry, in which the bacteria triggering ReA share some sequence homology with HLA-B27. A cationic outer membrane protein of *Salmonella*, OMP H, has been found to share homology with 5 amino acids of HLA-B27 in a non-linear fashion[36]. We need to investigate OMP H further in our patient group to determine the role of HLA-B27 in the disease pathogenesis. Recently, HLA-B27 (309–320) peptide has also been shown to have significant homology with at least 6 proteins from *S. typhimurium*[37].

We conclude that outer membrane protein is T cell stimulatory in *Salmonella* ReA, and further studies are needed to define the target epitope and its part in the pathogenesis of this disease. It may also help to serve as a diagnostic test, as in *Yersinia* triggered ReA[38].

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