Evidence of Cellular Immune Response to Outer Membrane Protein of *Salmonella typhimurium* in Patients with Enthesitis-related Arthritis Subtype of Juvenile Idiopathic Arthritis

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**ABSTRACT.** Objective. Enthesitis-related arthritis subtype of juvenile idiopathic arthritis (JIA-ERA) clinically resembles reactive arthritis (ReA). In patients with ReA the immune response is targeted at the outer membrane protein (OMP) of *Salmonella typhimurium*. We studied the immune response in JIA-ERA to *S. typhimurium* OMP.

**Methods.** Synovial fluid mononuclear cells (SFMC) and peripheral blood mononuclear cells (PBMC) were isolated from blood and SF of patients with JIA-ERA. Lymphocyte transformation test was done with *S. typhimurium* OMP and crude bacterial lysates of *Yersinia enterocolitica*, *Shigella flexneri*, and *S. typhimurium*. IgG and IgA ELISA were performed in serum and SF using *S. typhimurium* OMP as antigen and compared with sera from healthy controls.

**Results.** In PBMC samples (n = 25) an antigen-specific proliferative response was seen in 13 patients and a cross-reactive response in 6. Among these 19 patients, 12 showed response to OMP. In SFMC (n = 15) antigen-specific responses were seen in 3 patients and cross-reactive responses in 9. Among these 12 patients, 11 showed response to OMP. The IgG and IgA anti-OMP antibody concentrations in serum and SF were similar in patients and controls.

**Conclusion.** In JIA-ERA, OMP is the major antigenic target recognized by both SFMC and PBMC. Response to OMP is independent of specific bacterial response, suggesting that OMP is the immunodominant antigen. In these patients, absence of significant humoral response suggests response to OMP is mainly T cell mediated. (First Release Nov 1 2010; J Rheumatol 2011;38:161–6; doi:10.3899/jrheum.100542)

**Key Indexing Terms:**
JUVENILE IDIOPATHIC ARTHRITIS
PATHOGENESIS
LYMPHOCYTE TRANSFORMATION TEST

Enthesitis-related arthritis (ERA) is the most common form of juvenile idiopathic arthritis (JIA) seen in India. The onset and clinical course of JIA-ERA have striking similarity to reactive arthritis (ReA). Both diseases share several features such as predilection of male sex, asymmetrical lower-limb arthritis, enthesitis, progression to ankylosing spondylitis, and a strong association with HLA-B27. It is therefore possible that this type of arthritis could be triggered by microbes as in ReA. This is supported by the HLA-B27 transgenic rat model, where gut bacterial flora influence disease development. HLA-B27 transgenic rats raised in germ-free conditions do not develop arthritis.

Previously we had shown a higher lymphoproliferative response of mononuclear cells from blood and synovial fluid (SF) to enteric bacteria in patients with ReA. Data further suggested that *Salmonella typhimurium* was the most common organism and was responsible for proliferative responses in at least one-third of patients with ReA. The immune response was more vigorous against the outer membrane protein (OMP) of *S. typhimurium* than the cytosolic proteins. Further fractionations have shown that low molecular weight fractions of OMP contained the immunodominant antigens. Even in other organisms that trigger ReA such as *Chlamydia* and *Yersinia*, OMP have been found to generate a good immune response.

In patients with JIA-ERA, we had earlier shown that synovial fluid mononuclear cells (SFMC) proliferated significantly to enteric bacterial antigens in vitro, suggesting that the bacteria could have triggered the arthritis in half of these children. A study by Sieper, et al had similar observations. However, it is not known whether the response is directed against the OMP or not.
OMP are present in the outer membrane of bacteria. There are 100–250 proteins in the outer membrane of enteric bacteria\(^{16,17,18}\). They can belong to the porin family or be non-porin proteins. They are conserved across species among the gram-negative enteric bacteria, and antigenic cross-reactivity of major OMP in Enterobacteriaceae species is well known\(^{19,20,21}\).

As OMP is the major antigenic target, we evaluated both cellular and humoral response to S. typhimurium OMP in patients with JIA-ERA to test our hypothesis that, both clinically and immunologically, JIA-ERA shares several features with ReA.

MATERIALS AND METHODS

Patients with JIA-ERA satisfying ILAR (International League of Associations for Rheumatology) criteria were included in the study after providing informed consent\(^{22}\). PB was collected in heparin vials for the lymphocyte transformation assay (LTT) and in plain vials for serum separation. SF was collected in heparin tubes at the time of intraarticular corticosteroid injection. Part of the SF was centrifuged immediately at 840 g and cell-free supernatant was stored in aliquots at −80°C for ELISA. The remaining SF was used for the LTT assay. The sera were stored at −80°C until analysis. We included 25 patients; of these, 15 had paired samples (both SF and PB); the remaining 10 were unpaired samples (PB). Sera samples from 10 healthy volunteers (of similar age and sex) were collected with consent and used as controls.

The study was approved by the institutional review board.

Antigen preparation. Bacterial lysates were prepared according to a described protocol\(^{9}\). Briefly, S. typhimurium, Shigella flexneri, Yersinia enterocolitica, and Escherichia coli were grown overnight at 37°C in Luria Broth (HiMedia, Mumbai, India). The bacteria were pelleted and washed 3 times with phosphate buffered saline (PBS, 0.15 M, pH 7.4) and lysed using a pulse sonicator (Branson Sonifier, Frankfurt, Germany). 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, stored at −40°C, and used as a source of bacterial antigens.

OMP preparation. OMP from S. typhimurium was prepared by sarcosine-insoluble outer membrane fractionation as described\(^{40}\). Briefly, crude lysate of S. typhimurium was centrifuged at 100,000 g for 1 h to obtain the cytoplasmic supernatant and membrane pellet. The membrane pellet was treated with 2% sodium lauryl sarcosine for 20 min at 37°C, then centrifuged at 100,000 g to obtain OMP pellet. The OMP pellet 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, aliquoted, and stored at −80°C.

Lymphocyte proliferation assay (LTT). SFMC and PB mononuclear cells (PBMC) were separated by density gradient centrifugation using Histopaque (Sigma, St. Louis, MO, USA). The mononuclear cell layer was collected and washed 3 times with PBS at 470 g for 15 min, 300 g for 12 min, and 210 g for 10 min. The cell number was adjusted to 1×10^6/ml in complete RPMI-1640 (RPMI contains 10% fetal calf serum 10 mg/ml, amphotericin 0.25 µg/ml, penicillin 10,000 U/ml, pH 7.2; Sigma). A total of 1×10^5 SFMC were plated in 96-well culture plates and cultured 5 days with bacterial antigen in triplicate in 3 dosages: 2.5, 5, and 10 µg/ml. Phytohemagglutinin (PHA) 5 µg/ml (Sigma) was used as positive control in a 3-day culture. Wells without antigen were used as a control. Tritiated thymidine 0.5 µCi (BARC, India) was added per well during last 18 h of culture. The cells were harvested on fiberglass filters (Advanced Microdevices Pvt., Ambala Cantt, India) using a multiwell harvester (Skatron, Lier, Norway) and washed 3 times with RO water during harvesting. The filter discs were washed with distilled water 3 times during harvesting and then dried at 60°C for 2 h. Dried filter discs were placed in scintillation vials (Tarsons, New Delhi, India) containing 2 ml scintillation Cocktail-O (SRL, New Delhi, India). Radioactivity was counted in a liquid scintillation counter (Beckman, Fullerton, CA, USA). Thymidine uptake was measured as counts per minutes (cpm) and the mean cpm of triplicate wells was calculated for each concentration of antigens. The results are expressed as stimulation index (SI) = cpm in the antigen well/cpm in the control well. Antigen-specific proliferation was considered significant when either SI value was > 2.5 to a single antigen or in cases of response to more than one antigen the highest SI was 2.5 times more than the highest value, as taken by Sieper, et al in patients with juvenile chronic arthritis\(^{15}\).

ELISA for anti-OMP antibody. IgG and IgA antibodies to OMP were measured by ELISA in serum and SF using an in-house assay. All samples were run in duplicates. Briefly, 96-well flat-bottom ELISA plates (Nunc, Roskilde, Denmark) were coated with 100 µl OMP (10 µg/ml) in carbonate and bicarbonate buffer (pH 9.5) and incubated at 37°C for 1 h, then at 4°C overnight. Next morning, plates were washed with PBS (0.15 M, pH 7.4) and blocked with 300 µl/ml of PBS containing 4% bovine serum albumin (BSA) for 2 h at 37°C. Then the plates were washed with PBS and 100 µl of diluted serum or cell-free SF was added in duplicate to the wells and incubated 2 h at room temperature. In serum samples for IgA 1:100 dilution was used, and for SF 1:250. For IgG 1:500 dilution was used for both SF and serum samples. Then plates were washed 4 times with PBS-Tween and then with PBS and 100 µl of 1:4000 anti-human IgA horseradish peroxidase (Dako, Heidelberg, Germany) and 1:5000 anti-human IgG horseradish peroxidase (Dako) in PBS-1% BSA was added and incubated 1 h. Plates were washed 4 times with PBS-Tween and once with PBS. The color was developed with 100 µl of TMB (BD Biosciences, San Jose, CA, USA); the reaction was stopped after 20 min with 2 N H₂SO₄ and absorbance was taken at 450 nm. Mixture of SF samples providing high absorbance was taken as standard and run with every assay in doubling dilutions; 100 arbitrary units (AU) were assigned to the value where the curve plateaued. All patient sample values were calculated using the standard curve and are expressed as arbitrary units, and values exceeding the mean + 2 SD of control were taken as positive.

HLA-B27 typing. HLA-B27 was typed by the amplification refractory mutation system (ARMS)-polymerase chain reaction (PCR) technique\(^{23}\).

Statistical tests. Chi-square test was used for proportion analysis in HLA-B27-positive and negative groups; Mann-Whitney U test was used to compare groups in ELISA.

RESULTS

Of the 25 patients, 24 were male. Median age at assessment was 17 years (range 13–22 yrs), median disease duration was 5 years (range 0.5–10 yrs). HLA-B27 was positive in 80% of the patients (20/25). Most patients had active peripheral arthritis (22/25). Active enthesitis was observed in 13 patients and inflammatory back pain in 12.

Proliferative responses of SFMC and PBMC to crude bacterial lysate. PBMC from 13 of the 25 patients had antigen-specific responses to enteric bacteria: 8 to S. typhimurium, 3 to Y. enterocolitica, and 2 to S. flexneri. PBMC from 6 patients showed a cross-reactive response to more than one bacterial antigen, and those from 6 patients showed no response (Table 1). SFMC from 3 of the 15 patients had antigen-specific responses to enteric bacteria: 2 to S. typhimurium, 1 to S. flexneri. SFMC from 9 patients showed a cross-reactive response to more than one bacterial antigen, and those from 3 patients showed no response (Table 1).
Proliferative responses of SFMC and PBMC to OMP. PBMC from 12/13 patients with an antigen-specific or cross-reactive response had a proliferative response to OMP. Of the 6 patients who had no response to any bacterial lysate, one had a response to OMP (Table 1). In PB, the SI varied from 2.7 to 32.6 (Figure 1).

SFMC from 11/12 of patients with an antigen-specific or cross-reactive response had a proliferative response to OMP (Table 1). None of the patients with absence of response to crude bacterial lysate had a proliferative response to OMP. In the SF, SI varied from 2.9 to 75 (Figure 1).

Anti-OMP antibody in serum and SF. IgG and IgA antibody levels in SF and serum were similar in patients and controls (Figure 2).

Relationship between HLA-B27, LTT response to OMP, and antibody response to OMP. In the PB, 11 of 13 patients who had an OMP response were HLA-B27-positive. In the SF, 10 of 11 patients who had OMP response were HLA-B27-positive. IgG antibody response to OMP was seen in 7 patients: 2 in SF and 5 in the serum. All 7 patients were HLA-B27-positive. However, there was no statistically significant difference between the HLA-B27-positive and negative groups with regard to either LTT or antibody responses.

DISCUSSION
Lymphoproliferative responses to enteric bacterial antigens were seen in both PB and SF. *S. typhimurium* was the most common organism responsible for antigen-specific response. Lymphoproliferative responses to OMP could be observed in more than 50% of patients with antigen-specific responses. Most of the patients with cross-reactive responses also had proliferative response to OMP. However, the antibody levels to OMP were similar between the patients and controls. HLA-B27 was present in 80% of patients. But there was no difference in the LTT responses between HLA-B27-positive and negative subgroups.

Since JIA-ERA shares a lot of similarity with ReA, enteric bacteria are thought to play a role in its pathogenesis. Our data of 50% of patients showing lymphoproliferative response to enteric bacterial lysates support this view. They are similar to previous data from India and Europe. Further, *S. typhimurium* was the most commonly identified organism responsible for proliferative responses. This supports our previous observations in children with JIA-ERA and also in patients with ReA, whereas, in a European population with late-onset pauciarticular juvenile chronic arthritis, *Y. enterocolitica* was the most common.

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OMP: outer membrane protein; SFMC: synovial fluid mononuclear cell; PBMC: peripheral blood mononuclear cell; NA: sample not available; CR: cross-reactive response; NR: nonresponder; R: responder.
organism (11/28) against which the proliferative response was seen. This is probably related to the difference in prevalence of microbial pathogens causing enteric infections in Indian and European populations.

In this study we had a higher prevalence of antigen-specific proliferative response to crude bacterial lysate in PBMC as compared to SFMC. This is in contrast to our earlier observation where the immune response was mostly limited to the synovial compartment in both JIA-ERA and ReA. In ReA, the proliferative response depends on the time between the onset of flare/arthritis and sample collection. Proliferative response in the synovial compartment is seen both during early and late disease, but proliferative responses in PB are seen in early disease usually within 2 to 4 weeks. Most of our patients presented to the hospital within 2 to 4 weeks of flare of arthritis and this can explain the higher prevalence of proliferative responses in PB.

The majority of the patients with proliferative responses to S. typhimurium also recognized OMP. OMP are present in the outer membrane and are thus most accessible to the immune system to generate an immune response. In our previous study, 19 of 20 patients with S. typhimurium-induced ReA had a proliferative response to the OMP. This suggests that, similar to S. typhimurium ReA, OMP may be one of the antigenic targets in patients with JIA-ERA. OMP represent important virulence factors and play essential roles in bacterial adaptation to host niches, which are usually hostile to invading pathogens. OMP are conserved across species and antigenic cross-reactivity of major OMP has been seen among Enterobacteriaceae species. The entire genome of S. typhimurium has been sequenced. There is striking similarity between the genomes of various enteric bacteria. If the DNA sequences of genes in the core genome of different enteric bacteria are compared, E. coli and S.

![Figure 1. The stimulation index (SI) with OMP in PBMC and SFMC. **Patient number 21 had SI value of 32.6 in the PBMC assay. *Patient 23 had SI value of 75 in the SFMC assay. NA: sample not available. Horizontal line represents the cutoff value for SI, 2.5.](www.jrheum.org)
enterica are found to differ by ~10%, and Salmonella serovars within S. enterica differ by ~1%. This 10% divergence between the core sequences of E. coli and S. enterica most likely represents evolutionary drift over the ~100 million years since the 2 species separated from a common ancestor.18,25 These data indicate common ancestry among the gram-negative bacteria, resulting in sharing of various components including the OMP. This explains our observation that S. typhimurium OMP was recognized by patients’ T cells recognizing other enteric bacterial antigens, as well as patients having response to multiple enteric bacteria (cross-reactive responses). Thus, OMP may be a common antigenic target against which the immune response is generated.

The exact protein in the S. typhimurium OMP responsible for this T cell response remains unknown. Data in ReA suggest that the low molecular weight fraction of the S. typhimurium OMP is more immunogenic. Proteomic analysis of these low molecular weight fractions has revealed 10 identifiable proteins including OMPA, OMPW, and OMPX.11 Of these OMP, OMPA is evolutionarily conserved, and cross-reactivity between gram-negative bacteria was described as early as 1982. OMPA functions as an adhesin, invasin, and immune evasin.27,28,29 It has a role in survival of the organism in macrophages/monocytes. OMPA mutants have reduced survival within macrophages.28 Bacteria expressing OMPA prevent macrophage apoptosis by inducing the antiapoptotic factor BcL-xL.20 This may result in persistence of the organism in the immune cell that in turn results in dissemination of the organism in the infected host. Salmonella species DNA has been detected in SF of patients with juvenile-onset ankylosing spondylitis.30

The lack of antibody response to OMP emphasizes that JIA-ERA is a T cell-mediated disease and a humoral response may have no role in the pathogenesis.15 HLA-B27 was positive in 80% of the patients. This prevalence is similar to figures quoted in the literature.3,4 However, HLA-B27 did not seem to influence proliferative responses to OMP. These results are similar to those of Sieper, et al.15 However, it would be difficult to draw any conclusions from these findings as the number of samples was small.

The LTT response to OMP may be used as a surrogate marker for an enteric bacterial trigger in patients with JIA-ERA. A study has shown that, in patients with enteric ReA treated with ciprofloxacin for 3 months, there was less chance of having uveitis, chronic rheumatic disease, and ankylosing spondylitis.31 Thus, identifying an enteric bacterial trigger may provide a basis to prescribe antibiotic therapy in patients with JIA-ERA.

In summary, in JIA-ERA, OMP are the major antigenic target recognized by both SFMC and PBMC. Response to OMP is independent of specific bacterial response, suggesting that OMP are the immunodominant antigens. In these patients, the absence of significant humoral response suggests that response to OMP is mainly T cell-mediated. Further, it suggests that JIA-ERA not only shares clinical features with ReA but probably has a similar immunopathogenesis.

REFERENCES


