Avidity of antibodies against released proteins of *Yersinia* spp: comparison of patients with or without reactive arthritis

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**SUMMARY** The avidity of IgM, IgG, and IgA class antibodies against virulence plasmid encoded proteins of *yersinia*, so called released proteins (RPs), was studied in the serum samples of 22 patients with *yersinia* triggered reactive arthritis and 22 with uncomplicated yersiniosis. The avidity of anti-RP antibodies did not differ significantly between the patient groups, even though the total amount of IgA class anti-RP antibodies was significantly higher in patients with arthritis than in those with uncomplicated yersiniosis at the early stage of infection. Earlier results with whole bacterial extracts or lipopolysaccharide as the antigens have shown an increased avidity of IgA class anti-*yersinia* antibodies in patients with *yersinia* triggered reactive arthritis. This phenomenon was not observed in antibody response against RPs, and thus production of these proteins seems unimportant at later stages of infection for the *yersinia* organisms stimulating the persisting and maturing antibody response in the *yersinia* triggered reactive arthritis.

*Y enterocolitica* and *Y pseudotuberculosis* grown at 37°C produce several virulence plasmid encoded proteins. Moreover, plasmid bearing *Y enterocolitica* and *Y pseudotuberculosis* release into calcium deficient media proteins, so called released proteins (RPs), which are immunologically related to *yersinia* outer membrane proteins. These proteins are expressed in vivo as both patients and animals produce specific antibodies against them during *yersinia* infection.

It is not known why some patients develop reactive arthritis after acute yersiniosis, whereas others recover without any complications after the infection. Development of reactive arthritis is associated with the tissue antigen HLA-B27 and certain immunological features. Patients who develop *yersinia* triggered reactive arthritis are characterised by strong and persisting IgA antibody response against *yersinia*. Analysis of antibody avidity has shown that patients with reactive arthritis have significantly more high avidity IgA class antibodies than those with uncomplicated infection, — a difference which increases with time.

This was seen by using a bacterial extract and separately, purified lipopolysaccharide of *Y enterocolitica* O:3 as the antigen. In this study of the role of RPs in the pathogenesis of reactive arthritis we investigated avidity of the anti-RP antibody response in patients with and without *yersinia* triggered reactive arthritis.

**Patients and methods**

**Patients and Serum Samples**

The avidity of anti-*yersinia* antibodies was studied in the serum samples of 44 patients with recent yersiniosis. Diagnosis was based on the clinical picture, clearly increased levels of anti-*yersinia* antibodies detected by enzyme linked immunosorbent assay (ELISA), and in 15 cases, additionally, by isolation of the pathogen from the stools. Twenty two patients (age 37 (SD 13) years, 14 men, eight women) developed reactive arthritis as a complication after infection and the other 22 (age 29 (11) years, 13 men, nine women) had uncomplicated yersiniosis. Two samples from each patient were studied; one taken at less than two months and another at about six months after onset of the infection. Serum pairs from the two patient groups were chosen so that the time interval after onset of
infection differed by only 2.6 (SD 2.1) days between each pair.

**Antigens**

The bacterial strain used for production of RPs was *Y. enterocolitica* serotype O:8 (WA-314 NaI†, containing the 42 megadalton virulence plasmid). Isolation and analysis of the proteins have been described recently in detail. Briefly, bacteria were first grown in BHI broth at 37°C for 90 minutes, followed by incubation under calcium restriction by addition of 10 mM magnesium-EGTA (ethyleneglycolbis (aminoethylether)tetra-acetate) for an additional 90 minutes. The bacteria were removed by centrifugation and the proteins precipitated from culture supernatant by ammonium sulphate followed by three washings of the insoluble protein precipitate with water. Analysis of the RPs by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting and purity controls was performed as described earlier.

**ELISA for Anti-RP Antibodies**

An ELISA for anti-yersinia antibodies was performed as described earlier. The polystyrene microtitre plates (Titertek, Flow Laboratories, Scotland) were coated with 6 μg/ml of RPs in phosphate buffered saline (0.1 M, pH 7.5) by overnight incubation at 37°C, and saturated with 1% normal sheep serum in phosphate buffered saline. Optimal dilutions were chosen to be on the log-linear part of the antibody dilution curves. Thus serum samples taken at six months were diluted two-fold from 1:20 onwards for IgA, IgM, and IgG. Serum samples taken at 0–2 months were diluted from 1:160 onwards for IgM and from 1:320 onwards for IgG. Samples were incubated on the plates for two hours at 37°C and the plates were washed three times with 0.9% saline containing 0.05% Tween 20. Alkaline phosphatase conjugated antihuman IgM, IgG, and IgA (Orion Diagnostica, Helsinki, Finland) were added and incubated on the plates overnight at room temperature (the specificity of these antihuman antibodies had been confirmed previously). The plates were washed, substrate added, and after an enzyme reaction of 30 minutes at 37°C the optical density was measured at a wavelength of 405 nm (OD405). A blank OD405 given by buffer alone was subtracted from the OD405 values of the samples.

**Determination of Antibody Avidity**

Different antibody dilutions with a constant amount of antigen were used to construct a dose-response curve for each sample, and the curves were analysed by a computerised curve fitting method. The method gives in arbitrary units two variables, which indicate the amount of antibodies binding either in antibody-antigen excess. The estimate of high avidity antibodies reflects the amount of antibodies binding as extrapolated antibody excess—that is, OD405 of undiluted sample—and the estimate of the total amount of specific antibodies gives the end point titre at a cut off level of OD405=0.05, indicating the amount of antibodies binding in antigen excess. It has been shown experimentally that these estimates do correlate with the amount of high avidity antibodies and the total amount of antigen specific antibodies respectively.

For evaluation of the specificity and sensitivity of the avidity determinations the following properties of yersinia RPs are relevant: (a) all patients with yersinia infection develop antibodies against RPs; (b) RPs of different serotypes of enteropathogenic yersiniae are serologically closely related (common virulence associated antigens); (c) purified RPs contain insignificant amounts of lipopolysaccharide (<0.5 μg/mg protein); (d) the residual reactivity of serotype O:3 specific anti-lipopolysaccharide antibodies with traces of lipopolysaccharide in RP preparation can be excluded by using RPs isolated from *Y. enterocolitica* serotype O:8; (e) serotype O:8 strains are common in the United States but are rare in Finland; there is no cross reactivity between antigens of serotypes O:8 and O:3.

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Fig. 1 Left: Coomassie stained band pattern of released proteins (RPs) of *Y. enterocolitica* serotype O:8 obtained by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (11%); the molecular weights of the major antigenic proteins are denoted as kilodaltons. Right: IgG immunoblot patterns of representative patients with yersinia infection (1–4). Salm: IgG immunoblot showing no reactivity against RPs with serum of a patient suffering from Salmonella typhimurium infection (negative control).
Antibodies against released proteins of Yersinia spp 1005

Statistics

Patient groups were compared using linear discriminant analysis.18

Results

Antigens

Analysis of RPs on SDS-PAGE and immunoblotting (Fig. 1) shows that RPs are recognised by serum samples from patients with yersinia infection and not by serum from a patient with salmonella infection.

Total amount of anti-RP antibodies

At the early stage of infection the total amount of IgA class anti-RP antibodies was significantly higher in patients with arthritis than in those with uncomplicated yersiniosis (p<0.0001, Table 1). Such a difference was not seen in the samples taken at six months after onset of the infection. The total amount of IgM and IgG class anti-RP antibodies did not differ between the patient groups.

Avidity of anti-RP antibodies

The avidity of anti-RP antibodies did not differ significantly in patients with or without yersinia triggered reactive arthritis. This was true for all three immunoglobulin isotypes and for samples taken at the early and later stages of the infection. Whereas the avidity of IgA and IgG antibodies was lower in the six months' samples than in those taken at two months, the avidity of IgM anti-RP antibodies increased with time; the increase was observed in both patient groups (Table 1). The results were the same whether the antigen was used at a concentration of 6 μg/ml or in a 1:50 dilution of that.

Discussion

We have recently shown that the difference in IgA response between patients with or without yersinia triggered reactive arthritis concerns predominantly antibodies of high avidity.13 This result was obtained with bacterial cell extracts or lipopolysaccharide. In this study the avidity against RPs of Y enterocolitica was investigated. Owing to the antigenic similarity between RPs of Yersinia spp, antibodies raised against the RPs of Y enterocolitica O:3 react equally well with the RPs of Y enterocolitica O:8.3,5 To exploit this fact Y enterocolitica O:8 was chosen as the source of RPs, thereby excluding the influence of serotype O:3 specific anti-lipopolysaccharide antibodies on the results.

In contrast with our earlier studies using other antigens, this study did not show any differences in the avidity of anti-RP antibodies between the two patient groups either at the early stage of the infection or at six months. The total amount of IgA class anti-RP antibodies was significantly higher in patients with arthritis, however, than in those with uncomplicated yersiniosis at the early stage of the infection (p<0.0001). Furthermore, our previous results indicated that the avidity of IgA class antibodies against yersinia lipopolysaccharide decreased less in patients with arthritis than in those without arthritis.13 This was not observed in antibody response against RPs. In fact, as estimated by OD₄₀₅ values, most patients had a considerably decreased level of IgA antibodies against RPs at six months in comparison with either the level at the start of the disease or the level against lipopolysaccharide at six months (data not shown). An explanation could be that RPs are functionally important for the bacteria invading tissues and trying to escape the

Table 1  Estimates of serum total specific (ETSA) and high avidity (EHAA) anti-yersinia antibodies against 'released proteins' in serum samples of patients with (A+) and without reactive arthritis (A−) after recent yersinia infection. Values are means (SD) in arbitrary units

<table>
<thead>
<tr>
<th>Antibody class</th>
<th>0–2 Months</th>
<th>6 Months</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>A+</td>
<td>A−</td>
</tr>
<tr>
<td>ETSA*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgM</td>
<td>3.49 (0.38)</td>
<td>3.26 (0.26)</td>
</tr>
<tr>
<td>IgG</td>
<td>3.94 (0.52)</td>
<td>3.62 (0.34)</td>
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<tr>
<td>IgA</td>
<td>3.98 (0.31)</td>
<td>3.28 (0.65)</td>
</tr>
<tr>
<td>EHAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgM</td>
<td>1.09 (0.67)</td>
<td>0.80 (0.54)</td>
</tr>
<tr>
<td>IgG</td>
<td>1.33 (0.40)</td>
<td>1.10 (0.43)</td>
</tr>
<tr>
<td>IgA</td>
<td>0.65 (0.31)</td>
<td>0.57 (0.48)</td>
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*The number of patients in both groups was 22 at 0–2 months and 18 at 6 months.
host's defence mechanisms—that is, at the early stage of the infection. Plasmid encoded outer membrane proteins of yersinia are known to increase the serum resistance of bacteria and the ability to resist C3b mediated phagocytosis by granulocytes. Thus the increased anti-RP IgA in arthritic patients might reflect the increased production of RPs. This view is also concordant with the finding that high concentrations of antigen favour formation of low avidity antibodies. Large amounts of RPs are released when plasmid bearing yersiniae grow at 37°C.

Altogether, these results indicate that at the early phase of disease patients with reactive arthritis have an increased level of IgA class antibodies against RPs, probably reflecting increased RP production by the bacteria invading the host tissues. At the later stages of the infection secretion of RPs seems not to be important for the yersinia organisms persisting in the host.

We thank Mrs Annika Inna for excellent technical assistance. This study was supported by the Sigrid Juslius Foundation and the Yrjö Jahnsson Foundation.

References