Immunoblot analysis of IgM, IgG, and IgA responses to plasmid encoded released proteins of *Yersinia enterocolitica* in patients with or without yersinia triggered reactive arthritis

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SUMMARY The IgM, IgG, and IgA antibody responses of patients with *Yersinia enterocolitica* O:3 infection were studied by immunoblotting with plasmid encoded released proteins of *Y enterocolitica* as the antigens. The results indicate that antibodies of all three classes are most consistently directed against the proteins of molecular weights 25 000 and 36 000. Less than two months after the onset of infection 18 of the 19 patients with yersinia triggered reactive arthritis had IgA class antibodies against the released protein of mol. wt 36 000, whereas only eight of the 17 patients with non-arthritic yersiniosis had these antibodies. The same difference between the arthritic and non-arthritic patients was observed also 8-12 months after the onset of infection.

Yersinia enterocolitica is an important causative agent of enteric infections in humans. Occasionally, this primary infection triggers secondary complications such as reactive arthritis.¹⁻³ The mechanisms of the development of yersinia triggered arthritis are not known, but evidently both host related and bacteria related factors are involved.

Human pathogenic strains of the genus *Yersinia* harbour closely related plasmids of 42-46 megadaltons. Association of the plasmid with the bacterial virulence is firmly established. Several temperature induced outer membrane proteins have been found to be plasmid mediated in *Y pseudotuberculosis* and *Y enterocolitica*.^{4 5} Virulent strains of enteropathogenic yersiniae are also known to release plasmid associated proteins to the surrounding medium when grown in calcium deficient conditions at 37°C.⁶ It seems that at least some of the temperature induced outer membrane proteins share antigenic determinants or are identical with the released proteins; these proteins are either integrated into the outer membrane or released to the surrounding medium, depending on the growth conditions used.

Several quantitative differences in the humoral immune response against *Y enterocolitica* O:3 have been shown between patients developing reactive arthritis and those recovering without complications.^{7 8} When the IgM, IgG, and IgA responses against a whole-cell lysate of a plasmid bearing strain of *Y enterocolitica* were qualitatively studied by immunoblotting an examination of single bands did not show any definite differences between the patients with and without arthritis.⁹

In this study we analysed IgM, IgG, and IgA responses against the released proteins of *Yersinia enterocolitica* in patients developing or not developing arthritis after yersinia infection. The results indicate that patients with yersinia triggered reactive arthritis have IgA class antibodies against the released protein of mol. wt 36 000 significantly more often than the non-arthritic patients with yersiniosis.

Patients and methods

PATIENTS

A diagnosis of *Y enterocolitica* O:3 infection was established by bacterial isolation or serology, or both with a serotype specific enzyme linked immunosorbent assay (ELISA).^{7 8} Table 1 summarises the

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Table 1 Clinical features of the patients. * Number of patients is given

Total number of patients	Clinical symptoms				Female/male	HLA-B27‡ (+/-)	Age§ (years)	Stool culture
	Arthritis	Other sequelae†	Diarrhoea	Abdominal pain				
24	No	0	15	19	9/15	1/18	31.8 (9.9)	8
24	Yes	4	18	13	9/15	16/7	38.8 (12.5)	9

*All patients showed a clear response—that is, greater than the mean +2SD of the values obtained for 50 healthy controls, in terms of specific IgM, IgG, or IgA class antibodies against *Yersinia enterocolitica* O:3, determined by ELISA.

†Erythema nodosum or iritis.

‡Not determined for all patients.

§Mean (SD).

||With *Y enterocolitica* O:3 isolated.

clinical features of the patients. Serum samples were obtained <2 months and 8–12 months after the onset of infection and stored in aliquots at -20°C until use. Additionally, serum samples from six uninfected persons without a history of yersiniosis were studied as controls. Not all samples were available in sufficient quantities for all analyses.

ISOLATION OF RELEASED PROTEINS

A plasmid positive strain, WA-314, of *Y enterocolitica* O:8 was used as the source of released proteins. This strain was chosen as it releases the proteins in high quantities. As antigens, these proteins are known to be identical with those of *Y enterocolitica* O:3,^{10–14} and a 75% homology has been shown by DNA-DNA hybridisation between the plasmids of *Y enterocolitica* O:3 and O:8.¹⁰ An 8.4 kbp-BamH1 fragment of the O:8 plasmid did not hybridise with the O:3 plasmid,¹⁰ but restriction mapping has shown that this fragment does not encode for released proteins.¹⁴

The bacteria were grown as described.¹⁰ In brief, an overnight culture (grown in brain heart infusion broth at room temperature) was diluted 1:20 with fresh broth and then incubated at 37°C for 90 minutes (to an optical density of approximately 0.4 at 600 nm). Next, the medium was supplemented with EGTA (ethyleneglycol-bis(β -aminoethylether)- N,N,N',N' -tetraacetic acid disodium salt), the incubation was continued for 90 minutes at 37°C , and the bacteria were harvested by centrifugation. The culture supernatant was filter sterilised and the proteins precipitated with solid ammonium sulphate (40 g/100 ml supernatant). After overnight incubation at 4°C the brown-yellow precipitate was collected by centrifugation (15 minutes at 4000 g). Precipitates of the 300 ml culture were resuspended in 1 ml of a buffer containing 10 mM TRIS-HCl (pH 8) and 0.1 mM EDTA. The suspensions were recentrifuged, and the pellets obtained were dissolved in 1.5 ml of

the sample buffer and used for sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting.

SDS-PAGE AND IMMUNOBLOTTING

The protein pellet was treated with sodium dodecyl

Fig. 1 Immunoblot analysis of serum IgM, IgG, and IgA antibody responses against plasmid encoded released proteins of *Yersinia enterocolitica* at (a) 0–2 months and (b) 8–12 months after the onset of infection (in a patient with yersinia triggered reactive arthritis). The arrows indicate the released proteins of molecular weights (1) 58 000, (2) 46 000, (3) 38 000, (4) 36 000, and (5) 25 000. The three control lanes represent IgM, IgG, and IgA responses of a person without a history of yersinia infection.

sulphate and 2-mercaptoethanol, underwent SDS-PAGE, and was immunoblotted as described.⁹

Results

Serum samples obtained 0–2 months and 8–12 months after onset of infection were studied. Figure 1 shows an example of the reaction patterns against the released proteins of mol. wts 25 000, 36 000, 38 000, 46 000, and 58 000 by a patient with yersinia triggered reactive arthritis. None of the six patients without a previous history of yersinia infection reacted with any of the released proteins in any of the three immunoglobulin classes studied.

IgM ANTIBODIES

IgM class antibodies of 23 out of 27 patients studied recognised in a varying degree the released proteins of mol. wts 25 000, 36 000, 38 000, and 58 000. Serum samples of only a few patients reacted with the protein of mol. wt 46 000. The IgM antibodies were most consistently directed against the proteins of mol. wts 25 000 and 36 000, giving a positive

reaction in 23/27 patients (Fig. 2). The strength of the reactions against the released proteins of mol. wt 36 000 was much stronger than against the other proteins (Fig. 1).

IgG ANTIBODIES

All 33 patients studied had detectable levels of IgG antibodies to at least some of the released proteins of mol. wts 25 000, 36 000, 38 000, and 58 000 (Fig. 2). One third also had IgG antibodies directed against the released protein of mol. wt 46 000. The most pronounced reactions were elicited against the released proteins of mol. wts 25 000, 36 000, and 58 000 (Fig. 1). Some of the patients had a very strong reaction against all five released proteins.

IgA ANTIBODIES

Serum samples from 36 patients were examined. IgA class antibodies were most consistently directed against the released proteins of mol. wts 25 000, 36 000, and 38 000 (Fig. 2); those of higher mol. wt were only detected by the serum samples of a few patients. The strength of the reactions against the proteins of mol. wts 36 000 and 38 000 was much stronger than that against the protein of mol. wt 25 000 (Fig. 1).

Fig. 2 Percentage of patients with serum IgM, IgG, and IgA class antibodies against released proteins of a plasmid bearing strain of *Y. enterocolitica*, shown by immunoblotting at (a) 0–2 months and (b) 8–12 months after the onset of infection. At 0–2 months the numbers of patients with IgM, IgG, and IgA responses were 27, 33, and 36 respectively. The corresponding numbers at 8–12 months were 28, 28, and 34.

COMPARISON OF IgM, IgG, AND IgA RESPONSES

The different immunoglobulin classes generally recognised most consistently the released proteins of mol. wts 25 000 and 36 000. In addition, the protein of mol. wt 38 000 was often recognised by both IgG and IgA class antibodies. Those of mol. wts 46 000 and 58 000 were detected only in a few cases by the different immunoglobulin classes; an exception was the frequent reaction of IgG class antibodies against the protein of mol. wt 58 000 and less commonly against that of mol. wt 46 000 (Fig. 2). IgA and especially IgM patterns were much weaker than those for IgG (Fig. 1).

COMPARISON OF EARLY AND LATE RESPONSES

All released proteins were recognised by antibodies of at least one immunoglobulin class in the acute and convalescent phases (0–2 and 8–12 months respectively after the onset of infection) (Fig. 2). The protein of mol. wt 46 000 was, however, recognised only by the IgG antibodies in the serum samples of two patients at the late stage. The strength and incidence of positive reactions varied, IgA and especially IgM showing faint bands with the serum samples of a few patients at the convalescent stage. The IgG reactive bands generally appeared to be quite strong even at the convalescent stage.

Table 2 Immunoblot analysis of serum antibody responses against plasmid encoded released proteins of *Yersinia enterocolitica* after *Y enterocolitica* O:3 infection. The serum samples were taken less than two months after the onset of infection. Number of patients is given

Isotype	Arthritis	Total	With antibodies to released proteins of mol. wt:				
			25 000	36 000	38 000	46 000	58 000
IgM	No	14	12	11	6	2	3
	Yes	13	11	12	7	2	6
IgG	No	16	14	15	11	7	11
	Yes	17	16	17	10	4	15
IgA	No	17	6	8	13	3	3
	Yes	19	8	18*	13	1	4

* $p<0.005$ when compared with patients not developing arthritis (χ^2 test; $df=1$).

Table 3 Immunoblot analysis of serum antibody responses against plasmid encoded released proteins of *Yersinia enterocolitica* after *Y enterocolitica* O:3 infection. The serum samples were taken 8–12 months after the onset of infection. Number of patients is given

Isotype	Arthritis	Total	With antibodies to released proteins of mol. wt:				
			25 000	36 000	38 000	46 000	58 000
IgM	No	14	6	2	0	0	0
	Yes	14	7	2	1	0	1
IgG	No	12	6	8	3	1	7
	Yes	16	8	14	2	1	8
IgA	No	16	1	1	1	0	0
	Yes	18	3	8*	3	0	0

* $p<0.05$ when compared with patients not developing arthritis (χ^2 test; $df=1$).

COMPARISON OF SERUM SAMPLES FROM ARTHRITIC AND NON-ARTHRITIC PATIENTS A significant difference was seen in the IgA class antibody response against the protein of mol. wt 36 000. At the acute stage 18 of 19 arthritic patients had IgA antibodies against this protein, whereas it was recognised by only eight of 17 non-arthritic patients ($p<0.005$) (Table 2). The same difference was also seen at the convalescent stage: eight of 18 arthritic patients and one of 16 non-arthritic patients elicited an IgA class antibody response against the protein of mol. wt 36 000 ($p<0.05$) (Table 3). No significant differences between the arthritic and non-arthritic patients with yersiniosis were observed in the other antibody classes or in the responses against other released proteins.

Discussion

In this study we used immunoblotting to compare IgM, IgG, and IgA responses against the plasmid

encoded released proteins of *Y enterocolitica* after natural infection in man. The results indicate that IgA response against the released protein of mol. wt 36 000 was more consistent and persisted for longer in the arthritic than in the non-arthritic patients with yersiniosis ($p<0.005$). It remains to be elucidated whether the released protein of mol. wt 36 000 and the antibody response against it play a part in the pathogenesis of reactive arthritis. The difference between arthritic and non-arthritic patients was restricted to IgA and to this particular protein, which may indicate pathogenetic significance. Possibly this finding might be applied in the diagnosis of yersinia triggered reactive arthritis.

It has been shown that release of plasmid encoded proteins is a common feature of enteropathogenic serotypes of *Y enterocolitica* and *Y pseudotuberculosis*. It is also known that released proteins of different strains share common antigenic determinants or are identical.^{4 10 11} Probably, within the limits of molecular weight determinations, the

proteins of mol. wt 25 000, 36 000, and 46 000 of *Y enterocolitica* correspond to the temperature induced outer membrane proteins 5, 4 (mol. wt 34 000), and 2 of *Y pseudotuberculosis* respectively (Ståhlberg *et al*, unpublished results).^{4 10}

When the antibody responses against the released proteins in this study are compared with those observed against plasmid encoded antigens in a whole-cell lysate of *Y enterocolitica* O:3⁹ and with those against the temperature induced outer membrane proteins of *Y pseudotuberculosis*¹⁵ it is obvious that the antibody responses are most strongly directed against antigens of mol. wts 25 000 and 36 000/34 000. These antigens are also most consistently recognised by antibodies against *Y pestis*.¹⁶ Thus proposals to use these two proteins as antigens for the primary screening of the yersiniae infections^{13 15 17} are justified by the present study.

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