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Virulence-plasmid is associated with the inhibition of opsonization in *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*

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SUMMARY

Plasmid-cured variants of virulent strains of *Yersinia enterocolitica* and *Y. pseudotuberculosis* were obtained by selection after growth in calcium-deficient medium. To obtain antigen preparations consisting of whole bacteria the original plasmid-containing strains and the plasmid-cured variants were grown in conditions favouring expression of the temperature-inducible outer membrane proteins of *Yersinia* (YOP) (37°C, calcium-deficient culture medium). The presence or absence of the YOP on the bacteria was verified by immunoblotting. Opsonophagocytosis of YOP-negative *Yersinia* preparations (YOP−) was compared to that of YOP-containing ones (YOP+) in human polymorphonuclear leukocyte (PMN) chemiluminescence (CL) assay. The attachment of complement C3b on the surface of the bacteria after opsonization with normal human serum was determined by using a fluorescent anti-C3c-antibody and flow cytometry. YOP+ bacteria resisted opsonization in the absence of specific antibodies, as indicated by diminished C3b-fixation on bacteria and weaker CL response. This implies that virulence-plasmid-coded structures provide *Y. enterocolitica* and *Y. pseudotuberculosis* with an ability to avoid complement-mediated opsonization and phagocytosis.

Keywords *Yersinia enterocolitica* *Yersinia pseudotuberculosis* opsonization chemiluminescence

INTRODUCTION

The most common and well-known disease caused by *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* is gastroenteritis, often associated with mesenteric lymphadenitis. It is occasionally followed by complications such as arthritis, iritis and urethritis, to which people with HLA-B27 tissue antigen are particularly susceptible (Aho *et al.*, 1974; Mattila, Granfors & Toivanen, 1982). Studies on different arms of the host's defence have revealed several interesting features in the immune response against the micro-organism in people who develop reactive arthritis (Toivanen *et al.*, 1985; Vuento *et al.*, 1983). Nevertheless, *Yersinia* itself remains the decisive triggering factor, and it is possible that some strains are especially prone to lead to reactive arthritis (Tertti *et al.*, 1984). In the pathogenesis of yersiniosis a well established virulence factor is a 72 kb plasmid (Gemski, Lazere & Casey, 1980; Portnoy & Martinez, 1985) associated with the production of the Vwa^+ virulence determinant (Brubaker, 1983), tissue invasiveness *in vitro* (Zink *et*

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al., 1980) and production of several temperature inducible outer membrane proteins (YOP) (Bölin, Norlander & Wolf-Watz, 1982).

Phagocytosis and killing of invading bacteria is an important first-line defence. Correspondingly, inhibition of the opsonophagocytic function is a virulence factor for many bacteria. The M-protein of *Streptococcus* interferes with the activation of complement (Whitnack & Beachey, 1985). Alginate obtained from a mucoid strain of *Pseudomonas aeruginosa* inhibits opsonization and also appears to function as a barrier, preventing the attachment of macrophages to bacteria (Oliver & Weir, 1985). Non-virulent *Pseudomonas aeruginosa* strains with defective lipopolysaccharide (LPS) are more easily opsonized than the virulent ones (Engels, Endert & van Boven, 1985; Engels *et al.*, 1985), and studies with smooth, serum-resistant *Salmonella montevideo* suggest that the long O-polysaccharide chains of bacterial lipopolysaccharide (LPS) sterically hinder the access of complement C3b to the bacterial surface saving the micro-organism from efficient deposition of C3b (Joiner *et al.*, 1986). The resistance to opsonization of *Escherichia coli* provided by its capsule can be overcome by high serum concentrations, but then intracellular killing of some strains may be ineffective (Rozenberg-Arska *et al.*, 1985). The virulence-plasmid-coded outer membrane proteins of *Yersinia enterocolitica* (YOP) have been shown to diminish phagocytic activation of the polymorphonuclear leukocytes (PMN) as measured by chemiluminescence (CL) assay (Lian & Pai, 1985). In order to gain more information about the resistance of *Yersinia* to this part of host defence, we studied the role of YOP in the opsonization of *Y. enterocolitica* and *Y. pseudotuberculosis*. An inhibition of complement C3b-attachment and human PMN CL response was seen in the presence of YOP, when the bacteria were opsonized in normal human serum. Our findings suggest that inhibition of opsonization is a virulence factor in *Yersinia*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Two strains of *Y. enterocolitica* serotype O:3 (YSa, YHo) and one strain each of *Y. pseudotuberculosis* serotypes IIB and III were studied. All the strains were positive in the autoagglutination test (AA^+) indicating the presence of the virulence plasmid (Laird & Cavanaugh, 1980). The two *Y. enterocolitica* strains were isolates from clinical stool samples and their virulence-plasmids have been analysed in detail (Pulkkinen *et al.*, 1986). Both *Y. pseudotuberculosis* strains were isolates from stool samples taken during an outbreak (Tertti *et al.*, 1984). To select plasmid-cured variants, bacteria were grown on magnesium-oxalate agar at 37°C, which favours emergence of Ca^{2+}-independent, plasmidless bacteria (Higuchi & Smith, 1961; Lian & Pai, 1985). Single larger colonies were taken and cultured on lactose agar at room temperature. At this point of the study the loss of the virulence-plasmid was verified by the autoagglutination test. Thus we had altogether eight strains: the four original, plasmid-containing strains, and their isogeneic, plasmid-cured variants. To get bacterial preparations for CL and C3b-attachment assay single colonies were cultured overnight in brain-heart infusion (Difco, Detroit, MI, USA) at room temperature. After pelleting, the bacteria were inoculated in Higuchi minimal medium (Higuchi & Smith, 1961) supplemented with 1% tryptone (Difco) 0·5% yeast extract (Oxoid Ltd, Basingstoke, UK) and 0·2% glucose. The cultures were grown on a shaker, 250 rev/min at 37°C for 5 h. These culture conditions promote the expression of plasmid-coded outer membrane proteins (YOP). After washing the bacteria twice with phosphate-buffered saline supplemented with 0·1% gelatin (gel-PBS) the suspension was divided in small aliquots into 20% (vol/vol) glycerol-trypticase soy broth and stored at −70°C. The purity of the cultures was verified by inoculating test samples on lactose plates. The number of bacteria was adjusted to approximately 10^9 bacteria/ml by using the most probable number method (Koch, 1981).

SDS-PAGE and immunoblotting. To verify the presence or absence of the YOP, the bacterial preparations were studied by SDS-PAGE and immunoblotting. The bacterial pellet was treated with sodium dodecyl sulphate (SDS) and 2-mercapto-ethanol, electrophoresed on SDS-PAGE gradient gels and immunoblotted. The antigens were separated on a gradient gel of 10–12·5% and electrotransferred to nitrocellulose sheets. Nonspecific binding sites were blocked by incubating the nitrocellulose sheets in phosphate-buffered saline containing 10% horse serum (HS-PBS) at +4°C

overnight. After washes, the sheets were cut into vertical strips and reacted with a rabbit anti-*Yersinia* serum, known to recognize the plasmid-coded outer membrane proteins of *Yersinia*, diluted 1:300 in HS-PBS at 4°C overnight. The strips were then washed and incubated for 3 h at room temperature with horseradish peroxidase-labelled swine immunoglobulins to rabbit immunoglobulins (DAKO Immunoglobulins, Copenhagen, Denmark), diluted 1:1,000 in HS-PBS. After washing three times, the strips were developed with 4-chloro-1-naphthol.

Opsonization. Serum was collected from five healthy HLA-B27 negative and anti-*Yersinia*-antibody negative individuals, pooled and stored immediately at −70°C. All the individual sera were studied for anti-*Yersinia*-antibodies of IgG, IgM and IgA class using EIA (Granfors *et al.*, 1980). In some of the experiments the complement was inactivated by incubating the serum at 56°C for 30 min. Stored bacteria were thawed and washed with gel-PBS at 4°C. Opsonization was carried out in Hanks' balanced salt solution supplemented with 0·1% gelatin (gel-HBS) containing opsonizing serum (2·5%–20%) at 37°C for 30 min on a shaker, 250 rev/min. The other steps were carried out at 4°C in order to retard bacterial growth. After the opsonization the bacteria were washed twice and resuspended in gel-PBS.

C3-fixation. The presence of C3b on the surface of bacteria after the opsonization was tested by using fluorescent anti-C3c-antibody (fluorescein-conjugated anti-human-C3c, Behringwerke AG, Marburg, FRG). Anti-human-C3c-antiserum diluted 1:40 in gel-PBS was allowed to adhere for 30 min at 4°C on bacteria. Bacteria were washed with gel-PBS and fixed with 94% ethanol. The fluorescence of the individual bacteria was analysed by using an EPICS-C flow cytometer (Coulter Electronics, Hialeah, Fl, USA). Bacterial suspensions of approximately 10^7 bacteria/ml were used as samples. Bacterial aggregates were excluded from the analysis by setting an electronic gate in the detection of forward angle light scatter to correspond to the size of single bacteria. The fluorescence was excited by an argon-laser 488 nm band with 400 mW power. The results for the fluorescence of single stained bacteria are presented as fluorescence histograms.

Cell preparation. For the preparation of polymorphonuclear leukocytes (PMN) a modified method of Bøyum (1968) was used. Thirty millitres of blood from healthy B27-negative individuals was heparinized and the erythrocytes were sedimented using dextran T500 (Pharmacia Fine Chemicals AB, Uppsala, Sweden). Mononuclear cells were discarded after Ficoll-Paque (Pharmacia Fine Chemicals AB, Uppsala, Sweden) gradient centrifugation and residual erythrocytes were lysed with 0·83% NH_4Cl. PMN were washed twice and resuspended in gel-HBS without phenol red to a final concentration of $2{\cdot}0 \times 10^6$/ml. When tested by trypan blue exclusion the cell viability was always more than 95%.

Chemiluminescence (CL) assay. A modified CL assay (Robinson *et al.*, 1984) was performed using Luminol-dependent CL measured by a computer controlled luminometer (Luminometer 1251, LKB Wallac, Turku, Finland) for at least 45 min. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, Sigma, St Louis, Mo, USA) (0·1 ml, of 5×10^{-5}M) was added to 0·5 ml of PMN, and the cells were preincubated at 37°C in test vials for 30 min. The CL assay was started by adding 0·1 ml suspension of opsonized or non-opsonized bacteria into the test vial. The experiments were run in duplicates or triplicates.

HLA typing. The HLA-B27 antigen on lymphocytes was assayed by the cytotoxicity method (Terasaki & Park, 1976) (Histognost-B27, Behring Institut, Marburg, West Germany).

Antibody responses. Quantification of IgM, IgG and IgA class antibodies against *Yersinia* was made by EIA (Granfors *et al.*, 1980). Whole bacteria of the *Yersinia enterocolitica* serotype O:3 and *Yersinia pseudotuberculosis* serotype III were used as antigens.

RESULTS

Demonstration of plasmid-encoded outer membrane proteins (YOP) on the bacterial preparations. The plasmid-containing strains (pYV+) of the two *Y. enterocolitica* strains and the two *Y. pseudotuberculosis* strains were compared to their plasmid-cured derivatives (pYV−). Only the pYV+ strains were autoagglutinable, indicating the presence of a plasmid-specified outer membrane protein with the molecular weight 150,000, known to be involved in the autoagglutina-

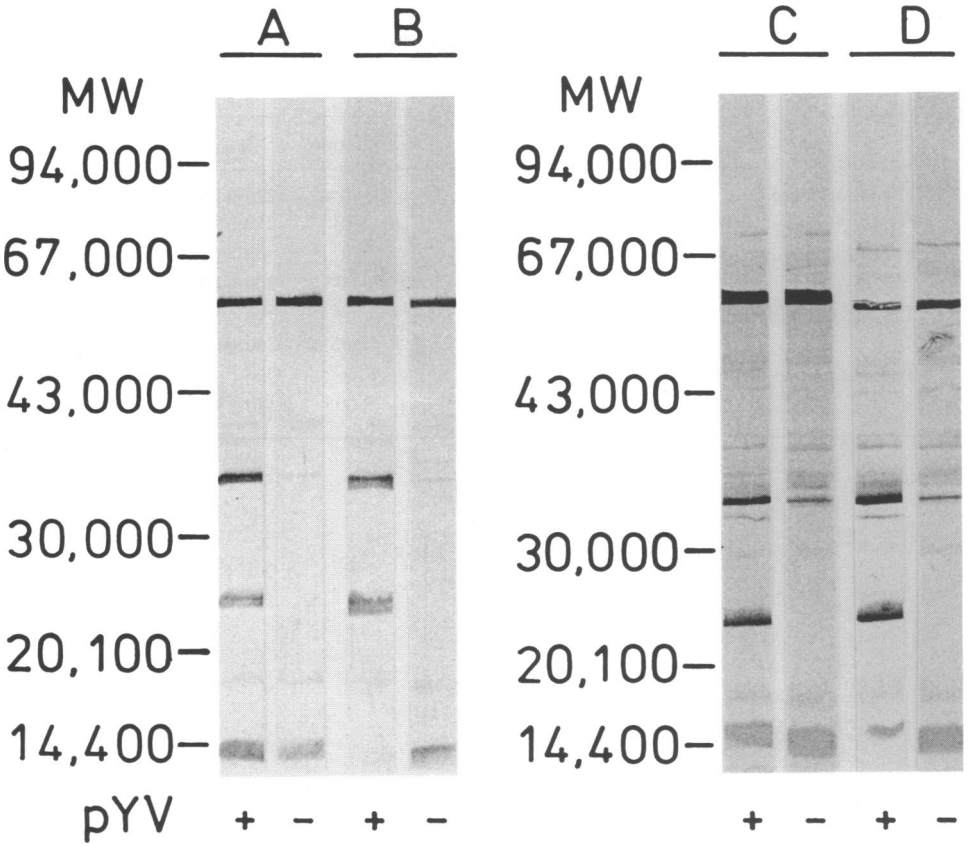

Fig. 1. Immunoblotting analysis of *Y. enterocolitica* O:3 strains (A and B) and *Y. pseudotuberculosis* IIB and III strains (C and D), grown in plasmid-expressive conditions. A rabbit anti-*Yersinia* serum was used for the detection of antigens expressed in the plasmid-containing strains, pYV+, and their plasmid-cured derivatives, pYV−. MW, molecular weight in daltons.

tion (Skurnik *et al.*, 1984). Furthermore, this polypeptide was exclusively seen in the pYV+ as a very strong band, when whole-cell lysates of different strains were separated in SDS-PAGE and stained by Coomassie brilliant blue (data not shown). When rabbit anti-*Yersinia* serum was immunoblotted against the pYV+ and pYV− strains of *Y. enterocolitica* (Fig. 1, A & B) and *Y. pseudotuberculosis* (Fig. 1, C & D), several plasmid-specified antigens (YOP) were recognized on the pYV+ preparations; the most prominent had the molecular weights of 26,000 and 32,000. The *Y. enterocolitica* and *Y. pseudotuberculosis* had similar YOP-profiles.

CL assay. To study the PMN response against *Y. enterocolitica* and *Y. pseudotuberculosis* the CL assay was used. Opsonized bacteria stimulated CL over a wide range of bacterial concentrations. In the experiments presented here the CL response was measured at a ratio of *ca* 50 bacteria to one PMN.

When opsonized YOP+/YOP− pairs were tested in the CL assay, YOP− variants of both *Y. enterocolitica* and *Y. pseudotuberculosis* elicited a significantly higher CL response (Table 1). This was a consistent finding in all the individual experiments using PMN from different donors, indicating that the opsonophagocytic function was inhibited by the plasmid-coded structures, as YOP+ and YOP− differed only in this respect. Non-opsonized bacteria and bacteria treated with inactivated serum caused a low CL stimulation. In these experiments, in the absence of active complement, no plasmid-related inhibition of CL was seen. On the contrary, YOP+ bacteria showed a slight tendency to lead to higher CL response (Table 1). This finding suggests that YOP-

Table 1. Peak chemiluminescence values stimulated with *Yersinia*

		Opsonized		Non-opsonized	
		CL_{max}	% inhibition	CL_{max}	% inhibition
Y. enterocolitica O:3 (YSa)	YOP+	680±180	81±7	220±100	12±24
	YOP−	3420±1270	$P=0{\cdot}01$	250±90	NS
Y. enterocolitica O:3 (YHo)	YOP+	620±210	83±3	200±140	0±70
	YOP−	3550±1070	$P=0{\cdot}005$	200±150	NS
Y. pseudotuberculosis IIB	YOP+	380±200	63±27	290±170	−38±69
	YOP−	1030±280	$P=0{\cdot}05$	210±140	$P=0{\cdot}02$
Y. pseudotuberculosis III	YOP+	290±100	66±6	200±80	−18±27
	YOP−	860±280	$P=0{\cdot}005$	170±40	NS

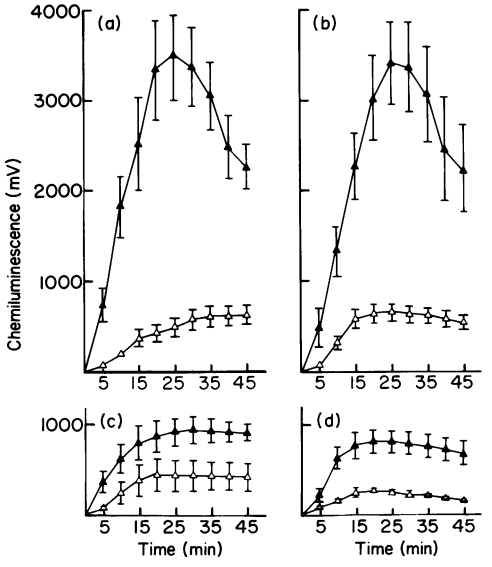

Fig. 2. Chemiluminescence time/response curves of different strains of *Yersinia* (the mean of five experiments ±s.e.m.). (a) *Y. enterocolitica* O:3 (strain YSa); (b) *Y. enterocolitica* O:3 (YHo). (c) *Y. pseudotuberculosis* IIB, (d) *Y. pseudotuberculosis* III. (△) YOP+; (▲) YOP−.

associated inhibition of opsonization rather than phagocyte inactivation is the explanation of the difference observed in the CL assay, and prompted us to further studies on the attachment of C3b on bacteria.

Regarding the CL time-response curves of opsonized YOP− bacteria, *Y. pseudotuberculosis* caused lower response with a less marked peak than *Y. enterocolitica* (Fig. 2). Non-opsonized and inactivated serum-treated bacteria did not show this difference. When the serum concentration was increased from 2·5% to 20%, the peak of the CL against YOP+ was achieved earlier and it was higher. It did not, however, quite reach the level reached by CL of plasmid-cured variants (Fig. 3).

C3b-fixation. Because it seemed possible that the weaker PMN CL response against YOP+ *Yersinia* could be attributed to the inhibition of complement-mediated opsonization in the presence of virulence-plasmid-coded structures a C3b-fixation assay was developed for flow cytometry.

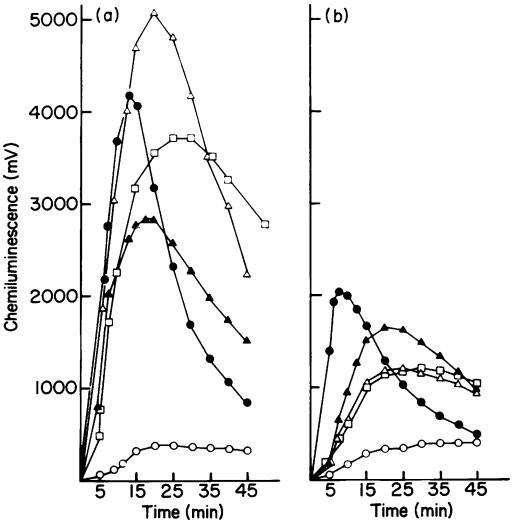

Fig. 3. Chemiluminescence time/response curves of *Y. enterocolitica* O:3 (YHo): (a) YOP−, (b) YOP+, from one representative experiment using different serum concentrations: (●) 20%; (▲) 10%; (△) 5%; (□) 2·5%. (○) 2·5% heat-inactivated serum.

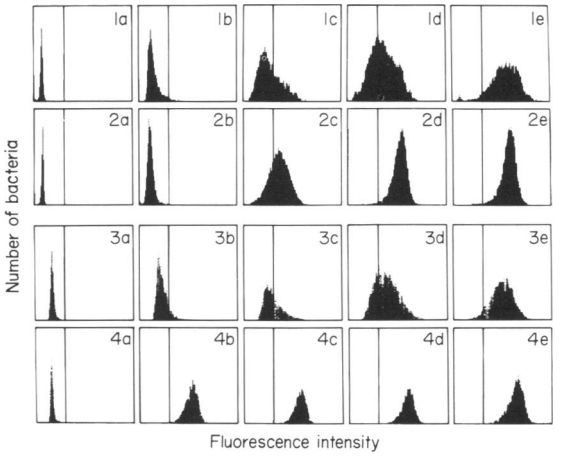

Fig. 4. Typical result of C3b-fluorescence analysis by flow cytometry. Each figure represents the fluorescence nistogram of one stained sample. Approximately 10–50 × 10^3 bacteria were analysed for each sample. 1, *Y. enterocolitica* O:3 (YHo) YOP+; 2, *Y. enterocolitica* O:3 (YHo) YOP−; 3, *Y. pseudotuberculosis* IIB YOP+; 4, *Y. pseudotuberculosis* IIB YOP−. Serum %: a, 2·5% heat-inactivated serum; b, 2·5% serum; c, 5% serum; d, 10% serum; e, 20% serum.

Indeed, it was consistently discovered with all the strains that less complement C3b was attached on YOP+ than YOP− bacteria, when analysed with fluorescent anti-human-C3c-antibody. Figure 4 presents typical fluorescence analysis results of *Y. enterocolitica* and *Y. pseudotuberculosis*. Each frame represents the fluorescence histogram of one stained sample. Approximately 10–50 × 10^3 bacteria were analysed in each sample. Non-opsonized bacteria and bacteria treated with serum inactivated for 30 min at 56°C were totally negative for anti-C3c-fluorescence (Fig. 4: 1a, 2a, 3a, 4a). The rise in the serum concentration from 0% to 20% increased the amount of fluorescence positive

cells from 0 to approximately 100% (Fig. 4). This phenomenon was seen with all bacteria, both plasmid positive and negative. The major finding of the fluorescence analysis was that the fluorescence intensity, detecting the C3c-binding, and the amount of positive cells were higher in the YOP− strains, when compared to the YOP+ strains (Fig. 4: 1b, 1c, 2b, 2c). When the serum concentration was raised up to 20%, this effect became less clear with all bacteria showing strong fluorescence intensity (Fig. 4: 1e, 2e). In C3b-attachment requirements both *Y. enterocolitica* strains (Sa, Ho) resembled each other, as well as both *Y. pseudotuberculosis* strains (IIB, III) respectively (results not shown). When *Y. enterocolitica* and *Y. pseudotuberculosis* are compared, it can be noted that YOP− *Y. pseudotuberculosis* shows strong C3b-attachment in 2·5% serum, whereas YOP− *Y. enterocolitica* requires 5% for this.

DISCUSSION

Resistance to opsonophagocytic function is probably an important virulence factor in many pathogenic micro-organisms. In *Y. enterocolitica*, PMN CL has been demonstrated to be diminished in the presence of the plasmid-encoded YOP (Lian & Pai, 1985). In the present work we demonstrate that the YOP are associated with the inhibition of opsonization as measured by diminished C3b-attachment on bacteria, when they are opsonized in serum containing active complement without specific anti-*Yersinia*-antibodies. Attachment of C3b/C3b$_i$ is crucial for C3-receptor mediated phagocytosis, and the weaker PMN CL response against YOP+ *Y. enterocolitica* and *Y. pseudotuberculosis* observed here can as well be regarded as an indirect evidence of ineffective opsonization.

When the serum concentration is increased, strong C3b-fluorescence can be achieved in YOP+ yersiniae, though some difference in fluorescence histograms can be seen between the YOP+ and YOP− bacteria. Using higher serum concentrations up to 20% the C3b-fixation to the YOP− bacteria can be increased, too. This does not enhance the PMN CL response any more. This indicates that plasmidless *Yersinia* is effectively opsonized and then phagocytized in low serum concentrations. The CL response against YOP+ bacteria does not achieve the level of YOP− bacteria despite the strong C3b-attachment at high serum concentrations, which may suggest that the YOP interfere additionally with the defence mechanisms inside the phagocyte.

When C3b-fixation fluorescence histograms are compared, it is noted that the both YOP− *Y. pseudotuberulosis* strains show a strong C3b-attachment in 2·5% serum but YOP− *Y. enterocolitica* require the minimum of 5% serum for this. However, the plasmid-associated inhibition of opsonization is consistent in all the four YOP+ strains. Also the CL response profiles obtained with YOP− *Y. enterocolitica* and YOP− *Y. pseudotuberculosis* are slightly different; both YOP− *Y. pseudotuberculosis* strains cause weaker CL response with less marked peak than YOP− *Y. enterocolitica* strains despite being effectively opsonized as demonstrated by C3b-fixation. This suggests that besides plasmid-coded structures additional factors, such as LPS structure, may be important in resistance to PMN phagocytosis of yersiniae.

Considering the mechanism of the C3b-fixation inhibition, we have two suggestions. First, plasmid-encoded outer membrane proteins may be protruding, complement non-activating structures on bacteria that sterically hinder the access of complement on the bacterial surface and by this mechanism block the complement amplification. Second, complement may be activated, but effective C3b attachment is not accomplished.

When using non-opsonized bacteria as well as bacteria treated with heat-inactivated serum a low but significant stimulation of the CL is observed. The PMN may recognize some lectin-like structures on bacteria as demonstrated in the gonococcus (Rest, Lee & Bowden, 1985). Without opsonization, no difference between plasmidless and plasmid-containing bacteria could be seen, which stresses the importance of inhibition of the complement function in association with the virulence-plasmid of *Yersinia*. Indeed, the YOP+ non-opsonized bacteria tend to lead to somewhat higher CL response than the YOP−, though statistical significance is only seen with *Y. pseudotuberculosis* IIB. It is possible that without opsonization, plasmid-containing *Yersiniae* are more adhesive to the PMN and by this mechanism stimulate the CL response.

The ability to induce post-infection complications makes *Yersinia* an especially interesting pathogen. Our earlier results (Toivanen *et al.*, 1985) suggest that the organism or parts of it may persist in patients with reactive arthritis. The present results demonstrate one mechanism by which *Yersinia* may escape the host's defence mechanisms.

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