Physical and Antigenic Heterogeneity in the Flagellins of *Listeria monocytogenes* and *L. ivanovii*

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Listeria monocytogenes serotypes 4a, 4b and 7, and *L. ivanovii*, all grown at 20 °C, were negatively stained and examined by electron microscopy. Crude extracts of the cell surface of *L. monocytogenes* serotypes 1/2b, 3b, 3c, 4a, 4b, 4d and 7 and of *L. ivanovii* (all grown at 20 °C) were examined by SDS-PAGE and Western blotting using (i) affinity-purified polyclonal monospecific antibody, and (ii) monoclonal antibody, each raised against 29 kDa flagellin of serotype 4b. No flagella were seen on serotype 7 by electron microscopy and no flagellin was detected in crude cell surface extracts of serotype 7 either in silver-stained gels or in Western blots. The monospecific polyclonal antibody detected flagellins of approximate molecular mass 29 kDa in each of the seven flagellate strains including *L. ivanovii*. The monoclonal antibody detected 29 kDa flagellin in serotypes 1/2b, 3b, 4a, 4b and 4d, but not the flagellins of serotype 3c or *L. ivanovii*, which had a slightly lower molecular mass. Following prolonged electrophoresis of crude flagellar extracts the 29 kDa complex was resolved into three closely migrating bands. In a heterologous system using serotype 1/2b crude flagellar extract, all three bands were detected using the polyclonal antibody whereas only two bands were detected by the monoclonal antibody. It is concluded that polyclonal anti-flagellin antibodies are not useful tools with which to distinguish serotypes of *L. monocytogenes sensu lato* in immunoblotting, but that differences can be determined using a monoclonal antibody directed against particular components of the flagellar complex. These differences did not fully correspond to those anticipated from results of agglutination tests.

INTRODUCTION

Listeria monocytogenes sensu lato is an important pathogen of both man and domestic animals (Gray & Killinger, 1966) and is serotyped by agglutinating reactions between bacterial cell antigens and serotype-specific antisera (Seeliger & Hohne, 1979). Flagellar antigens first described by Paterson (1939) contribute to this serotyping. In cross-agglutination tests using washed formol-killed cells of five serotypes of *L. monocytogenes sensu lato* grown at 25 °C and unabsorbed antiserum, Paterson demonstrated a common flagellar antigen designated antigen B. A further three flagellar antigens (A, C and D) were variably present. In the same study monospecific antisera against factors A, C, and D were used to analyse a total of 27 isolates which all fell into one or other of three antigen combinations, namely AB, ABC and BD.

In the ensuing years no other method of antigenic analysis of *L. monocytogenes* flagella has been undertaken, and no further antigen combinations have been determined. Thirteen of the seventeen serotypes defined by agglutination bear the ABC combination (Seeliger & Hohne, 1979). The serotypes comprising *L. monocytogenes sensu lato* were redefined by Rocourt *et al.* (1982) on the basis of DNA/DNA hybridization studies to include five genomic groups, only one of which is now known as *L. monocytogenes* (*L. monocytogenes sensu stricto*). As a result of that study, serotype 5 was redesignated *L. ivanovii*. This paper reports the comparison of the crude

Abbreviation: CFE, crude flagellar extract.

flagellar extracts (CFEs) from *L. ivanovii* and seven serotypes of *L. monocytogenes* using SDS-PAGE and immunoblotting. A polyclonal monospecific antibody and a monoclonal antibody, both directed against purified flagellin of serotype 4b, were used to detect antigenic similarities between the CFEs.

METHODS

Bacteria. *L. monocytogenes* serotypes 4a (768/88) and 4b (787/88) were isolated from the brains of sheep with listeric encephalitis. Serotypes 1/2b (788/88), 3b (789/88), 3c (790/88), 4d (791/88), 7 (799/88) and *L. ivanovii* (793/88), were obtained from Mr M. Gitter, Central Veterinary Laboratory, Weybridge, Surrey, UK. All strains were stored on Dorset egg agar slopes. *L. ivanovii* was differentiated from *L. monocytogenes sensu stricto* strains on the basis of a positive CAMP test (with *Rhodococcus equi*) and the fermentation of D-xylose. Bacteria were grown at 20 °C in tryptic soy broth (TSB, Gibco), without shaking, to the stationary phase of growth (24 h). Cultures were stored by lyophilization in skimmed milk.

Preparation of CFEs. Bacteria (1 litre cultures) grown at 20 °C were harvested by centrifugation at 5000 g, washed twice in phosphate-buffered saline (PBS: 0.01 M-sodium phosphate, 0.15 M-sodium chloride; pH 7.4), and resuspended in 5 ml PBS. Volumes (5 ml) of this bacterial suspension were added to stoppered glass universal bottles together with 12 glass beads (2 mm diameter). The bottles were clamped vertically on a microid wrist-action flask shaker (Griffin & George) and shaken vigorously for 30 min at 20 °C. The bacterial suspension was centrifuged at 5000 g (4 °C) for 30 min and the supernate retained. The bacterial pellet was washed twice with PBS by vigorous pipetting to remove sheared flagella trapped within the cell mass. The supernate and cell washings were pooled and centrifuged at 14000 g (4 °C) for 40 min to clear remaining bacteria. The supernate from this final centrifugation represented the CFE, which was heated in a water bath for 60 min at 60 °C, and stored at -20 °C. The protein content of each CFE was determined (Peel *et al.*, 1988) and adjusted so that a standard protein loading of each CFE could be applied to gels.

SDS-PAGE and Western blotting. The protocols and reagents used were as described by Peel *et al.* (1988). Western blots were developed using (a) an affinity-purified rabbit antibody, and (b) a mouse monoclonal antibody (isotype IgG2). Both antibodies were directed against 29 kDa flagellin of serotype 4b.

In some experiments SDS-PAGE was done using pre-stained molecular mass markers (Sigma) and prolonging electrophoresis until chymotrypsinogen A (25.7 kDa) had reached the bottom of the gel. This took approximately 6 h, twice the time normally taken for electrophoresis.

Biotin-avidin peroxidase technique. For enhanced staining of Western blots using monoclonal antibody, a biotin-avidin amplification step was used (Sera-Lab, Sussex). Mouse antibody was followed by incubation with an affinity-purified biotinylated horse anti-mouse IgG immunoglobulin, which was succeeded by incubation with avidin D conjugated to horse-radish peroxidase. Incubation steps were for 1 h at 37 °C. The enzyme substrate was diaminobenzidine.

Dot-blotting. For dot-blots, 0.5 µg quantities of crude flagellar protein were applied directly to nitrocellulose, allowed to air dry and developed with polyclonal or monoclonal antibody as for Western blots.

Electron microscopy. Electron microscopy was performed on *L. monocytogenes* serotypes 4a, 4b and 7, and *L. ivanovii*, as described by Peel *et al.* (1988).

Estimation of protein and molecular mass. The methods were those described by Peel *et al.* (1988).

RESULTS

Comparison of L. monocytogenes serotypes 4a, 4b and 7, and L. ivanovii, by transmission electron microscopy

When late-exponential-phase broth cultures grown at 20 °C were examined by electron microscopy no differences were observed between *L. monocytogenes* serotypes 4a and 4b and *L. ivanovii* with regard to structure, quantity or distribution of flagella. However, no flagella were visible on *L. monocytogenes* serotype 7 cells (data not shown).

Comparison of CFE by SDS-PAGE, silver staining and Western blotting

When the CFEs of *L. monocytogenes* serotypes 1/2b, 3b, 3c, 4a, 4b, 4d and 7, and of *L. ivanovii*, were run in SDS-PAGE and stained with silver, the prominent protein band observed in all strains except serotype 7 had a molecular mass in the region of 29 kDa (Fig. 1a, b). This was similar to the molecular mass already described for type 4b flagellin (Peel *et al.*, 1988). It was not clear from the gel shown in Fig. 1(a) exactly how much presumptive flagellin was expressed by

Fig. 1. SDS-PAGE and silver stain of CFEs (*a*, protein loaded at 5 μ g per lane; *b*, protein loaded at 0.5 μ g per lane) of *L. monocytogenes* serotypes 7 (lane A), 4b (C), 4a (D), 4b (E), 4d (F), 3c (G), 1/2b (H) and 3b (I), and of *L. ivanovii* (B). The arrows denote the position of 29 kDa flagellin polypeptides. Note the slight difference in molecular mass between flagellin of serotype 3c (lane G) and the four other serotypes in (*b*). MM, molecular mass markers.

serotype 7, but only a minor protein was present in the 29 kDa region for this serotype (lane A). In a Western blot of CFEs from serotypes 4a, 4b and 7 and *L. ivanovii* an affinity-purified antibody against serotype 4b flagellin reacted strongly with 29 kDa polypeptides of serotypes 4a, 4b, and *L. ivanovii* whereas no polypeptides could be detected in serotype 7 (Fig. 2).

The results of SDS-PAGE (Fig. 1*a*), showed serotypes 4a and 4b and *L. ivanovii* to have approximately the same amount of 29 kDa material per μ g of loaded protein, and that the presumptive flagellin was composed of two closely migrating polypeptides. The proteins stripped from each of the strains shown in Fig. 1(*b*) contained a similar proportion of flagellin, and the double banding of the 29 kDa complex was more obvious. An additional observation from Figs 1(*a*), 1(*b*) and 2 was that the flagellins of serotype 3c and *L. ivanovii* had slightly lower molecular masses than those of the six other serotypes examined. Calculations showed that the molecular mass of serotype 3c flagellin was approximately 1 kDa lower than those of the other serotypes shown in Fig. 1(*b*).

Prolonged electrophoresis was used to determine whether subtle differences in molecular mass existed between flagellins of different serotypes. Application of this technique to serotypes 1/2b, 3b, 4d and 4b resolved each flagellar preparation into three rather than two distinct bands. Again the flagellin of serotype 3c had an apparently lower molecular mass. Prolonged co-electrophoresis of serotype 4b CFE together with CFE of serotype 1/2b, 3b or 4d yielded a three-polypeptide complex, as seen following prolonged electrophoresis of individual serotypes (Fig. 3). However, when serotype 3c CFE was co-electrophoresed with that of serotype 4b an extra band was obtained (Fig. 3, lane C), confirming differences in molecular mass of at least one of the polypeptide components; similar results were obtained when *L. ivanovii* CFE was co-electrophoresed with 4b CFE.

Monoclonal antibody to serotype 4b flagellin reacted against flagellins of all serotypes except 3c and 7, and *L. ivanovii*. Polyclonal antibody reacted with all CFEs tested (Fig. 4*a*, 4*b*).

Fig. 2

Fig. 3

Fig. 2. SDS-PAGE and Western blot analysis of CFEs of *L. monocytogenes* serotypes 7 (lane A), 4b (C) and 4a (D), and of *L. ivanovii* (B), developed using affinity-purified antibody raised against serotype 4b flagellin. Each lane was loaded with 5 µg protein. The arrow denotes the position of 29 kDa flagellin.

Fig. 3. Prolonged SDS-PAGE followed by silver staining of the combined CFEs of serotypes 1/2b and 4b (lane A), 3b and 4b (B), 3c and 4b (C), and 4d and 4b (D). Each component was loaded at 0.5 µg of protein per lane. Note that when serotype 3c is mixed with serotype 4b (lane C) the combined flagellins are resolved as a four-band complex (arrows).

Fig. 4 (a, b). Dot-blots of crude flagellar extracts of serotype 4a (dot A), 4b (B), *L. ivanovii* (C), 7 (D), 4d (E), 3c (F), 3b (G), 1/2b (H) and 4b (I) developed with : (1, 3) mouse monoclonal antibody to serotype 4b flagellin followed by anti-mouse IgG horse-radish peroxidase (HRP) conjugate; (2, 4) anti-mouse HRP conjugate alone; (5) affinity-purified rabbit anti-flagellin antibody plus anti-rabbit HRP conjugate, (6) anti-rabbit HRP conjugate alone. Antigen applied at 0.5 µg protein per spot.

Fig. 5. SDS-PAGE and Western blot analysis of crude flagellar extracts of serotype 1/2b developed with (A) monoclonal antibody, (B) monoclonal antibody followed by biotinylated anti-mouse IgG, and (C) affinity-purified rabbit anti-flagellin antibody. Note that one, two and three bands, denoted by arrows, are visible in strips A, B and C respectively.

The most successful separation of the three flagellar bands (F1, F2, F3) was effected by prolonged electrophoresis through a 12% resolving gel. The well loading compatible with maximum resolution of the three bands of type 1/2b flagellin was low (0.5 µg per mm² gel area) but readily detected in Western blots by the affinity-purified rabbit anti-flagellin antibody (Fig. 5, lane C). The monoclonal antibody clearly detected the middle band, F2 (Fig. 5, lane A), and further scrutiny revealed a much fainter band of lower molecular mass corresponding to F3. This was confirmed when a further strip was developed using monoclonal antibody enhanced with biotin-avidin, in which both F2 and F3 bands were detected and F1 remained unstained (Fig. 5, lane B).

DISCUSSION

The flagellins of *L. monocytogenes* serotypes 1/2b, 3b, 4a, 4b and 4d can be distinguished from the flagellins of slightly lower molecular mass of serotype 3c and *L. ivanovii* by SDS-PAGE. In addition, the results obtained by immunoblotting using a monoclonal antibody directed against serotype 4b flagellin provide evidence for an antigenic difference between the flagellins of serotypes 3c and *L. ivanovii* and the other serotypes examined.

The differences in flagellar structure and antigenicity observed for serotype 3c are not unexpected as the traditional agglutination test assigns a BD antigenic profile to it while the rest of the strains examined have an ABC antigen composition (Ivanov, 1962; Seeliger & Hohne, 1979). Although *L. ivanovii* has an ABC antigenic combination (Ivanov, 1962) the differences observed here between it and the other *L. monocytogenes* serotypes reflect its recent re-designation as a separate species.

Extended electrophoresis revealed the flagellin preparation to be composed of three distinct bands. A reasonable interpretation of this observation is that the F1 protein, with the highest molecular mass, represents flagellin and that the two proteins of lower molecular mass, F2 and F3, represent modifications of flagellin brought about by proteolytic action of bacterial proteases acting at the terminal -COOH or -NH₂ ends of 29 kDa flagellin, and released from organisms during the shearing stage of flagellar harvest. However, the result obtained using monoclonal antibody suggests that the F1 and F2 components are unrelated proteins, and thus it is unlikely that the F2 protein has been derived by proteolytic digestion of the larger F1 component. The presence of the epitope bound by the monoclonal antibody in the F2 and F3 components suggests that F3 could be a closely related modified product of F2.

From the electron microscopic examination of whole organisms and SDS-PAGE of crude extracts it is concluded that the serotype 7 isolate used in this study does not elaborate surface flagellins. The absence of flagella from serotype 7 is probably not remarkable since other workers have demonstrated the existence of stable, non-flagellated variants of *L. monocytogenes* (Leifson & Palen, 1955). Further work is required to determine whether aflagellation is a common feature of this serotype.

In conclusion, an affinity-purified monospecific polyclonal antibody raised by inoculation of 29 kDa flagellin of *L. monocytogenes* serotype 4b was equally able to detect flagellins of six other flagellate serotypes, including representatives of the serogroups commonly isolated from pathogenic material, viz. 1/2 and 4, and *L. ivanovii*. However, flagellins of serotype 3c and *L. ivanovii* could be distinguished by (1) a lower molecular mass, and (2) lack of reaction with monoclonal antibody raised against serotype 4b flagellin. Using a battery of monoclonal antibodies, Newell (1986*a, b*) postulated six different epitopes on the flagella of *Campylobacter jejuni*, and described the serotype specificity of such monoclonal antibodies and the development of an ELISA capture technique for the detection of flagellar antigen in faecal material. In our study, an epitopic difference between serotypes of *L. monocytogenes* could be detected using a single monoclonal antibody. This gives some optimism that a panel of monoclonal antibodies would reveal other differences which could be exploited in an alternative method of serotyping.

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