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Comparative study of histopathological alterations during intestinal infection of mice with pathogenic and non-pathogenic strains of *Yersinia enterocolitica* serotype O:8

A. Grützkau*, C. Hanski, M. Naumann**

Klinikum Steglitz, Abteilung für Innere Medizin mit Schwerpunkt Gastroenterologie, der Freien Universität Berlin, Berlin, Germany

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Abstract. *Yersinia enterocolitica* is an invasive pathogen capable of causing a wide spectrum of gastrointestinal diseases in man. While there is a considerable body of data on the invasiveness of *Y. enterocolitica* in vitro, little is known about the events in vivo leading to the translocation of the bacteria from the intestinal lumen into the ileal tissue. There is no detailed ultrastructural information describing the course of infection of pathogenic *Y. enterocolitica* in comparison with an avirulent strain. We compared a virulent plasmid-bearing strain and an isogenic avirulent plasmid-free derivative strain of *Y. enterocolitica* serotype O:8 at the ultrastructural level, in the established model of murine yersiniosis. At 12 h post-inoculation we found no indications of an active invasion of the intestinal epithelium, although microcolonies of the pathogenic strain were detectable closely under the follicle-associated epithelium of the Peyer's patches. The plasmid-bearing strain of *Y. enterocolitica* affected the gut-associated lymphoid tissue which was destroyed 36 h post-infection. Unlike the pathogenic strain of *Y. enterocolitica*, the nonpathogenic plasmid-free strain caused no detectable morphological alterations in the ileal tissue by this time. Morphological evidence is provided that *Yersinia* does not invade the ileal epithelium in an active manner, as has been observed in vitro, but appears to be transported across the epithelial barrier by M-cells.

Key words: *Yersinia enterocolitica* – Peyer's patches – Ultrastructure – M-cell – Invasiveness

Introduction

Yersinia enterocolitica causes a broad range of gastrointestinal syndromes including acute enteritis, enterocolitis and terminal ileitis (Vantrappen et al. 1977; Attwood et al. 1989). Pathogenic strains of the genus *Yersinia (Y. pseudotuberculosis, Y. enterocolitica* and *Y. pestis)* possess closely related plasmids with molecular weights in the range 42–47 megadaltons, which are essential for expression of full virulence, as has been shown in a number of in vitro and in vivo models (Ben-Gurion and Shafferman 1981; Heesemann et al. 1983; Portnoy and Martinez 1985; Cornelis et al. 1987).

The histopathological alterations determined during infection of mice and other laboratory animals, including rabbits and gnotobiotic piglets, have been examined mainly at the light microscopic level. They include formation of micro-abscesses, epithelial ulceration and inflammatory oedema with neutrophils (Carter and Collins 1974; Carter 1975; Une 1977a; Robins-Browne et al. 1985). Recently published results from our laboratory described the interaction of mouse intestinal tissue with plasmid-bearing *Y. enterocolitica* serotype O:8 by immunocytochemistry and electron microscopy, and identified Peyer's patches as the main route of infection (Hanski et al. 1989a). Limited attention has been focused so far on the mechanisms responsible for the ability to invade intestinal tissue. In vitro it has been shown that the penetration of cultured epithelial cells is dependent on two chromosomal loci called *inv* and *ail* (Isberg et al. 1987; Miller and Falkow 1988). Although the *ail* gene correlates with the pathogenicity of *Yersinia* strains (Miller et al. 1989) the importance of the *inv* and *ail* gene products during epithelial penetration in vivo has not yet been elucidated. The primary objective of this study was to compare a virulent plasmid-bearing strain and an isogenic avirulent plasmid-free derivative strain of *Y. enterocolitica* serotype O:8 at the ultrastructural level to determine the manner in which they invade the intestinal epithelium. Ability to cross the epithelial barrier was shown for the pathogenic as well as for the non-patho-

**Present address:* Schering AG, Zelluläre Pharmakologie (S107/12. OG), Müllerstrasse 170–178, D-13342 Berlin, Germany

***Present address:* Max-Planck-Institut für Molekulare Genetik, Otto-Warburg-Laboratorium, Ihnestrasse 73, D-14195 Berlin, Germany

Correspondence to: A. Grützkau

genic strain (Hanski et al. 1989b). It is uncertain whether yersiniae are capable of invading epithelial cells in an active manner (Lee et al. 1977; Une 1977b; Devenish and Schiemann 1981; Vesikari et al. 1982; Isberg et al. 1987; Miller et al. 1988) or whether bacteria are mainly phagocytosed and transported from the lumen into the lamina propria by M-cells (Grützkau et al. 1990). These specialized cells in the follicle-associated epithelium of Peyer's patches are actively involved in antigen uptake and could be used by pathogenic bacteria to cross the epithelial barrier (Owen et al. 1986; Sneller and Strober 1986; Walker et al. 1988; Wassef et al. 1989; Kraehenbuhl and Neutra 1992). Data obtained by scanning electron microscopy in particular might give new insights into the patho-physiological changes during experimental yersiniosis and supplement recently publisbed histological and ultrastructural investigations (Hanski et al. 1989a; Grützkau et al. 1990).

Materials and methods

Pathogen-free 6 to 8-week old female CD–1 mice weighing about 30 g were used. They were bred at the Zentrales Tierlaboratorium der Freien Universität Berlin, Berlin, Germany and were routinely checked for bacterial infection and were found free of common murine pathogens, including *Y. enterocolitica*. They were deprived of water for 24 h before infection, but had free access to food. To prevent reinfection by coprophagy, the animals were placed in separate cages with grid bottoms.

The plasmidless strain NCTC 10598 and the isogenic plasmid-bearing strain NCTC 10938 of *Y. enterocolitica* serotype O:8 were obtained from the National Collection of Type Cultures, Central Public Health Laboratory, London, England. The bacteria were grown overnight at 24° C in brain heart infusion broth (Oxoid, London, England).

Oral administration was carried out as described previously (Hanski et al. 1989a). Briefly, prior to dosing the bacteria were spun down, washed twice with saline, and diluted with water to approximately 1×10^7 plasmid-bearing organisms/ml and 1×10^9 plasmidless bacteria/ml respectively. We applied 5 ml of each of the bacterial suspensions individually to 12 separately kept mice. The mice drank the suspensions within 30 min.

At specified times (12 h, 24 h and 36 h), groups of two mice were killed by CO_2 asphyxia and all the Peyer's patches of the small intestine, as well as specimens of ileal and jejunal tissue, were excised. These were freed of luminal contents without washing and then fixed in Karnovsky's fixative (3% glutaraldehyde and 3% formaldehyde in 0.1 M cacodylate buffer, pH 7.2) for 24 h at 4° C. Postfixation was carried out in 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.2 for 1 h at 4° C. The tissue was then washed in 0.1 M cacodylate buffer, and dehydrated in a graded series of ethanol solutions at room temperature. For transmission electron microscopy (TEM) preparation the dehydrated plaques were transferred to propylene oxide and embedded in Epon 812 resin (Serva, Heidelberg, Germany). Semi-thin sections (0.5 µm) were mounted on glass slides and stained with toluidine blue for quick evaluation. Ultrathin sections (50 nm) were picked up on copper grids and stained with uranyl acetate and lead citrate. Specimens were examined with a Philips 410 transmission electron microscope at 80 kV. For scanning electron microscopy (SEM) preparation the dehydrated specimens were immersed in hexamethyldisilazane (Sigma, Deisenhofen, Germany) twice for 10 min each as described by Nation (1983), air dried at room temperature, and mounted on aluminium discs. The samples were then coated with gold-palladium and examined using a Philips EM 505 scanning electron microscope at 15 kV.

In three separate experiments at least 213 tissue samples were obtained from 36 animals. All these blocks were selected for thin sectioning and were examined by TEM or SEM, respectively.

Results

Figure 1 represents a scanning electron micrograph of the follicle-associated epithelium of an uninfected murine ileal Peyer's patch. Three single lymphoid follicles without surface villi or crypt mouths were seen among grouped villi. Higher magnifications of these follicles and the neighbouring villi as well as the jejunal tissue showed no signs of infection or histological alterations (micrographs not shown).

Yersiniae identified in the tissue of infected mice 12 h after infection were consistent in appearance with the structure of the organism in pure culture. The size of the bacteria, the structure of the outer membrane and the electron density of the cytoplasm were used as special features for identification of *Y. enterocolitica* in the ileal tissue. Twelve hours after oral administration of the pathogenic plasmid-bearing bacteria only a few yersiniae were located on the follicle-associated epithelium of Peyer's patches (Fig. 2) and in the area of villi grounds. None of the bacteria resting on the Peyer's patch exhibited active invasion of the epithelial surface. The surface of the ileal villi showed no adhering bacteria. Furthermore, numerous leucocytes, presumably neutrophils, were detectable in the close vicinity of adhering bacteria (Fig. 2). However, these cells showed no signs of phagocytic activity. The integrity of the follicle-associated epithelium was not disturbed by the adhering bacteria. The cracked structure of the epithelium, visible in Fig. 2, is a result of dehydration and drying procedures during specimen preparation for SEM and is unambiguously distinguishable from bacterially induced histological alterations.

TEM evaluation of Peyer's patches 12 h after infection revealed distinct micro-colonies of *Y. enterocolitica* close under the follicle-associated epithelium (Fig. 3). The histological structure of the patch as well as the integrity of the overlying epithelium showed no discernible alterations (Fig. 3).

Figure 4 represents a similar detail of follicle-associated epithelium, to that shown in Fig. 2, with the difference that distinct histopathological changes in the follicle-associated tissue were only detectable 24 h after infection. These focal lesions were interspersed with mucin, cell debris, leucocytes, and numerous bacteria. In the remaining ileal tissue there were no discernible alterations.

Extensive progression of the early lesions occurred only 36 h after infection. These advanced alterations ranged from limited ulceration (Fig. 5) to total destruction of the morphological integrity of Peyer's patches (Figs. 6, 7). Figure 5 demonstrates partial removal of follicle epithelium with no discernible effects on the integrity of the surrounding villi. Similar results obtained after proteolytic digestion of the follicle epithelium were presented by Pappo et al. (1988). The area of detached follicle epithelium reveals a porous basal lamina covered

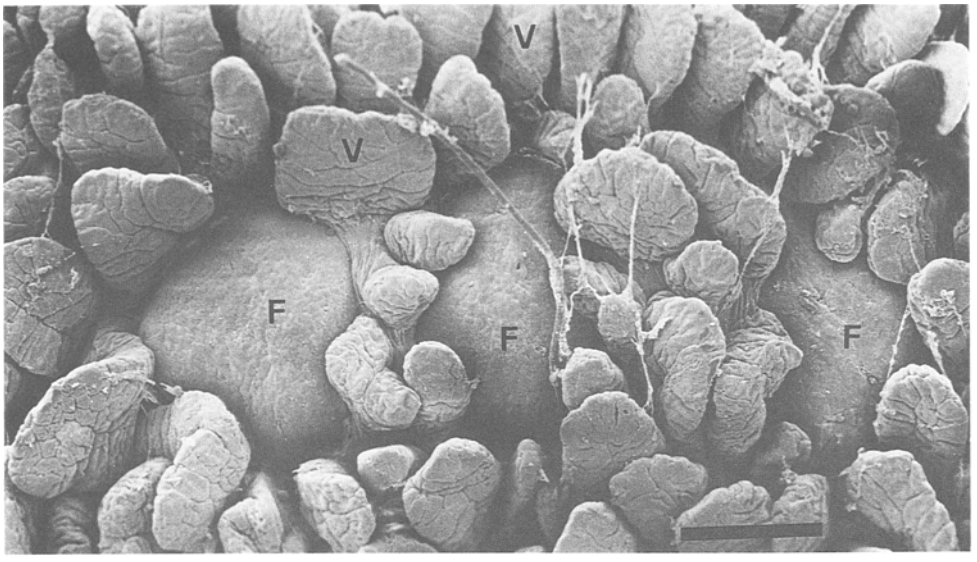


Fig. 1. Scanning electron micrograph of an uninfected, ileal Peyer's patch composed of three lymphoid follicles (*F*) and surrounded by numerous villi (*V*); × 120. *Bar* = 200 μm

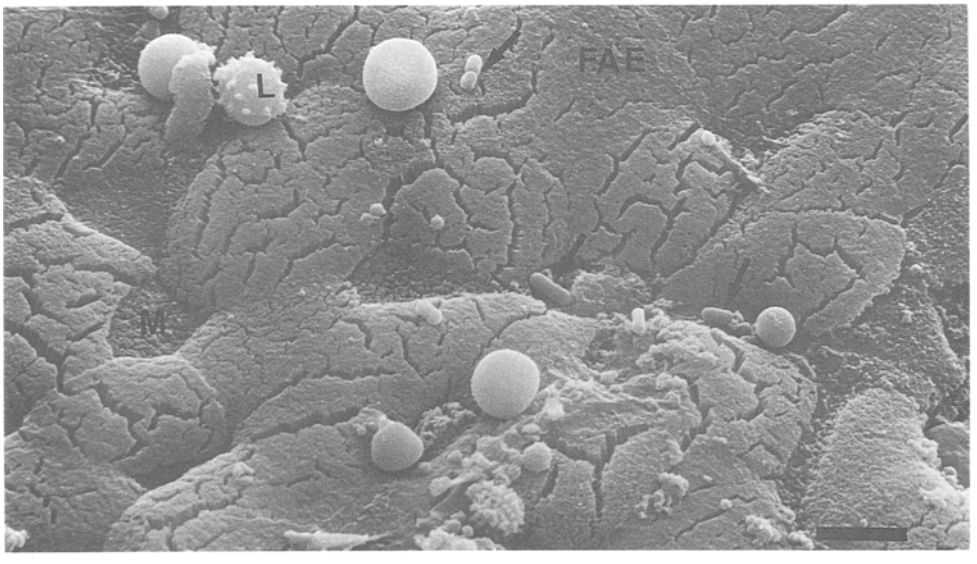


Fig. 2. Scanning electron micrograph of the follicle-associated epithelium of an ileal Peyer's patch 12 h after infection with a pathogenic strain of *Yersinia enterocolitica* serotype O:8. Only a few bacteria are located on the epithelium *(arrows)*. Numerous leucocytes (*L*) are in the close vicinity of adhering bacteria. The integrity of the follicle-associated epithelium is not disturbed by the adhering bacteria. *FAE*, Follicle-associated epithelium; *M*, M–cell; × 3200. *Bar* = 5 μm

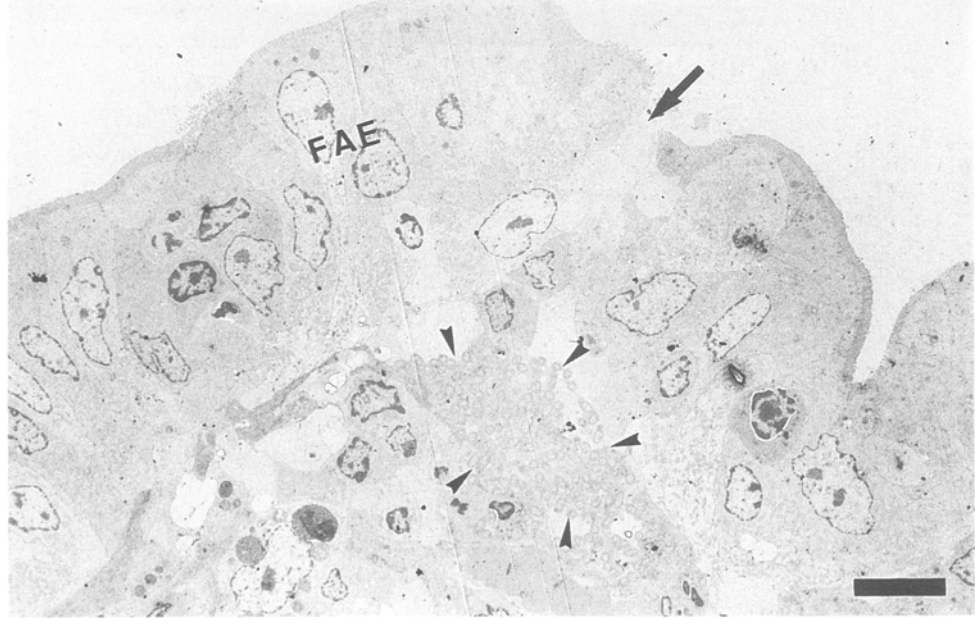


Fig. 3. Transmission electron micrograph of the follicle-associated epithelium of an ileal Peyer's patch 12 h after infection with a pathogenic strain of *Y. enterocolitica* serotype O:8. A microcolony *(short arrows)* is visible close under the intact epithelium. In the right upper corner the epithelium is interrupted by a M-cell *(long arrow)*. *FAE*, Follicle-associated epithelium; × 1650. *Bar* = 10 μm

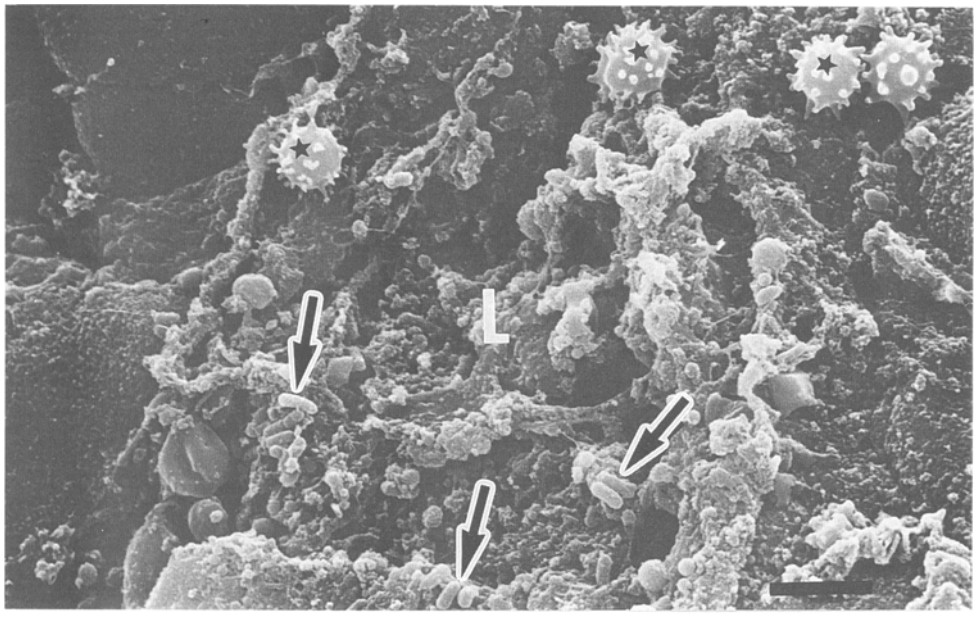


Fig. 4. Scanning electron micrograph of the follicle-associated epithelium of an ileal Peyer's patch 24 h after infection with a pathogenic strain of *Y. enterocolitica* serotype O:8. A focal lesion (*L*) is interspersed with mucin, cell debris, leucocytes *(asterisks)*, and numerous bacteria *(arrows)*; × 3600. *Bar* = 5 μm

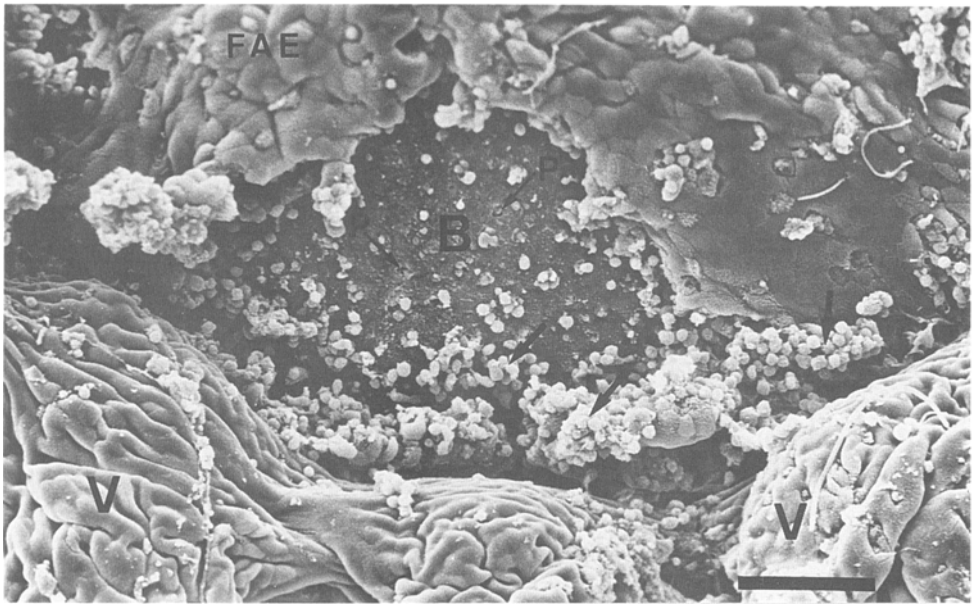


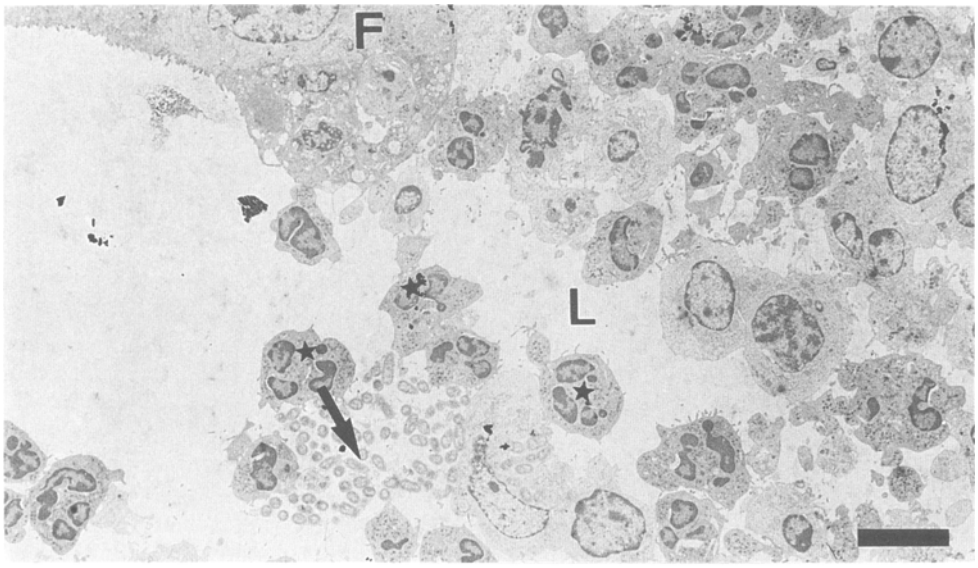
Fig. 5. Scanning electron micrograph of the follicle-associated epithelium of an ileal Peyer's patch 36 h after infection with a pathogenic strain of *Y. enterocolitica* serotype O:8. At this time a limited ulceration of the follicle epithelium is detectable. The area of detached epithelium reveals a porous basal lamina (*B*) covered by numerous leucocytes *(thick arrows)*. *FAE*, Follicle-associated epithelium; *V*, villi; *P*, pores in the basal lamina; × 480. *Bar* = 50 μm

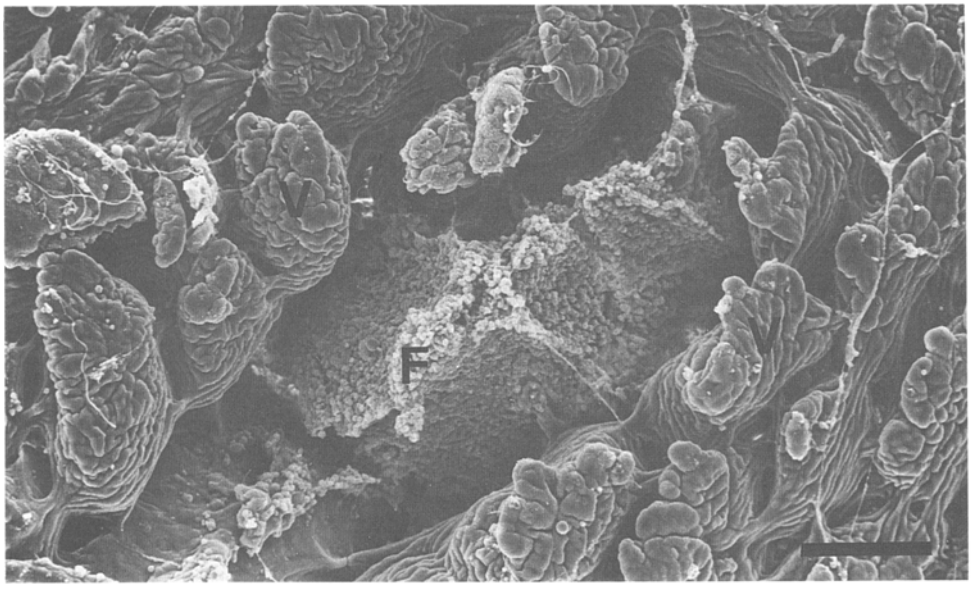
by numerous leucocytes indicating that the erosion is a result of released inflammatory mediators. The extent of the most serious histopathological alterations 36 h after infection is demonstrated in Figs. 6 and 7. The complete architecture of the follicle-associated epithelium together with the underlying basal lamina has been destroyed and the abscess has ulcerated into the intestinal lumen. High power magnifications of these extensive ulcerations by SEM and TEM investigations revealed necrotic and mucinous material as well as huge inflammatory infiltration with granulocytes, macrophages and lymphocytes in the proximity of numerous yersiniae (Figs. 6, 8).

During the first 12–24 h after infection with plasmid-free bacteria we found only a few isolated bacteria in the proximity of the follicle-associated epithelium of Peyer's patches and the base of villi, although we gave 100 times the dose of bacteria. Moreover, there was no evidence of a pathological interaction between adhering bacteria and ileal tissue. At 36 h postinoculation we could not detect any yersiniae in the prepared ileal specimens. The ileal tissue as well as the Peyer's patches remained intact.

Discussion

The primary objective of this study was to obtain insight into the way on which pathogenic as well as non-pathogenic yersiniae enter intestinal tissue. The use of SEM allows the observation of great areas of interest with the advantage of producing a three-dimensional effect in the image which is particularly suitable for the present study of infected intestinal villi and follicles. This technique





Figs. 6, 7. Transmission and scanning electron micrographs of the follicle-associated epithelium of an ileal Peyer's patch 36 h after infection with a pathogenic strain of *Y. enterocolitica* serotype O:8. The complete architecture of the patch is destroyed and the abscess is ulcerated in the intestinal lumen (*L*). *F*, Destroyed follicular tissue; V, villi; *arrow*, bacteria in the vicinity of numerous leukocytes *(asterisks)*. TEM × 3200. *Bar* = 5 µm. SEM × 230 *Bar* = 100 µm

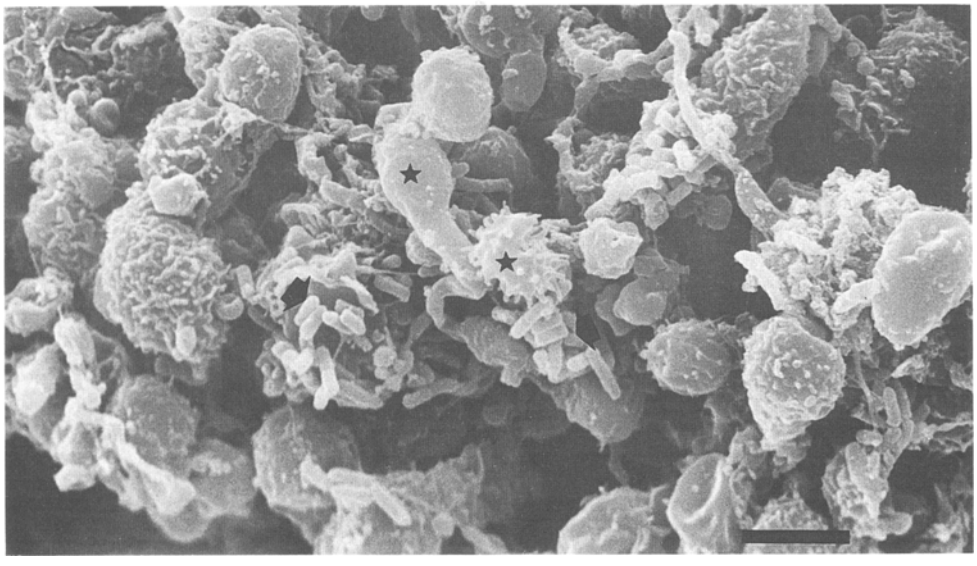


Fig. 8. Scanning electron micrograph at higher magnification of the extensive ulceration visible in Fig. 7, showing necrotic and mucinous material as well as huge inflammatory infiltration of various leucocytes *(asterisks)* in the proximity of numerous yersiniae *(arrows)*; × 3800. *Bar* = 5 µm

also facilitates the detection of bacterial micro-colonies and those minor histopathological changes which may occur in the infection by the non-pathogenic strain of *Y. enterocolitica*.

The results of the present investigation extend previous in vivo studies on the mechanism of pathogenicity of *Y. enterocolitica* (Carter 1975; Une 1977a; Pai et al. 1980; Robins-Browne et al. 1985; Lian et al. 1987; Hanski et al. 1989a). Thus, the first remarkable findings in the ileum were made 24 h after infection probably caused by proliferating bacterial micro-colonies directly located under the basement membrane overlaying the dome of Peyer's patch. Such colonies were demonstrated by TEM only 12 h after infection (Fig. 3). They might be the result of a single invading organism crossing the epithelial barrier via M-cells (Grützkau et al. 1990). Having reached the lamina propria the bacteria proliferate and cause an inflammatory response resulting in epithelial disruptions observed 24 h after infection. The ability of pathogenic *Y. enterocolitica* to evade cellular defence mechanisms of infiltrating phagocytes, mainly neutrophils and macrophages (Fig. 8) (Heesemann and Laufs 1985; Lian and Pai 1985; Rosqvist et al. 1988; Hartiala et al. 1989; Hanski et al. 1991), enables the bacteria to survive and to spread into other organs, like the spleen, kidneys, mesenteric lymph nodes or liver (Carter and Collins 1974; Falcao et al. 1984).

The images obtained 36 h after challenge (Figs. 5, 7) show in an impressive manner the extent of histopathological changes at this time, leading to the total destruction of Peyer's patches. The extensive histopathological alterations concerning the whole intestinal tissue observed by various laboratories (Carter 1975; Une 1977a; Robins-Browne et al. 1985) are probably due to systemic infection appearing at a later time after infection.

Unlike the findings revealed by Lian et al. (1987) in rabbits after intra-gastric inoculation of plasmid-free bacteria, serotype O:3, we detected no histopathological changes of epithelium after infection with plasmid-free *Y. enterocolitica*, serotype O:8 and O:3, in mice (data not shown). Therefore, it seems reasonable to assume that non-pathogenic yersiniae which have crossed the epithelial barrier via M-cells are subsequently eliminated without disturbing the histological structure of the intestinal tissue.

The present and recently published results of Simonet and Falkow (1992) do not support the hypothesis that yersiniae are capable of penetrating the intestinal epithelium in the way demonstrated by various in vitro tissue culture models (Lee et al. 1977; Une 1977b; Devenish and Schiemann 1981; Vesikari et al. 1982; Isberg et al. 1987; Miller et al. 1988). This conclusion is based on the following observations: we found no signs of extensive interaction between pathogenic yersiniae and the intestinal mucosa 12 h after infection, although micro-colonies could already be detected at this time close under the follicle-associated epithelium (Fig. 3); there were no signs of histopathological alterations at this time (Figs. 2, 3); clinical findings obtained within 12 h–36 h of infection of mice with a 100 times greater dose of non-pathogenic yersiniae also failed to reveal any signs of pathological interaction between these bacteria and the intestinal mucosa; to our knowledge, in vivo, only yersiniae transported by M-cells have been observed crossing the epithelial barrier (Grützkau et al. 1990). Simonet and Falkow (1992) also observed a discrepancy between the ability of *Y. pseudotuberculosis* to invade cultured epithelial cells and the ability to translocate from the intestinal lumen after intragastric inoculation. We conclude, therefore, that mainly the transport of the bacteria through M-cells, the resistance to professional phagocytes and the following proliferation of pathogenic bacteria in the lamina propria are the events responsible for the clinical manifestations observed 36 h after infection (Figs. 5, 7).

The use of SEM offered new insights into the course and extent of histopathological alterations during murine yersiniosis. We did not detect any signs of active epithelial invasion by virulent or avirulent yersiniae, as has been observed in cultured epithelial cells. Consequently, the assumed capability of *Yersinia* for invasion in an active manner seems questionable.

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