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Actin Accumulation at Sites of Attachment of Indigenous Apathogenic Segmented Filamentous Bacteria to Mouse Ileal Epithelial Cells

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Selective attachment of indigenous, apathogenic, segmented filamentous bacteria to follicle-associated epithelia of terminal ileal Peyer's patches involves both the specialized M cells and normal enterocytes. A striking, highly localized cytoplasmic accumulation of polymerized actin, which resembles that associated with adherence of enteropathogenic *Escherichia coli* to cultured epithelial cells, occurs at attachment sites of segmented filamentous bacteria. The epithelial response to the attachment of microorganisms may thus involve mechanisms which are the same for diverse microorganisms.

Apathogenic, segmented, filamentous bacteria (SFBs) are indigenous to the ilea of mice and rats (10, 21), where they have been shown to preferentially colonize the follicle-associated epithelium (FAE) of Peyer's patches (13, 19). SFBs have not been cultured in vitro and, therefore, have not been taxonomically classified. The morphology, attachment, and complex life cycle of SFBs have been described in detail (3, 5, 7, 10, 17, 22) and have been reviewed recently (13). Initial attachment of SFBs to the intestinal epithelium occurs via specialized holdfast segments. Transmission electron microscopy has revealed that attachment of SFBs to epithelial cells causes an invagination of the plasma membrane and displacement of microvilli at the site of attachment. A characteristic accumulation of filamentous material in the cytoplasm adjacent to the protruding appendage of attached SFB holdfast segments is observed (7, 10, 17). The origin of this cytoplasmic filamentous material has not been established. Morphologically similar cytoplasmic alterations at the site of attachment of enteropathogenic *Escherichia coli* (EPEC) to intestinal epithelium have been described (16) and have been shown to be associated with a localized accumulation of polymerized actin in cultured epithelial cells (15). In this study, we have investigated the possibility that the attachment of apathogenic SFBs to murine intestinal epithelia is also associated with accumulation of polymerized actin at the sites of attachment.

Previous studies have failed to show direct attachment of SFBs to the apical surface of membranous cells (M cells), despite extensive colonization of Peyer's patch FAE (19). We have reexamined the distribution of SFB attachment sites within the Peyer's patch FAE to determine whether any interaction between these organisms and M cells does occur, as this would be of particular interest in view of the specialized role of this cell type in antigen sampling (reviewed in reference 18) and the recent observation that monoassociation with SFBs stimulates the mucosal immune system of mice (14).

Studies of the terminal ileal Peyer's patch and the neighboring normal intestine of adult BALB/c mice (10 to 12 weeks old), housed under conventional animal house conditions and sacrificed by cervical dislocation, were performed. Tissue was rapidly removed, rinsed in phosphate-buffered saline (PBS), pH 7.4, and either fixed in 2% glutaraldehyde (in 100 mM sodium phosphate buffer [pH 7.3]) for 3 h at 4°C and processed for scanning electron microscopy (11) or fixed in freshly prepared 2% paraformaldehyde in PBS for 1 h at 4°C prior to whole-tissue cytochemical staining.

Scanning electron microscopy revealed extensive colonization of the murine terminal ileum by SFBs with preferential attachment to Peyer's patch FAE (Fig. 1a), confirming previous reports (13, 19). The basis of the selective attachment of SFBs is unknown but may involve the increased expression or accessibility of receptors on the FAE surface. Close examination revealed that although most SFBs were attached either to FAE enterocytes displaying typical microvillous structure or at the junction between enterocytes and M cells, the latter being identified by their characteristic irregular brush border, occasional SFBs were observed to attach directly to the apical surface of M cells (Fig. 1b). It should be noted that it was not possible to identify all cells to which SFBs were attached, as the epithelium was partially obscured where colonization was most extensive.

To determine whether in vivo attachment of SFBs to ileal epithelial cells is associated with accumulation of polymerized actin, whole paraformaldehyde-fixed tissue preparations were washed thoroughly in PBS, immersed in 0.1% Triton X-100 for 30 min at room temperature, and, after a second washing in PBS, transferred to a solution containing 2 μg of phalloidin-tetramethylrhodamine isothiocyanate (Sigma, Poole, United Kingdom) per ml of PBS for 1 h at room temperature. After being washed in PBS, the tissue was mounted under glass coverslips with Vectashield mountant (Vector, Burlingame, Calif.) and examined with a Nikon Diaphot inverted epifluorescent microscope (Nikon, Telford, United Kingdom) and a Bio-Rad MRC-600 confocal laser scanning imaging system (Bio-Rad Microscience, Hemel Hempstead, United Kingdom) equipped with an argon-ion laser.

Confocal laser scanning imaging of phalloidin-stained tissue facilitated the accurate determination of points of attachment of SFBs and the distribution of polymerized actin within epithelial cells associated with SFBs. SFB segments above the epithelium could be located by their obstruction of

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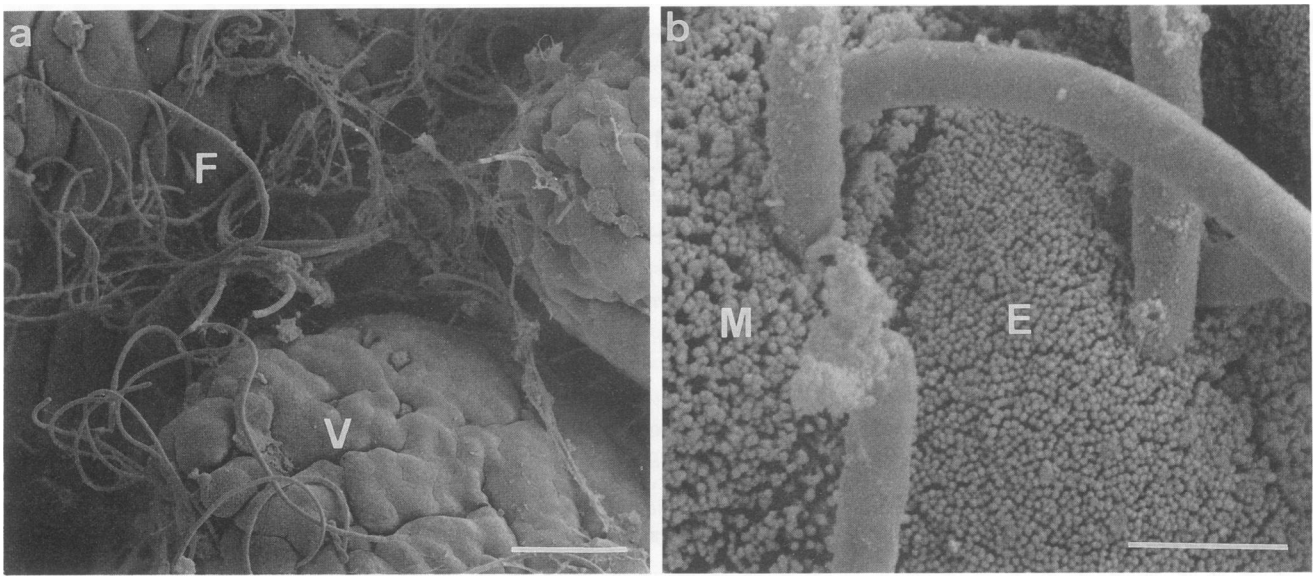

FIG. 1. Scanning electron micrographs of a mouse Peyer's patch. (a) At low magnification, SFB colonization of FAE (F) can be seen to exceed that of neighboring villi (V). Magnification, ×610; bar, 25 μm. (b) SFB attachment to an FAE enterocyte (E) exhibiting normal densely packed microvilli and to an M cell (M) with characteristic irregular microvilli. Magnification, ×10,100; bar, 2 μm.

fluorescence from the epithelial surface, which produced a shadowing effect (Fig. 2 and 3). Confocal optical sections at the level of the apical epithelial surface revealed points of attachment of SFBs as gaps in the brush border phalloidin staining which corresponded to the profile of SFBs (Fig. 2 and 3b). Attachment of SFBs had no detectable effect on the normal distribution of polymerized actin within the epithelial brush border other than that consistent with the physical displacement of microvilli at the point of attachment. Optical sections immediately beneath the apical surface revealed a striking accumulation of polymerized actin in the form of narrow rings with internal diameters of 0.8 to 1.2 μm, which correspond to the diameters of the SFBs (Fig. 2 and 3c). These actin structures extend to a depth of 2 to 3 μm below the apical surface, where they taper to a point presumably beneath the attached SFB (Fig. 3d). All SFB attachment sites on villous epithelium and FAE were associated with accumulations of polymerized actin, and similar structures were never observed in the absence of attached SFBs.

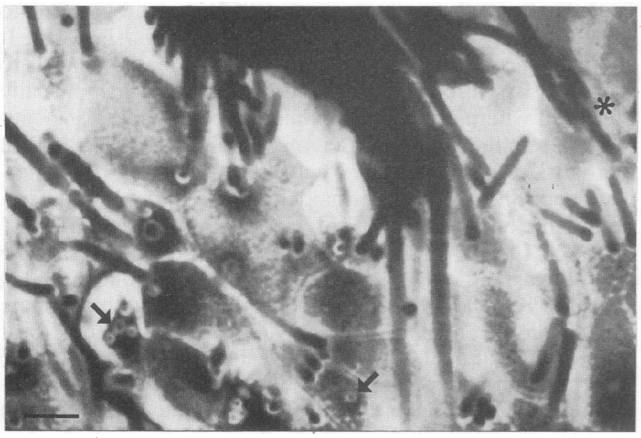

FIG. 2. Confocal laser scanning image of mouse Peyer's patch FAE stained with phalloidin-tetramethylrhodamine isothiocyanate to localize polymerized actin. The optical section is at the level of the apical epithelial surface (upper part of the figure) and passes through the apical cytoplasm (lower part). The attachment of many SFBs is evident, and brush border actin (*) does not appear to be redistributed. Localized accumulations of polymerized actin forming ring-like aggregates around SFB holdfast segments are evident in the apical cytoplasm of epithelial cells (arrows). Bar, 5 μm; magnification, ca. ×1,500.

Some phalloidin-stained tissue samples were immersed in 10 μg of fluorescein isothiocyanate-labelled *Ulex europaeus* agglutinin I (Sigma) per ml for 1 h at room temperature to reveal the positions of M cells, which are specifically labelled by this lectin (4). Confocal laser scanning imaging of these dually-labelled tissues revealed SFB attachment to central regions of the apical surface of M cells and at junctions between enterocytes and M cells (Fig. 4). SFB attachment to M cells was associated with actin accumulation indistinguishable from that seen in normal enterocytes (Fig. 4).

Although previous studies have demonstrated that SFBs attach to central regions of the enterocyte apical membrane or attach at the junction between M cells and enterocytes, they have failed to show direct attachment of SFBs to the apical surface of M cells (19). We have shown that, when colonization is extensive, SFB attachment to the apical surface of M cells is a frequent occurrence. Interaction of SFBs with M cells suggests that these specialized antigen-sampling cells may be involved in the immune response to SFBs, which has recently been demonstrated with mice monoassociated with these organisms (14).

The accumulation of polymerized actin in the host cell cytoplasm adjacent to the plasma membrane in contact with SFBs closely resembles the localized accumulation of polymerized actin which is characteristic of the attaching and effacing lesion induced by EPEC in cultured epithelial cells (8, 15) and is distinct from the more diffuse and widespread actin polymerization associated with *Salmonella* invasion of

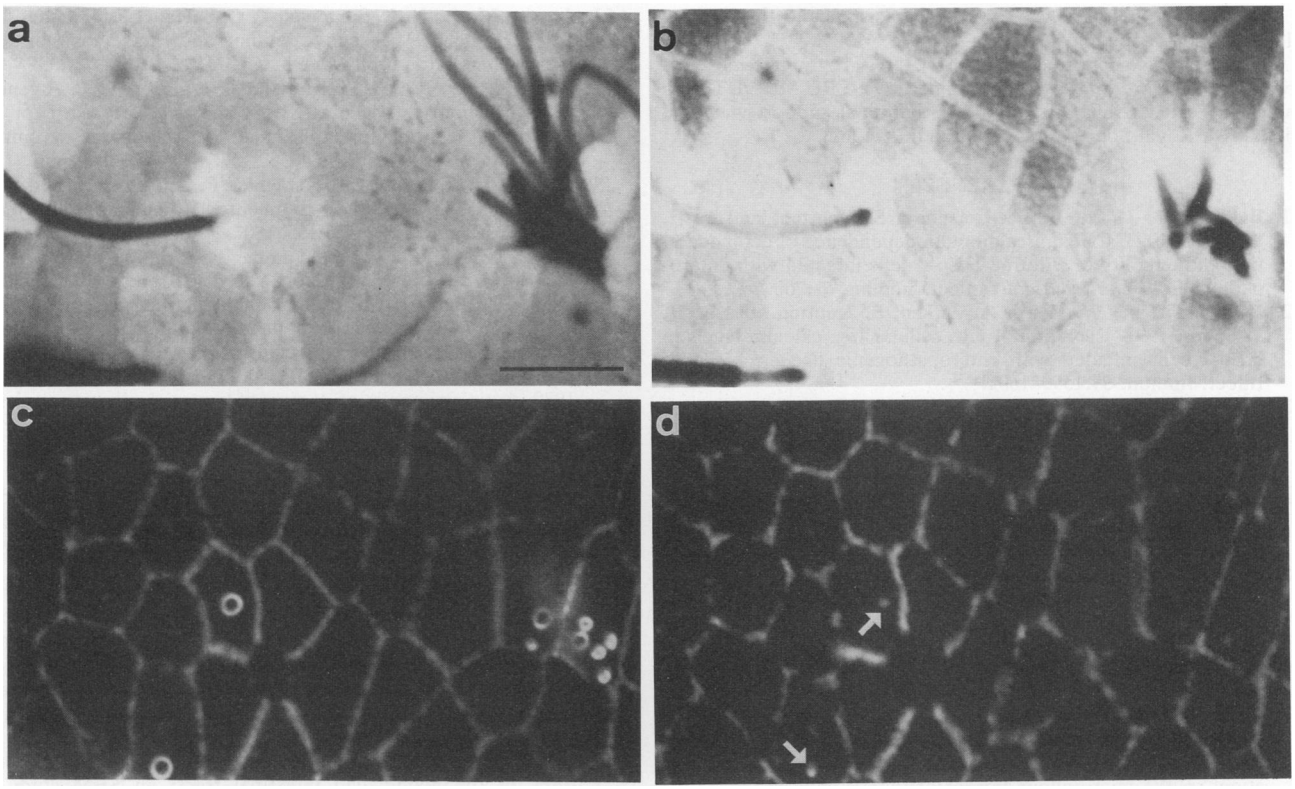

FIG. 3. Confocal images of mouse Peyer's patch FAE at 2-μm intervals, showing structure of actin aggregates at sites of SFB attachment. Attachment of SFBs to epithelial cells showing normal brush border actin distribution is evident in optical sections at brush border level (a and b). Accumulation of polymerized actin in apical cytoplasm immediately adjacent to SFB holdfast segments appears as ring-like structures (c) which taper to a point below SFB holdfast segments (d) (arrows). Bar, 10 μm; magnification, ×1,660.

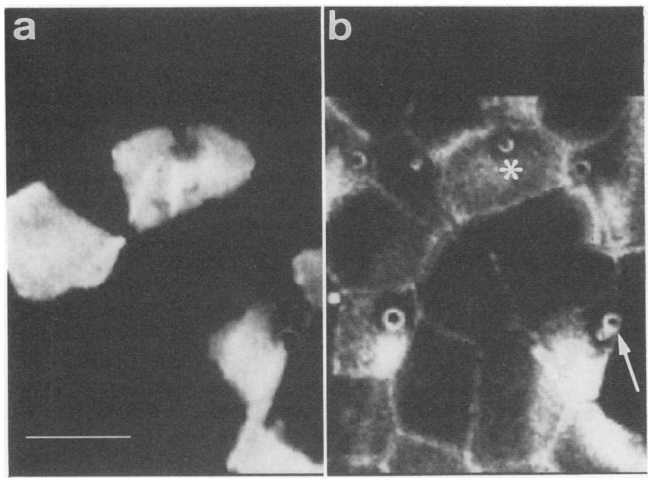

FIG. 4. Dual labelling of mouse Peyer's patch FAE with fluorescein isothiocyanate-labelled *U. europaeus* agglutinin I to localize M cells (a) and phalloidin-tetramethylrhodamine isothiocyanate to localize polymerized actin (b). The confocal optical section at the level of the apical surface reveals M cells intensely labelled with *U. europaeus* agglutinin I (a), and the optical section 2 μm below (b) displays actin aggregates around SFB holdfast segments, one of which is attached directly to an M cell (*) and another of which is attached to the cell junction between an M cell and an enterocyte (arrow). Bar, 10 μm; magnification, ×1,360.

cultured epithelial cells (8, 9). SFB attachment is associated with an invagination of the apical membrane around the SFB holdfast segments (7, 10, 17) and thus differs from EPEC adherence, which is characterized by pedestal formation (15). The actin accumulation associated with SFB attachment surrounds the invaginated membrane, forming a sheath-like structure which is morphologically distinct from the localized actin accumulations observed immediately beneath EPEC attached to cultured epithelial cells. SFB attachment causes minimal disturbance of normal brush border architecture and is apparently not harmful to the host, suggesting that actin accumulation may not be causally related to the brush border effacement associated with EPEC or to the pathogenicity of EPEC infections. Actin accumulation in the host cell cytoplasm may increase the stability of the SFB and EPEC attachment sites and may thus contribute to the pathogenicity of EPEC infections by promoting and/or maintaining close adherence.

EPEC adherence to cultured epithelial cells has been shown to cause an increase in concentrations of intracellular free calcium (2), which is involved in the induction of actin accumulation (1). Several EPEC genes appear to be involved in the induction of actin accumulation in cultured epithelial cells (6, 12), one of which causes tyrosine phosphorylation of host proteins which accumulate within the actin-containing aggregates and which are essential for their formation (20). It will be of interest to determine whether any part of the complex signal transduction mechanisms evoked by EPEC in cultured epithelial cells is also involved in the response of ileal epithelial cells to SFB attachment.

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