**Yersinia pseudotuberculosis** is a foodborne pathogenic bacterium that causes acute gastrointestinal illness, but its mechanisms of infection are incompletely described. We examined how host cell sterol composition affected *Y. pseudotuberculosis* uptake. To do this, we depleted or substituted cholesterol in human MDA-MB-231 epithelial cells with various alternative sterols. Decreasing host cell cholesterol significantly reduced pathogen internalization. When host cell cholesterol was substituted with various sterols, only desmosterol and 7-dehydrocholesterol supported internalization. This specificity was not due to sterol dependence of bacterial attachment to host cells, which was similar with all sterols studied. Because a key step in bacterial internalization differed from that of endocytosis, as monitored using antibody-clustered integrin and previous studies on other proteins, which had a strong dependence of wildtype *Y. pseudotuberculosis* internalization with that of Δinv, ΔyadA, and ΔinvΔyadA mutant strains. YadA deletion decreased bacterial adherence to host cells, whereas invasin deletion had no effect. Nevertheless, host cell sterol substitution had a similar effect on internalization of these bacterial deletion strains as on the wildtype bacteria. The ΔinvΔyadA double mutant adhered least to cells and so was not significantly internalized. The sterol structure dependence of *Y. pseudotuberculosis* internalization differed from that of endocytosis, as monitored using antibody-clustered β1 integrin and previous studies on other proteins, which had a more permissive sterol dependence. This study suggests that agents could be designed to interfere with internalization of *Yersinia* without disturbing endocytosis.

**Yersinia pseudotuberculosis** is an enteropathogenic bacteria that in humans can cause mild diarrhea, enterocolitis, mesenteric lymphadenitis, reactive arthritis, and occasionally sepsis (1). Infection typically occurs via ingestion of contaminated food. When the bacterium arrives at the terminal ileum, it enters into and translocates across M cells, thus breaching the intestinal epithelial barrier and leading to colonization of the subepithelial Peyer’s patches and lamina propria (2, 3).

Human *Y. pseudotuberculosis* infection occurs sporadically in all continents of the world, including in North America, Europe, Russia, and Japan (4). In Europe, the infection typically causes a self-limiting gastroenteritis, whereas in Russia and Japan, *Y. pseudotuberculosis* infection can also manifest itself in severe systemic inflammatory symptoms called Far East scarlet-like fever, making it a health problem (4). In addition, *Y. pseudotuberculosis* is considered a direct ancestor of *Yersinia pestis*, the causative agent of plague that is responsible for three major pandemics in human history. The phylogenetic analyses of various *Y. pestis* and *Y. pseudotuberculosis* strains have indicated that *Y. pestis* is a clone of *Y. pseudotuberculosis* that evolved as recently as 2,000–10,000 years ago (5).

The invasion mechanism of *Y. pseudotuberculosis* has been studied by several groups, but the role of cholesterol in the host cell plasma membrane for the infection has not been defined. Infections of diverse pathogens, such as *Escherichia coli*, *Campylobacter jejuni*, *Listeria monocytogenes*, and *Shigella flexneri*, were reported to be affected by lipid rafts (in which cholesterol is a main component) in the host cell plasma membrane (6–12). The cell entry of diverse viruses and protozoan parasites has also been shown to be affected by host cell cholesterol (13–18). We wished to investigate whether cholesterol has a role in the infection of mammalian cells by *Y. pseudotuberculosis*.

The *Y. pseudotuberculosis* adhesins, invasin and YadA (encoded by *inv* and *yadA* genes, respectively), are largely responsible for the adhesion to and internalization into epithelial cells (19). Invasin promotes internalization of *Y. pseudotuberculosis* into intestinal cells immediately after oral infection (20–22). In host cell plasma membranes, invasin binds to β1 integrin complexed with any of several α integrins (23). High densities both of invasin in *Y. pseudotuberculosis* outer membrane and of β1 integrin in host cell plasma membrane are needed for efficient bacterial internalization into host cells. At lower densities, there is adhesion without internalization (24–25). YadA can enhance adhesion and internalization of *Y. pseudotuberculosis* under circumstances in which invasin expression is suppressed (26, 27). YadA also interacts with β1 integrin. However, unlike the direct interaction between invasin and β1 integrin, the interaction between YadA and β integrin occurs indirectly though extracellular matrix (24, 26, 28, 29). Invasin and YadA compete for binding to β1 integrin, so interaction of host cells with *Y. pseudotuberculosis* can depend on the expression level of each adhesin (30).
Sterol effect upon Yersinia internalization

The interaction of invasin and YadA with β1 integrin is likely to play a key role in *Y. pseudotuberculosis* uptake into host cells. Their binding to β1 integrin induces its clustering within the host cell plasma membrane (24, 30, 31). This clustered integrin interacts with extracellular matrix and cytoskeleton, and adhesion complexes involved in cell movements along a substrate control this process by transmitting signals between the outside and inside of cells (32, 33). Disassembly of the adhesion complexes, which is important for regulation of cell movements, might be controlled by rapid endocytosis of β1 integrin (34–36). An analogous set of events may occur during bacterial uptake. Clustered β1 integrin induces cytoskeletal rearrangements and a phagocytosis-related signaling pathway, promoting internalization of *Y. pseudotuberculosis* (20, 26).

Little is known about the role of cholesterol in this process, although it has been reported that cholesterol enhances intracellular growth of the bacterium (37). In addition, cholesterol may impact infection via its effect on β1 integrin. There have been reports that endocytosis of β1 integrin is lipid raft–mediated (38), and that β1 integrin expression increases the amount of raft domain–forming lipids in cell plasma membranes (39). This suggests the possibility that host cell lipids, including cholesterol, could affect phagocytosis of *Y. pseudotuberculosis*.

Using a sterol substitution method, we found that sterol structure had little effect on *Y. pseudotuberculosis* adhesion to host MDA-MB-231 cells. In contrast, internalization of *Y. pseudotuberculosis* into host cells was only observed when host membranes contained cholesterol, 7-dehydrocholesterol, or desmosterol. The effect of sterol type upon the antibody-clustered β1 integrin endocytosis was different from its effect upon uptake of *Y. pseudotuberculosis* into cells, even though uptake of the latter is mediated by β1 integrin-binding adhesins YadA and invasin. β1 integrin endocytosis had a more permissive sterol structure dependence, with a sterol dependence pattern very similar to that for endocytosis of other proteins in this cell line (40). This suggests that β1 integrin-mediated internalization of *Y. pseudotuberculosis* into host epithelial cells requires more specific sterol properties than does membrane protein endocytosis.

**Results**

**Effect of sterol substitution in host cell plasma membrane upon internalization of *Y. pseudotuberculosis***

Diverse sterols (Fig. 1) were employed using the protocol shown in Fig. 2 to determine the effect of sterol substitution and/or cholesterol depletion upon *Y. pseudotuberculosis* internalization into MDA-MB-231 cells. Because we previously used MDA-MB-231 cells to study the effect of sterols upon clathrin-mediated and clathrin-independent endocytosis (40), this allowed us to compare the effect of sterols upon various types of endocytosis with their effect upon bacterial uptake. MDA-MB-231 cells are epithelial, as are the cells infected by *Y. pseudotuberculosis*. The *Y. pseudotuberculosis* contained an IPTG2-inducible mCherry expression plasmid, to allow facile detection of the bacteria by microscopy. To differentiate *Y. pseudotuberculosis* outside and inside cells, immunofluorescence was performed using anti-*Yersinia* primary antiserum SB349 (41) and an FITC-conjugated goat anti-rabbit secondary antibody. Because immunofluorescence was carried out without cell permeabilization, antibody labeled only the bacteria outside of host cells. Thus, bacteria outside of cells exhibited both red and green fluorescence, whereas bacteria inside of cells only exhibited red fluorescence. Bright-field images were also taken to define cell areas and avoid counting bacteria not interacting with cells. The micrographs in Fig. 3 illustrate some representative results under conditions that either support or inhibit internalization. Note the presence of red bacteria, showing that they are partially or totally internalized in untreated samples or after substitution with 7-dehydrocholesterol or desmosterol, but that there are virtually no red bacteria after depletion of cholesterol or substitution with allocholesterol.

When the images from internalization experiments were quantified, it was found there was no significant effect of cholesterol depletion or sterol substitution upon the apparent amount of *Y. pseudotuberculosis* adhering to host cells (with the exception of somewhat reduced adhesion in the case of substitution with epicholesterol) (Fig. 4A). However, *Y. pseudotuberculosis* internalization was sensitive to sterol manipulation (Fig. 4B). Cholesterol depletion decreased internalization to <10% of the untreated control. Replenishment of cholesterol or substitution with 7-dehydrocholesterol or desmosterol restored internalization to levels (90–120%) similar to that in the untreated control. Substitution with other sterols did not restore internalization.

**Adhesin and sterol dependence of adhesion and internalization**

Adhesins (YadA and invasin) of *Y. pseudotuberculosis* are required for internalization of the bacterium into host cells in a process probably involving their ability to bind to β1 integrin (19, 30). To study whether the effects of sterol upon internalization were dependent upon adhesins, four strains of *Y. pseudotuberculosis* with different adhesin expressions (wildtype adhesins, ΔyadA, Δinv, and a ΔyadAΔinv double deletion) were studied.

Adherence of bacteria to host cells and internalization of bacteria were measured in untreated cells, in cholesterol-depleted cells, and in sterol-substituted cells. Significant effects of adhesin deletions upon both adherence and internalization were observed (Fig. 5). Adherence of *Y. pseudotuberculosis* was not decreased by deletion of invasin, but it was greatly decreased by deletion of YadA and even more in the double YadA/invasin deletion (Fig. 5A). For all strains studied, adhesion was similar in untreated, cholesterol-depleted, and sterol-substituted cells. Thus, host cell sterol content does not appear
Sterol effect upon Yersinia internalization

Figure 1. Structures, names, and abbreviations of sterols used in this study.

to significantly influence *Y. pseudotuberculosis* adhesion to host cells.

Internalization and its sterol dependence were also evaluated (Fig. 5B). In the double \(\Delta yadA\Delta inv\) mutant, internalization was abolished no matter what the sterol content. (The apparent small amount of internalization in the presence of desmosterol is not statistically significant.) This suggests that adhesin interaction with integrin is required for internalization. In contrast, internalization was observed in both single deletion mutants, indicating that only one adhesin is needed for internalization. There was a modest decrease in the absolute level of internalization in the single mutants. Normalization shows that the internalization/adherence ratio was the same in the wildtype and \(\Delta yadA\) strains (~15%) and only modestly reduced in the \(\Delta inv\) strain (10%) (Fig. 5C). Interestingly, the dependence on sterol structure in both \(\Delta yadA\) and the \(\Delta inv\) single mutants was similar to that of the wildtype bacterium, indicating that sterol influences *Y. pseudotuberculosis* internalization in a mechanism that involves both YadA-dependent and invasin-dependent processes. In other words, both of these adhesion pathways lead to uptake processes that are sterol-dependent.

**Effect of sterol substitution upon endocytosis of antibody-clustered \(\beta 1\) integrin**

The adhesin invasin interacts directly with \(\beta 1\) integrin, whereas the adhesin YadA interacts indirectly with \(\beta 1\) integrin via interactions with \(\beta 1\) integrin-binding extracellular matrix molecules (23, 24, 26, 28, 30). In addition, these adhesins trigger clustering of \(\beta 1\) integrin, and this mediates onset of signaling pathways and cytoskeletal rearrangements that induce internalization of *Y. pseudotuberculosis* (20, 26).

Thus, it is possible that the specific sterol dependence pattern for *Y. pseudotuberculosis* internalization reflects the sterol dependence pattern for endocytosis of \(\beta 1\) integrin. To test this hypothesis, the sterol structure dependence of the endocytosis of \(\beta 1\) integrin clustered using antibodies (38, 42) was studied. After cholesterol depletion or sterol substitution, \(\beta 1\) integrin in the plasma membrane was clustered with anti-\(\beta 1\) integrin IgG
Sterol effect upon Yersinia internalization

Figure 2. Protocol to study the effect of sterol substitution in host cells upon Y. pseudotuberculosis internalization. MDA-MB-231 cells were cholesterol-depleted by incubation with 10 mM MβCD for 30 min at 37 °C. Then sterol substitution was performed by incubation with sterol-loaded 2.5 mM MβCD for 2 h at 37 °C. Y. pseudotuberculosis expressing mCherry to impart red fluorescence was added to the sterol-substituted host cells at MOI of 10 and incubated for 30 min at 37 °C. Most of the unbound bacteria were then washed out with DPBS, and samples were fixed with 4% PFA. Anti-Yersinia antiserum SB349 and FITC-conjugated goat anti-rabbit antibody were then added. Y. pseudotuberculosis outside of cells appeared yellow-green (merge of red and green), whereas those internalized appeared red, as the anti-Yersinia antibodies in the antiserum cannot penetrate inside the host cells.

Figure 3. Adherence and internalization of Y. pseudotuberculosis after sterol substitution in host cell plasma membrane. Experiments were performed following the protocol shown in Fig. 2. Micrographs were taken using a Zeiss 510 Meta NLO confocal microscope. Y. pseudotuberculosis outside of host cells have green and red fluorescence and appear yellow-green, whereas bacteria inside of host cells have red fluorescence only. Note that some bacteria seem to be partly engulfed by cells (this was also counted as internalized). Scale bar, 20 μm.

and fluorescence-conjugated secondary antibody. Endocytosis was then induced by incubation of the cells at 37 °C for 20 min (Fig. 6A).

Cholesterol depletion and sterol substitution were found to influence endocytosis of the antibody-clustered β1 integrin. The images of some representative examples are shown in Fig. 6B, and the quantified data for the full range of sterol substitutions are shown in Fig. 7A. Cholesterol depletion strongly inhibited β1 integrin endocytosis (by >80%). Endocytosis was fully restored after cells were cholesterol-replenished. Substitution of the two sterols that supported internalization of Y. pseudotuberculosis, desmosterol and 7-dehydrocholesterol, also fully supported endocytosis. However, in contrast to Y. pseudotuberculosis internalization, several other sterols (lathosterol, cholesta-4,6-dien-3-ol, and dihydrocholesterol) were able to support β1 integrin endocytosis to a significant degree, and some sterols (zymosterol, lanosterol, and allocholesterol) may have at least weakly supported endocytosis relative to that in cholesterol-depleted cells. Other sterols (epicholesterol, 4-cholesten-3-one, or coprostanol) did not support endocytosis above the cholesterol-depleted baseline level.

Previously, we studied the effect of sterol type upon the clathrin-independent endocytosis of antibody-clustered placental alkaline phosphatase (PLAP) and the clathrin-mediated endocytosis of transferrin in MDA-MB-231 cells stably expressing PLAP (40). Interestingly, the effect of sterol type on antibody-clustered β1 integrin endocytosis was similar to that for endocytosis of antibody-clustered PLAP and transferrin (Fig. 7, B and C). This indicates that endocytosis of membrane-associated proteins is less sensitive to sterol type than is internalization of Y. pseudotuberculosis.

To determine whether the amount of β1 integrin located in the plasma membrane was changed by cholesterol depletion or sterol substitution, cells were fixed after sterol manipulation in the cells and stained for β1 integrin without cell permeabilization. The results showed that there were no significant changes in β1 integrin level except when substitution was carried out with 4-cholesten-3-one (Fig. 8). Although substitution with 4-cholesten-3-one reduced the β1 integrin level in plasma membrane about 30% (compared with cholesterol-replenished cells), the decrease of β1 integrin endocytosis by the 4-cholesten-3-one substitution was much larger (~90%, compared with cholesterol-replenished). Thus, decreased endocytosis of β1 integrin could not be fully explained by a decrease in the amount of plasma membrane β1 integrin.

Amount of substituted sterols in host cells

Sterol level after sterol substitution is an important variable that can influence how much endocytosis/uptake is observed. In prior studies, the sterol substitution levels were evaluated in...
MDA-MB-231 cells expressing PLAP (40). Because MDA-MB-231 was also the host cell used for *Y. pseudotuberculosis* uptake studies, similar levels of sterol substitution would be expected in the present study. Confirming this, cholesterol and substituted sterol levels were similar in cells expressing PLAP and those that did not express PLAP (Fig. 9A). In both cases, cholesterol depletion decreased cholesterol to about 40% of untreated control. After sterol substitution, the cholesterol decreased further, to 15–30% of the untreated control. This probably resulted from additional cholesterol removal by the sterol-loaded MJCD used in the substitution procedure. For the sterols tested, except for lanosterol, substituted total sterol levels were above that in control samples. It should be noted that dihydrocholesterol has the same mobility on TLC as cholesterol, so the sterol band on the high-performance TLC (HP-TLC) plate in this case corresponds to total sterol. Nevertheless, because cholesterol level in the cells after sterol substitution is quite low (40), we can assume that most of the band reflects the dihydrocholesterol level. It should also be noted that although
differences in sterol levels after substitution can influence the exact level of endocytosis, the effect is too small to affect conclusions about which sterols support or do not support endocytosis (40).

Because the two sterols other than cholesterol that permit the uptake of \textit{Y. pseudotuberculosis}, 7-dehydrocholesterol and desmosterol, are the immediate biosynthetic precursors of cholesterol, there was a possibility that it was their conversion into cholesterol that allowed bacterial uptake. This is unlikely because the sterols predominantly located in the plasma membrane (40), but the reductases that convert them to cholesterol are located in the endoplasmic reticulum (43–45). Furthermore, the sterols were not converted to cholesterol during the lipid exchange incubation (Fig. 9A). We also confirmed that 7-dehydrocholesterol was not converted into cholesterol during the additional 30-min incubation at 37 °C for bacterial uptake (Fig. 9B).

**Discussion**

We found that cholesterol depletion severely inhibits uptake of \textit{Y. pseudotuberculosis} by cultured cells. To study the effect of sterol properties upon uptake of \textit{Y. pseudotuberculosis}, we then carried out sterol substitution experiments. The results showed that sterol type strongly affects the internalization of \textit{Y. pseudotuberculosis} into host cells. Other than cholesterol, only 7-dehydrocholesterol and desmosterol could support internalization of the bacterium and did so at levels similar to that supported by cholesterol. This suggests that cholesterol, 7-dehydrocholesterol, and desmosterol share some specific property needed for the internalization of the bacterium. Interestingly, 7-dehydrocholesterol and desmosterol are the immediate precursors of cholesterol during its biosynthesis (46) and so are similar in structure and properties. All three possess a double bond between the 5- and 6-carbons of the sterol B-ring. None of the studied cholesterol precursors formed in earlier steps of the cholesterol biosynthesis pathway (lanosterol, zymosterol, and lathosterol) have a 5–6 double bond (46) or support internalization. Thus, it is possible that a 5–6 double bond is important for a specific sterol-protein interaction required for internalization. A different, but related, possibility is that 7-dehydrocholesterol and desmosterol have more functionally important cholesterol-like physical characteristics than earlier precursors or the other sterols tested. This would be consistent with the observation that of all the sterols tested, cholesterol, 7-dehydrocholesterol, and desmosterol also supported the maximum level of antibody-clustered β1 integrin endocytosis.
In this regard, it should be noted that among the sterols we tested, epicholesterol also has a 5–6 double bond but could not induce internalization of the bacterium. The only difference in structure between cholesterol and epicholesterol is the orientation of a hydroxyl group attached to the 3-carbon in the A-ring (β-orientation in cholesterol but α-orientation in epicholesterol). Thus, the orientation of the hydroxyl group seems to be also critical for an internalization of the bacterium. Changing the orientation of the hydroxyl group from /H9252 to /H9251 also inhibited both clathrin-mediated and clathrin-independent protein endocytosis (40).

Phytosterols also contain both a 5–6 double bond and the 3 /H9252-hydroxyl group, but have modified aliphatic tails relative to cholesterol, so their effect on uptake would be of interest. Unfortunately, we found very low substitution efficiency for the major phytosterol /H9252-sitosterol and were not able to study its effect on endocytosis (40). Therefore, it was not included in the bacterial uptake studies. Future endocytosis and bacterial uptake studies examining a wider range of phytosterols would be worthwhile if it is possible to identify

Figure 7. Quantitation of endocytosis of antibody-clustered β1 integrin and comparison with endocytosis of other proteins. A, the antibody-clustered β1 integrin endocytosis level is shown as a percentage of the endocytosis in cholesterol-replenished cells. Sterol abbreviations are as in the legend to Fig. 4. W/O-I, without internalization (incubation on ice instead of 37 °C). Mean values and S.D. are shown from three experiments except for coprostanol, which was done twice. B, correlation of sterol effects between antibody-clustered β1 integrin endocytosis and antibody-clustered placental alkaline phosphatase endocytosis (40). C, correlation of sterol effects between antibody-clustered β1 integrin endocytosis and transferrin endocytosis from (40). *, p < 0.05; **, p < 0.01 compared with cholesterol-replenished (CH) (unpaired, two-tailed Student’s t test). Error bars, S.D.

Figure 8. β1 integrin amounts in plasma membrane of MDA-MB-231 cells after cholesterol depletion or sterol substitution. After sterol manipulations were performed, the cells were fixed, and β1 integrin in the plasma membrane was measured by immunofluorescence (without permeabilization) using a Zeiss 510 Meta NLO confocal microscope. Mean values and S.D. (error bars) are shown from three experiments. *, p < 0.05 compared with cholesterol-replenished (CH) (unpaired, two-tailed Student’s t test).
phytosterols that can be substituted for cholesterol at high levels.

One of the important physical properties of sterols is their raft-forming ability (54–57). However, the results from our study do not by themselves specify the importance of ordered domain (lipid raft domain) formation for Y. pseudotuberculosis internalization. Although all of the sterols that support internalization support some level of ordered domain formation, 7-dehydrocholesterol and cholesterol have an ability to promote raft formation significantly stronger than that of desmosterol (54, 58). In addition, neither other strongly raft-promoting sterols (dihydrocholesterol, lathosterol, and epicholesterol) nor other sterols with intermediate raft-forming ability (zymosterol, allocholesterol, and cholesta-4,6-dien-3-ol) supported Y. pseudotuberculosis uptake (54–57). Thus, high or intermediate raft-forming ability is not sufficient for sterol to support Y. pseudotuberculosis internalization. On the other hand, raft-disrupting sterols (coprostanol and 4-cholesten-3-one) (56) did not support Y. pseudotuberculosis internalization. Thus, it is still possible that raft-forming ability is necessary but not sufficient for a sterol to support Y. pseudotuberculosis internalization.

The effect of sterol structure on internalization of Yersinia was not due to an effect of sterol upon adhesion, which was independent of sterol type. It should be noted that the decreased adherence by YadA deletion that we observed does not agree with a previous study using macrophages (30). This might be due to using different cell lines (epithelial non-professional uptake versus professional phagocytosis by a macrophage). Also, we used a shorter infection time (30 min in this study versus 60 min previously (30)) and a lower multiplicity of infection (MOI) (MOI of 10 in this study versus MOI of 20 previously (30)), which might increase the sensitivity of bacterial adherence to the presence of YadA.

It was interesting that the dependence of β1 integrin endocytosis on sterol structure was much less restricted than that for internalization of Y. pseudotuberculosis, although β1 integrin is a receptor (directly via invasin or indirectly via extracellular matrix proteins and YadA) for Yersinia. The endocytosis of β1 integrin showed a pattern of sterol dependence similar to that of other proteins we tested previously (40). An intriguing outstanding question is why uptake of Y. pseudotuberculosis is so much more sterol-specific than protein endocytosis. It is possible that it is an efficiency issue, arising because the host cell–
Sterol effect upon Yersinia internalization

bound bacterium engages many more receptors than in the case of antibody-clustered proteins, so that if a sterol does not promote molecular uptake into cells as well as cholesterol (e.g. due to some impaired protein-sterol interaction), the effect on uptake is much greater for a bacterium than for an antibody-clustered protein. It is also possible that some other issue involving the much larger size of a bacterium relative to a protein cluster is involved. For example, the amount of bent membrane necessary to engulf a bacterium would greatly exceed that for a protein or protein cluster. Another issue might be that the extent of clustering of β1 integrin by Y. pseudotuberculosis might be very sterol structure-specific. However, because in the case of transferrin receptor, endocytosis has a sterol structure dependence very similar to that of β1 integrin but does not involve clustering, it is unlikely that sterol effects on clustering can explain the sterol structure dependence of internalization in general.

In any case, the difference between protein endocytosis and bacterial uptake might have some practical or biomedical applications. Because some sterols that interfere with bacterial uptake do not greatly inhibit endocytosis, it may be possible to find other molecules that interfere with bacterial infection by interfering with bacterial uptake into cells without blocking endocytosis, a process needed for basic cell functions.

Experimental procedures

Materials

DMEM, Dulbecco’s phosphate buffered saline (DPBS; containing 200 mg/liter potassium chloride, 200 mg/liter potassium phosphate monobasic, 8 g/liter sodium chloride, and 2.16 g/liter dibasic sodium phosphate) for tissue culture, Hanks’ balanced salt solution (HBSS, containing 140 mg/liter calcium chloride, 100 mg/liter magnesium chloride, 100 mg/liter magnesium sulfate, 400 mg/liter potassium chloride, 60 mg/liter potassium phosphate monobasic, 350 mg/liter sodium bicarbonate, 8000 mg/liter sodium chloride, 48 mg/liter sodium phosphate dibasic anhydrous, and 1000 mg/liter D-glucose), antibiotics for tissue culture (10,000 units/ml penicillin and 10 mg/ml streptomycin in 0.85% saline), and trypsin-EDTA were obtained from Gibco. Calf serum was iron-fortified/supplemented bovine calf serum from SAFC Biosciences (Lenexa, KS) or Atlanta Biologicals (Flowery Branch, GA). Dihydrocholer sterol, 7-dehydrocholer sterol (Fluka brand), lanosterol, MβCD, and paraformaldehyde (PFA) were obtained from Sigma-Aldrich. Coprostanol, desmosterol, epicholesterol (5-cholen-3α-ol), allocholesterol (4-cholen-3β-ol), cholesta-4,6-dien-3β-ol, androstenediol (5-androst-3β-ol), and cholestenerone (4-cholen-3-one) were obtained from Steraloids Inc. (Newport, RI). Cholesterol, lathosterol, and zymosterol were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). The sterols generally appeared as single bands on thin layer chromatography, but lanosterol was purchased as a mixture with ~65% lanosterol, the remainder likely being dihydrolanosterol. Bradford protein assay reagent and 10X PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.4) used for immunofluorescence were bought from Bio-Rad. BSA was bought from Millipore (Kankakee, IL). Alexa Fluor 488 (AF488) goat anti-mouse IgG (A11001), ProLong Gold™ antifade mountant, and CellMask™ Deep Red plasma membrane stain were Molecular Probes brand obtained from Life Technologies, Inc. Azide-free anti-β1 integrin IgG (PSD2, sc-13590L) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX). HP–TLC plates (Silica Gel 60) were bought from VWR International (Batavia, IL). Hexanes, isopropyl alcohol, chloroform, methanol, citric acid, and sodium chloride were purchased from Fisher. VECTASHIELD® mounting medium was purchased from Vector Laboratories (Burlingame, CA).

Methods

When temperature is not stated, procedures were carried out at room temperature (21–23 °C).

Bacterial strains

The bacterial strains used in this study are listed in Table 1. Y. pseudotuberculosis strains IP2666 ΔyadA, IP2666 Δinv, and IP2666 ΔinvΔyadA were kind gifts of Dr. J. Mecsas (59). Y. pseudotuberculosis translocates anti-phagocytic factors known as Yersinia outer proteins (Yops) into the host cells. To prevent Yops from complicating our analysis of bacterial internalization, yopB, a gene required for the translocation of Yops into the host cells, was inactivated in the above strains by allelic exchange, as described previously (60). The allelic exchange was performed either with pSB890-yopB40 plasmid that introduces a stop codon followed by a frameshift at codon 8 of YopB followed by a frameshift) or with pSB890-ΔyopB plasmid that introduces an in-frame deletion at nucleotides 496–774 of YopB (62). The plasmid-encoded mCherry under the control of a lactose-inducible promoter (pMMB207-mCherry) (63) was then introduced into these strains by conjugation.

Y. pseudotuberculosis strains were cultivated at 28 °C on Luria–Bertani (LB) agar plates or in LB broth with appropriate antibiotics with aeration.

Mammalian cell (host) preparation

The MDA-MB-231 human breast cancer epithelial cells were obtained from ATCC and maintained with DMEM supplemented with 10% calf serum and 100 units/ml penicillin plus 100 μg/ml streptomycin at 37 °C and 5% CO2. Cells were counted with a hemocytometer. A day before the experiments, 2 × 105 cells/well (containing a coverslip) of a 24-well plate

| Table 1 | Bacterial strains used in this study |
|---------|-----------------------------------|
| Y. pseudotuberculosis strain name and relevant characteristics | Reference/source |
| ΔyadA | This study |
| Δinv | This study |
| ΔinvΔyadA | This study |
| ΔyadA | Ref. 68 |
| ΔyadA | Ref. 69 |
| ΔyadA | Ref. 59 |
| ΔyadA | Ref. 59 |
| ΔyadA | Ref. 59 |

1474 J. Biol. Chem. (2018) 293(4) 1466–1479
Sterol effect upon Yersinia internalization

Y. pseudotuberculosis, 6 × 10^5 cells/35-mm dish containing a coverslip were plated for study of antibody-clustered β1 integrin endocytosis and detection of the amount of β1 integrin localized in plasma membranes, or 1.2 × 10^6 cells/60-mm dish were plated for HP-TLC analysis.

Sterol substitution in MDA-MB-231 cells

MβCD (to give a final concentration of 2.5 mM) was loaded with sterol (to a final concentration of 0.1 mM cholesterol or 0.4 mM for other sterols if all sterol loaded onto the MβCD). This sterol concentration difference was used because cholesterol loads onto MβCD better than other sterols.) To do this, MβCD in distilled water and sterols in chloroform were placed in a glass tube and vortexed. The mixture was dried under N₂ gas and then placed under high vacuum for 1–2 h. Next, 1 ml of serum-free DMEM was added, and the samples were sonicated using an Ultrasonic cleaner (model 8845-3, Cole-Parmer Instrument Co. (Chicago, IL)) for about 2 min. The sample was then incubated in a 37 °C shaking incubator overnight, and if necessary, serum-free DMEM was added (up to 4 ml) to bring the sample to the desired final concentration. The solution was filtered through 0.22-μm syringe filters (Sarstedt, Nümbrecht, Germany) to remove undissolved sterol (MβCD concentration was assumed to be unaltered by filtration).

Cells prepared 1 day before the experiments, as described above, were washed with 1–2 ml of DPBS twice, and then 10 mM MβCD (0.4 ml/well of a 24-well plate, 1 ml/35-mm dish, or 1.5 ml/60-mm dish) was added, followed by incubation for 30 min at 37 °C to deplete cholesterol. For sterol substitution, the cells were then washed once with 1–2 ml of DPBS and then incubated with the sterol-loaded MβCD (0.4 ml/well of 24-well plates, 1 ml/35-mm dish, or 1.5 ml/60-mm dish) for 2 h at 37 °C. The cholesterol-depleted or sterol-substituted cells were then used for studies of bacterial uptake and integrin endocytosis.

Infection assay and immunofluorescence of Y. pseudotuberculosis

A day before the infection assay, a single Y. pseudotuberculosis colony from a bacterial culture dish was inoculated into 5 ml of LB broth containing 100 μM IPTG (to induce mCherry expression) and 25 μg/ml chloramphenicol, placed in a 10-ml glass tube, and then cultured overnight in a 28 °C shaking incubator. The next day, the culture was diluted 1:40 into 10 ml of fresh LB medium with 500 μM IPTG and 25 μg/ml μM chloramphenicol, placed in a 50-ml flask, and incubated in a 37 °C shaker for ~2 h. Next, 1 ml of the diluted Y. pseudotuberculosis culture was centrifuged for 4 min at 4000 rpm, and the pellet was resuspended in 1.2 ml of DPBS. Optical density at 600 nm was measured (1 A_{600} of Y. pseudotuberculosis = 1.778 × 10^9 cells). The bacteria in the OD sample was further diluted into serum-free DMEM containing 100 μM IPTG and added to MDA-MB-231 cells that had been grown for 1 day in a 24-well plate as described above to give an MOI of 10, assuming that cell numbers did not change significantly overnight or after sterol manipulation.

The plate was centrifuged in a Sorvall RT6000B centrifuge at ~750 rpm (~50 × g) for 5 min to bring the bacteria into contact with the MDA-MB-231 cells and incubated for an additional 25 min in a 37 °C and 5% CO₂ incubator (i.e. a total of 30 min for bacterial infection). After incubation, the mammalian cells were washed with 1 ml of DPBS three times and fixed with 250–500 μl of 4% (w/v) PFA for 10 min at room temperature. The fixed cells were then washed with 1 ml of DPBS three times. After the addition and removal of two aliquots of 0.5 ml of 3% (w/v) BSA in HBSS, each incubated with cells for 10 min, the cells were incubated with 250 μl of 1:1000 diluted rabbit anti-Yersinia antiserum SB349 (41) in 3% (w/v) BSA in HBSS, at room temperature for 1 h on a shaker. Cells were washed twice with 1 ml of DPBS and then incubated with 250 μl of 1:1500 diluted FITC-conjugated goat anti-rabbit IgG (1.5 mg/ml; Jackson ImmunoResearch, West Grove, PA) in 3% (w/v) BSA in HBSS. The cells were incubated for 40 min at room temperature with shaking and then washed three times with 1 ml of DPBS. The coverslip in each well was removed and mounted on slides using 7–8 μl of ProLong Gold™ antifade mountant.

Analysis of internalization

Z-Stack fluorescence images of bacteria (total bacteria (red) and bacteria outside cells (green)) and z-stack bright-field images of MDA-MB-231 cells were taken at a magnification of ×630 (×63 from the objective × ×10 from the eyepiece) using a Zeiss 510 Meta NLO confocal microscope. FITC was detected using the 488-nm argon laser line, and mCherry was detected using a 543-nm HeNe1 laser. A total of three images each from three independent samples prepared on different days were taken for each condition. The total number of bacteria was measured in the red image, and then the bacteria not associated with cells was identified from the combined red, green, and bright-field image and then subtracted. The number of MDA-MB-231 cells in each bright-field image was also counted. Bacteria internalized into host cells did not bind the anti-Yersinia antiserum added outside of cells, thus showing only red mCherry fluorescence. The bacteria showing only red fluorescence on part or all of their surface were scored as internalized bacteria (i.e. both partially and totally internalized bacteria were counted as internalized). Bacteria associated with cells that exhibited FITC-conjugated goat anti-rabbit IgG green fluorescence (yellow-green fluorescence in the combined image when present together with mCherry) were scored as bacteria that had not been internalized. Total bacteria interacting (attached or internalized) with host cells per host cell, internalized bacteria per host cell, and internalized bacteria per host cell-interacting bacteria were then calculated.

To determine whether membrane permeabilization upon sterol substitution and fixation would allow immunostaining of internalized bacteria by externally added antibody, F-actin immunostaining was measured in sterol-substituted and fixed cells. Most sterols used did not show increased permeabilization relative to controls, although a raft-disrupting sterol (4-cholesten-3-one) increased membrane permeability to some degree. Thus, immunostaining of bacteria by externally added antibody mainly reflects a lack of bacterial internalization rather than antibody leakage into cells.
Sterol effect upon Yersinia internalization

Antibody-clustering and endocytosis of β1 integrin

After sterol substitution or cholesterol depletion, cells were washed with 1.5 ml of serum-free ice-cold DMEM twice on ice, and anti-β1 integrin IgG diluted in serum-free DMEM to 100 µl (1 µg/ml final antibody concentration) was loaded only onto a coverslip that had been placed in the 35-mm dish when the dish had first been inoculated with cells. Cells were incubated for 30 min at 4 °C and then washed twice with 1.5 ml of serum-free ice-cold DMEM on ice. Then AF488-conjugated goat anti-mouse IgG in serum-free DMEM diluted to 100 µl (40 µg/ml final concentration) was loaded onto the coverslip, and samples were incubated for 15 min at 4 °C. The cells were washed twice with 1.5 ml of serum-free ice cold DMEM on ice, and then 1.5 ml of prewarmed (to 37 °C) serum-free DMEM was added, and samples were incubated at 37 °C for 20 min to induce endocytosis of the β1 integrin. Next, cells were placed on ice to halt endocytosis. Fluorescence on the cell surface (antibodies remaining on the cell surface) was removed by acid washing at 16–18 °C (incubating cells three times a in 2 ml of acid solution for 3 min). The acid solution was 100 mM citric acid, 140 mM NaCl adjusted to pH 1.75 with HCl. The cells were then washed twice with 1.5 ml of serum-free ice-cold DMEM on ice and fixed with 1.5 ml of 3% (w/v) PFA for 30 min (10-min incubation on ice followed by 20-min incubation at room temperature). Fixed cells were washed three times with 1.5 ml of 1× PBS at room temperature. Cell membranes were counterstained with 100 µl of CellMask Deep Red membrane stain solution (diluted to 4 µl/ml (20 µg/ml) in HBSS containing 3% (w/v) BSA for 2–5 min at room temperature. Finally, the cells were washed with 1.5 ml of 1× PBS at room temperature, and the coverslip was taken from the dish and mounted on a slide using VECTASHIELD® mounting medium.

Image analysis of antibody-clustered β1 integrin endocytosis assay

Z-stack images of fluorescently stained cells were taken at ×630 magnification (×63 from the objective × 10 from the eyepiece) using the Zeiss 510 Meta NLO confocal microscope (laser lines: argon (458/477/488/514 nm, 25 milliwatts) and HeNe2 (633 nm, 5 milliwatts)). For each type of sample in an experiment, a total of 3–6 images were taken. Three independent experiments were carried out, analyzing ≥50 cells/sample type/experiment. The endocytosed β1 integrin was detected as fluorescent puncta located within the cells. We found that quantifying clustered β1 integrin endocytosis from the puncta number/cell ratio was more sensitive and reproducible than measurements of total internalized anti-β1 integrin antibody fluorescence. Thus, numbers of puncta and cells in an image were counted using the ImageJ version 1.50C program supplied by Wayne Rasband (National Institutes of Health), and the puncta number/cell ratio was calculated.

Immunofluorescence of β1 integrin in plasma membrane

After cholesterol depletion or sterol substitution, cells were washed three times with 1.5 ml of DPBS at room temperature and then fixed with 1.5 ml of 3% (w/v) PFA for 30 min (10-min incubation on ice followed by a 20-min incubation at room temperature). The cells were washed three times with 1.5 ml of 1× PBS at room temperature and blocked using 1.5 ml of 3% (w/v) BSA in HBSS for 30 min at room temperature. Then 100 µl of 1 µg/ml anti-β1 integrin IgG (1:1000 dilution in 3% (w/v) BSA in HBSS) was added to the cells, and they were incubated for 1 h at room temperature. Next, cells were washed three times with 1.5 ml of 1× PBS at room temperature, after which 100 µl of 2 µg/ml AF488 goat anti-mouse IgG in HBSS with 3% (w/v) BSA was added. After the cells were washed three times with 1.5 ml of 1× PBS at room temperature, cell membrane staining was performed using 100 µl of CellMask Deep Red membrane stain solution (diluted to 4 µl/ml (20 µg/ml) in HBSS containing 3% (w/v) BSA) for 2–5 min at room temperature. Finally, the cells were washed twice with 1.5 ml of 1× PBS at room temperature, and the coverslip was taken from the dish and mounted on a slide using VECTASHIELD® mounting medium.

Image analysis of β1 integrin immunofluorescence assay

After performing immunofluorescence, cells were observed, and z-stack images were taken using a Zeiss 510 Meta NLO confocal microscope. Two to three images of each cell sample were taken at ×630 magnification (×63 from the objective × 10 from the eyepiece), and fluorescence intensity of each cell in the images was analyzed using the ImageJ version 1.50C program as follows. First, cell area (visualized by CellMask fluorescence) was determined, and then the integrated density of green fluorescence (immunostained β1 integrin) within the cell was measured (integrated density = mean intensity of green fluorescence × cell area). In addition, green fluorescence intensity of background was measured from five spots outside of cells in the image and averaged. Then the corrected total cell fluorescence (CTCF) was calculated using the equation, CTCF = integrated density within a cell − (area of the selected cell × mean background fluorescence per unit area). CTCF from a total of 50 cells in each sample was calculated, and the results from one sample from each of three independent experiments were averaged.

Purification of lipid and protein from MDA-MB-231 cells

The untreated or sterol-manipulated cells were washed three times with 3 ml of DPBS at room temperature. Then 1 ml of mixed hexanes/isopropyl alcohol (2:1 v/v) was added to the dishes and incubated at room temperature for 30 min with gentle shaking. The solution with dissolved lipids was harvested in a glass tube and dried under N2 gas. Then the tube was sealed with Teflon tape and then paraffin and stored at −20 °C. The undissolved residue in each of the tissue culture dishes after lipid isolation was used to isolate protein. The dishes were air-dried, and then 1 ml of 1 N NaOH was added. The dishes were then incubated at room temperature for 1 h with gentle shaking, and the solution in each dish was transferred to a 1.5-ml plastic tube and stored at −20 °C (64–66).

HP-TLC analysis of lipids

200 µl of methanol/chloroform (1:1, v/v) was added to a glass tube containing the lipids purified from the mammalian cells. The tube was vortexed, and a 20-µl aliquot was loaded onto a HP-TLC plate alongside various amounts of sterol standards.
The plate was air-dried and then placed in a tank containing mixed hexanes/ethyl acetate (3:2, v/v). The lipids were chromatographed until the solvent reached the top of the plate, and then the plate was removed from the tank and air-dried. The dried plate was sprayed with a solution of 3% (w/v) cupric acetate, 8% phosphoric acid (v/v) dissolved in water and again air-dried. The plate was heated in a 200 °C oven until charred lipid bands were clearly noticeable. The plate was then cooled and scanned, and the intensity of each sterol band was measured using the ImageJ program. Cell sterol levels were quantified by comparison with a standard curve obtained from the intensities of various amounts of standard sterols loaded on the same HP-TLC plate, using the SlideWrite Plus program (Advanced Graphics Software, Rancho Santa Fe, CA). The sterol amount was normalized to the protein level obtained from the same dish from which the sterol was purified.

**Protein quantitation**

Proteins from cells were quantitated by the Bradford method (67). Protein extracted from cells as described above was mixed with water and Bradford solution reagent in a ratio of 79:20:1 (v/v/v) water/Bradford solution/protein extract. Various amounts (1.5–12.5 μg/ml, final concentration) of BSA in water mixed with Bradford solution were used to generate a standard curve. The solutions were incubated for 20 min at room temperature, and then their absorbance at 595 nm was measured using a spectrophotometer. Protein concentration in the samples was calculated by comparison with the absorbance versus protein amount in the standard curve.

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