INFECTION AND IMMUNITY, Nov. 1986, p. 347–353
0019-9567/86/110347-07$02.00/0

Vol. 54, No. 2

Entry of *Bartonella bacilliformis* into Erythrocytes

LEE ANN BENSON, SIDDHARTHA KAR, GERALD McLAUGHLIN,† AND GARRET M. IHLER*

Department of Medical Biochemistry and Genetics, Texas A&M College of Medicine, College Station, Texas 77843

Received 28 April 1986/Accepted 29 July 1986

***Bartonella bacilliformis*, which causes the human diseases Oroya fever and verruga peruana, binds to human erythrocytes in vitro and produces substantial and long-lasting deformations in erythrocyte membranes, including cone-shaped depressions, trenches, and deep invaginations. The deforming force is probably provided by the polar flagella of these highly motile bacteria. Deep invaginations containing bacteria are commonly seen, and membrane fusion at the necks of the invaginations leads to the formation of intracellular vacuoles containing bacteria. Fluorescent compounds present externally render the vacuoles fluorescent and, occasionally, lightly fluorescent cells are seen, suggesting that the vacuoles sometimes rupture to admit the bacteria to the cytoplasm. Vacuoles present in fluorescent erythrocytes prepared by preloading the erythrocytes with fluorescent compounds are seen as dark areas from which the fluorescent marker is excluded. Entry of the bacteria appears to be the result of a process of forced endocytosis.**

Bartonella bacilliformis is a flagellated, highly motile intracellular bacterial parasite responsible for the human diseases Oroya fever and verruga peruana. In Oroya fever, nearly 100% of the erythrocytes become parasitized, leading to severe anemia with its associated symptoms. In the preantibiotic era, mortality was about 40% (8). In verruga peruana, bacterial invasion of the capillary endothelial cells leads to their proliferation and to the formation of hemangiomalike papules or nodules. Mortality in this manifestation of the disease is low.

Most intracellular parasites, including viruses, bacteria, malarial parasites, and flagellated protozoa, gain entry into cells by mechanisms related to endocytosis. (For a recent review on intracellular parasites, see reference 4.) Infecting organisms first bind to the host cell, either directly to some specific component of the cell surface or indirectly through antibodies or complement. In some cases, the mechanism of entry is similar or identical to that used by the host for internalizing hormones, serum proteins, or other substances. Viruses, for example, bind to specific membrane receptor proteins, migrate to coated pits, and are transiently found in endosomes from which they escape before they can be destroyed in lysosomes. Some bacteria enter by phagocytosis and escape from intracellular vesicles before they can be destroyed; others are resistant to destruction in lysosomes.

Mature erythrocytes, lacking even coated pits, are not phagocytic and are the cell type least capable of endocytosis, although nucleated erythrocyte precursors are fully capable of endocytosis. Despite the absence of spontaneous endocytosis, mature human erythrocytes can be successfully infected by sporozoans and bacteria. The mechanism of entry cannot be one in which the microorganism remains completely passive, but rather parasite-specified functions must play a key role which may not be simple, as indicated by the specialized apical complex of *Plasmodium* spp.

Endocytosis of a kind can be induced in erythrocytes by certain membrane-active compounds or by entrapping ATP, Ca^{2+}, and Mg^{2+} in the cells while the membranes are made permeable by reversible hypotonic hemolysis (5, 6). It is not known whether these processes of induced endocytosis use remnants of the endocytotic systems present in erythrocyte precursors or whether endocytosis is simply a property of the mature erythrocyte membrane and cytoskeleton. The erythrocyte membranes invaginate and then pinch off, entrapping external fluid within intracellular vesicles. During induced endocytosis, bacteria which normally do not enter erythrocytes may be entrapped in these vesicles along with the external fluid (6). We thought it possible that some erythrocyte parasites might use this induced endocytosis system for entry. We expected that *B. bacilliformis* might be an especially useful microorganism for such studies since it can be grown as a free-living bacterium in cell-free media and since mutations for binding and entry could presumably be readily isolated. An understanding of the contribution of the parasite to the entry process in erythrocytes would be interesting in itself and might be applicable to a general understanding of bacterial entry since *B. bacilliformis* infects nucleated cells in addition to erythrocytes.

The experiments reported here demonstrate that startling indentations and deformations of the membranes of erythrocytes occur when *B. bacilliformis* binds to erythrocytes and that pinching off of these indentations leads to entry within intracellular vacuoles.

MATERIALS AND METHODS

Bacteria. *B. bacilliformis* was obtained from H. H. Winkler (Department of Microbiology and Immunology, University of South Alabama School of Medicine, Mobile) and is also available from the American Type Culture Collection.

Growth on solid agar. The procedure most recently described for growing *B. bacilliformis* is that of Walker and Winkler (7), which we generally followed for growth of the bacteria on solid medium. *B. bacilliformis* was grown at 28°C in petri dishes (60 by 15 mm) in a water-saturated atmosphere. Our standard medium was brain heart infusion agar (Difco Laboratories, Detroit, Mich.) prepared according to the directions of the manufacturer and containing 10% (vol/vol) human serum (GIBCO Laboratories, Grand Island, N.Y.) and 10% (vol/vol) human blood lysate, prepared by

* Corresponding author.
† Present address: Malaria Branch, Division of Parasitic Disease, Centers for Disease Control, Atlanta, GA 30333.

the addition of 3 volumes of sterile distilled water to 1 volume of packed, outdated human blood cells obtained from the American Red Cross, Waco, Tex. Tryptone-yeast extract agar could be used, but clumping of the bacteria was much more pronounced. Erythrocyte extract could be replaced with Bacto-Hemoglobin (Difco), but the doubling time increased when dialyzed Bacto-Hemoglobin, purified hemoglobin (Sigma Chemical Co., St. Louis, Mo.), or hemin was substituted. We initially used fresh human serum, but found that commercial human serum (GIBCO) gave equivalent results. With erythrocyte lysate, serum could be replaced by human or bovine serum albumin (Sigma; 0.5%, final concentration), Casamino Acids (Difco; 2%, final concentration), or a complete amino acid mixture. Plates contained 4 to 5 ml of agar overlaid with 2 ml of phosphate-buffered saline (PBS) at pH 7.8 to keep the surface from drying. Bacteria grew in the fluid phase and also associated with the agar surface. After 48 h, cultures were harvested at late log phase by pouring off the overlay and rinsing the surface of the agar.

Growth in liquid medium. Bacteria were grown in petri dishes (60 by 15 mm) or in tissue culture flasks with the tops loosened. Our standard medium consisted of heart infusion broth (Difco) containing 10% serum and 10% centrifuged erythrocyte lysate, which was sterilized by filtration through a 0.22-μm-pore membrane filter (Millipore Corp., Bedford, Mass.). The doubling time during exponential growth was about 6 to 8 h and, by subculturing, exponential growth could be maintained indefinitely.

Motility. Motile bacteria grew in aggregates of 1 to 20 organisms to approximately 8×10^8 to 1.5×10^9 bacteria per ml. Nonmotile bacteria grew in aggregates of 20 to 100 organisms to 8×10^8 to 1.5×10^9 bacteria per ml. When the bacteria entered stationary phase at a concentration of 10^9/ml, they formed progressively larger clumps and became nonmotile. If nonmotile cultures were subcultured, the bacteria remained nonmotile even though they resumed exponential growth with a doubling time and a morphology identical to those of the motile form. The nonmotile bacteria were flagellated, as demonstrated both microscopically and by electrophoretic analysis of flagellar proteins. These nonmotile cultures occasionally become spontaneously motile, but it was fairly easy to maintain cultures in either the motile or the nonmotile form. During the stationary phase, the bacterial morphology changed from a bacillary form to a nonflagellated coccoid form, and the bacteria became difficult to subculture. They then underwent autolysis, with the disappearance of both turbidity and microscopically visible bacteria.

A similar situation was observed within colonies of bacteria on agar by scanning electron microscopy (data not shown). When the colonies first became visible or were rather small (2 to 4 days), flagellated bacilliform bacteria predominated, as seen by scanning electron microscopy of the colony, but in old colonies the bacteria were primarily coccoid in form and embedded in a matrix. Colonies over 3 weeks old could seldom be subcultured.

Isolation of large-colony-size variants. Nearly all the colonies from either motile or nonmotile cultures were very small. Occasionally, from nonmotile cultures a larger colony type was found which formed large irregular colonies upon restreaking. These variants differed in several respects from the parental bacteria. They were motile, despite being isolated from cultures of nonmotile bacteria. Their cultures contained a higher fraction of motile cells than did cultures of the motile parental line, and these bacteria appeared to move more rapidly than the parental type. In addition, aggregates of bacteria formed by these variants tended to contain fewer bacteria. The larger colony size possibly results from increased outward migration of the bacteria due to their higher motility and the smaller size of the aggregates. A higher frequency of binding to erythrocytes was observed for these variants, and several clones, L2 and L9, were used for most of the binding experiments. Qualitatively similar results, however, were obtained for the parental strain of *B. bacilliformis*.

Binding and agglutination. Binding to erythrocytes was routinely done in Geiman medium (2). Fresh erythrocytes (type O Rh^+) which had been collected in sodium heparin and washed three times in PBS containing 10 mM glucose and 5 mM inosine or in Hanks buffered saline were added, followed by bacteria to about 10^8/ml. Samples were removed for observation under supported cover slips at 5- to 15-min intervals.

Complexes of erythrocytes with bound bacteria could be isolated by Percoll (Sigma) gradient separation. A 1-ml 70% Percoll gradient in 154 mM NaCl was preformed by 15 min of centrifugation at 10,000 × *g* in a 1.5-ml Eppendorf tube and kept at 4°C until needed. A 100-μl volume of the solution containing bacteria and erythrocytes was added to the top of the gradient, and the tube was centrifuged for 5 min at 1,500 × *g*. The top layer contained free bacteria. The erythrocyte band from the middle of the tube was removed and washed twice in PBS containing 10 mM glucose and 5 mM inosine. Bound bacteria could be quantitated after staining with Giemsa.

Fluorescent vacuoles were visualized by incubating the bacteria in the usual medium, but with 8 mg of fluorescein isothiocyanate-bovine serum albumin (Sigma) per ml instead of human serum. After incubation for 6 to 24 h, the erythrocytes were washed three times with PBS and examined with a Leitz microscope by using a xenon lamp and the fluorescein filter.

Fluorescent erythrocytes were prepared by the preswell loading procedure as described by Humphreys et al. (3) by using 2 mM calcein (Sigma), incubated with bacteria (10 bacteria per erythrocyte) for 6 h, and washed three times with PBS containing 10 mM glucose and 5 mM inosine. Photomicrographs were taken with both phase-contrast and dark-field optics, using Ektachrome 200 film (Eastman Kodak Co., Rochester, N.Y.).

Microscopy. Binding was observed by light microscopy with either phase or Namarski optics. Intracellular bacteria were examined by using a Leitz microscope and a rhodamine filter after the addition of 500 μl of 200 μg of ethidium bromide per ml to washed erythrocytes, incubating for 10 min at 21°C, and washing three times with PBS. Free *B. bacilliformis* cells were viewed by transmission electron microscopy after negative staining with neutral 1% phosphotungstic acid. Thin sections were fixed in 2% glutaraldehyde in PBS, washed twice in PBS, and dehydrated in an ethanol series. Samples were embedded in Epon, and thin sections were cut and stained with uranyl acetate and lead citrate. Samples were examined on an electron microscope operated at 50 kV. Samples examined by scanning electron microscopy were fixed in 2% glutaraldehyde in PBS, washed in PBS, dehydrated in an alcohol series (50, 70, 95, and 100%), critical-point dried, and coated with gold-palladium. The buffer washes and ethanol dehydrations were performed on Nuclepore filters by allowing solutions to slowly drain through the filter holder from a syringe cylinder or gentle rinsing in petri dishes.

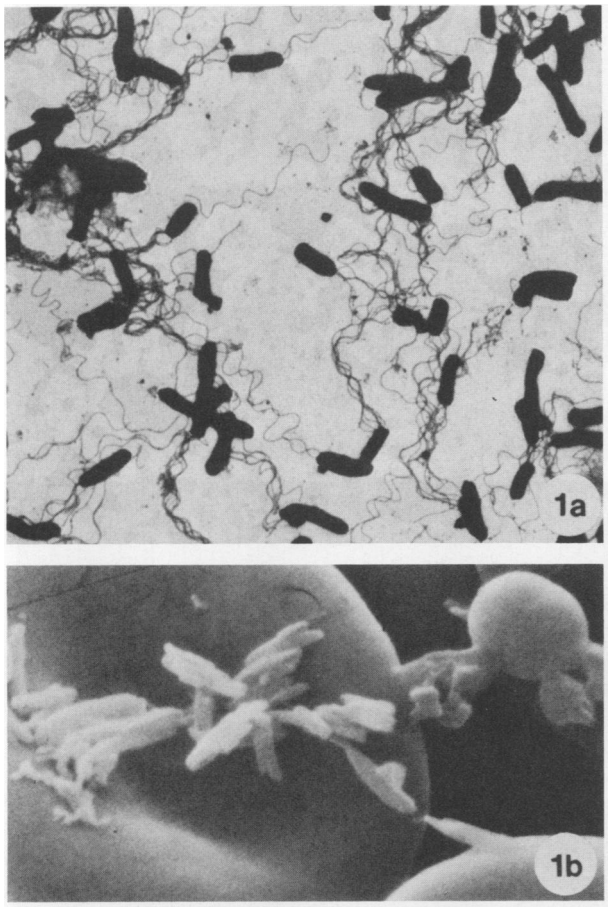

FIG. 1. Aggregates of *B. bacilliformis*. (a) Entangled flagella of centrifuged bacteria viewed by transmission electron microscopy. (b) Clumps of bound bacteria viewed by scanning electron microscopy.

RESULTS

Aggregates of *B. bacilliformis*. Two types of bacterial aggregates were observed. One occurred when the flagella became entangled after the bacteria were centrifuged (Fig. 1a). This type of aggregation was readily reversed by dilution and gentle agitation. Another type of aggregation occurred during growth, especially with bacteria growing at an agar-liquid interface or in liquid media containing serum or bovine serum albumin. During exponential growth, small clumps of bacteria formed, and these clumps were themselves motile, although their movements were of course much more ponderous than the motion of the individual motile bacteria. These small clumps could not be disaggregated by gentle agitation but could be disaggregated by vortexing, by repeated vigorous pipetting, by several vigorous resuspensions after centrifugation, or by pronase or proteinase K treatment. Electrophoretic analysis demonstrated that the flagellar protein was not degraded by protease treatment, although a 30- and a 20-kilodalton peptide disappeared (data not shown). Possibly one or both of these proteins might constitute part of the fibrous mesh linking the bacteria together. Clumps of bacteria could bind to and agglutinate erythrocytes (Fig. 1b), but the individual bacteria liberated from the clumps could not agglutinate erythrocytes, although they could bind. During the stationary phase much larger clumps formed which were not motile and did not bind to erythrocytes but which could be broken up by gentle agitation into small clumps.

Binding to erythrocytes. Erythrocytes could be agglutinated within 15 min when incubated with clumps of motile *B. bacilliformis* but could not be agglutinated by individual bacteria. The agglutinated erythrocytes could be disaggregated by the same treatments that disaggregated the bacterial clumps. Nonmotile or azide-killed bacteria failed to bind to erythrocytes to any significant extent, and bound bacteria could be released by azide treatment. Walker and Winkler (7) previously reported that UV-irradiated bacteria or those treated with KCN or a thiol reagent adhered less well to erythrocytes.

Individual bacteria or small clumps of bacteria bound to erythrocytes were able to push the erythrocytes around, as previously described (M. Cuadra, Abstr. 12th Int. Congr.

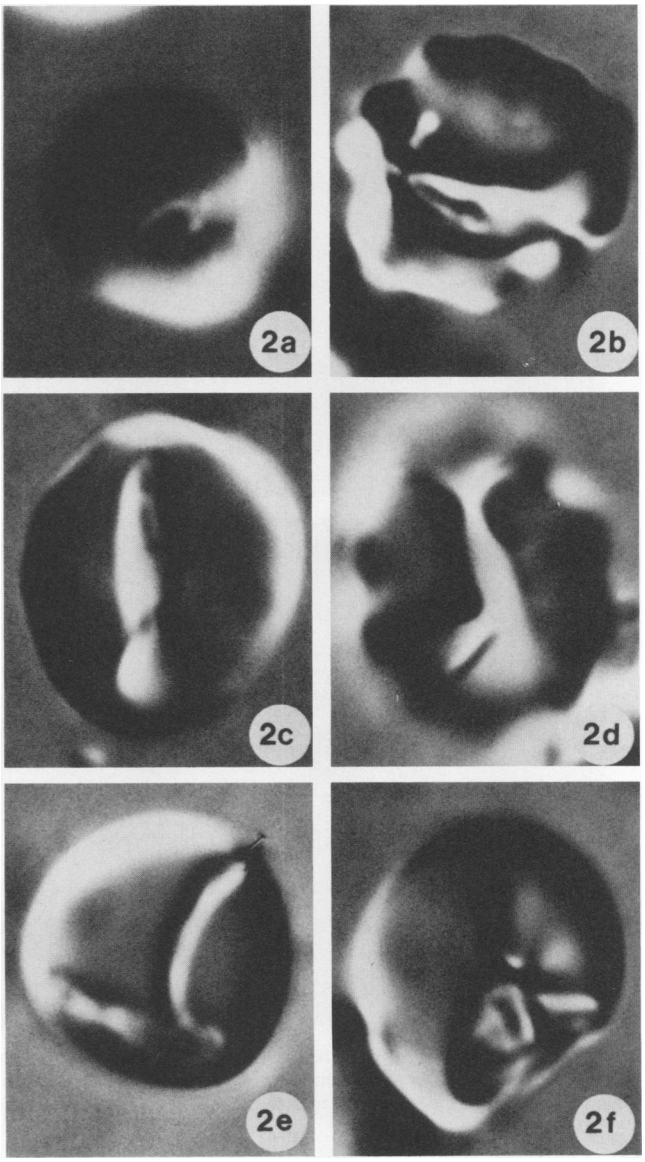

FIG. 2. Deformation of erythrocytes as seen by Namarski optics. (a) In many cases, the bacteria were actively twirling during photography, blurring the image of the bacteria. (b to f) Trenchlike structures of the erythrocyte are seen.

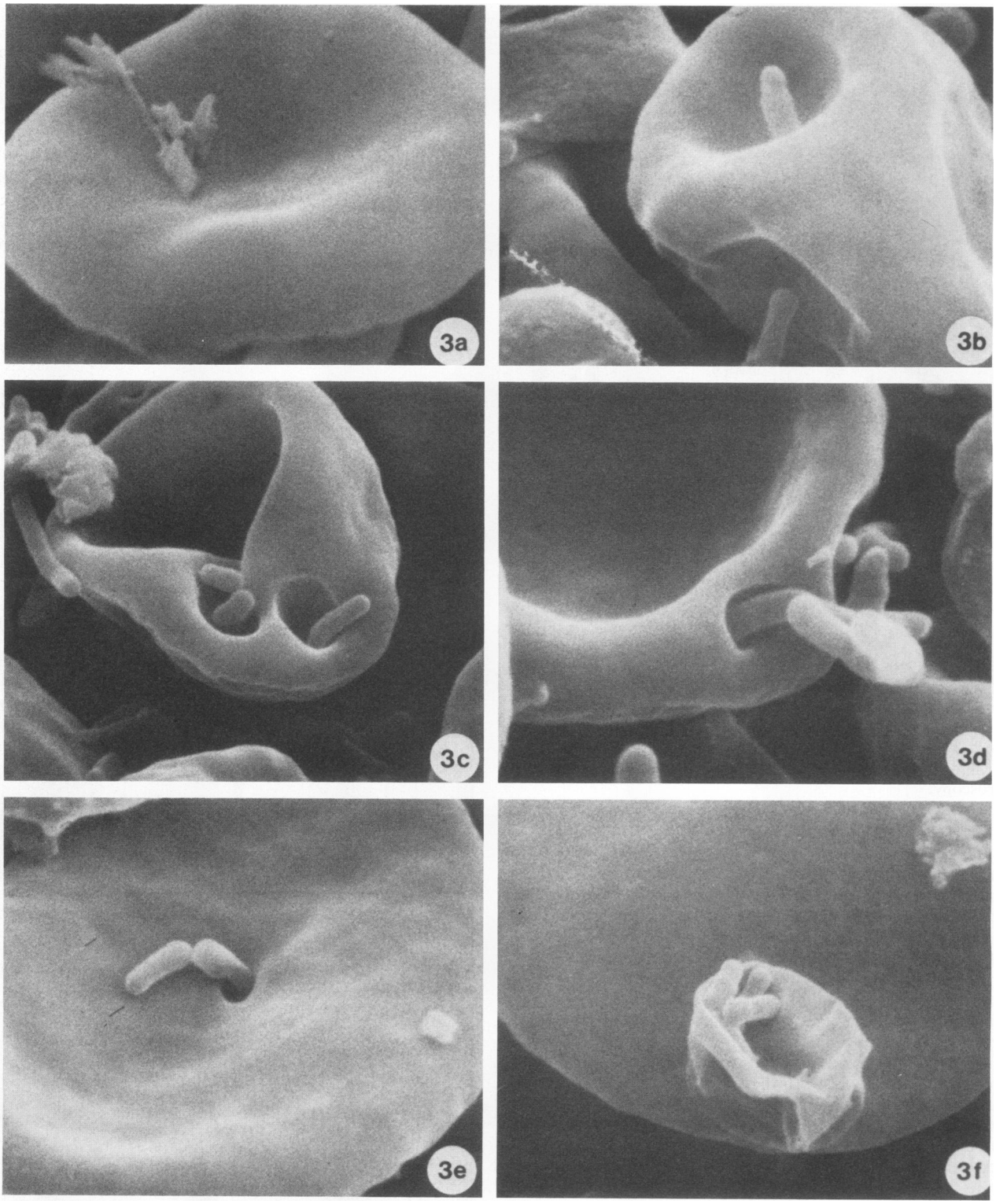

FIG. 3. Deformation of erythrocytes as seen by scanning electron microscopy. (a) Clumps of bacteria and (b) single bacterium bound at indentations. (c to e) Deep pits which result from bacteria pushing into the erythrocyte membrane. (f) Membrane of the erythrocyte apparently pulled up from the cytoskeleton and twisted.

Microbiol., p. 178). A twirling or rotational motion was usually observed with small clumps. By using Namarski optics, it was possible to clearly visualize indentations in the erythrocyte membranes where single bacteria which wobbled or precessed were attached (Fig. 2a). These indentations became deeper with time. In other cases, clumps of twirling bacteria were attached at indentations. Trenchlike structures on the surface of the erythrocyte were often seen

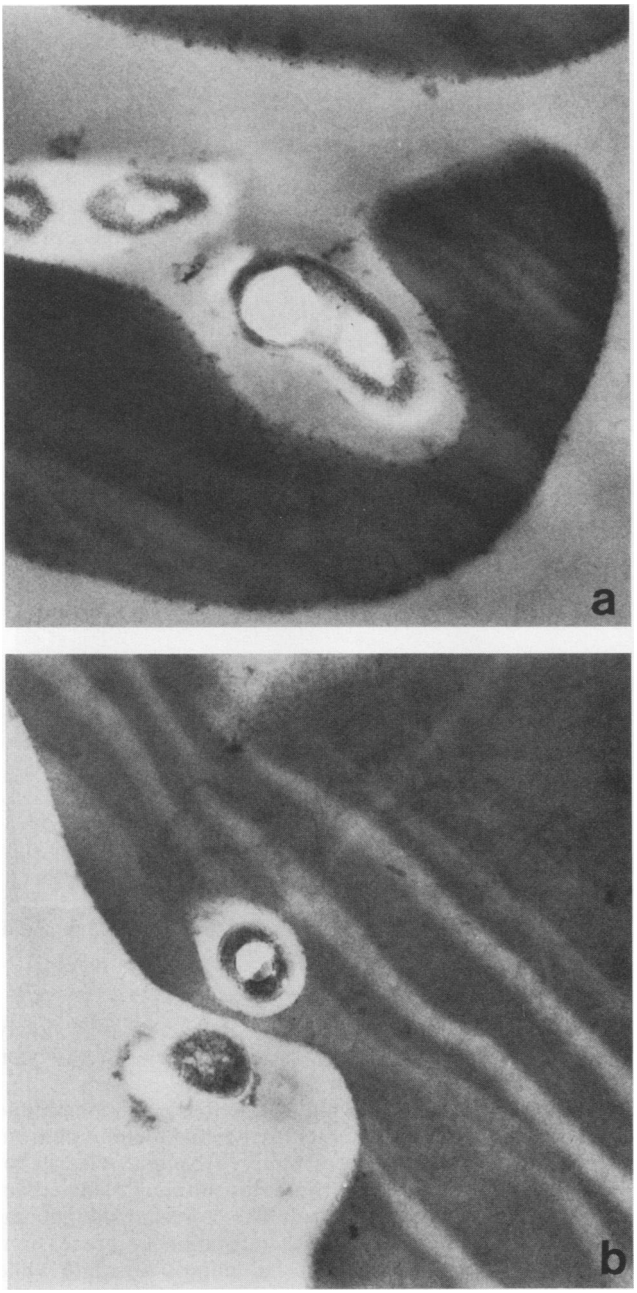

FIG. 4. Transmission electron microscopy of thin sections of complexes of bacteria and erythrocytes. (a) Bacteria lying clearly outside of the erythrocyte. (b) Bacteria apparently within an endocytotic vesicle.

(Fig. 2b to f). Occasionally, the outer lips of the trench were folded over a single bacterium or small groups of bacteria.

Scanning electron microscopy revealed clumps of bacteria (Fig. 3a) and single bacteria (Fig. 3b) bound at indentations which were sometimes fairly shallow, but very deep pits were often seen which were not much wider than the bacteria, as might result from the bacteria pushing directly into the erythrocyte membrane (Fig. 3c to e). Sometimes the membrane of the erythrocyte appeared to have been pulled up from the underlying cytoskeleton and twisted (Fig. 3f). Flagella cannot be seen in these photomicrographs, but by using a modified fixation procedure, bacteria with flagella could occasionally be visualized. We did not observe any clear instances to indicate whether flagella were directly involved in binding the bacteria to the erythrocytes.

Examination of sections by transmission electron microscopy revealed *B. bacilliformis* lying in indentations of the erythrocyte membrane but clearly still outside the cell (Fig. 4a). In other cases, the bacteria appeared to be within an endocytotic vesicle (Fig. 4b), but as this structure could still be open to the outside, it could not be ascertained from the transmission electron micrographs whether the bacteria were in vesicles or in invaginations of the membrane. Occasionally, bacteria were seen which appeared not to be surrounded by a membrane which might correspond to intracellular bacteria which escaped from or ruptured the vacuole.

Vacuoles. A simple pinching off of the invaginations seen by scanning electron microscopy would leave the bacteria in intracellular vacuoles. When fluorescein isothiocyanate-bovine serum albumin was included in the medium during the incubation of the bacteria with erythrocytes, fluorescent vacuoles were clearly observed in the washed erythrocytes. In a complementary series of experiments, the erythrocytes were first loaded with a fluorescent compound, calcein, and then resealed. After incubation of the fluorescent erythrocytes with bacteria for 6 h, vacuoles could be detected as dark spots in otherwise uniformly fluorescent erythrocytes. In most cases, bacteria could be seen in the vacuoles when observed by ordinary light microscopy (Fig. 5). Bacteria could be detected in most of the vacuoles, which were in many cases vibrating or moving with small jerks, their motion presumably being constrained by the limits of the vacuole. After removal of the free bacteria by centrifugation and continued incubation of the erythrocytes, the number of bacteria in each vacuole increased markedly. It seems likely that in some cases the bacteria escaped from the vacuoles, since occasionally erythrocytes were seen which were very lightly and uniformly fluorescent.

DISCUSSION

Erythrocytes became markedly deformed after motile *B. bacilliformis* bound to them but not during incubation with nonmotile bacteria. The highly motile bacteria repeatedly collided with erythrocytes before formation of stable complexes, but an incubation period of at least 15 to 30 min was required before any significant number of complexes formed, as previously observed by Walker and Winkler (7), and at least 6 h of incubation was required to form the maximum number of complexes resistant to Percoll gradient centrifugation. Once formed, stable binding did not reverse readily, and complexes could persist for hours without detachment of the bacteria. Binding occurred most extensively with the most motile cultures of *B. bacilliformis* and did not occur to any significant extent with cultures of nonmotile bacteria, whether the nonmotile bacteria were in exponential growth or not. It may be that motile bacteria collide more often or more effectively with the erythrocytes, facilitating binding, or possibly nonmotile bacteria lack the surface components required for binding.

By light microscopy, individual bacteria and clumps of bacteria could be seen pushing erythrocytes around and twirling on the surface of erythrocytes. Individual bacteria were not seen to clump erythrocytes, as noted previously by Cuadra (12th Int. Congr. Microbiol.), who suggested that there might be a single attachment site on the bacteria or that binding sites on erythrocytes are rare, although other expla-

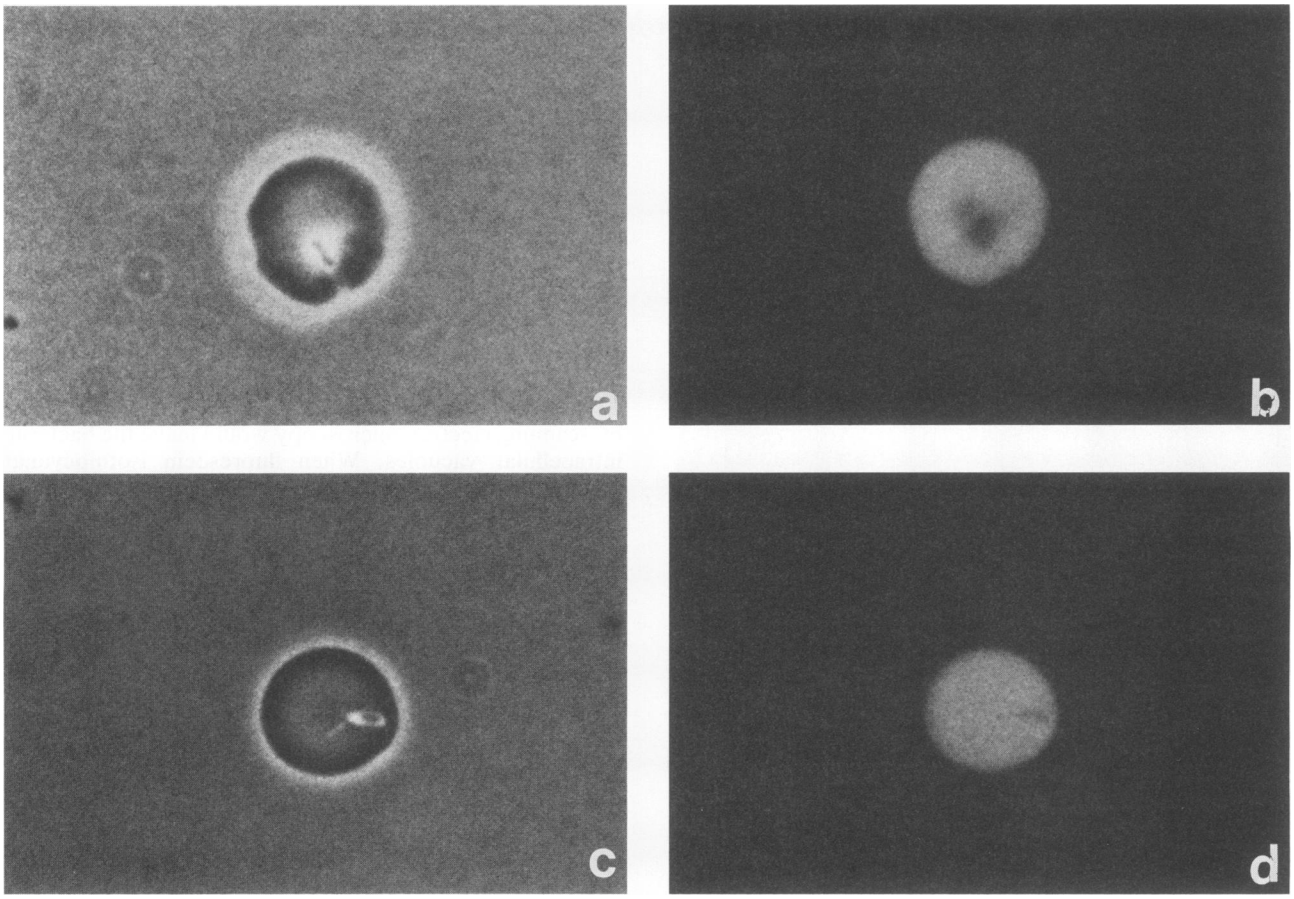

FIG. 5. Micrographs of erythrocytes preloaded with calcein and infected with bacteria. (a and c) Visualized by dark field and (b and d) visualized by fluorescence.

nations for this observation are possible. Erythrocytes could be agglutinated by clumps of bacteria, and these were disaggregated by the same treatments, mild mechanical shear or protease treatment, which disaggregated bacterial clumps into individual bacteria. As growth of the bacteria in cultures approached saturation, the number of bacteria in a clump increased to 50 to 100. These large aggregates composed of bacteria entering stationary phase, however, generally did not agglutinate the erythrocytes, and the individual bacteria were found to have also lost the ability to bind to the erythrocytes when the clumps of stationary phase bacteria were disrupted either mechanically or enzymatically.

Binding of the motile bacteria led to a remarkable deformation of the surface of the erythrocytes. Initially, small pits in the surface were seen as the bacteria pushed against the erythrocytes with a boring or twisting motion. By Namarski optics, deep trenches and pits were seen on the surfaces of the erythrocyte, and usually bacteria were bound to the cells in or near these pits. Many of the trenches were too large for the bacteria to be actively maintaining the deformation, and this conclusion is confirmed by observing that bacteria occasionally detached from the cells without reversal of the deformations. After prolonged incubation under conditions where many of the bacteria detached, the erythrocytes did recover their normal shape.

Erythrocytes are well known to undergo shape changes reversibly under the influence of biochemical changes or mechanical deforming forces. The observation that *B. bacilliformis* causes erythrocyte deformation which is only slowly reversible suggests that the internal cytoskeleton has been altered in some way.

Rotation of the bacteria while bound to the cell membrane seemed in some cases to pull up the twisted membrane from its underlying cytoskeleton. Clumps of bacteria clearly rotated while bound to the erythrocytes, although single bacteria may simply have precessed. The cone-shaped indentations could be the result of either rotation or precession. Since it is possible for the bacterial clumps to rotate while attached to the erythrocytes, there may be a swivel at some point in the complex, either at the site of membrane attachment, perhaps the membrane receptor rotating in the fluid erythrocyte membrane, or within the bacterial clump. The scanning electron micrographs suggest that rotation exerts a twisting force on the erythrocyte membrane, and the cones and trenches indicate the presence of a force pushing in. Apparently, the combination of a rotary force and an indenting force delivered more or less perpendicular to the cell surface causes the bacterium to behave like a drill.

Extremely deep invaginations of the erythrocyte membranes containing bacteria were routinely seen by scanning electron microscopy. Presumably, the continued rotation and pushing of the attached bacteria caused the erythrocyte membranes to indent until the bacteria were sheathed within invaginated erythrocyte membranes.

Membrane fusion at the necks of the invaginations left the bacteria contained in intracellular vacuoles. Cuadra and

Takano (1) reported finding by electron microscopy *B. bacilliformis* both in vacuoles and free in the erythrocyte cytoplasm. We have found that fluorescent compounds present externally to the erythrocytes were entrapped in vacuoles along with the bacteria, and fluorescent compounds loaded into resealed erythrocytes before the addition of the bacteria were excluded from the vacuoles. Bacteria can replicate within the vacuoles, since each of the vacuoles initially contained a single bacterium, and with continued incubation of the infected erythrocytes, an increasing number of bacteria were found within each vacuole. Despite an increase in number, the bacteria were still contained within apparently larger vacuoles, indicating that bacterial replication did not lead to rupture of the vacuoles and release of the bacteria into the cytoplasm. Rather, it appears that the vacuoles grew larger, but the source of the additional membrane has not yet been determined. Possibly, the vacuoles fused and refused with the erythrocyte membrane, thereby incorporating additional membrane as needed.

After hemolysis of the erythrocytes by dilution, internalized highly motile bacteria could be seen traveling rapidly in circles along the inside of the erythrocyte ghost membranes, occasionally taking short cuts across the diameter of the erythrocytes. Osmotic shock presumably ruptured the vacuolar membrane, releasing the bacteria into the cytoplasmic space. This may happen spontaneously on occasion, since a few faintly and uniformly fluorescent cells were seen after entry of the bacteria from a fluorescent external medium.

ACKNOWLEDGMENTS

We thank Ron Davis for assistance with the electron microscopy and Karl Aufderheide for his generous assistance with the Namarski microscopy.

This research was supported in part by National Science Foundation grant PCM-8214595.

LITERATURE CITED

1. **Cuadra, M., and J. Takano.** 1969. The relationship of *Bartonella bacilliformis* to the red blood cell as revealed by electron microscopy. Blood **33**:708–716.
2. **Geiman, Q. M.** 1941. New media for the growth of *Bartonella bacilliformis*. Proc. Soc. Exp. Biol. Med. **47**:329–332.
3. **Humphreys, J. D., T. D. Edlind, and G. Ihler.** 1981. Entrapment of viral vectors for recombinant DNA in erythrocytes. J. Appl. Biochem. **3**:199–211.
4. **Moulder, J. W.** 1985. Comparative biology of intracellular parasitism. Microbiol. Rev. **49**:298–337.
5. **Schrier, S. L., K. G. Bensch, M. Johnson, and I. Junga.** 1975. Energized endocytosis in human erythrocyte ghosts. J. Clin. Invest. **56**:8–22.
6. **Tsang, H., H. Mollenhauer, and G. Ihler.** 1982. Entrapment of proteins, viruses, bacteria and DNA in erythrocytes during endocytosis. J. Appl. Biochem. **4**:418–435.
7. **Walker, T. S., and H. H. Winkler.** 1981. *Bartonella bacilliformis*: colonial types and erythrocyte adherence. Infect. Immun. **31**:480–486.
8. **Weinman, D.** 1944. Infectious anemias due to bartonella and related red cell parasites. Trans. Am. Philos. Soc. **33**:243–287.