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Deformation Factor: an Extracellular Protein Synthesized by *Bartonella bacilliformis* That Deforms Erythrocyte Membranes

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***Bartonella bacilliformis*, a hemotropic bacterium and the causative agent of the human disease bartonellosis, when incubated in a tryptone-based medium produces an extracellular factor, termed deformation factor (DF), which induces extensive indentations and trenches in trypsinized erythrocyte membranes. The factor is stable during storage at 4°C. It can be inactivated by proteases or brief heating to 70 to 80°C, can be precipitated by ammonium sulfate, is nondialyzable, and is retained by membranes with a 30,000-molecular-weight cutoff. These properties suggest that DF is probably a protein. Incubation of erythrocytes with phospholipase D renders them resistant to deformation by DF.**

Bartonella bacilliformis is an interesting and unique bacterial pathogen which causes bartonellosis (Carrion's disease) in humans. Transmission occurs through the bite of a sandfly which is indigenous to remote valleys on the western slopes of the Andes in Columbia, Ecuador, and Peru (13). Because of its restricted geographic distribution, bartonellosis has received only sporadic research attention outside Peru. However, it remains a health problem in its endemic area, as indicated by a recent epidemic in the Peruvian Andes in which 14 patients died and another 14 were seriously affected (9). Humans are the only known natural reservoir for *B. bacilliformis*, which suggests the possibility of eradicating the disease.

Both erythrocytes and vascular endothelial cells are parasitized in vivo by these highly motile hemotropic bacteria. Tropism for these cell types gives rise to the distinct hematic (Oroya fever) and tissue (verruga peruana) phases of the disease (13). The acute anemia of Oroya fever, during which 100% of the erythrocytes may become infected, is in some respects analogous to that of malaria, where premature destruction of parasitized erythrocytes occurs. It has been suggested that bartonella-parasitized cells are selectively phagocytized by cells of the reticuloendothelial system which recognizes them as altered (16), thus accounting for the dramatic decrease in erythrocyte number.

The tissue (verruga) stage of bartonellosis is characterized by hemangiomatous dermal eruptions resulting from marked endothelial cell proliferation. These lesions are reported to be strikingly similar both clinically and histologically to those of bacillary (epithelioid) angiomatosis, a recently recognized bacterial complication seen in AIDS patients (3). Garcia et al. (7) have recently shown that *B. bacilliformis* synthesizes a protein which stimulates endothelial cell proliferation in an in vitro system and also proliferation of new blood vessels in an in vivo model for angiogenesis, supporting an earlier suggestion by Arias-Stella et al. (1) that *B. bacilliformis* possesses angiogenic potential.

In vitro, the bacteria adhere to and gain entry into erythrocytes, initially enclosed within intracellular vacuoles (2, 4, 15). (*B. bacilliformis* has also been shown to enter cultured nucleated mammalian cells [11], as has *Escherichia coli* containing a cloned 6.2-kDa fragment of *B. bacilliformis* DNA [12]). The in vitro interaction of *B. bacilliformis* with erythrocytes is accompanied by a dramatic, yet reversible, deformation of the cell surface with prominent pits, trenches, and invaginations (2). In vivo, *B. bacilliformis* may employ a similar mechanism of entry, since the bacteria have been detected in vacuolelike structures in erythrocytes from clinically infected patients (5). *Anaplasma marginale*, a hemotropic bacterium of veterinary importance which is related to *Bartonella* spp., is also known to attach to erythrocytes and to induce membrane invagination at the point of attachment which culminates in the formation of parasitic vacuoles (6, 14).

Erythrocytes are an exceptionally suitable host for the study of intracellular parasitism, since they are markedly nonendocytic. The finding of *B. bacilliformis* contained within vacuolelike structures indicates that the bacteria must initiate and mediate the process leading to their uptake by the erythrocytes. Formation of deep invaginations seen during entry is probably aided by mechanical forces generated by the highly motile bacteria, but in this report, we show that an extracellular bacterial product, termed deformation factor (DF), can produce extensive morphological changes in the erythrocyte surface independently of the bacteria.

MATERIALS AND METHODS

Strains and growth. *B. bacilliformis* was originally obtained from H. H. Winkler (Department of Microbiology and Immunology, University of South Alabama School of Medicine, Mobile, Ala.). Stock cultures were stored at −70°C with dimethyl sulfoxide added to a final concentration of 10%. For growth, frozen cultures were rapidly thawed in a 37°C water bath and transferred to brain heart infusion agar (BBL, Cockeysville, Md.) containing 10% (vol/vol) human serum (GIBCO Laboratories/BRL, Grand Island, N.Y.) and 10% (vol/vol) human erythrocyte lysate prepared as described by Benson et al. (2) from outdated blood bank erythrocytes (American Red Cross, Waco, Tex.). A 2.5- to 3-ml overlay of phosphate-buffered saline (PBS) (8 mM Na_2HPO_4, 1 mM KH_2PO_4, 3 mM KCl, 117 mM NaCl, pH

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7.4) was then added to each plate, and the cultures were incubated at 30°C. This overlay was used as an inoculum for incubation in liquid media. All assays were performed with cultures in exponential growth.

Deformation and entry assays. Bacterially induced erythrocyte deformation was assessed by incubating bacteria and erythrocytes at 30 to 35°C in modified Geiman's medium (8). Assays were carried out in petri dishes (35 by 10 mm). In a typical assay, 200 μl of a log-phase culture (~2×10^8 bacteria) of *B. bacilliformis* was mixed with 50 μl of a 50% erythrocyte suspension in 1 ml of medium containing, in final concentration, 1% autoclaved tryptone broth (Difco, Detroit, Mich.), pH 7.8; 0.5% NaCl; and filtered supplements of 0.5% human serum albumin (wt/vol) (Sigma Chemical Co., St. Louis, Mo.), 10 mM glucose, 5 mM inosine, and 0.2% (vol/vol) of a 10× amino acid-ascorbic acid-glutathione solution. After incubation, erythrocyte deformation was determined microscopically with a Zeiss microscope equipped with Nomarski optics. Cells scored as positive either had visible bacteria rotating on their surfaces or were extensively scarred with prominent pits, trenches, or invaginations. Two to three hundred cells were routinely counted. Entry of *B. bacilliformis* into erythrocytes was determined after 24 h by incubating washed cells with ethidium bromide (200 μg of PBS per ml, pH 7.4) for 45 min in a 37°C water bath. After incubation, cells were pelleted, washed, and observed by fluorescent microscopy. Ethidium bromide intercalates into DNA, and, since mature erythrocytes are anucleated, fluorescent intracellular bacteria were easily detected microscopically.

Erythrocyte deformation was also studied by using cell-free filtrates. Filtrates were prepared by removal of bacteria with 0.2-μm-pore-size filters (Amicon, Beverly, Mass.) from tryptone-based medium which had been inoculated at a 1:5 (vol/vol) ratio (about 2×10^8 bacteria per ml) with PBS overlays from overnight agar plate culture and grown for 2.5 to 3 h at 35°C. Control filtrates were prepared in a similar manner except that the overlays containing bacteria were replaced with sterile PBS overlays incubated overnight on agar plates.

Erythrocytes. Erythrocytes were collected from human volunteers by venipuncture into heparinized Vacutainer tubes. The cells were pelleted by centrifugation, washed three times, and resuspended to 50% packed cell volume in PBS, pH 7.4, containing 10 mM glucose and 5 mM inosine. Refrigerated cell suspensions could be used for approximately 2 weeks. If cells were washed weekly and resuspended in fresh PBS-glucose-inosine, they could be used for at least a month.

For experiments involving enzymatically treated cells, the erythrocytes were diluted to ~2×10^8 cells per ml in PBS-glucose-inosine and incubated for 30 min in a 37°C water bath with an equal volume of PBS or enzyme. A 10-ml volume of PBS-glucose-inosine was then added, and the cells were pelleted by centrifugation. After three additional washes, the cell pellet was resuspended in 250 μl of PBS-glucose-inosine. Cells prepared in this manner were subsequently exposed to either *B. bacilliformis* or sterile PBS overlay after being mixed with tryptone medium. For experiments using bacterial or control filtrates, a 50% erythrocyte suspension was mixed 1:1 (vol/vol) with tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (1 mg/ml of PBS), incubated, and washed as described previously and then resuspended to its original volume with PBS-glucose-inosine. These cells were subsequently incubated with filtrates at a 1:20 (vol/vol) ratio.

TPCK-trypsin (Worthington Biochemicals, Freehold, N.J.) and trypsin type XI from bovine pancreas (Sigma) were both used with equal effectiveness to sensitize erythrocytes to DF. These enzymes were used at a concentration of 1 mg/ml in PBS, pH 7.2, unless otherwise indicated. All additional enzymes were obtained from Sigma and used at the concentrations indicated. Those used included α-chymotrypsin type VII derived from bovine pancreas (500 μg/ml of PBS); *Clostridium perfringens* neuraminidase type V (1 U/ml of PBS, pH 7.2, containing 1 mM $CaCl_2$); phospholipase A_2 from bovine pancreas (500 μg/ml of PBS); phospholipase B from *Vibrio* species (500 μg/ml of PBS); phospholipase C type I (lecithinase) from *Clostridium welchii* (200 μg/ml of PBS); and phospholipase D types I, IV, and V from cabbage, type II from peanut, type VI from *Streptomyces chromofuscus*, and type VII from *Streptomyces* species (500 μg/ml of PBS). All phospholipases, with the exception of phospholipase C, were used at a concentration of 500 μg/ml. Phospholipase C was diluted to 200 μg/ml.

RESULTS

Kinetics of the interaction of *B. bacilliformis* with erythrocytes. Erythrocytes incubated with motile, log-phase *B. bacilliformis* became extensively deformed, beginning between 1 and 2 h after mixing (Fig. 1A). Erythrocytes incubated without *B. bacilliformis* or separated from the bacteria by a dialysis membrane retained their normal morphology (Fig. 1B). The percentage of cells deformed, as well as the degree of deformation, reached maximum levels after about 6 h (Fig. 2). Cell deformation was reversible since, after prolonged incubation (24 h), relatively few deformed cells were observed. Deformation did not require the continued presence of bacteria bound to the cells, and in fact most of the deformed cells had no bacteria on their surfaces. Entry of bacteria into erythrocytes lagged behind deformation by 2 to 4 h (Fig. 2), indicating that the process leading to deformation precedes entry and may be a prerequisite for entry. Although levels of deformation frequently reached 50% or more, only 10 to 20% of the erythrocytes ultimately contained intracellular bacteria, further indicating that deformation is not a consequence of entry. When the number of bacteria added per erythrocyte was increased, more deformed cells were seen, but the number of cells with intracellular bacteria decreased, apparently because of decreased bacterial motility at higher bacterial concentrations (data not shown).

Effect of temperature and pH. Temperatures of 28 to 37°C were optimum for deformation and entry (Table 1). Below 24 or above 40°C, little or no deformation occurred. Entry of *B. bacilliformis* appeared to be more sensitive to low temperatures than deformation; for example, in one experiment, when plates were held at 20°C throughout the incubation period, a 3-fold decrease in deformation and a 30-fold reduction in entry were observed. The effect of low-temperature inhibition was reversible, and normal levels of erythrocyte deformation and entry were observed following transfer of plates from a 6-h low-temperature preincubation to 30°C. If plates were first incubated for 4 h at 30°C and then placed at 4°C, a significant (fivefold) reduction in entry was seen, although the inhibitory effect of low temperatures was most pronounced when plates were transferred to the lower temperatures within the first 3 h of incubation. No deformation or entry occurred with continuous incubation at 4°C. The absence of deformation and entry above 40°C was most likely due to a direct, deleterious effect of the high temper-

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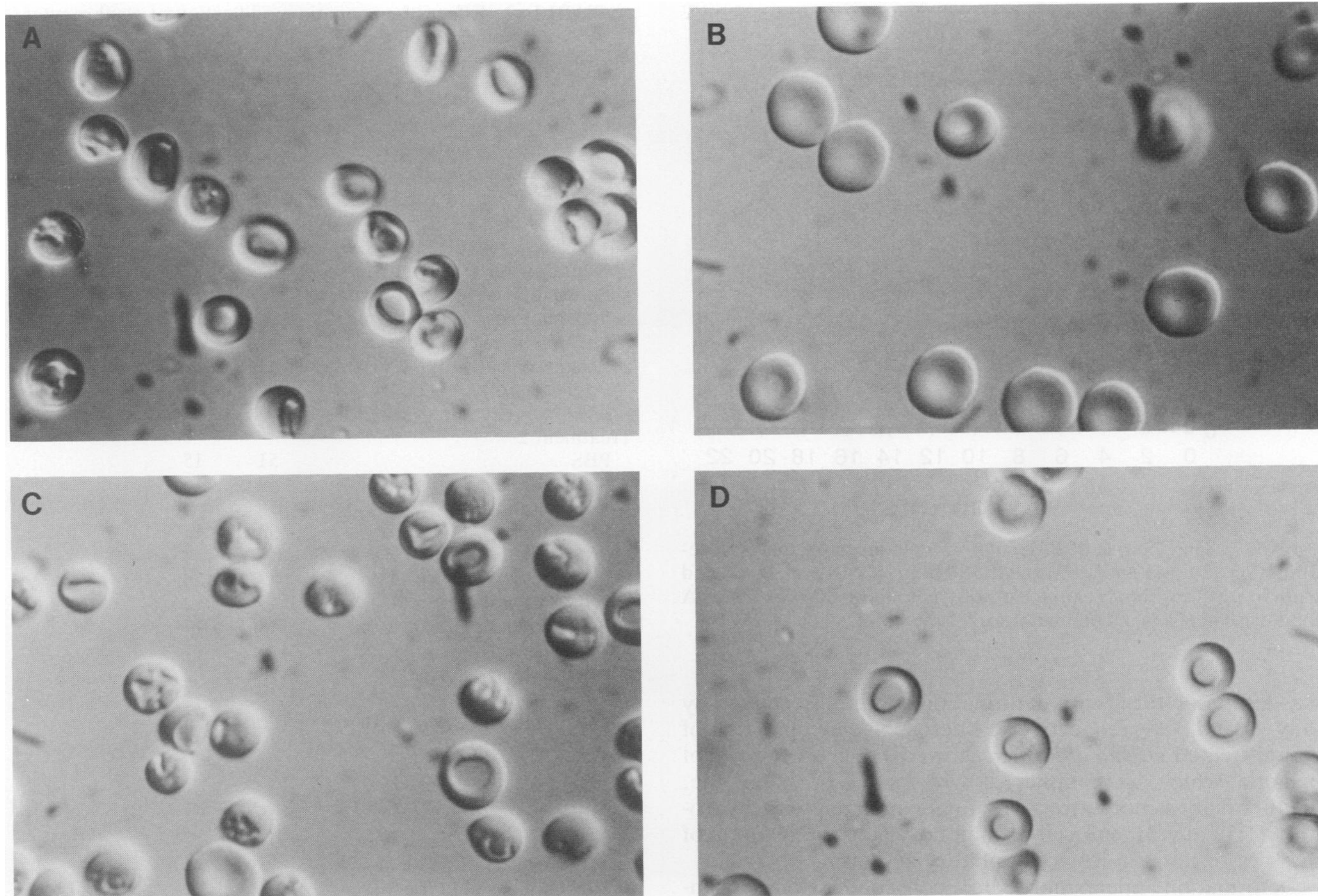

FIG. 1. *B. bacilliformis*-induced deformation of human erythrocytes. Nontrypsinized erythrocytes after 5 h of incubation with *B. bacilliformis* (A) or sterile PBS overlay (B). Trypsinized cells after 5 h of incubation with a *B. bacilliformis* filtrate (C) or uninoculated, incubated filtrate (D).

ature on the bacteria, since the inhibitory effect was not reversible when the temperature was lowered.

A pronounced pH effect was also noted. Deformation and entry were maximal at about pH 7.4 and considerably reduced at pH values of 6 and 8 (Table 1).

Effect of enzymatic modification of erythrocytes. Treatment of erythrocytes with trypsin or neuraminidase prior to incubation with *B. bacilliformis* greatly increased the extent of deformation and entry (Table 2). The effect of trypsin was concentration dependent, since lower percentages of erythrocytes were deformed with decreasing trypsin concentrations. In one experiment, 96% of erythrocytes pretreated with 1 mg/ml were deformed and 86% were deformed at 0.1 μg/ml, as compared with 34% for untrypsinized erythrocytes. Trypsin alone at 1 mg/ml generally caused <5% erythrocyte deformation, an insignificant amount. Trypsinization of the bacteria, followed by removal of the enzyme by centrifugation and washing, did not affect deformation or entry, a result consistent with the view that enhanced deformation was due to an enzyme-dependent change in the erythrocyte membrane.

In contrast to the effect of trypsin, specific phospholipases significantly reduced deformation and abolished entry. Incubation of erythrocytes with phospholipase D type VI for 5, 30, and 60 min reduced deformation to 20, 5, and 3%, respectively, of control values. Importantly, cells treated for only 5 min with the enzyme were completely refractory to entry. If phospholipase D was added to bacteria and erythrocytes being incubated together after deformation was well under way (~3 h), the phospholipase produced little effect. In this experiment the bacteria retained their motility throughout the observation period, so the enzyme's effect is unlikely to be bactericidal in nature. Of several phospholipase enzymes tested, only those phospholipase D types derived from *Streptomyces* species were effective.

Effect of chemical and physical agents. Erythrocytes exposed to 0.032% glutaraldehyde for 5 min were completely refractory to deformation and entry, indicating that the bacteria are unable to deform or enter erythrocytes whose membranes are rendered more rigid by cross-linking.

Pre-treatment of *B. bacilliformis* for 30 min with various chemical and physical agents, such as heat (52 or 100°C), 50 mM NaN_3, or 3% formaldehyde, which killed the bacteria, abolished their ability to deform erythrocytes. Continuous presence of 1 mM KCN or 50 mM NaF inhibited deformation as measured at 6 h, but if the bacteria were washed following treatment, neither inhibited deformation. KCN added at 6 h did not reverse the deformation which had already occurred (Table 3).

Pretreatment of *B. bacilliformis* with normal rabbit, horse, or human serum or with anti-*B. bacilliformis* rabbit serum prevented both deformation and entry. Fetal bovine serum

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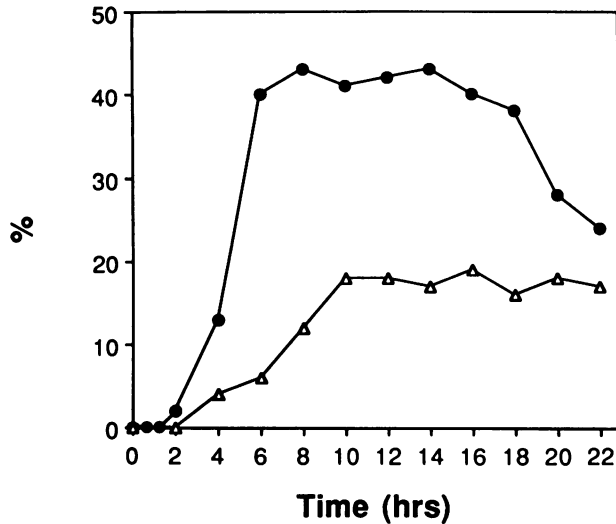

FIG. 2. Time course of *B. bacilliformis*-induced erythrocyte deformation (●) and entry (△). Culture fluid (200 μl) was incubated with 50 μl of erythrocytes in 1 ml of tryptone medium at 30°C. A total of 300 cells was counted at each time point.

was less inhibitory, with deformation and entry reduced by only 40 and 30%, respectively. Microscopic examination of serum-treated *B. bacilliformis* showed large aggregates of bacteria which were either slowly motile or nonmotile. Neither dialysis nor extraction of human serum with chloroform (1:5 vol/vol) removed its inhibitory activity. Dilution of the sera resulted in loss of inhibitory activity.

Deformation appears not to be a consequence of mechanical distortion of the erythrocyte membrane by motile bacteria since nonmotile *B. bacilliformis* could also induce erythrocyte deformation, although at much lower levels. However, nonmotile bacteria never gained entry into the cells. This observation indicates that binding, deformation, and the requirement for motility are separable but related aspects of entry, and it suggests that deformation might be the result of a biochemical factor rather than a purely mechanical event.

TABLE 1. Effect of temperature and pH on *B. bacilliformis*-induced erythrocyte deformation[a]

Temp (°C) or pH	% of erythrocytes:	
	Showing deformation	With intracellular bacteria
Temp		
4	0	0
15	0	0
21	0	0
23	0	5
28	41	13
30	41	15
37	37	20
40	30	7
43	8	1
pH		
6	6	1.5
7	12	5
7.4	27	8
7.8	20	7
8	3	6

[a] Cells were scored for deformation after 6 h of incubation and for intracellular bacteria after 24 h.

TABLE 2. Effect of enzymatic modification of erythrocytes on *B. bacilliformis*-induced deformation

Erythrocyte treatment	Erythrocytes with *B. bacilliformis*		Erythrocytes with *B. bacilliformis* filtrate (% deformation)
	% Deformation	% Entry	
Treatment 1[a]			
PBS	38	20	
Trypsin (1 mg/ml)	98	32	
Neuraminidase (1 U/ml)	75	22	
Phospholipase A_2 (500 μg/ml)	43	22	
Phospholipase B (500 μg/ml)	50	22	
Phospholipase C (200 μg/ml)	—[b]	—[b]	
Phospholipase D type VI (500 μg/ml)	3	0.6	
Treatment 2[a]			
PBS	51	15	
Phospholipase D type I (500 μg/ml)	49	16	
Phospholipase D type II (500 μg/ml)	50	16	
Phospholipase D type IV (500 μg/ml)	47	16	
Phospholipase D type V (500 μg/ml)	48	17	
Phospholipase D type VI (500 μg/ml)	6	2	
Phospholipase D type VII (500 μg/ml)	6	0.2	
Treatment 3[c]			
PBS	46		0.6
PBS/PBS	47		1.3
Trypsin (1,000 μg/ml)	95		44
Phospholipase D type VI (500 μg/ml)	3		0.8
Trypsin and phospholipase D type VI	26		4

[a] Erythrocytes were incubated with PBS or enzymes for 30 min at 37°C, washed, resuspended, and mixed with *B. bacilliformis*. Erythrocytes were scored for deformation after 5 h and entry after 24 h.
[b] —, cells lysed.
[c] Erythrocytes were incubated either singly or sequentially with PBS and enzymes as described above. Cells were mixed with *B. bacilliformis* or with *B. bacilliformis* filtrate and scored for deformation after 5 h.

DF. When *B. bacilliformis* was incubated in tryptone-based medium and then removed by filtration, the filtrate was found to contain a factor which could deform erythrocytes which had been pretreated with trypsin at 1 mg/ml. Subsequent experiments by You-Han Xu (17) demonstrated that neuraminidase could also sensitize the cells to DF. The appearance of these deformed erythrocytes was identical to that of erythrocytes which had been incubated with *B. bacilliformis* (Fig. 1A and C). Deformation was time dependent, and a short lag was observed before significant deformation was observed (Fig. 3). At the concentrations used, deformation was not seen with untrypsinized red cells, although subsequent experiments by You-Han Xu (17) have shown the untrypsinized cells can be deformed by high concentrations of DF. Deformation was not observed when filtrates of uninoculated growth medium were used (Fig. 1D).

When *B. bacilliformis* were incubated in sterile tryptone-

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TABLE 3. Effect of KCN and kanamycin on *B. bacilliformis*-induced erythrocyte deformation[a]

Time of addition (h)	% Inhibition[b] with:				
	KCN		Kanamycin		
	Deformation	Entry	Deformation	Entry	Deformation by filtrate
0	100	56	100	100	81
1					35
2	96	70	95	96	8
3					8
4	46	64	42	64	
6	0	0	12	26	
8			0	0	

[a] One hundred microliters of 1 mM KCN or PBS or 250 μl of kanamycin (10 mg/ml) or PBS was added to a suspension of bacteria and erythrocytes at zero time or after incubation. Cells were scored for deformation at 6 h (KCN) or 8 h (kanamycin) and for intracellular bacteria at 24 h. For filtrates, kanamycin or PBS was added at the indicated times after mixing *B. bacilliformis* culture fluid and tryptone medium. Incubation was continued for a total of 3 h, and the bacteria were removed by filtration. DF activity in the filtrate was determined by incubating with trypsinized cells for 5 h. The three experiments were conducted separately.

[b] Percent inhibition was calculated as [(activity of PBS control − activity of KCN or kanamycin)/activity of PBS control] × 100.

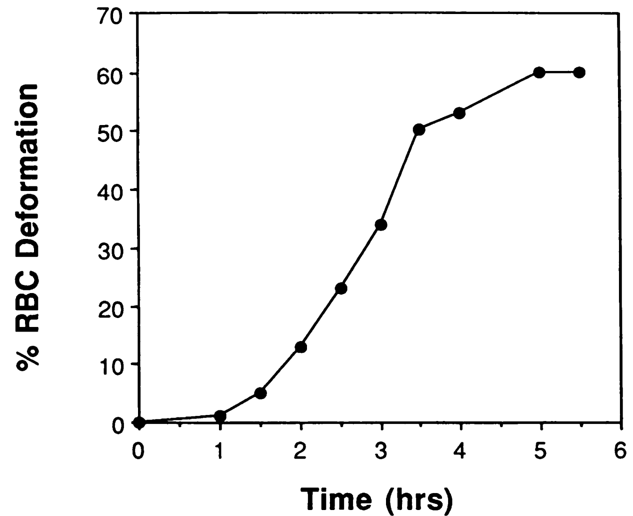

FIG. 3. Time-dependent deformation of trypsinized erythrocytes (RBCs) by a filtrate of medium in which *B. bacilliformis* was incubated. The filtrate was mixed with trypsinized erythrocytes, and the mixture was incubated at 35°C. Cells were scored for deformation at each time point. Trypsinized erythrocytes incubated with a similar filtrate from uninoculated medium were not deformed.

based medium, deforming activity increased in a time-dependent manner and required approximately 2 h of incubation to reach its maximum value. Only low levels of activity were present in filtrates prepared directly from cultures. Since no deforming activity was observed with bacteria incubated with brain heart infusion, RPMI, or PBS supplemented with human serum albumin and NaCl, it is likely that tryptone provides some nutrient essential for induction or synthesis of the DF. If the medium was diluted with PBS, the number of deformed erythrocytes decreased significantly, suggesting that some medium component required for DF synthesis or excretion became limiting. It is, however, possible that dilution simply slowed the bacterial metabolism, although the bacteria remained motile for 4 to 6 h. If the tryptone concentration was increased, more cells were deformed after 1 h and the maximum level of erythrocyte deformation was reached much earlier. Dialysis or heating of the tryptone-based medium for 2 min at 90°C or 100°C prior to the addition of bacteria greatly reduced or abolished deforming activity. Heating presumably affected the supplements, probably the human serum albumin fraction, since the tryptone had previously been autoclaved.

Kanamycin completely inhibited *B. bacilliformis*-induced deformation and entry if added when bacteria and erythrocytes were mixed and was less effective if the addition of kanamycin was delayed until after 2 h (Table 3). Addition of kanamycin to bacteria incubating in tryptone medium eliminated deformation activity in the filtrate prepared from the incubation mixture. Kanamycin added during the first 2 h of incubation markedly decreased deforming activity when the filtrates were subsequently assayed directly for deforming activity, suggesting that DF is synthesized during this period. Even when erythrocytes were incubated directly with DF, a period of time was required before deformation was seen. This lag period was generally shorter than that observed when bacteria were used, possibly in part because of the time required for synthesis or export of DF by whole bacteria. When untrypsinized erythrocytes and bacteria were incubated, addition of DF did not shorten the lag period, presumably because DF is relatively ineffective on untrypsinized erythrocytes. This suggests that the bacteria may cause some change in the erythrocyte membrane to render it more sensitive to DF.

Properties of DF. Filtrates containing DF could be stored for at least 2 weeks at 0, −20, or −70°C without loss of activity, and repeated freezing (−70°C) and thawing (37°C) did not alter the level of activity. DF could be inactivated by heating 1-ml volumes of filtrate for 2 min prior to mixing with trypsinized erythrocyte and subsequent incubation for 5 h at 35°C. As shown in Fig. 4, deforming activity was destroyed between 70 and 80°C. DF activity could also be depleted from filtrates by sequential incubation with batches of trypsinized erythrocytes, suggesting the possibility that DF binds directly to the erythrocytes. After two rounds of incubation with erythrocytes, deforming activity was reduced by 30%, and after three rounds, it was decreased by 70%.

DF could be precipitated from filtrates with saturated $(NH_4)_2SO_4$ at a final concentration of 60% (vol/vol), suggesting that it may be a protein or possibly a compound bound to a protein. Precipitates from both bacterial and control filtrates were collected by centrifugation, resuspended to 10% of the original volume in PBS, and dialyzed. For assay, the solubilized precipitates were mixed with tryptone medium (1:10, vol/vol) and trypsinized erythrocytes.

All of the deforming activity in filtrates was retained by an Amicon Centriprep 30 membrane, suggesting a molecular weight greater than 30,000. Filtrates were centrifuged for 30 min at 300 × *g*, after which both filtrates and retentates were tested for deforming activity by mixing with trypsinized erythrocytes and incubating 5 h at 35°C. The activity of DF in filtrates was sensitive to proteases such as α-chymotrypsin which, at final concentrations of 500 μg/ml, reduced deforming activity to approximately 5% of the control value.

Treatment of either normal or trypsinized erythrocytes with phospholipase D type VI greatly reduced the cells' capacity for deformation by live bacteria or by filtrate (Table 2). This suggests that the erythrocyte target for DF is

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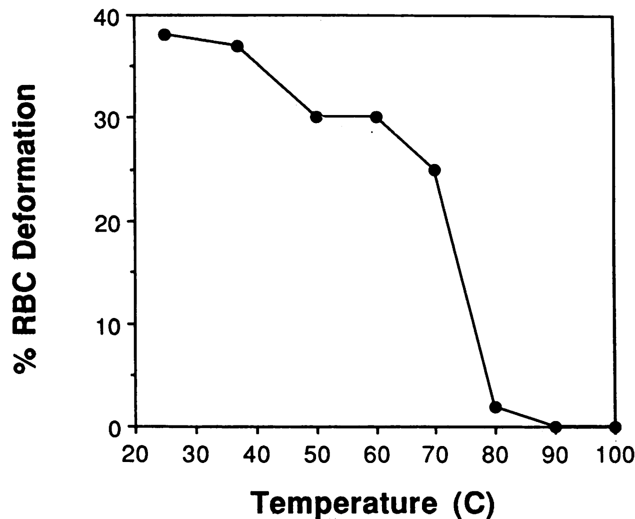

FIG. 4. Effect of temperature on deforming activity of filtrates. *B. bacilliformis* filtrates were heated for 2 min at various temperatures and mixed with trypsinized erythrocytes (RBCs), and the mixture was incubated at 35°C. After 5 h, cells were scored for deformation. Trypsinized erythrocytes incubated with a filtrate from uninoculated medium heated correspondingly showed no deformation.

disrupted by the action of phospholipase D and that the factor produced by *B. bacilliformis* during incubation with erythrocytes and the factor present in filtrates have a similar target.

DISCUSSION

Experiments reported here demonstrate the existence of an extracellular factor produced by *B. bacilliformis*, termed DF. This factor deforms trypsinized erythrocytes and reproduces the morphological changes seen in erythrocytes incubated with live bacteria during the invasive process, leading to intracellular parasitism of these cells.

When *B. bacilliformis* was added to erythrocytes, reversible binding of bacteria to the erythrocytes was observed. Actual attachments clearly occurred, as opposed to transient collisions, since the erythrocytes' visibility rotated in response to the attached motile bacterium. However, the association frequently was observed to terminate with an abrupt separation of the two. No deformation or invagination or other residual evidence of binding was visible in this case. Binding can therefore occur in the absence of visible deformation. A few deformed erythrocytes could be seen after about an hour of incubation, and the frequency increased as the incubation was continued, so that after about 6 h extensive deformation and invagination of the erythrocytes were seen. Some deformed erythrocytes with bound bacteria could be observed (and subsequently bacteria in internal vacuoles were found), but most erythrocytes, although deformed and similar in appearance, were free of bound bacteria. This observation shows that maintenance of the deformed state does not require the continuous presence of bound bacteria and suggested that binding of bacteria might not be necessary for deformation to occur.

The physical and chemical characteristics of DF are consistent with its being a protein. DF was stable during storage at 4°C or after freezing and was not inactivated by several cycles of freezing and thawing. DF could be inactivated by heating to 70 to 80°C. It could be precipitated by ammonium sulfate, was nondialyzable, and was retained by a membrane with a 30,000-molecular-weight cutoff. It could be inactivated by proteases, and its synthesis could be prevented by antibiotics such as kanamycin or by metabolic inhibitors. The lag period observed after bacteria were added to erythrocytes and before maximal deformation occurred may be accounted for largely by the time required for synthesis and/or export of DF; however, there was also a lag after addition of DF to trypsinized erythrocytes before deformation was observed. After removal of the bacteria by centrifugation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the supernatant demonstrated the presence of several distinct polypeptides synthesized by *B. bacilliformis*, including major polypeptides with molecular weights of 66,000 and 38,000 and smaller quantities of other polypeptides of bacterial origin (17). A recent study by Knobloch and Schreiber (10) has identified a 65,000-dalton protein as one of the major antigens of *B. bacilliformis*.

Incubation of erythrocytes with phospholipase D renders them resistant to deformation by DF. The phospholipase D-sensitive membrane component has not yet been identified. Possibly the action of phospholipase D modifies membrane deformability, as does glutaraldehyde treatment, which abolishes the capacity for deformation and entry of *B. bacilliformis*. It is more likely, however, that either phospholipase D modifies a substrate for DF or inactivates a receptor for DF. There is some evidence that DF binds to erythrocytes, since activity is lost from the supernatant after serial addition and removal of erythrocytes.

Binding of *B. bacilliformis* is in some manner related to DF, perhaps indirectly, since treatment of the erythrocytes with phospholipase D affects both the ability of the bacteria to bind to the erythrocytes and the ability of DF to deform the erythrocytes. Bacteria bound after deformation begins appear to be more tightly bound and to dissociate less readily than bacteria bound before deformation commences. Possibly, bacteria simply have difficulty in withdrawing from deep invaginations as seen by Benson et al. (2); alternately, DF may be present both as an extracellular factor and on the surface of the bacteria, where it may assist in binding the bacteria to the erythrocyte membrane. Bacterial motility seems to be required for internalization, since entry of nonmotile *B. bacilliformis* centrifuged onto erythrocytes was not observed. Deep invaginations which conform approximately to the dimensions of the bacteria were previously seen by scanning electron microscopy (2) and may be the result of mechanical pressure applied to a erythrocyte membrane rendered more deformable by DF.

DF requires prior modification of the erythrocyte surface with trypsin to produce a significant effect. Entry of *B. bacilliformis*, much more so than binding, is facilitated by treatment of the erythrocytes with trypsin or neuraminidase. This suggests that removal of proteins, or possibly negatively charged sialic acid residues associated with proteins such as glycophorin on the erythrocyte surface, facilitates binding of DF or its ability to deform the membrane. Although trypsin-dependent sensitization of erythrocyte membranes to DF suggests that a bacterial trypsinlike or neuraminidaselike activity may be involved in entry, there is not enough sensitizing activity present in the filtrate to permit the erythrocytes to be extensively deformed by DF without the addition of trypsin. If such an extracellular activity exists, possibly it was inactivated, or possibly it is a surface component of *B. bacilliformis*.

Experiments to identify DF with a *B. bacilliformis* protein

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and to clone the gene responsible for deformation activity are currently in progress, as are studies to identify the putative receptor or substrate for DF defined by phospholipase D sensitivity.

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