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Purification of deformin, an extracellular protein synthesized by *Bartonella bacilliformis* which causes deformation of erythrocyte membranes

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Abstract

A factor capable of deforming erythrocyte membranes, found in the culture supernatants of *Bartonella bacilliformis*, was purified 1840-fold using hydrophobic, ion exchange and gel exclusion chromatography. The final fractions contained a single detectable polypeptide species, referred to as deformin, having a molecular weight of 67000 by SDS-PAGE and a native molecular weight of 130000 by gel exclusion chromatography or velocity sedimentation in a glycerol gradient. Erythrocytes treated with deformin acquire trenches, indentations, and invaginations which could be reversed by vanadate, dilauroylphosphatidylcholine (DLPC), or by raising the internal Ca^{2+} concentrations with the ionophore A23187. Internal vacuoles also form. Erythrocytes treated with trypsin or neuraminidase are much more sensitive to deformin than untreated erythrocytes; erythrocytes treated with phospholipase D are less sensitive to deformin. This protein may play a role in causing the severe anemia which can result as a consequence of infection by *B. bacilliformis*.

Keywords: Erythrocyte; Membrane; Erythrophagocytosis; Internalization; (*B. bacilliformis*)

1. Introduction

Human erythrocytes are well known to serve as hosts for malaria and babesia and they also serve as targets for the pathogenic bacterial species, *B. bacilliformis*. The route of infection is through the bite of a nocturnal sand fly and a single night of unprotected exposure in the endemic area, certain valleys of the Andes, mainly in Peru but also in Ecuador and Colombia, is apparently sufficient to contract bartonellosis in one of its two manifestations. Oroya fever, which is named after the town of Oroya, Peru where more than 7000 workers constructing the Central Railway between Lima and Oroya died in 1870, is characterized by a fulminant anemia in which the erythrocytes become parasitized by *B. bacilliformis*. Nearly 100% of the erythrocytes are affected and the patient may die of an anemia of rapid onset with red cell counts as low as 500000/mm^3. Survivors of the acute anemia of Oroya fever, as well as infected persons who did not display a noticeable hematic phase, may develop verruga peruana in which endothelial cells infected by the bacteria give rise to verrugas, highly vascular cutaneous miliary or nodular eruptions. Verruga peruana is not a fatal disease and the development of verrugas in Oroya fever was traditionally taken as an indication that the patient would recover. The mortality rate of Oroya fever in the pre-antibiotic era was very high, about 40%, but not high enough to save the Incas from Pizarro and his soldiers, many of whom died from a plague-like disease reminiscent of small-pox, and who were reported by Garcillasco de la Vega (1539?–1616) to have developed fever and mysterious and loathsome skin warts which were very prone to bleeding [1], almost certainly verrugas. For an interesting historical review, see Strong [2].

The anemia of bartonellosis is the result of splenic removal of abnormal erythrocytes. Incubation in vitro of

Abbreviations: BSA, bovine serum albumin; BHI, brain heart infusion; BCYE agar, buffered charcoal yeast extract agar; DLPC, dilauroylphosphatidylcholine; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline.

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B. bacilliformis with human erythrocytes resulted in marked changes in the surface of the erythrocytes, including trenches, indentations and invaginations, as well as binding of individual bacteria and clumps of bacteria to the erythrocytes [3]. Bacteria were often seen lying within deep indentations of the erythrocyte membrane. However, erythrocytes were routinely and frequently seen which did not have bacteria bound to their surfaces, but which displayed equivalent morphological changes. A factor, termed deformation factor, was previously found in the supernatant of *B. bacilliformis* cultures [4]. This factor was capable of producing all the morphological changes that resulted when living bacteria were incubated with the cells. Deformation factor was shown to be stable on storage at 4° C, but could be inactivated by proteinases or brief heating to 70° C–80° C and was retained by membranes with a 30000 molecular weight cutoff. This activity could be an agent partially responsible in Oroya fever for the changes in erythrocyte morphology which lead to erythrophagocytosis and anemia.

Deformation factor may play a role in permitting the bacteria to gain entry to erythrocytes through a process similar to endocytosis or phagocytosis. *B. bacilliformis* may provide a convenient model to study the process by which infectious agents gain entry into cells. Since erythrocytes are not phagocytic and have little or no residual capability for endocytosis after maturation, *B. bacilliformis* must actively gain entry into the cells, rather than entering passively by phagocytosis or cell controlled endocytosis.

Here we report the purification of deforming factor 1840-fold and the presumptive identification of the deforming activity with a dimer of a 67000 Da polypeptide, termed deformin.

2. Materials and methods

2.1. Bacteria and growth conditions

General procedures used for growing *B. bacilliformis* are described in [3,4]. Cultures used for the isolation of deformin were grown by one of two procedures. In the first, an inoculum of 0.6 ml unwashed bacteria grown in brain heart infusion (BHI) medium was added to 8 ml sterile phosphate-buffered saline (PBS, 8 mM Na_2HPO_4, 1 mM KH_2PO_4, 3 mM KCl, 117 mM NaCl, pH 7.4) over BCYE agar (Difco) in a 100 × 15 mm petri dish. The cultures were incubated at 28° C for 3 days. The liquid overlay was centrifuged at 15000 rpm for 10 min and the supernatant was filtered through a 0.2 μm Acrodisk filter. Typically the supernatant contained 200–600 U/ml of deforming activity. An alternative procedure was used to minimize the amount of added protein to the growth medium. *B. bacilliformis* was centrifuged twice to remove protein from the BHI medium and resuspended in PBS. 0.6 ml was added to BCYE agar (Difco) overlain with 8 ml sterile PBS containing 0.5% BHI, 1% tryptone, and 0.004% hemin. The bacteria grew less well in this medium and the supernatant contained about 50–100 U/ml of deforming activity.

2.2. Enzyme and phospholipid assays

Sphingomyelin (40 μg) was dissolved in chloroform and the solvent removed by a stream of nitrogen. 1 ml of detergent buffer (0.5% Triton X-100, 1 mM Mg^{2+}) in PBS was added and samples vortexed until the solution cleared [5]. This was incubated for 1 h at 37° C with added enzyme (10 μl) *Staphylococcus aureus* sphingomyelinase C (Sigma) or purified deformation factor (1000 units/ml) and extracted with chloroform/methanol/HCl (100:50:0.5). Sphingomyelinase activity was assayed using thin-layer chromatography [6].

Erythrocytes were treated with 5 units of purified deformin and their lipids were extracted according to Turner [7]. Lipid standard (Avian) and samples of the lipid extracts were spotted onto HPTLC-alufolien Kiesel Gel 60 plates (E. Merck, Darmstadt, Germany) and the plates were developed in a solvent system composed of chloroform/methanol/ammonium hydroxide (65:35:8) and visualized with iodine vapors. The phospholipid was quantitatively determined after digestion of the spots with 70% perchloric acid by phosphorous analysis using the method of Lanzetta [8].

2.3. DLPC vesicles

A suspension of 10 mM DLPC in PBS was sonicated under N_2 until clear. Sonicated vesicles were incubated with deformed trypsinized erythrocytes for various times and aliquots of the cells were then fixed in 0.5% glutaraldehyde for 10 min at room temperature for light microscopic analysis [9].

2.4. Assay of deforming activity

One deforming unit was defined as the amount of deformin needed to deform 50% of the trypsinized erythrocytes under standard conditions. Specific activities were calculated using protein concentrations determined using the micro BCA assay kit (Bio-Rad) and BSA as standard. Erythrocytes were trypsinized by incubation at 50% hematocrit for 1 h with trypsin (1 mg/ml), or in some cases were treated with neuraminidase (0.5 units/ml). The erythrocytes were washed in PBSGI (PBS containing 10 mM glucose, 5 mM inosine) and resuspended in the same medium at 4° C at 50% hematocrit. 20 μl of trypsinized erythrocytes (final concentration 10^8 cells/ml) were added to 0.6 ml of binding medium (10 mM phosphate buffer, pH 7.4, 0.7% NaCl, 10 mM glucose, 5 mM inosine, and 0.5% human serum albumin) (Sigma). Human serum albu-

min was included in the binding medium to prevent loss of deformation activity at low concentrations. Aliquots of culture filtrate or fractions from column chromatography were then added. After incubation at 37° C, generally for 2 h, 10 μl of the red cell suspension was fixed for 10 min with an equal volume of 1% glutaraldehyde in PBS. At least 200–300 erythrocytes were examined using a Zeiss microscope equipped with phase optics; those scored as positive had prominent invaginations, pits or trenches.

2.5. Electron microscopy

3 μl of packed erythrocytes were resuspended in 1% glutaraldehyde for 20 min at room temperature, washed with distilled water three times and resuspended in 1 ml of distilled water. 2 μl of the cell suspension were air dried on a glass coverslip and samples were sputter-coated with 150 Å gold-palladium about 1 min before viewing with the scanning electron microscope.

For transmission electron microscopy, after glutaraldehyde fixation, the erythrocytes were fixed in 1% OsO_4 in PBS buffer for 30 min and then serially dehydrated in acetone-water. Samples were embedded in Epon and thin sections were stained with lead citrate and uranyl acetate. Thin sections were observed by transmission electron microscopy using a Zeiss 10C electron microscope.

2.6. Fluorescence microscopy

Trypsinized erythrocytes were incubated in isotonic buffer containing 1% FITC-BSA for 2 h at 37° C with or without deformation factor. The erythrocytes were washed with PBS and fixed with 1% glutaraldehyde. To reduce fading, the sample was mounted in glycerol (pH 8.0) containing antifade (*p*-phenylenediamine) for viewing by epifluorescence microscopy using the 100 × oil immersion objective [10].

2.7. Column chromatography

Filtrate (360 ml, 200--400 U/ml deforming activity) was centrifuged at 35 000 rpm for 1 h and the supernatant was applied to a 1.5 cm × 6 cm phenyl-Sepharose CL-4B column (Pharmacia), pre-equilibrated with 5 bed volumes of PBS buffer. The column was washed with PBS and then with 10 mM Tris buffer, pH 9.0 until the absorbance of the eluate returned to the base line. Most the added protein but none of the added deforming activity was recovered in the column wash. Deforming activity was eluted with 10 mM Tris, pH 9.0, 2% cholate at a flow rate of 15 ml/h.

The active fractions were pooled and loaded onto a 1.5 × 3 cm) DEAE-Sephacel column (Pharmacia) pre-equilibrated with 10 mM Tris buffer, pH 8.0 and washed with 10 mM Tris, pH 8.0, followed by 10 mM Tris, pH 8.0, 0.1 M NaCl, 1% cholate. The column was then eluted with a linear gradient of 10 mM Tris, pH 8.0, 1% cholate, and NaCl from 0.1 M to 0.5 M. Deforming activity was found to be eluted between 0.3 M and 0.4 M NaCl. The active fractions were pooled and concentrated with centriprep (Aminco, 30 000 molecular weight cut off). The concentrate was loaded onto an FPLC Superose 6 column equilibrated with 50 mM Tris, pH 8.0, 0.4% cholate and eluted at a flow rate of 0.5 ml/min. Fractions were assayed using 5–10 μl aliquots added to 0.6 ml of assay. Final concentrations of cholate were below 0.01%, which did not cause any deformation.

2.8. Molecular weight determination

Polypeptides were detected by SDS-PAGE (10%) visualized with Daichi silver stain (Daichi Pure Chemicals) or Coomassie brilliant blue, and their molecular weights determined by comparison with standard proteins. Native molecular weights were obtained by comparison with standard proteins on Superose 6 gel filtration and by glycerol gradient centrifugation. 0.5 ml of filtrate was loaded on the top of a 20%–5% glycerol gradient and centrifuged for 16 h at 40 000 rpm at 5° C in an SW50.1 rotor. The gradient was fractionated and assayed for deforming activity and the locations of standard marker proteins was determined on Coomassie brilliant blue SDS-PAGE.

3. Results

3.1. Semi-quantitative assay for deformation activity

Incubation of erythrocytes with bacteria-free filtrates of the culture medium used to grow *B. bacilliformis* resulted in extensive deformation of the erythrocytes, as shown in Fig. 1B–D. Based on this property, a standard assay for deformation activity was developed, which permitted yields to be quantitated and the location of deforming activity to be determined in the various fractions. Several potential problems needed to be considered before this biological assay could be used to monitor deformation activity during purification. First, individual erythrocytes are deformed to various extents. We chose to consider only two states for the cells, normal and deformed, without consideration of the extent of deformation, because we found that as increasing amounts of filtrate were added, the clearest consequence was a decrease in the fraction of undeformed cells and this decrease was simple to quantitate. Second, several different morphological changes are observed, including pits, trenches, invaginations and depressions, as can be seen in Fig. 1. We did not observe any obvious transition from one type of morphology to another as increased amounts of filtrate were added, and so we regarded all forms of deformed morphology as equivalent. Third, apparently undeformed erythrocytes coexist with extensively deformed erythrocytes, suggesting that not all erythrocytes are equally sensitive to deforming factor. The sensitivity of

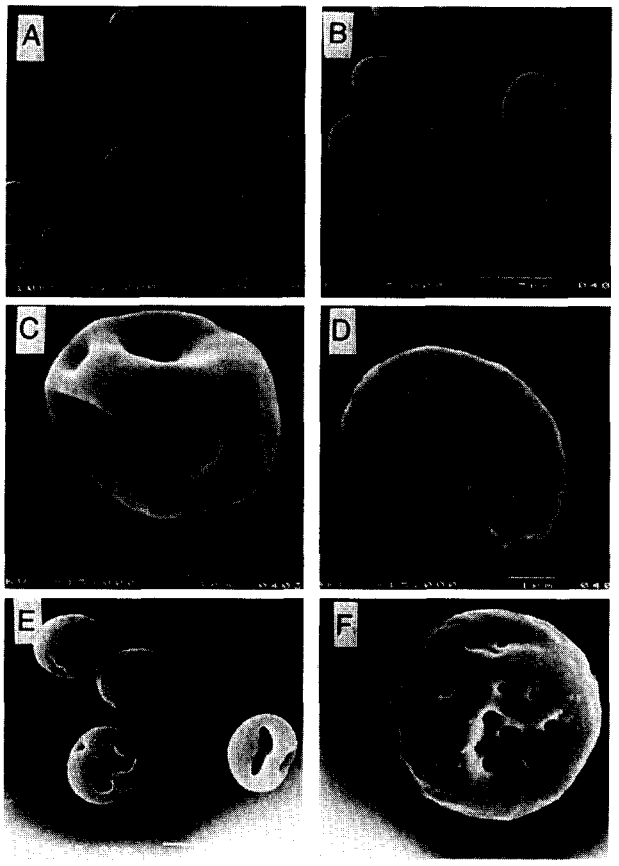

Fig. 1. Scanning electron micrograph of erythrocytes. (A) Trypsinized normal erythrocytes. (B–D) Trypsinized erythrocytes incubated with filtrate for 2 h at 37° C. (E–F) Trypsinized erythrocytes incubated with purified deforming factor for 2 h at 37° C.

the erythrocytes can be greatly increased by preincubation of the erythrocytes with trypsin or, to a lesser extent, with neuraminidase. Preincubation of the erythrocytes with 500 μg/ml phospholipase D for 30 min at 37° C converted part of the phosphatidylcholine to phosphatidic acid and decreased the fraction of cells deformed by 5 units of deforming factor from 80% to 20% (data not shown). Erythrocytes were routinely trypsinized, since preincubation with trypsin seemed to yield a more homogenous population of erythrocytes and, in any case, made the assay more sensitive.

Finally, conversion of the normal morphology to the deformed was not related in a simple or linear way to experimental variables such as time or filtrate concentration. After incubation of trypsinized erythrocytes with filtrate, the appearance of deformed cells occurred only after a lag time of a few minutes to more than an hour. Similar lag periods were observed with purified deformin, as discussed below. The length of the lag time was dependent on the concentration of filtrate added to the assay system. With increasing times of incubation, the fraction of erythrocytes seen to be deformed increased, up to some maximal value which was dependent on the amount of filtrate added. At high concentrations of filtrate, nearly 100% of the cells became deformed, indicating that there was not a fraction of erythrocytes resistant to the action of deformation factor. At low concentrations of deforming factor, several hours of incubation was needed for the extent of deformation to become maximal and even then only a minority of the erythrocytes became deformed. These considerations lead us to routinely employ an incubation time of 2 h as the standard. Prolonged incubation increased the sensitivity of the assay and eliminated the lag time as a significant consideration. The reproducibility of the assay and the purification described below was very good; all the experiments reported here were generally repeated at least three times with equivalent results.

Addition of increasing amounts of filtrate caused higher percentages of erythrocytes to be deformed. However, the percent deformed was not related to the amount of filtrate added in a simple linear way; instead a sigmoidal dose–response curve was observed. We did not consider the assay to be very reliable when either a small fraction or a large fraction of the erythrocytes were deformed and so we took 50% deformation after 2 h of incubation to be the desired end point of the assay. During purification, each fraction

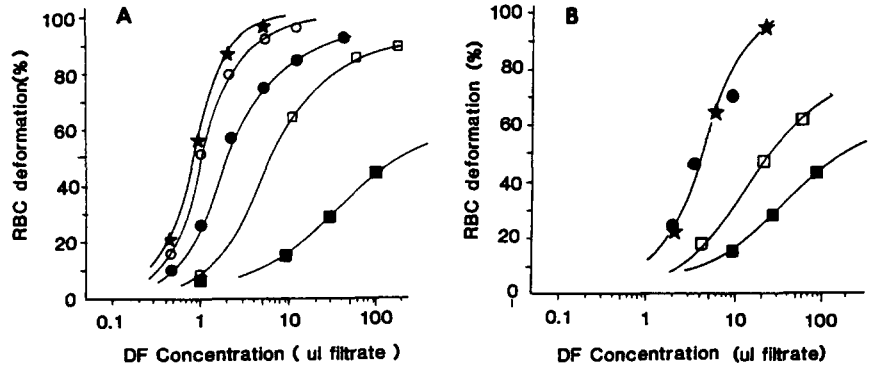

Fig. 2. Sensitivity of erythrocytes to deformation after trypsin or neuraminidase treatment. (A) A 50% erythrocyte suspension was treated with trypsin for 0 (■), 1 (□), 10 (●), 30 (○), and 60 (★) min. (B) Erythrocytes were treated with neuraminidase (0.5 U/ml) for 0 (■), 1 (□), 10 (●), and 60 (★) min.

needed to be assayed at several different concentrations in order to find an amount of activity which resulted in approximately 50% deformation. We defined 1 unit of deformation activity to be the amount which caused 50% deformation under the standard conditions using trypsinized erythrocytes. Fig. 2 shows the percent deformation plotted against the amount of filtrate added for erythrocytes incubated for various times with trypsin (Fig. 2A) or neuraminidase (Fig. 2B). The sensitivity of the assay is increased about 100-fold by trypsin treatment and about 25-fold by neuraminidase treatment, using 50% deformation as the assay standard. For example, in Fig. 2A, about 100 μl of filtrate is required to reach 50% deformation of untrypsinized cells, whereas fully trypsinized cells are 50% deformed by about 1 μl of filtrate. The response of the trypsin-treated erythrocytes is more homogenous than that for untreated erythrocytes or neuraminidase-treated erythrocytes, in the sense that only a small increase in filtrate added is needed to convert the trypsinized erythrocytes from mostly undeformed to mostly deformed.

3.2. Purification of deformin

Purification of deformin was complicated by the fact that the culture medium contained significant amounts of human serum and erythrocyte lysate. Much of the purification needed could have been accomplished simply by not adding these complex mixtures of proteins to the growth medium. A further complication was that human serum contains an inhibitor of deforming activity. We devoted considerable time to develop a satisfactory growth medium lacking added proteins, but we were unsuccessful. Although we were able to grow *B. bacilliformis* in protein free medium, the bacteria grew much more slowly and the deforming activity of the filtrate was greatly diminished.

Deforming factor proved to be a relatively stable protein. Not only did it remain active in the supernatant over several days of incubation while the bacteria were growing, but also the crude filtrate could be stored for more than 3 months at $-20°$ C with little or no loss of activity.

The culture medium was centrifuged to remove bacteria and filtered through a 0.2 μm Acrodisk filter, centrifuged again at 100 000 $\times g$ for 1 h, and loaded onto a phenyl-Sepharose CL-4B column. After washing the column, bound protein was eluted with 10 mM Tris buffer, pH 9.0 and 2% cholate. Essentially no deforming activity was present in the flow through, nor was any activity eluted from the column in low ionic strength buffer (10 mM Tris, pH 9.0). The fact that the deforming factor bound strongly to this column and required cholate to be eluted indicates that deformin is a very hydrophobic protein.

The deforming factor was further purified using DEAE-Sephacel. In the initial experiments, deforming factor was eluted with 0.15 M NaCl, along with many other proteins. Purification was improved markedly by equilibrating the column with buffer containing 1% cholate and

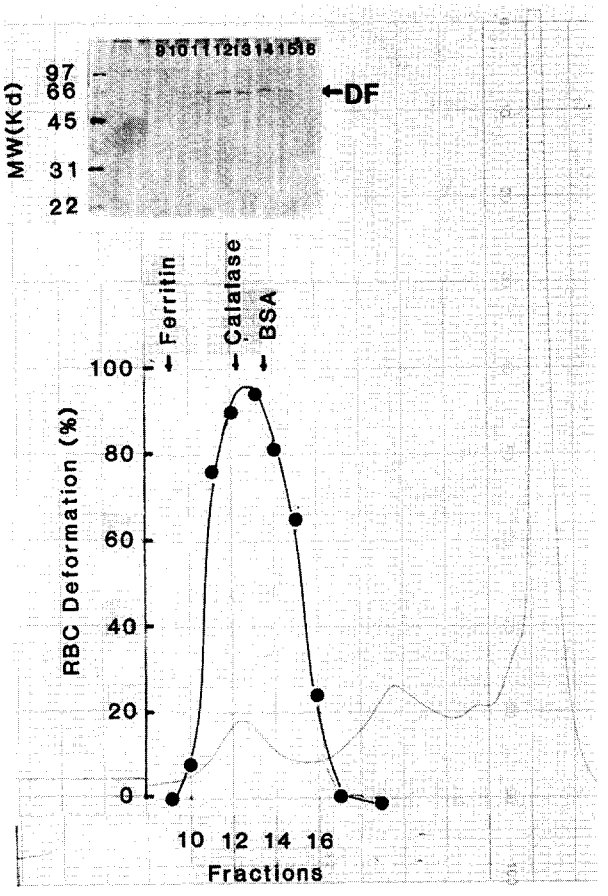

Fig. 3. Superose 6 chromatography profile. The symmetric first protein peak, which has an apparent molecular weight of 130 000, contains all the deforming activity (specific activity 227 U/μg). Only a 67 000 molecular weight protein is seen by silver stain (inset).

washing the column with 10 mM Tris (pH 8.0), 0.1 M NaCl, 1% cholate. Under these conditions most of the impurities eluted from the column, but deforming factor remained bound and was subsequently eluted with 0.35 M NaCl.

After concentration, the deforming factor was applied to an FPLC Superose 6 column equilibrated with 50 mM Tris buffer (pH 8.0), 0.4% cholate. Cholate was necessary to prevent irreversible adsorption of the protein to the column. The deformation activity co-eluted with the first symmetric protein peak at a position corresponding to a molecular weight of about 130 000 (Fig. 3). The fractions corresponding to the deforming activity contained only one detectable polypeptide with an apparent molecular weight of 67 000 on SDS-PAGE using silver staining. Glycerol gradient sedimentation was used to provide a second estimate of the native molecular weight, using fumarase, lactic dehydrogenase, BSA, and carbonic anhydrase as known molecular weight standards. The deforming activity sedimented slightly slower than lactic dehydrogenase (140 000 molecular weight), with an $S_{20,w}$ of 6.5 and an apparent molecular weight of about 130 000 (Fig. 4).

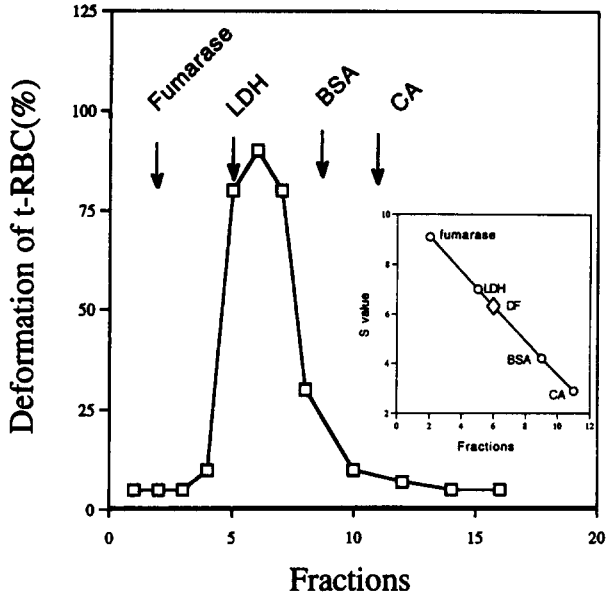

Fig. 4. Sedimentation of deforming factor in a glycerol density gradient. The position of deforming factor relative to fumarase (9.1S, 192 kDa), lactate dehydrogenase (7.0S, 140 kDa), BSA (4.2S, 61 kDa), and carbonic anhydrase (2.9S, 31 kDa) is indicated.

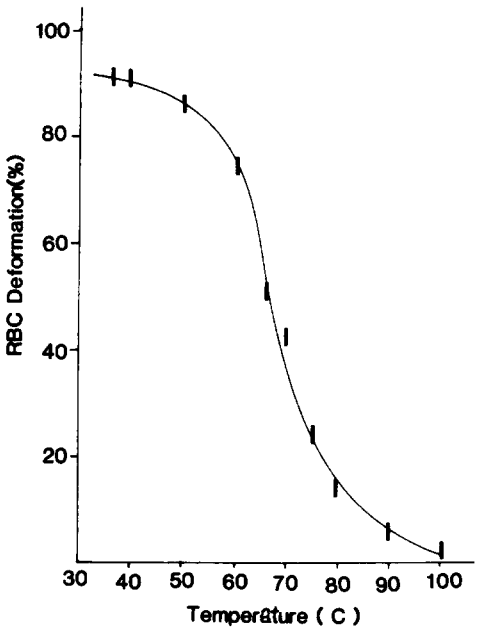

Fig. 5. Heat inactivation of deforming factor. The protein was heated for 5 min at various temperatures and the residual activity determined. The results given are the average of three experiments.

The results of this purification process are summarized in Table 1. Similar results were obtained in five separate purifications. It can be seen that each step resulted in considerable purification, but also considerable loss in recovered activity. Deformation factor prepared by two separate purifications was sent to two different protein sequencing laboratories, but in neither case could an N-terminal sequence be obtained, either because the amount was too small or because the N-terminus was blocked.

3.3. Properties of purified deformation factor

In all respects tested, the properties of the purified deformation factor were equivalent to those observed with the filtrate of the culture supernatant. This indicates that a second factor is not present in the culture filtrate and that the activity of deforming factor is not altered by purification. The morphology of erythrocytes incubated with the purified deformation factor, as shown in Fig. 1E and 1F are very similar to erythrocytes incubated with the culture supernatant (Fig. 1B–D). Fig. 5 shows that the purified deformation factor was completely heat inactivated by incubation for 5 min at temperatures above 80° C, as was found for the deforming activity in the culture filtrate [4] and is partially inactivated by incubation for 5 min at temperatures between 60° C–70° C.

The kinetics of deformation of trypsinized erythrocytes is shown in Fig. 6. There is a lag period, the length of which decreases with increasing concentration of deforming factor, before obviously deformed erythrocytes were seen. At higher concentrations of deforming factor, 100% of the erythrocytes could be deformed, but when lower concentrations of deforming factor were used, the percentage of deformed cells eventually reached a plateau. The overall shape of the time course was sigmoid at each of the concentrations examined.

The concentration dependence of deformation for both purified deformation factor and filtrate is shown in Fig. 7. In both cases, there is a concentration range below which only a small percentage of cells are deformed and above which essentially all the cells are deformed. This concentration range is rather narrow when trypsinized erythrocytes are used, and the plot of % deformation versus added deforming activity is sigmoidal. In an experiment where

Table 1
Purification of DF protein from *Bartonella*-free culture filtrate

Purification step	Volume (ml)	Activity (U/ml)	Protein (μg/ml)	Total activity (kUnits)	Specific activity (kUnits/mg)	Purification (x-fold)
Culture filtrate	360	200	4000	72	0.075	1
Phenyl-Sepharose	24	750	125	18	6	80
DEAE-Sephacel	15	200	4	3	50	666
Gel filtration	15	100	0.44	1.5	227	1840

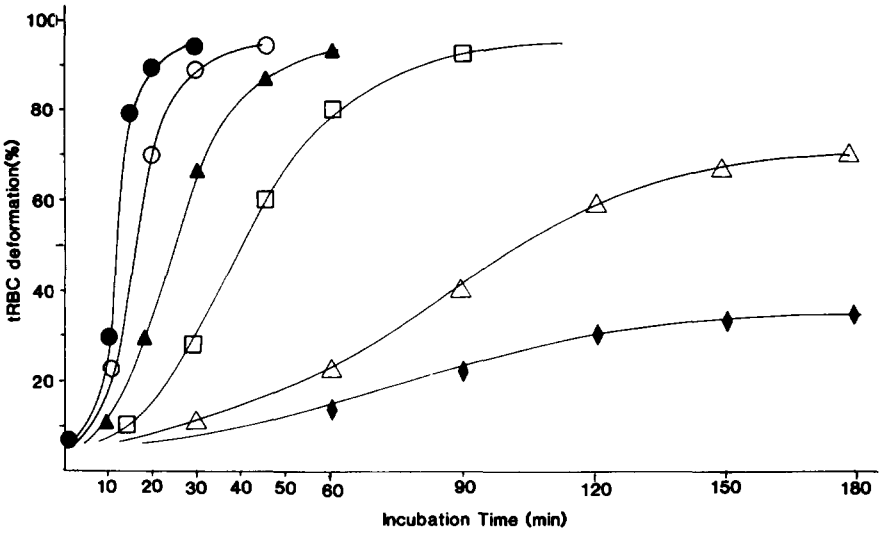

Fig. 6. Kinetics of deformation of trypsinized erythrocytes by purified deformin. Concentrations used are 0.25 (♦), 0.50 (Δ), 1.25 (□), 2.5 (▲), 5 (○), and 10 (●) units.

the concentration of deformation factor was constant (using in this experiment the filtrate) and the number of erythrocytes varied, the percentage of erythrocytes deformed was nearly 100% at low concentrations of erythrocytes and decreased with increasing concentrations of erythrocytes (Fig. 8B).

Erythrocytes treated with *S. aureus* sphingomyelinase C or *Corynebacterium pseudotuberculosis* sphingomyelin phospholipase D develop a morphology similar to that observed with deformation factor [11,12]. When trypsinized erythrocytes were incubated with 0.4 μg of *S. aureus* sphingomyelinase C (Sigma), about 95% of the cells were deformed in 1 mM Mg^{2+}, but less than 5% in 1 mM EDTA after 2 h at 37° C. With 4 units of deformation factor, about 95% of the erythrocytes were deformed both in 1 mM Mg^{2+} and in 1 mM EDTA. More than 95% of the erythrocytes treated with sphingomyelinase C were sensitive to cold lysis (5 min at 4° C) whereas less than 5% of the erythrocyte treated with deformin lysed. Phospholipid was extracted from the treated erythrocyte membranes and analyzed by thin-layer chromatography. There was no change in the amounts of phosphatidylcholine or sphingomyelin seen by thin-layer chromatography or in the number or location of spots on the chromatogram for cells treated with deformin, whereas with cells treated with *S. aureus* sphingomyelinase C about 1/3 of the sphingomyelin was degraded (data not shown).

Direct assay using thin-layer chromatography of 4 units of deformin for sphingomyelin or phosphatidylcholine did not reveal any phospholipase activity.

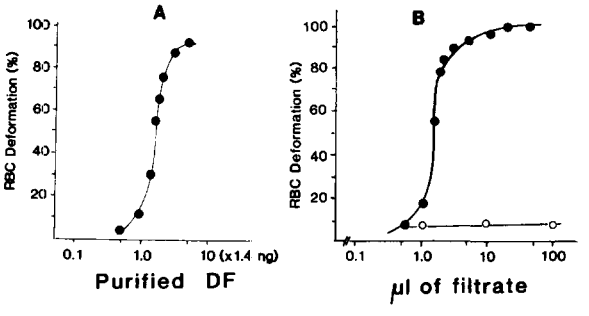

Fig. 7. Concentration dependence of deformation of trypsinized erythrocytes by purified deformin (A) or filtrate (B). 20 μl of erythrocytes (50% hematocrit) in 0.6 ml of binding medium was mixed with the indicated amounts of filtrate or deforming factor and incubated 2 h at 37° C. In B, a control filtrate (○) form uninoculated growth medium did not deform erythrocytes. The results given are the average of three experiments.

3.4. Depletion of activity

Assuming that deforming factor might bind to erythrocytes, erythrocytes were incubated with deforming factor and then removed by sedimentation to determine how

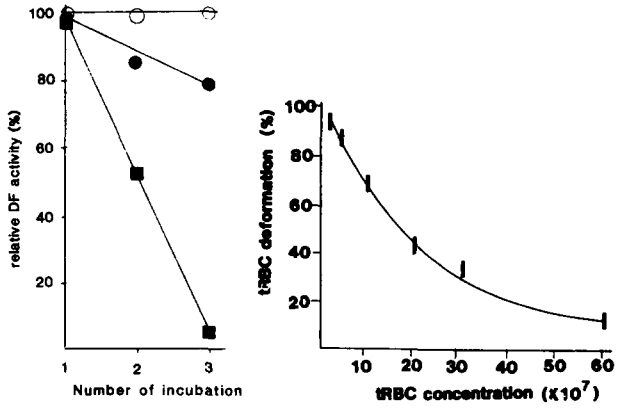

Fig. 8. Depletion of deforming activity by trypsinized erythrocytes. (A) After incubation for 2 h with filtrate erythrocytes were removed by centrifugation at 2000× *g* for 5 min; the supernatant was then incubated with the same volume of fresh erythrocytes for 2 h and again the erythrocytes were removed by centrifugation. 1) no erythrocytes added (○), 10 μl of erythrocytes (●), 40 μl of erythrocytes (■). (B) The percent deformation observed with various concentrations of trypsinized erythrocytes.

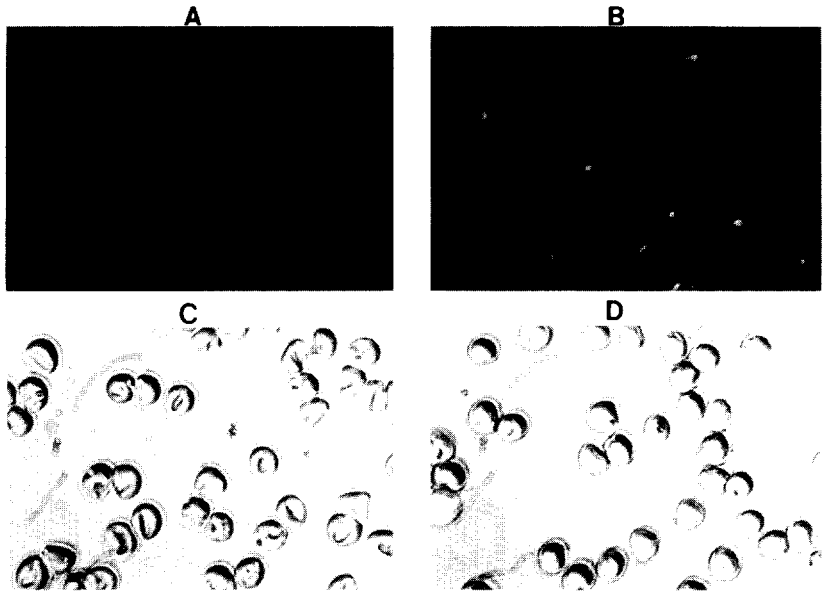

Fig. 9. FITC-BSA was added to erythrocytes in the absence (A,C) or presence (B,D) of deforming factor. Fluorescence microscopy (A,B) showed the presence of small fluorescent vacuoles after addition of deforming factor (B). FITC-BSA by itself deformed some of the cells (C).

much deforming activity remained in the supernatant. If little deforming activity remained in the supernatant when erythrocytes were incubated with an amount of deforming factor capable of deforming a much larger number of erythrocytes, we perhaps could conclude that more deforming factor can bind to erythrocytes than the minimum needed to deform the cells, and might be able to estimate the number of binding sites per cell. Fig. 8A shows that when 10 μl of erythrocytes was incubated for 2 h with filtrate and removed by centrifugation for 5 min, substantial deforming activity remained in the supernatant and the incubation could be repeated again with fresh erythrocytes with little depletion of the deforming activity remaining in the supernatant. On the other hand, when the same experiment was done with four times as many erythrocytes, only about 50% of the original deforming activity remained after the first incubation and none after the second. This experiment demonstrates that deforming activity is lost from the supernatant after interaction with cells, either because it binds to the cells or because it is somehow used up in the process, and also that the activity is lost in proportion to the number of cells added.

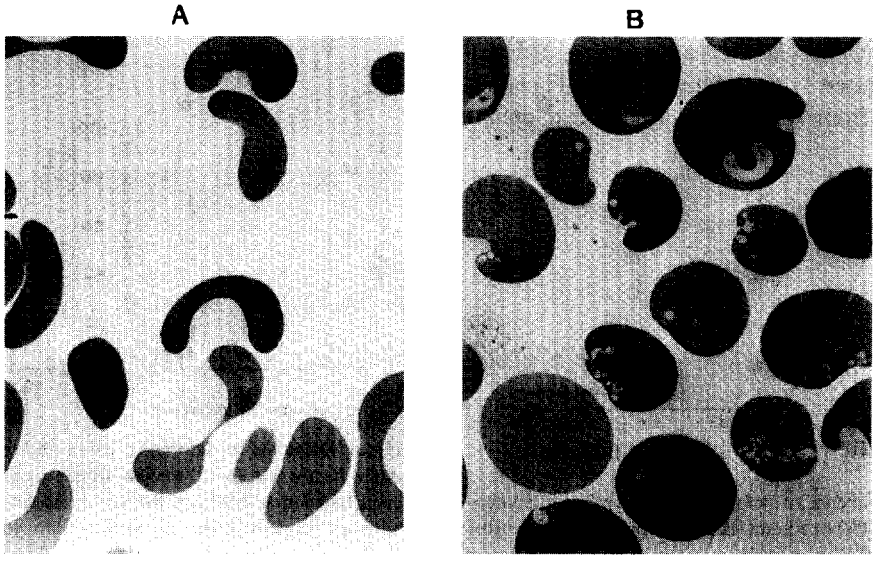

Fig. 10. Transmission electron microscopy of deformed red cells. Trypsinized erythrocytes were incubated in isotonic buffer containing 1% FITC-BSA without deformation factor (A) or with deformation factor (B) for 2 h at 37° C. Numerous small vacuoles are seen in the erythrocytes treated with deformation factor (B).

3.5. Internalization

Deformation factor alone, in the absence of bacteria or other added substances, is capable of causing the formation of internal vesicles in erythrocytes. When FITC-BSA was present in the medium, some of it became entrapped in intracellular vesicles and could not be washed away (Fig. 9). The entrapped fluorescence appeared to be contained in small fluorescent clusters underneath the membrane. In the electron microscope, the vacuoles were of variable size and most of them appeared to be close to the membrane (Fig. 10). FITC-BSA alone deformed some of the cells (Fig. 9C) but none was internalized (Fig. 9A). This experiment demonstrated that deforming factor not only alters the surface topography of the erythrocyte membrane, but also is capable of causing internalization of extracellular fluid by the creation of closed internal vesicles (for a review of entrapment in erythrocytes, see Schrier [13]).

3.6. Reversibility of deformation

Deformed cells incubated overnight were found to have spontaneously reverted to the biconcave shape. Several chemicals could revert the deformed cells very quickly. Vanadate (10–300 μM), raising the intracellular Ca^{2+} concentration using A23187 [14] (Fig. 11A–C), or acquisition of DLPC from lipid vesicles by the outer leaflet of erythrocyte membranes (Fig. 11D–F) quickly reverted the deformed red cells to biconcave discs, within 30 s after addition of A23187 or 1 min using DLPC vesicles. Further incubation with these agents caused the formation of echinocytes. Low concentrations of vanadate (1–10 μM) which by themselves did not cause any observable morphological change and were too low to reverse deformation, nevertheless were capable of preventing deformation by low concentrations of deformation factor (3 U) (Fig. 12).

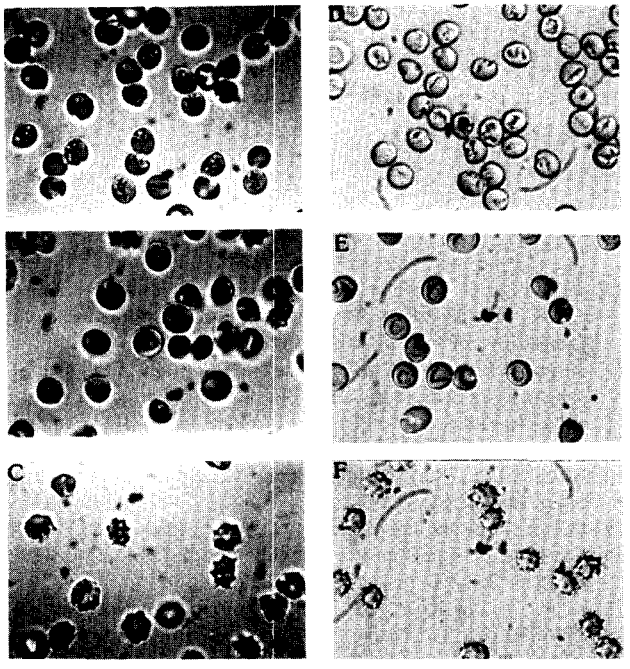

Fig. 11. Deformed erythrocytes were incubated with 5 μM of ionophore A23187 in 0.1 mM Ca^{2+} for 30 s (A), 1 min (B), or 5 min (C) before fixation in 0.5% glutaraldehyde; deformed erythrocytes were incubated with 2.5 μl of 10 mM DLPC vesicles for 0 min (D), 1.5 min (E) or 3 min (F) before fixation.

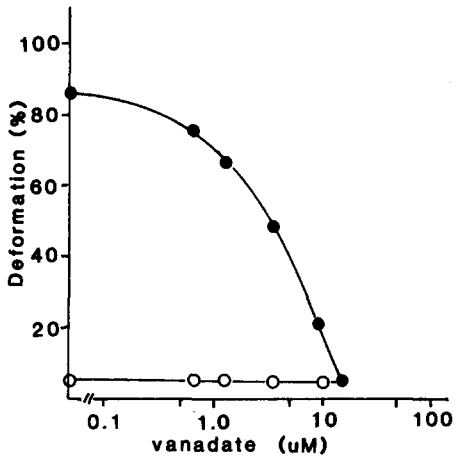

Fig. 12. Inhibition of deformation by vanadate. Trypsinized erythrocytes were incubated with various concentrations of vanadate and with 3 units of deforming factor with (●) or without (○) deforming factor. Concentrations of 1 μM or above inhibited deformation.

4. Discussion

We report here the purification of deformation factor activity from the bacteria-free supernatants of cultures of *B. bacilliformis*. The deformation activity was purified about 1840-fold, using hydrophobic, ion exchange and gel exclusion chromatography. Deformation activity sediments in glycerol gradient centrifugation and elutes from Superose 6 with an apparent molecular weight of 130000. The only polypeptide species which we could detect in these fractions migrated on SDS-PAGE with a molecular weight of 67000, suggesting that deformin is a dimer.

The supernatant from cultures of *B. bacilliformis* contains significant amounts of many of the outer membrane proteins of *B. bacilliformis* (Arevalo, J., unpublished observations). These appear to be present as membrane fragments or vesicles. In view of this, it is not clear whether deformation factor is secreted from the bacteria as an extracellular protein or, alternately, is a component of the bacterial outer membrane and is present in the supernatant along with other membrane proteins as the result of some vesiculation process. A protein of about 67000 molecular weight can be faintly detected in outer membrane preparations. One argument that the activity might be surface-associated is that the serum contains an inhibitor of deforming factor, so that deforming factor on the bacterial surface

in close proximity to the erythrocyte membrane would be expected to be more active and less inhibitable by serum. Despite considerable effort we were unable to characterize the nature of the inhibitory activity in serum.

Although substantial purification of deformin was achieved by this purification scheme, the overall yield was only about 2%. We believe the low yield of purified protein is the result of its hydrophobic properties. Cholate is necessary to prevent non-specific loss of deformin during purification and in the absence of cholate, losses are very high, up to 100%. It was not possible to obtain an N-terminal amino acid sequence on the 67000 molecular weight polypeptide, either because the N-terminus is blocked or, more likely, because it was not possible to purify adequate amounts of the protein for sequencing. Since we were unable to obtain an amino acid sequence, it was not possible to isolate a clone of the gene for nucleotide sequencing using the amino acid sequence to configure a DNA probe, or to compare a partial amino acid sequence with the protein database. Without some additional evidence that the 67000 protein is related to other membrane active proteins, the identification of this polypeptide with the deforming activity rests mainly on our inability to detect some other protein in the fractions containing deforming activity.

We considered the possibility that deformin might be an enzyme catalyzing a reaction involving a component of the membrane, but we could obtain no direct evidence for this possibility. No obvious changes were seen in the phospholipid components of membranes of erythrocytes treated with deforming factor. Deformin did not appear to have sphingomyelinase or other phospholipase activities when assayed directly. Whether deformin is an enzyme or acts in some other way on a membrane component, it might bind to erythrocyte membranes, possibly to a specific receptor in the membrane. The heterogeneous response of the red cells to deformin, particularly the untrypsinized cells, suggest that the red cell population itself is heterogeneous, possibly due to red cell age. However, this is a complicated question since storage of the red cells reduces their sensitivity and since red cells freshly trypsinized are more sensitive than stored trypsinized red cells. It is interesting that incubation of a relatively large number of erythrocytes with deformin followed by centrifugation of the erythrocytes leaves the supernatant deficient or devoid of deforming activity. However, incubation and centrifugation of one-fourth as many erythrocytes under similar conditions leaves most of the deforming activity in the supernatant. This suggests that there may be a relatively limited number of targets per erythrocyte for deformin. We estimate that in the standard assay, only about 500 molecules of deformin per erythrocyte are present and perhaps 80% of these remain in the supernatant when the cells are removed by centrifugation.

Several agents – increased intracellular Ca^{2+}, DLPC and vanadate – all reversed the action of deformin. The action of vanadate is believed to inhibit a Mg^{2+}-ATPase which functions as an aminophospholipid translocase [15,16]. DLPC is directly transferred to the erythrocyte membrane [9]. The ability of these compounds to reverse deformation suggests that deformin may act to alter the lipid composition or asymmetry of the membrane, according to the hypothesis of Sheetz and Singer [17,18]. For a review, see Backman [19].

When increasing concentrations of deformin are added to trypsinized erythrocytes, the percentage of deformed cells changes abruptly from near zero to nearly 100% with an S-shaped curve. It is not possible to infer anything about the state of association of deformin however, since the width of this transition, as well as the sensitivity of the erythrocytes to deformation, is very much determined by how completely the erythrocytes have been trypsinized. It is clear that 100% of the erythrocytes are sensitive to deformation if enough deformin is added, but that much more deformin is required for cells that are not trypsinized or only lightly trypsinized than for cells extensively trypsinized. Similarly it is difficult to interpret the observed time course, with its lag period and apparent saturation, since this may reflect more the variable resistance of a heterogeneous population of erythrocytes than any mechanistic considerations.

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