Molecular Cloning and Characterization of *Bacillus alvei* Thiol-dependent Cytolytic Toxin Expressed in *Escherichia coli*

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A chromosomal DNA fragment from *Bacillus alvei*, encoding a thiol-dependent haemolytic product known as alveolysin (M_r 60000, pI 5.0) was cloned in *Escherichia coli* SK1592, using pBR322 as the vector plasmid. Only a single haemolysin-positive clone was identified, by testing for haemolysis on blood agar plates. The haemolytic material was associated with the host bacterial cell. It was released by ultrasonic disruption and purified 267-fold. A 64 kDa polypeptide of pI 8.2 cofractionated with haemolytic activity during gel filtration chromatography and isoelectric focusing. It behaved identically to alveolysin in its activation by thiols, inactivation by thiol group reagents, inhibition by cholesterol, and neutralization, immunoprecipitation and immunoblotting by immune sera raised against alveolysin and streptolysin O.

INTRODUCTION

Alveolysin is an extracellular, cytolytic and lethal single-chain protein toxin (M_r ~60000) identified as a haemolysin in the culture fluids of *Bacillus alvei* (Bernheimer & Grushoff, 1967), a micro-organism isolated from soil and honey-bee larvae suffering from European foul brood (Katznelson & Lochhead, 1947; Buissière & Colobert, 1966). This toxin is a member of the group of thiol-activated (or SH-dependent) cytotoxins, which comprises at least 16 biologically and antigenically related proteins produced by Gram-positive bacterial species belonging to the genera *Streptococcus*, *Bacillus*, *Clostridium* and *Listeria* (Smyth & Duncan, 1978; Alouf, 1980; Alouf *et al.*, 1986; Bernheimer, 1986). The prototype of these toxins is streptolysin O (SLO), produced by streptococci belonging to Lancefield groups A, C and G (Alouf, 1980; Bhakdi *et al.*, 1984). The toxins damage eukaryotic cells by disruption of the cytoplasmic membrane and intracytoplasmic organelles (Smyth & Duncan, 1978; Launay *et al.*, 1984; Bremm *et al.*, 1985). Alveolysin and the other SH-dependent toxins (Alouf *et al.*, 1977; Geoffroy *et al.*, 1981; Thelestam *et al.*, 1981; Geoffroy & Alouf, 1983) share common biochemical properties such as activation by thiols and inactivation by oxidation or interaction with 3 β -hydroxysterols. Cholesterol is thought to be the toxin receptor and target at the surface of eukaryotic cells (Prigent & Alouf, 1976; Cowell & Bernheimer, 1978; Smyth & Duncan, 1978; Alouf & Geoffroy, 1984; Bernheimer, 1986). Cholesterol-containing artificial lipid membranes such as liposomes (Cowell & Bernheimer, 1978; Alving *et al.*, 1979; Geoffroy & Alouf, 1983) or mixed lipid monolayers at air-water interfaces (Alouf *et al.*, 1984; Blumenthal & Habig, 1984) are disrupted or penetrated, or both, by the SH-dependent toxins.

As a step towards the elucidation of the structural and antigenic relatedness of *B. alvei* alveolysin to the six other SH-dependent toxins purified so far (Alouf *et al.*, 1984, 1986; Parrisius *et al.*, 1986; Geoffroy *et al.*, 1987), the molecular cloning of the alveolysin determinant, its expression, and partial purification of the alveolysin-like product in *Escherichia coli* were

Abbreviations: HU, haemolytic unit; RH, recombinant haemolysin; SLO, streptolysin O.

undertaken. A comparative study of some biochemical, toxic and immunological properties of *B. alvei* alveolysin and the recombinant protein expressed in *E. coli* is also described.

METHODS

Bacterial strains. *Bacillus alvei* strain ATCC 6344 (Pasteur Institute Collection) was used for the preparation of genomic DNA (Geoffroy & Alouf, 1983). This strain produces large amounts of alveolysin (about 2500 haemolytic units, HU, per ml of culture supernate, equivalent to $2.5 \mu\text{g}$ protein ml^{-1}). *Escherichia coli* strain SK1592 (*tonA gal thi sbcB15 endA hsdR4 hsdM⁺*) (Rapoport *et al.*, 1979) kindly provided by G. Rapoport and A. Klier (Pasteur Institute, Paris) was used as the recipient strain in transformation experiments.

Media and growth conditions. *B. alvei* was cultured overnight at 30°C with shaking in minimal medium containing (per litre) 6 g KH_2PO_4 , 10.65 g K_2HPO_4 , 2 g $(\text{NH}_4)_2\text{SO}_4$, 2 g sodium citrate. $5\text{H}_2\text{O}$, 0.4 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ supplemented with 1% (w/v) proteose peptone no. 3 (Difco) and 2.5% (w/v) glucose. *E. coli* was cultured at 37°C with shaking in L-broth (Davis *et al.*, 1980) [1% (w/v) Bactotryptone (Difco), 0.5% (w/v) yeast extract (Difco), 1% (w/v) NaCl, supplemented when necessary with ampicillin ($100 \mu\text{g ml}^{-1}$)]. For agar plates, 15 g Bacto-agar (Difco) was added per litre of L-broth supplemented with either ampicillin ($100 \mu\text{g ml}^{-1}$) or tetracycline ($12.5 \mu\text{g ml}^{-1}$) (Sigma). Blood agar plates contained 5% (v/v) defibrinated sheep blood.

Alveolysin preparation. The toxin was purified as described by Geoffroy & Alouf (1983). One HU corresponds to 1 ng (1.66×10^{-5} nmol) protein.

Titration of haemolytic activity. The assay was based on the determination of the haemolytic activity of the toxin preparation (activated with 20 mM-cysteine) as described previously (Geoffroy & Alouf, 1983). Haemoglobin released from sheep erythrocytes (6×10^8 cells ml^{-1}) incubated at 37°C with 1 ml of appropriately diluted toxin in phosphate-buffered saline (PBS) pH 6.8 (37 mM- NaH_2PO_4 , 37 mM- Na_2HPO_4 , 77 mM-NaCl) was determined spectrophotometrically at 541 nm. One HU is that amount of toxin needed to release 50% of the haemoglobin content of the erythrocytes. The incubation times for haemolytic assays were 45 min for alveolysin and 3 h for the haemolytic gene product expressed from plasmid pCG100, referred to as recombinant haemolysin (RH).

Preparation of *B. alvei* chromosomal DNA. DNA was prepared and purified according to G. Rapoport & A. Klier (personal communication). Briefly, the bacterial pellet from an 800 ml culture was suspended in 3 ml Tris/EDTA/NaCl buffer pH 8.0 (100 mM-Tris/HCl pH 8.0, 10 mM-EDTA, 150 mM-NaCl) and incubated for 10 min at 37°C with 1.6 mg lysozyme (Sigma). The suspension was incubated for an additional 1 h at 37°C in the presence of 1.25 mg pancreatic ribonuclease A (Sigma), dissolved in 1 ml distilled water and heated at 80°C for 10 min prior to use. After addition of sodium dodecyl sulphate to 1% (w/v), the mixture was incubated at 70°C for 15 min and then allowed to react for 1 h at 37°C with 40 mg proteinase K (Boehringer). The lysate was dialysed overnight at 37°C and extracted twice with phenol/chloroform (1:1, v/v) and DNA was then precipitated by addition of 2 vols absolute ethanol and 0.1 vol. 3 M-sodium acetate pH 4.8. After overnight incubation at -20°C , DNA was recovered, washed in 70% ethanol, dissolved in distilled water and stored at -20°C .

Isolation of plasmid DNA. The vector plasmid pBR322 and its recombinant derivative (pCG100) were isolated from *E. coli* strain SK1592 and purified according to Humphreys *et al.* (1975). The method of Birnboim & Doly (1979) was used for rapid preparation of plasmid DNA.

DNA digestion with restriction enzymes. Restriction endonucleases were used as recommended by the manufacturers (Boehringer and New England Biolabs). The electrophoresis of DNA fragments was done in 0.8% (w/v) horizontal agarose gels using TBE buffer pH 8.0 (45 mM-Tris, 45 mM-boric acid, 1.25 mM-disodium EDTA). The agarose gels and running buffer contained $0.5 \mu\text{g}$ ethidium bromide (Sigma) ml^{-1} . Gels were photographed on a UV transilluminator (Vilbert Lourmat) with a Polaroid camera equipped with red and yellow filters, using type 665 Polaroid film.

Construction of hybrid plasmid and transformation. Purified *B. alvei* DNA ($3 \mu\text{g}$) partially digested with *Hind*III (2 h, 37°C) was added to $3 \mu\text{g}$ of *Hind*III-digested pBR322. Ligation of DNA fragments (1000–1000 bp) was done using T4 DNA ligase and standard procedures (Maniatis *et al.*, 1982). The products of ligation were used to transform *E. coli* SK1592 cells previously rendered competent (Bolivar *et al.*, 1977). *E. coli* transformants were selected on either L-agar plates or blood agar plates containing appropriate antibiotics.

Ultrasonic disruption of transformed *E. coli*. Washed organisms from an overnight culture of *E. coli* carrying either pBR322 or its derivatives were resuspended in a 1/50 to 1/200 volume of PBS. The cell suspensions were subjected to ultrasonic disruption at 4°C using a Branson Cell Disruptor B30 sonicator (Heat Systems Ultrasonic) fitted with a microtip operated at a setting of 7. The cells were sonicated for 3×10 s bursts and allowed to cool for 30 s between treatments. The suspension was centrifuged (15 min, 10000 g, 4°C) and the supernate taken for the determination of haemolytic titre and other assays. This treatment was repeated up to six times on the same cell pellet to maximize recovery of haemolysin.

Nick-translation and Southern blot hybridization. Plasmid pCG100 DNA was labelled by nick-translation according to Rigby *et al.* (1977), with α - ^{32}P dATP and α - ^{32}P dCTP (NEN; 400 Ci mmol^{-1} , 14.8 TBq mmol^{-1}).

Hybridization experiments were done as described by Southern (1975), using Schleicher and Schüll BA85 nitrocellulose filters. *Hind*III-digested chromosomal DNA isolated from *Streptococcus pyogenes* strain A78 (Alouf, 1980), *Bacillus thuringiensis* strain H1-30 (serotype 1), kindly provided by H. de Barjac (Institut Pasteur, Paris), *Listeria monocytogenes* strain EGD (Geoffroy *et al.*, 1987) and *Clostridium perfringens* strain 5265 type C, kindly provided by M. Sebald (Institut Pasteur, Paris) were probed for hybridization.

Immune sera. Hyperimmune horse antistreptolysin O serum no. 525 (Alouf *et al.*, 1965) and rabbit anti-alveolysin serum (Alouf *et al.*, 1977) were used in neutralization, precipitation and immunoblotting experiments. Neutralizing titres of the sera were determined as described previously (Alouf *et al.*, 1965).

Agarose double immunodiffusion. Immunodiffusion assays were done on glass slides in 1% (w/v) agarose in PBS, pH 6.8. Wells of 4 mm were cut in the agar gel and filled with appropriate antigens and sera. The distance between the wells was 6 mm.

Purification of the recombinant haemolysin expressed in *E. coli*. The washed cell pellet from a 2-litre overnight culture was resuspended in 10 ml buffer and submitted to ultrasonic disruption as described above. The disrupted cell suspension was immediately centrifuged (15 min, 10000 g, 4 °C) and the supernatant fraction collected. The pellet was resuspended in 5 ml PBS and disrupted again. Six successive disruptions were made on the same pellet with decreasing amounts of buffer. The six supernatant fractions were combined (crude extract) and purified as follows.

Nucleic acids in the extract were precipitated by adding 10 mg protamine sulphate (Sigma) ml⁻¹ and removed by centrifugation (15 min, 10000 g, 4 °C). The supernate was concentrated by ultrafiltration through an Amicon PM-10 membrane and subjected to gel filtration on a Sephacryl S-200 column (Pharmacia) (2.5 by 100 cm) equilibrated in PBS, pH 6.8 (flow rate 10 ml h⁻¹, 3.5 ml fractions). The eluted haemolytic material was recovered in two peaks, corresponding to elution volumes of 300–390 ml and 390–600 ml respectively. About 60% of the haemolytic activity was recovered in the second peak at the same elution volume as that of alveolysin (Geoffroy & Alouf, 1983).

The second peak pool was concentrated by ultrafiltration (fraction P2) and electrofocused (16 h, 2 °C, 1600 V) in a 110 ml column (LKB) using a pH 3.5–10 sucrose gradient as described previously (Loridan & Alouf, 1986). The column content was collected (1 ml fractions) and monitored for pH (4 °C), *A*₂₈₀ and haemolytic titre. The haemolytic material focused in two peaks at pH 8.1 and 8.3, which were pooled and concentrated.

Inhibition of haemolytic activity by thiol group reagents and cholesterol. Haemolysin samples (alveolysin or RH) in a volume of 900 µl (25 HU) were incubated at room temperature for 30 min with 100 µl of appropriate dilutions of HgCl₂, *p*-chloromercuribenzoate, tosyl-lysine chloromethylketone (TLCK) dissolved in PBS, pH 6.8, and tosyl-phenylmethylketone (TPCK) (Geoffroy & Alouf, 1982), epicholesterol or cholesterol dissolved in double-distilled absolute ethanol (Geoffroy & Alouf, 1983). All reagents were from Sigma. The mixtures were brought to a final volume of 5 ml with PBS and their residual haemolytic activity determined. The results were expressed as the amount of reagent required to inhibit the lytic activity of 1 HU of toxin.

Neutralization by immune sera. Samples of alveolysin or RH (2 HU) were allowed to react with appropriate dilutions of rabbit anti-alveolysin serum and horse anti-SLO serum in a final volume of 1 ml as described by Alouf *et al.* (1965). The results were expressed as the amounts (in µl) of immune serum required to inhibit the lytic activity of 1 HU of toxin.

Protein assay. Protein was determined on crude and purified fractions by the method of Bradford (1976), using the Bio-Rad protein assay kit with bovine serum albumin (Sigma) as a standard.

Slab SDS-PAGE and immunoblotting. Slab SDS-polyacrylamide gradient gel electrophoresis was done according to Laemmli (1970). Protein samples (20–50 µl) containing 2% (w/v) SDS, 10% (v/v) glycerol and 5% (v/v) 2-mercaptoethanol in 62.5 mM-Tris/HCl buffer, pH 6.8, were boiled in a water bath for 90 s before being loaded on the gels. Electrophoresis was done in a linear gradient of 7.5–25% (w/v) acrylamide at 5 mA for about 15 h until the tracking dye (0.001% bromophenol blue) reached the bottom of the gel. After electrophoresis, the separated proteins were detected by staining with 0.2% Coomassie brilliant blue R-250 in methanol/water/glacial acetic acid (5:4:1, by vol.).

For Western blot analysis (Towbin *et al.*, 1979) the proteins were electrophoretically transferred to nitrocellulose sheets (BA85, Schleicher & Schüll) in a Trans-Blot cell apparatus (EMBL, Heidelberg, FRG) with 25 mM-Tris/200 mM-glycine (pH 8.4)/20% (v/v) methanol as the blotting buffer. The sheets were incubated for 1 h at room temperature with shaking in 50 mM-Tris/HCl, 150 mM-NaCl (pH 8.0) and 5% (w/v) skim milk (Regilait, France) prior to a 1 h incubation in anti-alveolysin or antistreptolysin immune sera diluted (1:20) in the above buffer. The sheets were washed eight times in buffer before addition of 20 ml milk buffer containing 1 µCi (37 kBq) ¹²⁵I-labelled protein A kindly prepared by N. Guiso (Institut Pasteur, Paris). Shaking was continued for a further 1 h and then the filters were washed six times in buffer supplemented with 0.1% Triton X-100. The filters were dried for 30 min at 80 °C and then autoradiographed using Kodak X-O-Mat (SO-282) film.

Preparation of membrane-bound haemolysins and analytical procedures. The standard erythrocyte suspension (6 × 10⁸ cells ml⁻¹) in PBS, pH 6.8, was incubated (500 µl, 30 min, 37 °C) with either 50 µl alveolysin (10000 HU,

10 µg) or 100 µl RH preparation (~10000 HU, 170 µg). After complete lysis, the membranes were sedimented by centrifugation at 15000 *g* for 10 min at 4 °C and the supernate was discarded. The ghosts were then washed twice in PBS, suspended in 50 µl of the same buffer and incubated overnight with α -chymotrypsin (Worthington) at a final concentration of 200 µg ml⁻¹. Membrane-associated proteins were then analysed by SDS-PAGE (~100 µg per lane) as described above.

Lethal activity. Specific-pathogen-free male ICR Swiss mice (Charles River), 6 to 8 weeks old, were used. The mice were injected intravenously with 0.5 ml PBS containing various amounts (2500, 1500, 1000, 750, 650 HU) of alveolysin or of the purified RH. The lethality was recorded 1 h after injection since, at dose levels which killed only some of the animals, death generally occurred within minutes or the animals survived (Alouf, 1980). The 50% lethal dose (LD₅₀) was determined by the probit method. Control injections consisted of 0.5 ml (13 mg protein) of the supernate generated following sonication of *E. coli* SK1592 harbouring pBR322.

RESULTS

Cloning of alveolysin in E. coli

A gene library of *B. alvei* total genomic DNA was constructed in *E. coli* using pBR322 as the cloning vector. *Hind*III-generated partial digest products of *B. alvei* DNA were ligated with

Fig. 1. Restriction map of the insert from the recombinant plasmid pCG100 containing the *B. alvei* alveolysin determinant studied in *E. coli* SK1592. Restriction enzyme cleavage sites: B, *Bam*HI, E, *Eco*RI, H, *Hind*III, P, *Pst*I, X, *Xba*I. No restriction sites were found with *Cla*I, *Kpn*I, *Pvu*II or *Sma*I.

Fig. 2. SDS-PAGE of purification fractions (Table 1) and of sheep erythrocyte ghosts obtained by lysis with alveolysin and RH. Lanes: 1, crude extract; 2, protamine-precipitated fraction (P1); 3, concentrated fraction from Sephacryl S-200 gel filtration (P2); 4, pooled haemolytic material (RH) after isoelectric focusing (P3); 5, sheep erythrocyte ghosts after lysis with RH; 6, same as lane 5, after chymotrypsin digestion; 7, purified alveolysin; 8, sheep erythrocyte ghosts after lysis with alveolysin; 9, same as lane 8, after chymotrypsin digestion; 10, *M_r* standards (Pharmacia *lit*) – phosphorylase *b* (94000), bovine serum albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), soybean trypsin inhibitor (20000), α -lactalbumin (14500).

*Hind*III-cleaved vector, and the ligated mixture was used to transform *E. coli* SK1592. Transformants were selected on L-agar and recombinant clones identified by their sensitivity to tetracycline. Of 3000 transformants examined, 360 (12%) were tetracycline sensitive. One haemolytic clone was identified among these following transfer of the transformants onto sheep blood agar, as evidenced by the formation after 24 h of a small zone of complete haemolysis (3 mm in diameter) around the colony. The recombinant plasmid pCG100 was isolated from this positive clone and purified by CsCl gradient centrifugation.

The recombinant plasmid was about 7.3 kb in size, with an insertion of about 3 kb. A restriction map of the DNA insert from *B. alvei* was constructed by a series of double enzyme digestions (Fig. 1). The analysis identified two *Bam*HI, two *Eco*RI, one *Xba*I and one *Pst*I sites, whereas no restriction sites were found with *Kpn*I, *Pvu*II, *Cla*I and *Sma*I.

The 3 kb DNA fragment labelled by nick-translation was used as a probe in Southern hybridization analysis of *Hind*III-digested chromosomal DNA isolated from *S. pyogenes*, *L. monocytogenes*, *B. thuringiensis* and *C. perfringens* strains, under hybridization conditions at a stringency of about 80%. No homology was detected.

Expression of alveolysin in *E. coli*

E. coli SK1592 harbouring pCG100 gave rise to colonies surrounded by a 3 mm haemolytic zone after 24 h of incubation. An identical pattern was exhibited by *B. alvei* colonies under the same conditions. No haemolytic zones developed with *E. coli* SK1592 harbouring pBR322 only. When the transformed *E. coli* cells were allowed to grow in the agar layer, the zones of haemolysis were larger and developed earlier than those obtained with surface-grown organisms. *E. coli* SK1592 harbouring pCG100 showed no detectable haemolytic material in the culture supernatant fraction when cultured with or without shaking in L-broth at 37 °C for up to

Table 1. Purification of recombinant haemolysin from *E. coli* SK1592

Step*	Vol. (ml)	Total protein (mg)	Total haemolytic activity (HU)	Specific activity [HU (mg protein) ⁻¹]	Purification (-fold)	Recovery (%)
Crude extract	34	882	165 000	187	1	100
P1	12	492	150 000	304	1.6	90
P2	4	11.6	100 000	8 620	47	60
P3	4	1.7	85 000	50 000	267	51

* P1, protamine sulphate purification; P2, gel filtration on Sephacryl S-200; P3, isoelectric focusing in a broad pH gradient.

Table 2. Comparative properties of alveolysin and cloned haemolysin (RH)

Property	Alveolysin	RH
pI	5.0	8.1-8.3
M_r	60 000	64 000
Haemolytic activity*	+	+
Inhibitors (μ mol HU ⁻¹)†		
Cholesterol	10 ⁻⁸	10 ⁻⁵
pCMB	1	1
HgCl ₂	1	1
TLCK	1	1
TPCK	2	2
Inhibition by immune sera‡		
Anti-alveolysin (μ l)	2	10
Anti-streptolysin O (μ l)	0.1	0.3
Lethal activity LD ₅₀ (in HU)	300	1000

* Tested on horse, rabbit and sheep erythrocytes as described in Methods.

† Amount of reagent required to inhibit the lytic activity of 1 HU of toxin. pCMB, p-chloromercuribenzoate; TLCK, tosyl-lysine chloromethylketone; TPCK, tosyl-phenylmethylketone.

‡ Amounts in microlitres of immune sera required to inhibit the lytic activity of 1 HU of toxin.

18 h. Furthermore, the bacterial pellet suspended in PBS was not haemolytic. However, when the cell pellet from an overnight culture was submitted to ultrasonic disruption followed by centrifugation at 10 000 *g* for 10 min, the cells released a haemolytic product into the suspending buffer. This material was subsequently identified as an alveolysin-like product (see below) with a titre of about 150 HU (mg protein)⁻¹. *E. coli* SK1592 harbouring pBR322 yielded under the same conditions a weakly active haemolytic material with a titre of about 10 HU (mg protein)⁻¹.

Purification and identification of the recombinant haemolysin

The purification process summarized in Table 1 yielded fraction 3, referred to as the recombinant haemolysin material (RH). The specific activity of this fraction was 267-fold higher than that of crude material.

The various fractions (Table 1) were analysed by SDS-PAGE (Fig. 2). The RH preparation exhibited a major band (*M_r* 64000) and several minor bands.

Comparative properties of alveolysin and recombinant haemolysin

These properties are summarized in Table 2. The haemolytic titres of the two preparations were practically identical when tested on horse, rabbit, and sheep erythrocytes. Their lytic activities were inhibited by the same concentrations of thiol group reagents, TPCK and TLCK, as previously described for alveolysin (Geoffroy *et al.*, 1981). As expected, cholesterol inhibited the lytic activity of both products, at concentrations of 4×10^{-3} ng HU⁻¹ and 4 ng HU⁻¹ for alveolysin and RH respectively. No inhibition with epicholesterol (400 ng HU⁻¹) was observed.

The RH and purified alveolysin preparations proved to be antigenically related. Immune sera raised against alveolysin and SLO were used to probe both preparations by immunoblotting (Fig. 3*a, b*). The haemolytic activity of these preparations was neutralized by the two sera. However, about three- to five-fold higher amounts of sera were required to neutralize 1 HU of RH as compared to alveolysin. Both haemolytic preparations showed total antigenic identity by agar double immunodiffusion against equine anti-SLO serum (data not shown).

The lytic process with RH was much slower (3 h for 100% lysis of the haemolytic system by 2 HU) as compared to alveolysin (45 min). Sheep erythrocyte ghosts resulting from lysis by either haemolysin showed a similar pattern on SDS-PAGE (Fig. 2). Both haemolysins were detected by immunoblotting at positions corresponding to their respective *M_r* values of 60000 for alveolysin and 64000 for RH (Fig. 3*a, b*). This finding suggests that RH binds to target cells and elicits lysis (Fig. 2, lane 5) in the same way as alveolysin (Fig. 2, lane 8) and other SH-dependent toxins.

When bound to sheep erythrocyte ghosts, both alveolysin and RH were resistant to enzymic digestion by α -chymotrypsin (Fig. 2, lanes 6, 9), whereas in solution, both haemolysins were totally degraded (data not shown).

The LD₅₀ of RH was about 1000 HU, as compared to 300 HU for alveolysin (Table 2). Death occurred within less than 5 min and the symptoms (convulsions, opisthotonos) were identical to those observed with alveolysin, SLO and listeriolysin O (Alouf, 1980; Geoffroy *et al.*, 1987). Control mice injected with supernatant material from sonicated *E. coli* SK1592 bearing pBR322 died after 24 h, probably due to LPS toxicity.

DISCUSSION

The antigenic, biochemical and biological similarity of the thiol-activated bacterial cytolytins is well documented (see Introduction). The understanding of the mode of action of these toxins requires knowledge of their structure. The first 29 N-terminal amino acid residues of alveolysin have been determined (Alouf *et al.*, 1986). Apparent homology between this sequence and that of shorter N-terminal sequences of other related toxins has been observed (Alouf *et al.*, 1986). The best approach for the determination of the amino acid sequences of these proteins would be that inferred from the nucleotide sequence of their structural genes. The genes encoding SLO (Kehoe & Timmis, 1984), pneumolysin from *Streptococcus pneumoniae* (Paton *et al.*, 1986; Walker *et al.*, 1987) and listeriolysin O from *Listeria monocytogenes* (Mengaud *et al.*, 1987, 1988)

Fig. 3. Western immunoblots of sheep erythrocyte ghosts obtained by lysis with alveolysin and RH. (a) Lanes 5 to 8 of Fig. 2 tested with rabbit anti-alveolysin immune serum; (b) RH (lane 1) and purified alveolysin (lane 2) tested with equine antistreptolysin O immune serum.

have been cloned and expressed in *E. coli*, and the nucleotide sequences of the cloned pneumolysin (Walker *et al.*, 1987), SLO (Kehoe *et al.*, 1987) and listeriolysin O (Mengaud *et al.*, 1988) genes have been determined.

Striking homologies at the protein level between these three toxins have been inferred from these determinations. The region of strongest homology was in the C-terminal end. The longest continuous identical sequence consisted of 12 residues encompassing the single Cys residue close to the C-termini of the molecules. However, when the cloned genes were used as probes no detectable homologies were observed at the DNA level (Kehoe & Timmis, 1984; Kehoe *et al.*, 1987; Mengaud *et al.*, 1987, 1988). It has been suggested that these genes have originated from a common evolutionary source but have undergone considerable divergence (Kehoe *et al.*, 1987).

In the present work, hybridization experiments with the cloned alveolysin determinant also failed to detect homology between this determinant and DNA from *C. perfringens*, *L. monocytogenes*, *S. pyogenes* and *B. thuringiensis* strains expressing high levels of their respective immunologically related thiol-dependent toxins.

The recombinant plasmid was stably maintained in *E. coli* SK1592 and directed the synthesis of a haemolysin exhibiting characteristics of alveolysin, namely (i) haemolytic activity and binding to target cells; (ii) specific inhibition of the haemolytic activity by cholesterol but not by epicholesterol; (iii) inhibition by thiol group reagents as well as TPCK and TLCK; (iv) neutralization, immunoblotting and immunoprecipitation by immune sera raised against alveolysin or SLO; (v) lethal activity in mice.

The recombinant haemolysin was not secreted from *E. coli* and required ultrasonic disruption for release. Thus either *B. alvei* possesses a protein-secretory apparatus that is lacking in *E. coli* or the alveolysin secretory signal is not recognized by the *E. coli* apparatus. A similar situation has been reported for the genes of the three above-mentioned cytolysins, as was also observed when cloned genes for haemolytic toxins and other extracellular toxins or enzymes from a number of Gram-positive and Gram-negative bacterial species were introduced into *E. coli* (Coleman *et al.*, 1983; Kehoe *et al.*, 1983; Chakraborty *et al.*, 1986; Guidolin & Manning, 1987).

The low amount (4%) of RH expressed in *E. coli* compared to that secreted by *B. alvei* (Geoffroy & Alouf, 1983) may be attributable to the impairment of toxin processing and transport in its new host, to a degradative turnover inside the cell, or to the likelihood that Gram-positive promoters function poorly if at all in a Gram-negative background. It is also possible that the native promoter of the alveolysin determinant was not cloned or functioning in the *E. coli* host, and that the RH was expressed from a vector promoter. Similar low yields of toxin have been reported for cholera toxin expressed in *E. coli* (Pearson & Mekalanos, 1982). In contrast, the amount of pneumolysin synthesized in *E. coli* is approximately one-third of that which accumulates in the cytoplasm of *S. pneumoniae* (Johnson, 1977; Paton *et al.*, 1986).

The RH expressed in *E. coli* differed in M_r and pI from alveolysin (Figs 2 and 3, Table 2), suggesting that it probably corresponds to the alveolysin molecule linked to its signal peptide. The basic pI of RH is consistent with this notion because signal peptides of most secreted proteins have a positively charged N-terminal region, a central hydrophobic core, and a more polar C-terminal region (Oliver, 1985; von Heijne, 1985). The structural differences between alveolysin and RH may explain the higher amounts of cholesterol required for the inhibition of the latter, the threefold lower LD₅₀ of RH and the slower rate of lysis. However, RH shared common epitopes with both alveolysin and SLO.

The determination of the nucleotide sequence of the alveolysin determinant will certainly contribute to a better understanding of the evolutionary, structural and functional relationships between the thiol-dependent toxins.

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