BACTERIAL PATHOGENICITY

Bactericidal activity of rat lung lavage fluid against *Bordetella pertussis*

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Cell-free lung lavage fluid (LLF) from healthy normal rats killed phase I (wild-type, virulent) *Bordetella pertussis* at 37°C *in vitro*. *B. parapertussis* was also killed by the LLF, but phase IV (avirulent mutant) *B. pertussis* and some other common bacterial species, including *B. bronchiseptica*, were not. Transmission electron microscopy of thin sections of the phase I *B. pertussis* showed extensive structural damage and cell lysis. None of the other mammalian species tested had LLF with bactericidal activity against *B. pertussis* as high as that of the rat. Rats killed with halothane yielded LLF with higher bactericidal activity than when CO₂ was used. Ultracentrifugation of LLF at 55 000 g gave a surfactant (pellet) fraction that had c. 95% of the bactericidal activity and which was biochemically distinct from the 5% of activity in the supernate fraction. Phospholipids and fatty acids appeared to be involved in LLF bactericidal activity, but not complement or lysozyme. Arachidonic acid was the most active of the fatty acids tested. Artificial surfactant, as used in premature infants, had no bactericidal effect on *B. pertussis*.

Introduction

The natural habitat of *Bordetella pertussis*, the causative agent of whooping cough, is on the ciliated epithelium of the human tracheobronchial tree [1, 2]. There, the bacteria encounter the host respiratory secretions which may provide both nutrients and bactericidal factors – cellular, dispersed or dissolved. Because of the difficulty in studying respiratory tract secretions from healthy human infants, this study investigated the possible bactericidal activity of cell-free lung lavage fluid (LLF) or ‘tracheobronchial washings’ from normal rats. This species was chosen because of its utility in the coughing rat model of pertussis, the rat being the only species (apart from some primates), where a paroxysmal cough develops after intrabronchial infection with *B. pertussis* [3–8].

The bactericidal activity of cell-free LLF from the rat against *Streptococcus pneumoniae* was reported by Coonrod and co-workers [9, 10], who showed that the activity was located in the surfactant fraction and was due to long-chain fatty acids. These fatty acids,

although of low mol. wt, were associated with phospholipids in LLF and were sedimented during centrifugation for 20 min at 55 000 g, leaving a supernate fraction which had very little bactericidal activity. Other investigators have reported that pulmonary surfactant from sheep, although lacking direct bactericidal activity, enhanced the killing of *Pasteurella haemolytica*, *Escherichia coli* and *Klebsiella pneumoniae* by normal serum [11]. LaForce and Boose [12] found a peptide in rabbit LLF that facilitated killing of *E. coli* by deoxycholate, while O'Neill *et al.* [13] described a rat alveolar lining material that enhanced the killing of *Staphylococcus aureus* by alveolar macrophages. The object of the present work was to explore the possible bactericidal activity of normal rat LLF against *B. pertussis* and other bordetellae. A previous report from this laboratory described the ability of bordetellae, other than phase I *B. pertussis*, to use LLFs of various mammalian species (which did not include the rat), as growth media [14].

Materials and methods

Bacterial strains and bactericidal tests

Most of the work was done with phase I (wild-type, virulent) *B. pertussis* 18-323 (ATCC 9797) available as

a stock culture in this laboratory. In addition, the following strains of *Bordetella* were similarly obtained: phase I *B. pertussis* Tohama, L84, D30042, 77/18329, 44122/7S, 44122/7R and 18-334; phase IV (avirulent mutant) *B. pertussis* L84 and D30042; *B. pertussis* BP347 (avirulent, Vir⁻ transposon-insertion mutant); *B. parapertussis* NCTC 10520; *B. bronchiseptica* NCTC 5376. Other laboratory stock cultures were of *E. coli* Lilly (NCIMB 11888), *Micrococcus luteus* (NCTC 2665), *S. aureus* 'Oxford' (NCTC 6571) and *Str. pyogenes* (NCTC 5763). All *Bordetella* strains were grown at 37°C on Bordet-Gengou (BG) agar (Becton Dickinson), *E. coli* and *M. luteus* on brain heart infusion agar, *S. aureus* on blood agar and *Str. pyogenes* on MacConkey agar. *B. pertussis* 18-323 was cultured on BG agar for 3 days, subcultured for 24 h and then suspended in casamino acids (CAA) 1% prepared according to Hall *et al.* [6].

For bactericidal tests, suspensions of *B. pertussis* and other bacteria in CAA were standardised to absorbance $A_{540\text{ nm}} = 0.45$ in a 1-cm cuvette [14]. The subsequent dilution in either phosphate-buffered saline (PBS, prepared as described by Porter *et al.* [15]) or CAA varied according to the bacterial strain and species, and was in the range of 1 in 2000 to 1 in 40 000, to give *c.* 10^5 cfu/ml. Bactericidal tests with *B. pertussis* were done by delivering 240 µl of test fluid into 2-ml polypropylene plastic vials with 'O' ring screw caps (Elkay Lab Products UK) and adding 10 µl of *B. pertussis* suspension containing *c.* 10^3 cfu. After mixing, time-zero samples were removed and vials were incubated at 37°C with shaking at 110 rpm for 120 min. At intervals, 20-µl samples were plated on to BG and colonies were counted after the plates had been incubated for 5 days. Both PBS and CAA were used as control fluids. Cyclodextrin liquid (CL) medium was used as a non-bactericidal control and was prepared according to Imaizumi *et al.* [16], except that 50 µl of the vitamin supplement were added to 10 ml of CL-medium base instead of 100 µl. CL is a good growth medium for *B. pertussis*, supporting growth from small inocula, whereas CAA is a maintenance medium. The results of bactericidal tests with LLF and its fractions were expressed as the percentage survival, taking 100% as the viable count at time zero in PBS. Tests on other bacteria were done similarly, but with plating on the same culture medium as used for growth of the inocula.

Animals

Male and female barrier-reared Sprague-Dawley rats were obtained from Harlan Olac (Shaws Farm, Blackthorn, Bicester). Each batch of rats (Viral Antibody-Free Plus status) was certified as free from a range of protozoal, viral and bacterial pathogens including *B. bronchiseptica*. Rats were obtained at 3–5 weeks of age and were 6–12 weeks (150–200 g) at the time of obtaining blood and LLF.

Preparation of LLF and serum

Four different methods, A, B, C and D, were used to kill the animals before collecting blood and lung washings. In methods A and D, the rats were anaesthetised with halothane (RMB Animal Health, Dagenham) and killed either with CO₂ (method A) or by cervical dislocation (method D) after removal of blood by heart puncture. In methods B and C, the animals were killed with CO₂ and by overdose of halothane, respectively. In all cases lung washings were collected from the recently killed animals by tracheal cannulation and gentle rinsing of the airways with 5 ml of PBS, of which about 3.5 ml was recovered. The subsequent processing of the lung washings to yield LLF was as described by Coonrod and Yoneda [9], with a low speed spin (160 *g*) to remove cells and debris, and storage was at -70°C. Thus, LLF used in this study comprised diluted and cell-free respiratory secretions. Early batches of LLF were membrane-filtered (0.45 µm pore size), but this was omitted with later batches as it was found to be unnecessary. The surfactant (SUR) and supernate (SN) fractions were prepared by ultracentrifugation at 55 000 *g* for 20 min at 4°C as described by Coonrod *et al.* [10]. LLFs from other species (calf, chicken, dog, horse, mouse, rabbit, sheep and man) were collected as described previously [14].

Long-chain fatty acids and related compounds

Myristic and palmitic acids were obtained as sodium salts and others as free acids: arachidonic, elaidic, linoleic, oleic, palmitoleic (all from Sigma). Pentadecanoic and petroselinic acids were dissolved by brief sonication in 10 mM NaOH to a concentration of 10 mM and were then diluted in CAA to 1 mM–0.1 µM and stored at -20°C. Myristic and palmitic acids were dissolved in methanol and diluted with chloroform to 10 mM. The other fatty acids were dissolved to 10 mM in chloroform. The fatty acid solutions were then further diluted in chloroform to 1 mM–0.1 µM and stored at -20°C. When required for bactericidal tests, the chloroform solutions were placed in a container of warm water in a fume cupboard and dried under a slow stream of nitrogen delivered through a plugged pasteur pipette with its tip near to the top of the solvent surface. The dried fatty acids were reconstituted with 2.7 ml of CAA and exposed to brief sonication to aid dissolution. Methanol or chloroform solvents without fatty acids were subjected to the same procedures, to prepare the CAA control fluid. Phosphatidylcholine (Sigma) was diluted in chloroform to 0.6–0.02 mM, after which aliquots were dried under nitrogen, reconstituted with CAA to the original volume and sonicated briefly. Artificial lung surfactant (ALEC; Britannia Pharmaceuticals, Redhill, Surrey) consisted of dipalmitoylphosphatidyl choline and phosphatidylglycerol at a ratio of 7:3 [17]. The sterile freeze-dried powder (100 mg) was suspended in 1.1 ml of saline and vortex mixed.

Analytical procedures on LLF

Protein concentration was estimated by the bicinchoninic acid method of Smith *et al.* [18], with bovine serum albumin (BSA; Sigma) as standard. Fatty acids were determined by the procedure of Duncombe [19] with a standard consisting of equimolar amounts of all the fatty acids listed above. Lysozyme was assayed with *M. luteus* [20] against a standard of hen's egg white lysozyme, and bactericidal complement was assayed by killing of *E. coli* Lilly, as described elsewhere [21].

Lipid extraction

Lipids were extracted and separated from freshly prepared surfactant (SUR) from LLF as described by Bligh and Dyer [22] and modified by Coonrod *et al.* [10].

Transmission electron microscopy

Standardised ($A_{540\text{nm}} = 0.9$) *B. pertussis* 18-323 suspension (200 μl) was mixed with 400 μl of PBS, SUR or serum and incubated for 1 h at 37°C. Samples were centrifuged for 15 min at 10 000 rpm and the pellets were fixed in glutaraldehyde 3% and post-fixed in osmium tetroxide 0.2%, both made up in 0.2 M phosphate buffer. The material was then stained in uranyl acetate, rinsed in distilled water, embedded in agar, dehydrated and embedded in Spurr resin. Sections were cut with a diamond knife on an LKB III microtome at a thickness of 60 nm (silver coloured sections), picked up on copper grids (300 mesh), stained in uranyl acetate and lead citrate and examined on a Zeiss 902 transmission electron microscope.

Statistical analysis

Minitab statistical software (Macintosh version, release 8; Clecom Microsoft Specialists, Birmingham) was used to obtain mean values and SEM, median values and their 95% confidence limits. Analysis of variance, combined with the Dunnett procedure, was done to compare significance of strain means with that of strain 18-323, which was taken as standard.

Results

Detection of LLF bactericidal activity with *B. pertussis* 18-323

It was found early in this investigation that LLF from normal Sprague-Dawley rats had readily detectable bactericidal activity towards *B. pertussis* 18-323 during incubation at 37°C for 2 h. This strain was the main test organism throughout the present work for this reason and because of its use as the standard strain for intrabronchial infection in the coughing rat model of pertussis. Fig. 1 presents accumulated results from a large number of experiments with 16 batches of LLF from c. 160 normal rats, and with PBS, CAA and CL

medium. The bacteria lost viability much more rapidly in LLF than in PBS; the survival rates at 30 min were 30% and 80% respectively. At the same incubation time, CL medium gave the highest (100%) survival and CAA gave a survival similar to that in PBS. Incubation of *B. pertussis* in the serum of normal rats (data from 32 experiments, not shown) gave only 6% survival after 30 min. Thus LLF was not as rapidly bactericidal as serum.

Batch variation in bactericidal activity of LLF

A total of 16 batches of LLF, each constituting a pool from 10–20 rats, was tested for bactericidal activity against *B. pertussis* 18-323 (Fig. 2). Most of these batches were tested several times, so that altogether a large number of replicate tests was performed. Fig. 2 shows that there was considerable batch-to-batch variation in the percentage survivals at 30 and 120 min, and also that the method of killing the rats before collection of the LLF influenced the bactericidal activity. Method A, which involved halothane anaesthesia followed by killing with CO₂, was used for five batches – of which four were similar and one was distinctly more active. Method B, in which the rats were killed with CO₂, was used with four batches which were of consistently low activity, leading to the abandonment of this procedure. In method C, halothane alone was used, but only one batch of LLF was collected by this method. This batch had bactericidal activity similar to that of batches collected by method A. The procedure finally adopted was method D, in which halothane anaesthesia was applied before cervical dislocation. Fig. 2 shows that five of the six batches of LLF collected by this method reduced the *B. pertussis* viable count to zero at 120 min, and with three of the batches this reduction had occurred by 30 min.

The means of the 30-min survival values for strain 18-323 in LLF from the four different methods of euthanasia were 20%, 82%, 11% and 12%, respectively, and method D gave LLF that was significantly more active than those obtained by method A. This was confirmed by analysis of variance with method of killing as the factor and gave a *p* value of 0.002, which was highly significant. With the 120-min data, the means were 4%, 55%, 3% and 3%, respectively, and the *p* value from analysis of variance was 0.002, again highly significant. Therefore, it was concluded that the LLF obtained by method D had higher bactericidal activity than that yielded by the other methods.

Of the 16 batches of LLF shown in Fig. 2, eight were from male rats and eight from females, distributed randomly in the series. There did not appear to be any relationship between the sex of the rats and the bactericidal activity of the LLF, after allowing for differences in methods of killing. The possibility that

Fig. 1. Mean (SEM) percent survival of *B. pertussis* 18-323 in PBS (-○-; $n = 133$), CL-medium (-□-; $n = 118$), 1% casamino acids (-■-; $n = 13$) and LLF (-●-; $n = 92$) from normal rats after incubation for 120 min at 37°C.

the age, and therefore the size, of the animals might influence the bactericidal activity of LLF was also explored, but no significant trend emerged from comparisons of rats weighing <200 g and those that were heavier.

Other sources of LLF

LLF from other mammalian species were also tested against *B. pertussis* 18-323. Similar tests with incubation for up to 2 h at 37°C were done on single samples of LLF from calf, chicken, dog, horse, mouse, rabbit, sheep and man, collected by various methods, the human sample being from an adult patient undergoing bronchoscopy [14]. Bactericidal activity was detected at 2 h in the calf LLF (40% survival) and in the human LLF (30% survival), but the other species appeared to be totally inactive. Indeed, some of them gave better maintenance of *B. pertussis* viability (80–90%) than the 60–70% (Fig. 1) observed in PBS at 2 h.

Other *B. pertussis* strains

Eight other phase I, one Vir⁻ transposon-insertion mutant and two phase IV strains of *B. pertussis* were

tested in parallel with strain 18-323 and with the same batch of LLF. PBS was used as a non-bactericidal control. The results (Table 1) show that there was considerable variation between the *B. pertussis* strains in their sensitivity to killing by LLF. With strain 18-323 as the standard for comparisons, strains 441227/S, Tohama and L84 phase I were significantly ($p \leq 0.05$) more sensitive to the bactericidal activity of LLF, while the phase IV strains L84 and D30042 were more resistant. With these latter strains, the small decrease in viability was not significantly different from that in PBS. The other *B. pertussis* strains did not differ significantly from strain 18-323 in their sensitivity to LLF. All strains showed similar slight losses in viability during incubation for 120 min at 37°C in PBS.

Protein, lysozyme and long-chain fatty acids in LLF

Fifteen of the 16 batches of LLF collected from rats after the different methods of killing, and whose bactericidal activity results are presented in Fig. 2, were analysed for protein, lysozyme and long-chain fatty acids. Table 2 shows that the protein values ranged from 125 to 635 mg/ml, with an overall mean

Fig. 2. Bactericidal activity of 16 batches of LLF from rats exposed to four different methods of killing (A, halothane, CO₂; B, CO₂; C, halothane; D, halothane, cervical dislocation), towards *B. pertussis* 18-323 after incubation times of 30 (□) and 120 (●) min.

Table 1. Survival of different *B. pertussis* strains in rat LLF and PBS

<i>B. pertussis</i> strain	Phase	Mean percentage survival* (SEM) in			
		LLF		PBS	
		30 min	120 min	30 min	120 min
18-323	I	66.9 (7.7)	13.0 (7.7)	101 (6.2)	77.4 (1.9)
18334	I	59.6 (7.7)	39.7 (8.2)	96.0 (11.5)	69.8 (14.2)
44122/7S	I	20.3 (6.2) [†]	0 [†]	101.5 (6.8)	83.7 (6.2)
44122/7R	I	46.0 (5.5)	5.6 (1.2)	84.9 (5.1)	65.3 (7.0)
Tohama	I	24.9 (9.4) [†]	2.7 (2.1) [†]	92.5 (6.6)	84.0 (10.3)
BP 347	Vir ⁻	37.4 (20.2)	29.7 (17.4)	89.1 (9.3)	89.9 (5.4)
77/18319	I	32.0 (17.1)	22.0 (13)	85.4 (6.5)	82.3 (3.0)
L84	I	7.6 (4.4) [†]	0 [†]	125.9 (41)	84.5 (14.2)
L84	IV	98.7 (4.4)	89.8 (6.0) [†]	91.7 (3.2)	83.3 (4.2)
D30042	I	41.0 (16)	19.4 (9.7)	79.7 (7.9)	72.0 (4.7)
D30042	IV	94.4 (3.4)	90.8 (3.7) [†]	95.6 (3.9)	84.2 (1.4)

*Based on zero-time counts in PBS.

[†]Significantly different ($p \leq 0.05$) from strain 18-323.

(and SEM) of 382.1 (39.4) mg/ml. Analysis of variance, with euthanasia as the factor, revealed no significant differences ($p = 0.164$). With lysozyme, the range of values was from 16 to >108 µg/ml, with a

median of 84 (95% confidence limits = 59, 91). Here there were differences according to the method employed; method D (median = 102) gave results at least 50% higher than methods A, B and C, which had

Table 2. Protein, lysozyme and long-chain fatty acids in 15 batches of rat LLF, in relation to method of killing

Batch no.	Method of killing*	Protein $\mu\text{g/ml}$ (SEM)	Lysozyme $\mu\text{g/}$ (SEM)	Long-chain fatty acids μM (SEM)
1	A	125 (5)	16 (1)	26 (1)
2	A	322 (4)	57 (1)	50 (6)
3	A	477 (7)	76 (3)	NT
4	A	338 (4)	93 (1)	50 (4)
5	B	320 (3)	84 (3)	46.0
6	B	613 (6)	92 (1)	48 (4)
7	B	635 (10)	47 (1)	NT
8	B	355 (3)	44 (1)	31.0
9	C	140 (3)	40 (0)	16 (1)
10	D	215 (3)	65 (2)	13 (1)
11	D	338 (2)	99 (1)	60 (9)
12	D	480 (6)	88 (1)	57 (1)
13	D	400 (3)	>108 (0)	47
14	D	529 (6)	>108 (0)	32 (0)
15	D	445 (2)	105 (1)	170 (25)
Mean (SEM)		382.1 (39.4)	...	49.7 (10.8)
Median (95% confidence limits)		...	84 (59, 91)	...

NT, not tested.

*A, halothane, CO₂; B, CO₂; C, halothane; D, halothane, cervical dislocation.

medians of 67, 66 and 40 $\mu\text{g/ml}$ respectively. However, the differences did not quite reach statistical significance ($p = 0.095$) in a non-parametric analysis of variance. With long-chain fatty acids, the range was from 13 to 170 μM , with an overall mean (and SEM) of 49.7 (10.8) μM and with no apparent influence of the method of killing ($p = 0.695$). Extensive attempts were made to explore possible correlations between *B. pertussis* survival at 30 and 120 min and the levels of protein, lysozyme and fatty acids in the different batches of LLF; however, no significant correlations emerged.

Surfactant and supernate fractions

For ultracentrifugal separation of LLF by the method of Coonrod *et al.* [10], the fluids were collected from the rats by method D with sterile precautions. The LLF was not membrane-filtered and sterility tests showed that there was little or no bacterial contamination. Ultracentrifugation at 55 000 g and 4°C for 20 min yielded a pellet, the surfactant (SUR) fraction and a supernate (SN). After separation, the former was reconstituted to the original LLF volume with sterile

PBS. Table 3 presents the bactericidal activity of five batches of LLF and their SUR and SN fractions. There was considerable variation in the activity of the different batches of LLF, despite all having been collected by the same procedure and with pool sizes of between 10 and 20 rats. Thus, batch 13 of LLF, although giving significant killing, nevertheless left 16% of the *B. pertussis* viable after 2 h. In contrast, the other four batches tested in parallel had reduced the viable count to zero by this time. The middle columns of Table 3 show that the SUR fraction possessed much more bactericidal activity than the SN and might, if anything, be more active than the unfractionated LLF. All the batches of SN were active, but much less so than SUR.

Biochemical analyses of SUR and SN

Five different batches of LLF yielded SUR and SN fractions that contained, respectively, *c.* 8% (mean, 33 $\mu\text{g/ml}$) and 88% (mean, 381 $\mu\text{g/ml}$) of the protein originally present (mean, 432 $\mu\text{g/ml}$). The SUR protein was apparently aggregated to some extent by the ultracentrifugation, as over half of it was retained by a 0.45- μm pore size membrane filter. This corresponded to similar losses of bactericidal activity that were found after membrane filtration of this fraction. In contrast, the original LLF could be membrane-filtered without significant loss of either protein or bactericidal activity. Lysozyme assays revealed that >95% of the enzyme originally present in the LLF could be recovered in the SN fraction, which had only low bactericidal activity against *B. pertussis* 18-323. The mean yield of lysozyme in the SUR fraction was <5% of that in the LLF. With the long-chain fatty acids, the main part (*c.* 85%) was in the SUR fraction and only *c.* 15% in the SN.

Inactivation of bactericidal activity

To obtain information about the chemical nature of the bactericidal substances in the SUR and SN fractions, these were exposed to various physical and biochemical treatments. Generally, the bactericidal activity of SUR was much more stable than that of SN. Thus there was little or no loss of activity of SUR after the following treatments: brief sonication, freezing and thawing (10

Table 3. Bactericidal activity of five batches of rat LLF against *B. pertussis* 18-323, and the division of activity between the surfactant (SUR) and supernate (SN) fractions

LLF batch no.	Percentage survival* (number of observations)					
	unfractionated LLF		SUR		SN	
	30 min	120 min	30 min	120 min	30 min	120 min
11	0.6 SEM 0.6 (10)	0 (10)	0 (6)	0 (6)	11.3 SEM 7.3 (6)	0 (6)
12	7.5 SEM 7.5 (2)	0 (2)	0 (2)	0 (2)	61 SEM 13 (2)	0 (2)
13	62 SEM 7.0 (2)	16 SEM 7.0 (2)	36.5 SEM 0.5 (2)	0 (2)	76.5 SEM 14.5 (2)	11.5 SEM 6.5 (2)
14	0 (2)	0 (2)	0 (4)	0 (4)	27.5 SEM 10.1 (4)	4.5 SEM 2.7 (4)
15	0 (2)	0 (2)	0 (3)	0 (4)	10.3 SEM 8.9 (3)	0.8 SEM 0.8 (4)

*Based on zero-time counts in PBS.

cycles, from -70°C to $+20^{\circ}\text{C}$), dialysis, heating at 100°C for 15 min, 18 h at 4°C in NaOH or HCl to final concentrations of 0.1 M (followed by neutralisation), or addition of bentonite (Sigma) at 11 mg/ml, bovine serum albumin (Sigma) at 1 mg/ml or starch (BDH) at 1 mg/ml. However, there was complete loss of bactericidal activity after addition of charcoal (Sigma) at 17 mg/ml and partial loss of activity with 2,6-O-dimethyl- β -cyclodextrin (Teijin, Tokyo, Japan) at 1 mg/ml. In contrast, the SN fraction was extensively or completely inactivated by 15 min at 100°C , 18 h at 4°C with HCl to 0.1M (less so with NaOH) followed by neutralisation, bentonite, bovine serum albumin and starch, as well as by the charcoal treatment that inactivated the SUR. A problem as regards testing the SN fraction for BA was the variable spontaneous loss of activity during simple incubation at 37°C for 30 min.

The SUR and SN fractions were also tested for sensitivity to proteases and phospholipases (all from Sigma). The bactericidal activity of SUR towards strain 18-323 was not affected by exposure for 30 min at 37°C to trypsin, chymotrypsin, subtilisin or proteinase K, each at 2 mg/ml. These enzymes alone had no activity against *B. pertussis*. This suggested that the bactericidal activity of SUR did not involve a protein. On the other hand, there was extensive loss ($>94\%$) of bactericidal activity after treatment with phospholipase A2 (1 mg/ml), but not with phospholipase C (0.26 mg/ml). This provided evidence that the bactericidal activity of SUR might involve phospholipid. However, pure phospholipid, in the form of phosphatidyl choline, had no bactericidal activity on its own against *B. pertussis* 18-323. Likewise an artificial lung surfactant preparation (ALEC) that is given therapeutically to premature babies, also lacked activity towards *B. pertussis*.

Interpreting the effects of enzyme treatments of the SN fraction was hindered by the instability of the bactericidal activity in this fraction at 37°C . However, it appeared that trypsin had no effect, that chymotrypsin accelerated the loss of activity and that

subtilisin and proteinase K possibly protected the bactericidal activity against inactivation at 37°C . Phospholipase A2 had only a slight inactivating effect, while phospholipase C was, if anything, protective against 37°C inactivation. The SN fraction was not examined further because of its instability and relatively small contribution to killing by LLF.

SUR and SN activity towards other bacteria

Two different LLF batches (nos. 15 and 16) from which SUR and SN were obtained with similar bactericidal activity were used for testing their effect on other bacteria (Table 4); *B. pertussis* 18-323 was used as a control. Both the SUR and SN fractions from LLF batch no. 15 killed the *B. paraptussis* strain as effectively as they did *B. pertussis* after incubation for 1 h. In contrast, a *B. bronchiseptica* strain was completely resistant and, after overnight incubation (1440 min), had grown in both fluids. *Str. pyogenes* likewise survived and grew in both SUR and SN. *E. coli* Lilly was insensitive to killing by either fraction during 2 h at 37°C , indicating the absence of complement bactericidal activity. *S. aureus* grew well in SN, but died slowly in SUR. Thus, of the bacterial species tested, only *B. paraptussis* resembled *B. pertussis* in being sensitive to killing by the SUR and SN fractions of rat LLF.

Dilution assay

Although most bactericidal experiments with LLF and fractions derived from it were done with undiluted samples, several batches of LLF, and their SUR and SN fractions, were titrated to obtain 50% bactericidal activity end-points with *B. pertussis* 18-323 after incubation for 1 h at 37°C . The results shown in Fig. 3 with two representative batches show that LLF and SUR had very similar end-points at dilutions of *c.* 1 in 25 and 1 in 30, while the SN fraction was much less active and gave an end-point at a dilution of *c.* 1 in 2. The SUR and SN fractions of the two batches were so similar that composite data are plotted in the figure

Table 4. Bactericidal activities of the surfactant (SUR) and supernate (SN) fractions from two batches of rat LLF against different bacterial species

LLF batch and bacterial species	Percentage survival* after incubation in					
	SUR			SN		
	60 min	120 min	1440 min	60 min	120 min	1440 min
LLF batch no.16						
<i>B. pertussis</i> 18-323	0	0	NT	49	38	NT
<i>E. coli</i> Lilly	77	85	NT	376	750	NT
LLF batch no.15						
<i>B. pertussis</i> 18-323	0	0	0	0	0	0
<i>B. paraptussis</i> 10520	0	0	0	0	0	0
<i>B. bronchiseptica</i> 5376	84	101	650	86	119	1300
<i>Str. pyogenes</i> 5763	118	117	1360	140	184	2700
<i>S. aureus</i> 'Oxford'	46	36	1	98	131	3900

NT, not tested.

*Mean of duplicate tests that gave closely similar results and based on zero-time counts in PBS.

Fig. 3. Bactericidal dilution assay of two batches of LLF and fractions: mean (SEM) percent survival of *B. pertussis* 18-323 incubated for 1 h at 37°C in LLF (-O-; n = 2); surfactant (-■-; n = 12); supernate (-●-; n = 10); percent survival was calculated from the PBS time 1-h counts.

rather than showing the individual preparations. Here again, there was a preliminary indication that SUR might be slightly more active than the LLF from which it had been prepared.

Cytological changes

Thin sections of preparations of *B. pertussis* 18-323 treated in various ways were examined by transmission electron microscopy to obtain morphological information about the effect of rat SUR on the bacteria after incubation for 1 h at 37°C. Organisms similarly exposed to PBS and normal rat serum were included as negative and positive controls, respectively, for bactericidal activity. Fig. 4a shows that cells incubated in PBS for 1 h had mainly normal morphology, with a few apparently having lost their internal contents and others appearing irregular in shape. After exposure to normal rat serum (Fig. 4b) many of the cells were severely damaged, some having lost their internal structures. More severe damage was noticed with cells

incubated in SUR (Fig. 4c) which caused cell envelope rupture, irregular cell morphology and complete disappearance of the internal structure of some cells.

Activity of long-chain fatty acids

Because of the demonstration by Coonrod *et al.* [10] that the anti-pneumococcal activity of rat LLF was due in part to long-chain fatty acids, these substances were tested for their effect on *B. pertussis* 18-323. Tests were done with a selection of fatty acids, both saturated and unsaturated, and of chain length from 14 to 20 carbon atoms (14C–20C). The fatty acids were tested in CAA solution because they dissolved more easily than in PBS (after their primary solution in methanol or chloroform). They were tested individually at a range of molarities and as dilutions of a mixture of equimolar concentrations. The most bactericidally active fatty acid (Table 5) towards strain 18-323 was the polyunsaturated arachidonic (C_{20:4}) acid, which gave a 50% survival end-point at 20 µM. The least

Fig. 4. Transmission electron microscopy of thin sections of *B. pertussis* 18-323 after incubation for 1 h at 37°C in: a, PBS; b, serum; c, surfactant (× 25 500).

Fig. 4.

Table 5. Effect of long-chain fatty acids (LCFA) dissolved in casamino acids (CAA) on the viability of *B. pertussis* 18-323

LCFA	Carbon chain*	50% survival end-point† (μ M)
Myristic	14:0	500
Pentadecanoic	15:0	63
Palmitic	16:0	60
Palmitoleic	16:1(9c)	30
Elaidic	18:1(9t)	100
Petroselinic	18:1(6c)	60
Oleic	18:1(9c)	55
Linoleic	18:2(9c, 12c)	55
Arachidonic	20:4(5c, 8c, 11c, 14c)	20
LCFA mixture	-	55

*Number of carbon atoms: number of double bonds, and cis (c) or trans (t).

†Mean of duplicate tests that gave closely similar results and based on the 1-h counts in casamino acids (diluent for fatty acids).

active was the saturated myristic acid ($C_{14:0}$) with an end-point at 500 μ M. After arachidonic, the next most active acid was palmitoleic ($C_{16:1}$). Unsaturation of the fatty acids was not closely correlated with bactericidal activity, as the saturated palmitic ($C_{16:0}$) was quite similar to the unsaturated palmitoleic. The equimolar mixture of the fatty acids gave a 50% survival end-point at 55 μ M, very close to the mean concentration of fatty acids in LLF.

Solvent fractionation

To gain preliminary information on the association of bactericidal activity with long-chain fatty acids and with phospholipids, the SUR fraction in which most of the activity occurred was subjected to solvent fractionation, as described by Coonrod *et al.* [10]. After chloroform-methanol extraction, most of the bactericidal activity went into the chloroform layer, indicating the involvement of hydrophobic substances in this activity. This extract was then loaded on to a silicic acid column and eluted with chloroform, whereupon all the applied activity was recovered. According to Coonrod *et al.* [10] this fraction would be expected to contain neutral lipid and fatty acids. No further bactericidal activity was released from the silicic acid by successive acetone or methanol elutions, which should have eluted glycolipids and phospholipids, respectively.

Discussion

The traditional view of *B. pertussis* (and other *bordetellae*) as growing attached to ciliated epithelium in the respiratory tract of the host implies that the bacteria are bathed in the local secretions containing both nutrients and antibacterial substances. It was shown in previous work [14] from this laboratory that LLFs from seven vertebrate species (which did not include the rat) supported growth to varying extents from small inocula of *B. paraptussis*, *B. bronchisept-*

tica and *B. avium*. With *B. pertussis*, there was no growth in LLF from any of the vertebrate species tested. The focus in the present work was on bactericidal substances in lung washings from normal Sprague-Dawley rats, as used in the coughing rat model of pertussis [5–8]. With *B. pertussis* 18-323 as the routine test organism, LLF from these rats had readily detectable bactericidal activity; LLFs from other mammals were much less active. Only moderate bactericidal activity was detected in a single sample of human LLF from an adult patient with suspected carcinoma who underwent bronchoscopy. Clearly it would be desirable to test a range of samples of human LLF or, better still, undiluted respiratory tract secretions from healthy non-immune human infants. However, it would be difficult to obtain such normal secretions, both for ethical reasons and because of the high uptake of pertussis vaccine for routine immunisation of infants. Therefore, although rats are not natural hosts for *B. pertussis*, their high health status ensured absence of prior exposure to *B. pertussis* or other *bordetellae* that might have generated cross-reacting antibodies.

An important variable for collecting LLF with high bactericidal activity was the method of killing the rats before lung lavage. Of the several methods investigated, it appeared that CO_2 should be avoided, as it gave LLF with lower activity than other procedures. The best method was halothane anaesthesia followed by cervical dislocation. The possibility that halothane in LLF and its fractions contributed to the activity was shown to be unlikely. The boiling point of halothane is 50°C and the bactericidal activity of the SUR fraction of LLF was unaffected by boiling for 15 min. Coonrod *et al.* [10] also found halothane satisfactory for collecting rat LLF with bactericidal activity towards *Str. pneumoniae*. However, these authors administered it in overdosage, corresponding to method C in this work. In a previous paper [9], intraperitoneal pentothal had been used. This suggests that Coonrod and co-workers also considered the method of killing to be an important variable in collecting LLF from rats.

B. pertussis strain 18-323, which was used in most of the experiments with LLF, was chosen because of its use both in the coughing rat model of pertussis and also in the standard mouse protection test for pertussis vaccines [6, 23]. However, strain 18-323 was not the most sensitive strain of *B. pertussis* for susceptibility to killing by rat LLF. Among a range of phase I strains, there were some that were appreciably more sensitive than strain 18-323 and others of lower sensitivity. On the other hand, phase IV strains of *B. pertussis* were completely resistant to LLF. Indeed, with these phase IV strains LLF functioned as a maintenance medium. The single strain of *B. paraptussis* tested (on the surfactant fraction of LLF) had a sensitivity that was similar to that of strain 18-323,

while a strain of *B. bronchiseptica* was completely resistant. Among bacteria other than bordetellae, *E. coli* Lilly, a strain highly sensitive to complement [21], was not killed by LLF. This result indicated not only that the LLF had no detectable serum complement, but also that the process of lung lavage did not cause appreciable contamination of LLF with blood. The LLF contained significant amounts of lysozyme, as detected by lysis of *M. luteus*. However, this was not the substance responsible for the activity of LLF towards *B. pertussis*, as lysozyme alone was inactive against this species.

Following the method of Coonrod *et al.* [10], rat LLF was separated by ultracentrifugation at 55 000 *g* into a surfactant (SUR) fraction which sedimented and a supernate (SN). In agreement with these authors (who used *Str. pneumoniae* as the test organism), the present study found the bactericidal activity towards *B. pertussis* to be largely in the SUR fraction. The small part of the activity in the SN fraction was not due to incomplete separation of the bactericidal agent, as there were appreciable differences in stability towards heat and other treatments. For example, the activity in the SUR fraction was stable at 100°C, and to chymotrypsin and bentonite treatment, whereas that in the SN was destroyed. The principal dissolved or colloiddally dispersed substances in the SUR fraction were lipid (*c.* 85%) and most of the rest were protein. These values were closely similar to those for dog SUR, as reported by King and Clements [24]. Protein did not appear to be involved in bactericidal activity, as treatment of SUR with various proteases, or heating at 100°C, had no effect. However, there did appear to be an involvement of phospholipid, as treatment of SUR with phospholipase A2 greatly reduced the bactericidal activity.

Long-chain fatty acids were found by Coonrod *et al.* [10] to be the main factors responsible for the killing of pneumococci by rat LLF, and the present studies suggest that these substances are similarly important for the killing of *B. pertussis*. Thus, long-chain fatty acids were present in LLF at an average concentration of *c.* 50 µM, and artificial mixtures from C₁₄ to C₂₀ at 50 µM had demonstrable activity against *B. pertussis* 18-323. On the other hand, SUR could be diluted by *c.* 1 in 25 and still kill 50% of the bacterial inoculum in 30 min, whereas the artificial long-chain fatty acids mixture at 50 µM was not active after diluting 25-fold. It might be thought that because the fatty acids are of low mol. wt, they would not be sedimented at 55 000 *g*. However, both Coonrod *et al.* [10] and the present study found the fatty acids of LLF in the SUR fraction, implying some kind of combination with the phospholipid micelles, which are also complexed with protein and which sediment at 55 000 *g*. Therefore, these results are compatible with the view that long-chain fatty acids are important components of LLF responsible for killing of *B. pertussis*, as they are with

pneumococci. However, the quantitative expression of bactericidal activity in LLF may be amplified by complexing with, or acting in concert with, phospholipids and perhaps other components such as lysozyme that may be inactive on their own. Possibly for that reason, there was no correlation between the bactericidal activity of different batches of LLF, which was quite variable, and the measured concentration of fatty acids.

Further characterisation is required to define more precisely the exact nature and mode of action of the substances in rat LLF that determine its bactericidal activity towards *B. pertussis*. This may not be straightforward if the state of colloidal dispersion of long-chain fatty acids in phospholipid micelles is, as suggested here, important for the quantitative expression of bactericidal activity. Coonrod *et al.* [10] concluded that long-chain fatty acids had a detergent-like action on pneumococci and caused the release of phospholipid from the bacterial membrane, with consequent loss of membrane integrity. The electron micrographs of *B. pertussis* treated with SUR show cell envelope changes that are consistent with such effects.

One of the unsolved mysteries of the in-vitro physiology of *B. pertussis* is its extreme sensitivity to fatty acids as inhibitors in culture media [25–27]. This is overcome by addition of such fatty acid-binding agents as albumin [26], starch [28], charcoal [25], anion-exchange resins [29] and, more recently, 2, 6-O-dimethyl-β-cyclodextrin [16]. In contrast, phase IV *B. pertussis* is resistant to inhibition by fatty acids in culture media and, as found here, to killing by rat LLF. There has never been a satisfactory explanation as to why *B. pertussis* in the virulent phase I state is so sensitive to inhibition by fatty acids, yet the avirulent phase IV organisms are resistant. The evolutionary conservation of this sensitivity suggests that in the respiratory tract of the mammalian host – be it human, mouse or rat – a critical stage in the infection may be the lysis of *B. pertussis* by long-chain fatty acids bound to surfactant in the fluid in which the bacteria are growing. Such lysis would release cellular components such as heat-labile toxin, adenylate cyclase toxin and pertussis toxin, all of which may contribute to pathogenesis.

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