Cough production, leucocytosis and serology of rats infected intrabronchially with *Bordetella pertussis*

ELIZABETH HALL, R. PARTON* and A. C. WARDLAW

Department of Microbiology, University of Glasgow, Glasgow G12 8QQ

Summary. Adult Sprague-Dawley rats infected intrabronchially with *Bordetella pertussis* strain 18-323 encased in agarose beads (BP-beads), developed a paroxysmal cough and leucocytosis, both of which peaked at around day 10. When animals were exposed to ether for 2 min after delivery of the beads, there was an enhancement of the number of subsequent coughing episodes. Inclusion of carrageenan in the beads also enhanced coughing. Control rats, given sterile beads or left untreated, showed only a low level of coughing or no coughing, depending upon their source. Rats challenged by the same route with heat-killed *B. pertussis* in beads, or with live organisms in suspension (without beads) showed no cough induction or leucocytosis. However, intranasal delivery of *B. pertussis* suspension gave rise to a moderate amount of coughing and leucocytosis. Serum IgG responses to *B. pertussis* antigens were greatest in rats infected with BP-beads and antibodies against both pertussis toxin and filamentous haemagglutinin were detected. Since the rat is the only conveniently accessible laboratory animal species in which *B. pertussis* induces an intermittent paroxysmal cough, as in man, it merits further study for determining the mechanisms of pathogenesis and immunity in pertussis.

Introduction

Bordetella pertussis, the causative agent of pertussis or whooping cough, produces an array of putative virulence factors.^{1,2} These include pertussis toxin (PT), adenylate cyclase toxin (ACT), heat-labile toxin, tracheal cytotoxin, filamentous haemagglutinin, agglutinogens and pertactin. Most of these factors have been well-characterised, except for their role in human disease. One of the main impediments to understanding the mechanisms of pathogenesis in pertussis is the lack of a good experimental model for this purely human disease. Laboratory animals such as the mouse,³ rabbit⁴ and marmoset,⁵ all support growth of *B. pertussis* in their respiratory tracts, and mice in particular are widely used in protection tests and for virulence studies.⁶ For example, transposon-insertion mutants deficient in the production of either PT or ACT are markedly reduced in their virulence for infant mice when given intranasally⁷ and several purified antigens, alone or in combination, protect mice against respiratory challenge.⁶ However, the cardinal sign of the human disease, the paroxysmal cough, does not occur in these animals after respiratory infection.

There are isolated reports of coughing having been induced in some primates⁶ but such species are unlikely to become widely used. As long ago as 1939, in a study

of rats infected by intranasal instillation of a suspension of *B. pertussis*, Hornibrook and Ashburn⁸ described animals that developed paroxysms resembling coughing or sneezing, in which air was forcibly expelled from the mouth or nose. The cough appeared at about 8 days after inoculation and, in some cases, lasted for up to 60 days. Young rats (25–40 g) usually developed a fatal pneumonia whereas adults were more resistant. The adult rats often had a more vigorous cough than the surviving younger animals and coughing could be heard at a distance of 10–20 ft. This report appears to have been overlooked in the subsequent pertussis literature.

In 1989, Woods *et al.*⁹ described a rat model for respiratory infection with *B. pertussis*, by a method originally used to establish chronic respiratory infection with *Pseudomonas aeruginosa*.¹⁰ Ether-anaesthetised, adult (200–220 g) Sprague-Dawley rats were infected with Phase I (virulent) *B. pertussis* embedded in beads of agar which were injected into the lungs *via* a tracheotomy and a long, bead-tipped needle. The subsequent infection was non-invasive and non-lethal and the rats were reported to develop a “paroxysmal cough with whooping similar to that heard in humans” although no further details of coughing were given. The infected animals also exhibited leucocytosis, another of the characteristic signs of human pertussis, and hypoglycaemia. *B. pertussis* was re-isolated from lung homogenates for up to 7 days post-infection and then at 21 days but not at 10

Received 2 Aug. 1993; accepted 2 Sept. 1993.

* Correspondence should be sent to Dr R. Parton.

and 14 days. Phase III (avirulent) *B. pertussis* did not establish infection and was ineffective in producing any of the above features, indicating that the effects with Phase I organisms were not due to non-specific pulmonary irritation or blockage.

In a preliminary report,¹¹ we confirmed the findings of Hornibrook and Ashburn⁸ and of Woods *et al.*⁹ that rats infected with *B. pertussis* may develop a paroxysmal cough and leucocytosis. This paper presents a full account of these studies to further characterise and develop the coughing rat model of pertussis.

Materials and methods

Bacterial challenge suspension

B. pertussis strain 18-323 (NCTC 10739, Phase I) was grown on Bordet-Gengou (BG) Agar (Gibco-BRL, Paisley) containing defibrinated horse blood (Becton-Dickinson, Cowley) 20% v/v. The growth from plates incubated for 24 h at 37°C was suspended in casamino acids (CAA) solution, containing (/L): casein hydrolysate peptone 5 (Gibco-BRL) 10 g, NaCl 5 g, MgCl₂·6H₂O 0.1 g and CaCl₂ 0.016 g, pH 7.1, to a turbidity of 10 opacity units, equivalent to $c. 2 \times 10^9$ cfu/ml by comparison with the WHO 5th International Reference Preparation of Opacity.¹² Ten-fold serial dilutions were made in CAA and 0.1-ml volumes were plated on BG medium to provide viable counts. The undiluted suspension was either used directly for infecting the rats, or more commonly, was first incorporated into agarose beads.

Agarose bead preparation

All materials and equipment were sterilised by either autoclaving or disinfected by immersion in ethanol followed by rinsing in sterile water. Low-gelling-temperature agarose (Sigma) 2% w/v in phosphate-buffered saline (PBS) was melted at 100°C, transferred to a water bath at 37°C and allowed to cool. Meanwhile, 200 ml of liquid paraffin (Hays Chemicals Distribution Ltd, Glasgow), previously warmed to 37°C, was transferred to a 600-ml beaker and mixed with a Silverson mixer-emulsifier at the lowest speed setting. Five ml of the bacterial suspension at 2×10^9 cfu/ml were mixed with 5 ml of the agarose, rapidly transferred to a 10-ml syringe fitted with a 22 g × 1.5 in needle and expelled dropwise into the paraffin. After mixing for 5 min, the paraffin was placed on ice and mixing continued for a further 5 min during which time the agarose droplets solidified into beads of $c. 100 \mu\text{m}$ diameter. This mixing procedure was done in a microbiological safety cabinet. The beads were deposited by centrifugation at 7000 *g* at 4°C for 20 min, washed three times in CAA by further centrifugations under the same conditions, primarily to remove the paraffin oil, and resuspended to 5 ml of CAA. The process of bead preparation, from

harvesting of the challenge suspension, generally took $c. 2\text{--}3$ h and the beads containing *B. pertussis* were used within 2 h of preparation. For control purposes, agarose beads were prepared in the same way but without incorporation of bacteria.

In several experiments, carrageenan (carrageenan lambda, type IV; Sigma) was incorporated into the agarose beads at a final concentration of 1% w/v. In other experiments, heat-killed bacteria were used in place of live cells. This was done by heating the standardised bacterial suspension in a water bath at 56°C for 40 min after which 0.1 ml was plated out on BG medium as a check on sterility.

Preparation of antigens for ELISA

Whole-cell sonicates were prepared from *B. pertussis* strain 18-323 and *B. bronchiseptica* NCTC 5376 grown overnight on BG agar plates at 37°C. The bacteria were harvested in CAA, centrifuged at 7000 *g* for 15 min at 4°C and suspended in ELISA coupling buffer (Na₂CO₃ 1.59 g/L, NaHCO₃ 2.93 g/L, pH 9.6) to a concentration of $c. 2 \times 10^9$ cfu/ml. Cells were disrupted by sonication (MSE sonicator; 4–5 μm amplitude; 20 kHz), on ice, for 30 s with intervals of 30 s for cooling, for a total of 10 min, then frozen in small volumes until required.

Pertussis toxin (PT) and filamentous haemagglutinin (FHA) were prepared from *B. pertussis* transposon-insertion mutants BP 353 and BP 357, deficient in the production of FHA and PT respectively.¹³ Growth of the bacteria in cyclodextrin liquid medium,¹⁴ extraction of PT and FHA from the culture supernate by dye-ligand chromatography and estimation of the protein content of the antigens was as described by Christodoulides *et al.*¹⁵

Animals

Barrier-reared Sprague-Dawley male rats were obtained from Charles River UK Ltd (Manston Road, Margate) (VAF PLUS status) or from Harlan Olac Ltd (Shaws Farm, Blackthorn, Bicester). Health status reports obtained with each batch of rats certified the stock as being free from a range of viral, protozoal and bacterial pathogens including *B. bronchiseptica*. The starting weights of the animals in each experiment were generally $c. 200$ g. The rats were caged in pairs and allowed food and water *ad libitum*.

Infection procedures

Rats were anaesthetised with a mixture of Hypnorm (midazolam hydrochloride; Janssen Pharmaceuticals Ltd, Grove, Oxford) and Hypnovel (fentanyl citrate and fluanisone; Roche Products Ltd, Welwyn Garden City) in distilled water in the ratio 1:1:2 and given a dose of 1.5 ml/kg body weight by intraperitoneal injection. The animals were secured on an electrically heated pad (International Market Supplies, Congle-

ton) (IMS) and full sterile procedure was used for the surgery. The trachea was exposed by making a midline incision in the neck followed by blunt dissection through the connective tissue and sterno-hyoid muscle. Meanwhile, 0.2 ml of air space followed by 0.1 ml of the agarose bead suspension was drawn up into a 1-ml syringe fitted with a 22 g $\times$ 1 in curved, bead-tipped needle (IMS) and inserted into the trachea which had been punctured with a 21 g needle *c.* 1 cm below the larynx. The whole length of the bead-tipped needle was inserted, angled down to the left side of the animal, and the inoculum of 10^8 cfu of *B. pertussis* was delivered intrabronchially. The wound was closed, firstly with a single subcutaneous stitch of catgut (4/0 chromic; Ethicon Ltd, Edinburgh) and then with three or more stitches of polypropylene monofilament (2/0 prolene; Ethicon Ltd). Rats were allowed to recover from the anaesthetic in an incubator at 32°C for *c.* 1 h before being returned to their cages.

In some experiments, rats were subjected to a 2-min period of inhalation of anaesthetic ether (BDH) immediately after the surgical procedure and while still anaesthetised by the Hypnorm and Hypnovel mixture. In other experiments, rats were infected with 0.1 ml of a suspension of *B. pertussis* containing 10^9 cfu/ml in CAA (BP-sus), rather than in agarose beads (BP-beads), and the inoculum was given either intrabronchially, as above, or intranasally. For intranasal infection, the animals were anaesthetised with ether and 0.1 ml of BP-sus was dropped on to the nares whereupon it was drawn into the respiratory tract during inspiration.

Post-infection procedures

Coughing. The rats were housed in groups of eight, two per cage, in cubical recording booths (*c.* 1 m³) which were open-fronted for ventilation but otherwise surrounded by PVC-coated poly-ether sound insulation material *c.* 1 in thick (Caledonian Rubber Co., Glasgow) to reduce noise contamination from neighbouring groups. A unidirectional microphone (Zoom-Universal, RMZ-10, Hamaphot KG, D-8855 Monheim, Germany) was suspended above the four cages in each booth and connected to a voice-activated cassette recorder (Minisette-20, Tandy Corporation). Recordings were made, usually each night, for 21 days after infection. The recorders were fitted with 45-min tapes and recording was started at *c.* 5 p.m. A talking clock was used to mark each hour on the tapes, and recording was commonly completed within 3–5 h. The recorder was activated by normal rat noises such as eating, drinking, playing, fighting and also by abnormal respiratory sounds. These ranged in volume and pitch from chirping sounds to a hoarse, distinctive cough. The chirps and coughs were paroxysmal and usually repeated 5–10 times, but episodes of up to 80 were recorded. The data are presented as the total number of paroxysms per recording (45-min tape) divided by the number of rats per group.

Weights and white blood cell (WBC) counts. The rats were weighed at intervals of 3–4 days. Blood samples (40 μ l) were taken at intervals by tail venipuncture from rats anaesthetised with halothane (RMB Animal Health Ltd, Dagenham) and diluted immediately in 20 ml of Isoton II (Coulter Electronics Ltd, Luton). Erythrocytes were lysed with Zap-Oglobin (Coulter Electronics Ltd) and the numbers of WBC/mm³ were counted in a Coulter Counter model FN. Data are presented as the means of the log₁₀ counts and the standard errors of the means.

ELISA procedures

Each well in 96-well microtitration plates (Nunc Maxisorp, Life Sciences, Paisley) was coated with 0.1 ml of antigen preparation diluted in ELISA coating buffer. Whole-cell sonicate stock suspensions were diluted 1 in 1000 and FHA was adjusted to 2 μ g of protein/ml. A sandwich ELISA was used for PT and fetuin (Sigma) 0.1 ml at 1 μ g/ml was used for coating the plates which were incubated overnight in a moist box at 4°C and then washed three times in wash buffer (WB), containing (/L): NaCl 8 g, KH₂PO₄ 0.2 g, Na₂HPO₄ $\cdot$ 12H₂O 2.8 g, KCl 0.2 g, Tween 20 (Sigma) 0.05% v/v, pH 7.5. The plates were blocked with 0.1 ml of bovine serum albumin (BSA, Sigma) 2% w/v in WB for 1 h at 37°C except in the ELISA for PT in which 0.1 ml of PT 10 μ g protein/ml in WB was applied. After washing, 0.1 ml of test and reference serum samples serially diluted in WB were added to duplicate wells and plates were incubated for 1 h at 37°C in a moist atmosphere. After three washes, 0.1 ml of horseradish peroxidase (HRP)-conjugated goat anti-rat IgG (Sigma), diluted 1 in 5000, was added to each of the wells and incubated for 1 h as before. After a further three washes in WB, 0.1 ml of O-phenylenediamine substrate (OPD; Sigma) was added. This contained OPD (34 mg/ml) and 20 μ l of H₂O₂ in 100 ml of citrate-phosphate buffer, pH 5.0 (49 ml of 0.1 M citric acid and 51 ml of 0.2 M disodium phosphate). After 20 min in the dark at room temperature, the reaction was stopped by the addition of 50 μ l of H₂SO₄ 12.5% v/v. Absorbance was measured at 492 nm in a Titertek Multiscan MC ELISA reader (Flow Laboratories, Hertfordshire). The mean absorbance was calculated and plotted against the log₁₀ dilutions of the sample. The dilution at an end-point of A₄₉₂ = 0.5 was taken as the titre of the serum.

A reference serum was provided by a hyperimmune pool from two rats immunised intraperitoneally with 1 ml of *B. pertussis* whole-cell sonicate (equivalent to 2×10^9 cfu/ml) followed by two further injections at intervals of 3 weeks and bled out 12 days later. The readings obtained for the reference preparation run on ELISA for the four different antigens were calculated and enabled reproducibility to be checked. The reference preparation was included in every ELISA run, and a pool of normal rat serum, providing a negative control, was included on every plate. The non-specific

Fig. 1. The effects of different methods of infection on cough production (A, B) and leucocytosis (C, D) in rats: ●—●, rats given BP-beads intrabronchially (32); ■—■, BP-sus intranasally (16); ○—○, BP-sus intrabronchially (16); ○—○, sterile beads intrabronchially (16); ■—■, untreated (24). All except the last group were given ether treatment after surgery.

binding levels of the HRP conjugates were calculated from wells in which the incubation of samples was omitted. For HRP goat anti-rat IgG, the levels were around $A_{492} = 0.05$. These levels were reproducible and were not subtracted from sample readings.

Minitab statistical software (Macintosh version, release 8; Clecom Microcomputer Specialists, Birmingham) was used to estimate median values of ELISA titres, and their 95% confidence limits, for each treatment group and to compare medians by the Mann-Whitney U-test.

Results

Comparison of different methods of infection

We have already reported briefly¹¹ that intrabronchial infection of rats with *B. pertussis* strain 18-323 embedded in agarose beads (BP-beads) induced coughing, leucocytosis and retardation of normal weight gain. In the present investigation, these findings have been confirmed and extended. Fig. 1 shows the summarised data from several experiments in which

the effects of BP-beads delivered intrabronchially were compared with those of other methods of infection. There were groups of eight rats in each experiment and all groups, except untreated controls, received a 2-min period of ether inhalation immediately after surgery. In rats infected with BP-beads intrabronchially, there was a peak of coughing episodes, in terms of number of paroxysms/rat/daily tape recording, at around days 9–14 after infection (fig. 1A). Suspensions of *B. pertussis* (BP-sus) without beads, delivered intrabronchially, produced only a low level of coughing, similar to that in the control groups of untreated rats and rats given sterile beads intrabronchially (fig. 1B). Control rats showed only a fairly constant and low level of coughing throughout each experiment and each paroxysm usually contained fewer individual coughs (*c.* 3–5) than in the infected animals (*c.* 5–10 or more [20–50] during the peak period). There was more coughing when the BP-sus was administered intranasally, with a moderate peak between days 9 and 14. However, this was considerably less than the coughing paroxysms produced by the BP-beads given intrabronchially (fig. 1A).

Rats given BP-beads were the only groups to

Fig. 2. The influence of ether treatment on (A) cough production and (B) leucocytosis: ●—●, rats given BP-beads intrabronchially and exposed to ether for 2 min after surgery (32); ●---●, BP-beads intrabronchially, without ether treatment (32); ○---○, sterile beads plus ether treatment (16).

develop a marked leucocytosis, which peaked at day 10 after infection (fig. 1 C and D). Rats given BP-sus intranasally showed a moderate leucocytosis from day 3, the first sampling time, suggesting that delivery of *B. pertussis* by this route induced a more rapid leucocyte response than by the intrabronchial method.

As in the previous report,¹¹ significant retardation in weight gain, from about day 3 and for the rest of the observation period, was apparent only in those groups that had received BP-beads (not shown).

Influence of ether treatment

In the original procedure of Woods *et al.*⁹ ether was used as anaesthetic for the intrabronchial administration of BP-beads. In the present work, alternative anaesthetic agents were used but we decided to investigate whether ether might have some role in the establishment of infection or in cough production. Therefore, immediately after surgical delivery of the BP-beads, some animals were allowed to inhale ether for 2 min whereas others were not exposed. Fig. 2 shows the summarised data from four experiments in

Fig. 3. Effect of inclusion of carrageenan in agarose beads on cough production by rats infected with BP-beads intrabronchially: ●—●, BP-beads containing agarose; ●---●, BP-beads containing agarose plus carrageenan; ○—○, sterile beads containing agarose; ○---○, sterile beads containing agarose plus carrageenan (16 in each group).

which the effect of this treatment was explored. The recordings of the number of coughing paroxysms per rat (fig. 2A) showed a modest but consistent increase in coughing episodes throughout the observation period in ether-treated animals given BP-beads compared with those receiving BP-beads alone. Animals given sterile beads and ether treatment showed only a low, background level of coughing similar to that in the untreated controls described above (fig. 1B). Thus, although ether treatment appeared to enhance coughing, there was no difference in the pattern of leucocytosis in the two experimentally infected groups (fig. 2B). However, in the light of these results, the post-operative treatment with ether was included as part of the routine procedure for infecting rats intrabronchially.

Effect of carrageenan in the agarose beads

In another attempt to enhance cough production, the effect of incorporation of the inflammatory agent lambda carrageenan into the agarose beads was examined. The combined results from two experiments are shown in fig. 3. In the groups infected with BP-beads containing carrageenan, there was somewhat more coughing than in the groups infected with the normal BP-beads although the effect was not consistent throughout the observation period. The presence of carrageenan had no effect on leucocyte responses in the rats (not shown).

Coughing in control rats

It can be seen from figs. 1 and 2 that there was a low incidence of coughing in the control animals, including completely untreated rats, as well as in rats given sterile beads intrabronchially. The cause of this coughing was not determined. These rats were all from the same supplier and were received with clean health-status reports. In addition, however, we arranged for

Table I. Comparison of the effect of heat-killed Phase I *B. pertussis* with live Phase I organisms on cough production

Treatment*	Number of rats	Sum of coughing paroxysms/rat (days 9–14 post-infection)
Live Phase I <i>B. pertussis</i> in beads	24	11.30
Heat-killed Phase I <i>B. pertussis</i> in beads	16	0.31
Beads alone	16	0.25
Untreated	16	0.12

* Intrabronchial inoculation, including exposure to ether.

serological examination (The Microbiology Laboratories, North Harrow, Middlesex) of one group of untreated control rats, which were coughing, for evidence of infection with a range of bacterial and viral agents likely to cause respiratory disease in laboratory rats (*Mycoplasma pulmonis*, *M. arthritidis*, cilia-

associated respiratory bacillus, Sendai virus and Pneumonia virus of mice). All of these tests, on sera taken at day 28, gave negative results. Similarly, our ELISA results (see below) on individual rat sera showed little evidence of exposure of the various control groups to *Bordetella*. However, when rats of the same strain (Sprague-Dawley) were obtained from a different supplier, little if any coughing was heard in the control animals, as shown in fig. 3. Animals from this supplier were used in all subsequent experiments.

Effect of heat-killed phase I B. pertussis

To demonstrate a requirement for live *B. pertussis* for cough production, heat-killed cells were incorporated into the beads and compared with the usual BP-bead challenge. Table I shows the combined results from three experiments involving a total of 72 rats. The cough data are expressed as the sum of the number of paroxysms/rat recorded over the peak

Table II. Summary of IgG ELISA titres in sera from rats exposed 28 days previously to various treatments and tested with coating antigens consisting of sonicates of *B. pertussis* (BP) and *B. bronchiseptica* (BB) or pertussis toxin (PT) and filamentous haemagglutinin (FHA) from *B. pertussis*

Group no.	Rat treatment			ELISA titre with coating antigen							
				BP		BB		PT		FHA	
				sera + ve	median	sera + ve	median	sera + ve	median	sera + ve	median
				sera tested	(range) [95% CL]	sera tested	(range) [95% CL]	sera tested	(range) [95% CL]	sera tested	(range) [95% CL]
1	+	A	+	$\frac{50}{57}$	1400 ($< 10, 100\,000$) [507, 4667]	$\frac{35}{57}$	80 ($< 10, 25\,000$) [$< 10, 199$]	$\frac{46}{57}$	750 ($< 10, 100\,000$) [187, 4667]	$\frac{57}{57}$	9500 (160, 100 000) [7000, 12 334]
2	+	A	–	$\frac{22}{22}$	4450 (200, 100 000) [498, 7255]	$\frac{14}{22}$	220 ($< 10, 20\,000$) [$< 10, 2903$]	$\frac{15}{22}$	250 ($< 10, 5900$) [$< 10, 916$]	$\frac{22}{22}$	7000 (420, 100 000) [4340, 15 027]
3	+	A+C	+	$\frac{15}{15}$	300* (180, 1000) [207, 625]	$\frac{15}{15}$	200 (36, 850) [41, 370]	$\frac{14}{15}$	800 ($< 10, 60\,000$) [562, 8736]	$\frac{15}{15}$	1200† (150, 100 000) [431, 4716]
4	+	–	+	$\frac{13}{16}$	200† ($< 10, 1800$) [100, 512]	$\frac{3}{16}$	< 10 † ($< 10, 40$) [$< 10, < 10$]	$\frac{9}{16}$	12† ($< 10, 60$) [$< 10, 18$]	$\frac{7}{16}$	< 10 † ($< 10, 790$) [$< 10, 235$]
5	+	–	–	$\frac{8}{8}$	250 (150, 1900) [178, 609]	$\frac{0}{8}$	< 10 † ($< 10, < 10$) [$< 10, < 10$]	$\frac{4}{8}$	10† ($< 10, 440$) [$< 10, 159$]	$\frac{8}{8}$	775† (210, 3200) [294, 1516]
6	+	–	+	$\frac{8}{8}$	730 (600, 2000) [619, 1252]	$\frac{7}{8}$	33 ($< 10, 160$) [11, 52]	$\frac{3}{8}$	< 10 † ($< 10, 5300$) [$< 10, 366$]	$\frac{8}{8}$	1025† (395, 4200) [867, 1954]
7	+	A	+	$\frac{14}{15}$	60* ($< 10, 45\,000$) [27, 236]	$\frac{4}{15}$	< 10 * ($< 10, 4000$) [$< 10, 9$]	$\frac{0}{15}$	< 10 † ($< 10, < 10$) [$< 10, < 10$]	$\frac{4}{15}$	< 10 † ($< 10, 170$) [$< 10, 19$]
8	–	A	+	$\frac{8}{32}$	< 10 † ($< 10, 700$) [$< 10, < 10$]	$\frac{8}{32}$	< 10 † ($< 10, 130$) [$< 10, < 10$]	$\frac{0}{32}$	< 10 † ($< 10, < 10$) [$< 10, < 10$]	$\frac{1}{32}$	< 10 † ($< 10, 185$) [$< 10, < 10$]
9	–	A+C	+	$\frac{2}{15}$	< 10 † ($< 10, 19$) [$< 10, < 10$]	$\frac{1}{15}$	< 10 † ($< 10, 15$) [$< 10, < 10$]	$\frac{3}{15}$	< 10 † ($< 10, 400$) [$< 10, < 10$]	$\frac{0}{15}$	< 10 † ($< 0, < 10$) [$< 10, < 10$]
10	–	–	–	$\frac{21}{38}$	16† ($< 10, 380$) [$< 10, 32$]	$\frac{7}{38}$	< 10 † ($< 10, 1300$) [$< 10, < 10$]	$\frac{0}{38}$	< 10 † ($< 10, < 10$) [$< 10, < 10$]	$\frac{1}{38}$	< 10 † ($< 10, 190$) [$< 10, < 10$]

Bp, *B. pertussis*; IN, intranasal; HK, heat-killed; A, agarose; C, carrageenan.

* p value (%) = < 5 and † < 1 in Mann-Whitney U-test in comparison with median of Group 1.

coughing period, from days 9 to 14 after infection. A significant level of coughing was heard only in the animals given live, Phase I *B. pertussis*.

Serological responses to infection

The serological response of the rats to *B. pertussis* infection was measured by ELISA of individual sera taken at the termination of each experiment, on day 28 after challenge. IgG antibodies were assayed with four different antigens: *B. pertussis* whole-cell sonicate, FHA, PT and *B. bronchiseptica* whole-cell sonicate. The latter was included to determine the exposure of the rats to *B. bronchiseptica* either before or during the course of the experiment. The responses in the different treatment groups are shown in table II and results are expressed as the number of sera positive/number tested, the median titre, the range of titres and the 95% confidence limits (CL) of the median for each group. The medians were compared by the Mann-Whitney U-test with those of Group 1 (rats given *B. pertussis* in agarose beads intrabronchially, followed by ether treatment).

Table II shows that there was a high incidence of responders (116 of 126, 92%) to *B. pertussis* sonicate antigens in all groups challenge with live organisms (Groups 1–6) and median titres were generally high. In control groups (Groups 8–10), there were fewer (31 of 85, 36%) animals with antibodies reacting with *B. pertussis* sonicate and their titres were generally very low. A high percentage (74 of 126, 59%) of rats infected with *B. pertussis* also reacted with *B. bronchiseptica* sonicate antigens but with consistently lower titres than those against *B. pertussis*. Some control rats (16 of 85, 19%) also had low but detectable titres of antibodies to *B. bronchiseptica* antigens (median < 10, range < 10–1300, CL < 10, < 10) at the end of the experiments. However, when sera taken before challenge were examined for antibodies to *B. bronchiseptica*, 38 (24.6%) of 154 had detectable titres but these were also generally low (median < 10, range < 10–130, CL < 10, < 10).

Rats infected intrabronchially with *B. pertussis* contained in agarose beads (BP-beads) (Groups 1 and 2) had significantly higher titres to both antigen preparations than those challenged with BP-sus intrabronchially (Groups 4 and 5), irrespective of ether treatment. The high antibody titres to *B. pertussis* sonicate in Group 6 given BP-sus intranasally suggest that delivery of antigen by this route provided an immunising dose. Rats in Group 7, given heat-killed *B. pertussis* in agarose beads, had much lower titres than those receiving live organisms.

Table II also shows the antibody responses of the various groups of rats to two important virulence factors of *Bordetella* spp. Uninfected controls (Groups 8–10) showed negligible titres to either antigen. However, there was a high incidence of seropositivity to both PT (80%) and FHA (93%) in groups (1–6) challenged with live organisms, suggesting that these

components were being expressed as antigens *in vivo*. Negligible responses were obtained when heat-killed bacteria were used (Group 7) even though PT and FHA should not have been destroyed by the 56°C treatment. When bacteria were not incorporated in beads (Groups 4–6), the challenge was much less immunogenic, especially in response to PT, although FHA was stimulatory in Groups 5 and 6.

A possible immunosuppressive effect of carrageenan was noted in Group 3 animals, infected with BP-beads containing both agarose and carrageenan. These animals had lower antibody titres to *B. pertussis* sonicate and FHA than animals given BP-beads with agarose alone.

Discussion

We have confirmed the original observation of Hornibrook and Ashburn⁸ that rats infected with *B. pertussis* develop respiratory paroxysms resembling coughing. The infection procedure used by these earlier authors was *via* a simple intranasal instillation of bacterial suspension after ether anaesthesia. Such suspensions delivered intranasally in the present investigation also stimulated coughing but a much more pronounced effect was achieved after intrabronchial delivery of the organisms encased in agarose beads, by a procedure based on that of Woods *et al.*⁹ In this latter work, the rats were anaesthetised with ether for the surgery. In our study, however, injectable anaesthetics were used but the role of ether treatment as a crucial part of the process was investigated. In rats exposed to ether for 2 min immediately after surgery, there was a generally higher number of coughing episodes compared to animals without ether treatment. The reason for this is unknown but the treatment was judged sufficiently worthwhile to become part of the routine procedure for infecting the rats.

Our coughing rat model differs from that of Woods *et al.*⁹ in several other respects: (a) the use of *B. pertussis* strain 18-323, instead of strain Tohama; (b) low-gelling-temperature agarose, in place of Ionagar No. 2; (c) a temperature of 37°C rather than 50°C when suspending the bacteria in the agarose; and (d) a bacterial dose per rat of 10⁸ cfu instead of 5 × 10⁵ cfu. Furthermore, we have attempted to quantify the coughing paroxysms with sound-activated tape recorders, as suggested by Woods (personal communication) since coughing is seldom heard or observed by casual observation. The number of paroxysms per 45-min tape recording during the evening reached a peak incidence around days 9–14 in infected rats and had returned to normal around day 21. The coughing was accompanied by leucocytosis and a slight retardation of normal weight gain but otherwise the animals appeared healthy.

Apart from the reports^{8,9} mentioned above, coughing in rats has not been clearly described in the scientific literature. Acute respiratory disease in rats is

often described as being accompanied by symptoms such as "snuffling", "rales" and "dyspnoea"¹⁶ rather than coughing. Indeed, there is one report¹⁷ that mammals the size of a rat, and smaller, lack a cough reflex. The cause of the background coughing in some of the control rats was not determined but the need to screen animals for coughing before use and the inclusion of suitable control groups is apparent.

The experiments on cough production demonstrated a requirement for live *B. pertussis* and their encasement in agarose beads before delivery to the lungs. Intrabronchial delivery of heat-killed organisms did not induce coughing, cause significant leucocytosis or stimulate significant antibody production. Similar negative results were obtained when live *B. pertussis* in suspension, rather than in beads, was delivered intrabronchially. Presumably the suspension, in the absence of a physical barrier formed by the agarose beads, was cleared rapidly from the lungs. However, when live *B. pertussis* suspension was given by the intranasal route, a different result was obtained. There was some coughing, which agrees with the early observations of Hornibrook and Ashburn⁸, but this was not as marked as in the groups infected with BP-beads intrabronchially. There was also a moderate leucocytosis and stimulation of antibody responses to whole-cell sonicate antigens although responses to PT and FHA were insignificant. It is possible that the intranasal delivery allowed the bacteria to localise and multiply to some extent in the upper respiratory tract to produce an immunising dose of antigen(s) and also to produce the factor(s) responsible for cough induction.

The main objective of this work was to monitor cough production. The course of the infection was checked in only one experiment, in which rats given BP-beads intrabronchially were killed at intervals of 3–28 days after challenge, the lungs were excised and a cut surface from a consolidated region was smeared over a BG plate. *B. pertussis* was recovered only up to 10 days after infection. Woods *et al.*,⁹ with a different *B. pertussis* strain, recovered organisms at 3, 7 and 21 days after inoculation but not at days 10 and 14.

The serological data on serum IgG levels in individual rats provided a useful indicator of *B. pertussis* infection. The *B. bronchiseptica* sonicate was included as antigen to determine any exposure of the rats to this common laboratory animal pathogen before or during the experiments. However, the whole-cell sonicate presumably contained many antigens cross-reactive with those of other bacteria, including the normal rat flora, and these cross-reactions could account for the titres detected in some animals in the control groups. The antibody titres to PT and FHA provided more useful information on exposure of rats to infection with *Bordetella* spp. PT is unique to *B. pertussis* whereas *B. bronchiseptica* produces FHA which is immunologically related, if not identical, to FHA of *B. pertussis*.¹⁸ Uninfected control groups showed negligible titres to either antigen, indicating that the animals had not been exposed to *Bordetella* spp. either before or during the experiments. All groups deliberately exposed to live *B. pertussis* showed a high incidence of seropositivity to PT and FHA. It is of interest to note that the groups given BP-beads and with the highest titres to PT, generally considered to be a major virulence component of *B. pertussis*, also showed the most pronounced coughing.

Since the rat is the only conveniently accessible laboratory animal species in which *B. pertussis* induces an intermittent paroxysmal cough, as in the human disease, it merits further study in the context of analysis of the mechanisms of pathogenesis and immunity in pertussis. The coughing rat model will be useful for exploring cough induction by mutant strains of *B. pertussis* deficient in individual virulence factors, for further analysis of humoral, secretory and cell-mediated responses to infection and for protection studies with monocomponent and multicomponent acellular vaccines.

We thank Dr Alison Weiss for the *B. pertussis* Tn5-insertion mutant strains, Mrs Berit Adam for performing the ELISA tests and Mr G. N. Fellah for his assistance. This work was supported by research grant K/MRS/50/C1494 from the Scottish Home and Health Department.

References

1. Wardlaw AC, Parton R (eds) Pathogenesis and immunity in pertussis. Chichester, Wiley. 1988: 75–271.
2. Parton R. Changing perspectives on pertussis and pertussis vaccination. *Rev Med Microbiol* 1991; **2**: 121–128.
3. Pittman M, Furman BL, Wardlaw AC. *Bordetella pertussis* respiratory tract infection in the mouse: pathophysiological responses. *J Infect Dis* 1980; **142**: 56–66.
4. Ashworth LAE, Fitzgeorge RB, Irons LI, Morgan CP, Robinson A. Rabbit nasopharyngeal colonization by *Bordetella pertussis*: the effects of immunization on clearance and on serum and nasal antibody levels. *J Hyg* 1982; **88**: 475–486.
5. Stanbridge TN, Preston NW. Experimental pertussis infection in the marmoset: type specificity of active immunity. *J Hyg* 1974; **72**: 213–228.
6. Sato Y, Sato H. Animal models of pertussis. In: Wardlaw AC, Parton R (eds) Pathogenesis and immunity in pertussis. Chichester, Wiley. 1988: 309.
7. Weiss AA, Goodwin MSM. Lethal infection by *Bordetella pertussis* mutants in the infant mouse model. *Infect Immun* 1989; **57**: 3757–3764.
8. Hornibrook JW, Ashburn LL. A study of experimental pertussis in the young rat. *Public Health Rep* 1939; **54**: 439–444.
9. Woods DE, Franklin R, Cryz SJ, Ganss M, Peppler M, Ewanowich C. Development of a rat model for respiratory infection with *Bordetella pertussis*. *Infect Immun* 1989; **57**: 1018–1024.
10. Cash HA, Woods DE, McCullough B, Johanson WG, Bass JA. A rat model of chronic respiratory infection with *Pseudomonas aeruginosa*. *Am Rev Respir Dis* 1979; **119**: 453–459.
11. Wardlaw AC, Hall E, Parton R. Coughing rat model of pertussis. *Biologicals* 1993; **21**: 27–29.
12. Perkins FT, Sheffield FW, Outschoorn AS, Hemsley DA. An international collaborative study on the measurement of the opacity of bacterial suspensions. *J Biol Standard* 1973; **1**: 1–10.
13. Weiss AA, Hewlett EL, Myers GA, Falkow S. Tn5-induced mutations affecting virulence factors of *Bordetella pertussis*. *Infect Immun* 1983; **42**: 33–41.
14. Imaizumi A, Suzuki Y, Ono S, Sato H, Sato Y. Effect of

- heptakis (2,6-O-dimethyl) β -cyclodextrin on the production of pertussis toxin by *Bordetella pertussis*. *Infect Immun* 1983; **41**: 1138–1143.
15. Christodoulides M, Sidey FM, Parton R, Stewart-Tull DES. Acellular pertussis vaccine prepared by a simple extraction and toxoiding procedure. *Vaccine* 1987; **5**: 199–207.
16. Kohn FD, Barthold SW. Biology and diseases of rats. In: Fox JG, Bennett JC, Loen FM (eds) *Laboratory animal medicine*. Orlando, Academic Press. 1984: 100.
17. Richardson PS. The function of the airway mucosa. In: Donachie W, Griffiths E, Stephen J (eds) *Bacterial infections of respiratory and gastrointestinal mucosae* (Special publications of the Society for General Microbiology, vol 24). Oxford, IRL Press. 1988: 1–8.
18. Domenighini M, Relman D, Capiou C *et al*. Genetic characterization of *Bordetella pertussis* filamentous haemagglutinin: a protein processed from an unusually large precursor. *Mol Microbiol* 1990; **4**: 787–800.