Bovine erythrocyte-agglutinin as a possible adhesin of *Bordetella bronchiseptica* responsible for binding to porcine nasal epithelium

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Summary. The bovine erythrocyte-agglutinin (BEA) of *Bordetella bronchiseptica*, located on the cell surface in non-fimbrial form, has been identified as a possible adhesin responsible for binding to porcine nasal epithelium in studies with BEA-negative (BEA⁻) mutants and in competitive studies with bovine erythrocytes.

Introduction

The ability of bacteria to attach to mucosal surfaces has been recognised as an important determinant influencing the successful colonisation of the host tissue (Jones and Rutter, 1972; Fader and Davis, 1980; Beachey, 1981). Many gram-negative bacteria possess surface structures which may serve as adhesins and are often associated with the ability of the organisms to cause haemagglutination. Bacterial haemagglutination correlates well with the adherence of the organisms to host cells (Punsalang and Sawyer, 1973; Koransky *et al.*, 1975; Burrows *et al.*, 1976; Finkelstein and Hanne, 1982; Svenson *et al.*, 1983; Goldhar *et al.*, 1984; Pruzzo *et al.*, 1984), and thus has been widely used as a model of host-parasite surface interactions (Svenson *et al.*, 1983; Korhonen *et al.*, 1984). Virulent bacteria tend to be more adhesive (Jones and Rutter, 1972; Punsalang and Sawyer, 1973; van den Bosch *et al.*, 1980) and haemagglutinating activity has been used often as a marker for identifying pathogenic isolates (Burrows *et al.*, 1976).

Bordetella bronchiseptica, an important cause of respiratory tract disease and a frequent cause of atrophic rhinitis in swine, experiences phase variation (from phase I to rough phase) (Nakase, 1957*b*; Ishikawa and Isayama, 1986), and phase-I cells grown at low temperature undergo a phenotypic alteration termed antigenic modulation (from X to C mode) (Nakase, 1957*b*; Ishikawa and Isayama, 1987*b*). Phase variants and C-mode cells are

characterised by loss or attenuation of capsular antigen (Nakase, 1957*a* and *b*; Éliás *et al.*, 1982; Ishikawa and Isayama, 1986, 1987*b*), as well as loss of virulence (Nakase, 1957*c*; Yokomizo and Shimizu, 1979). Other studies have demonstrated that the pathogenic organisms in X mode attach to the surface of porcine nasal mucosa (Yokomizo and Shimizu, 1979; Ishikawa and Isayama, 1987*a* and *b*). On the contrary, non-pathogenic organisms, either in C mode or in degraded phases (phases II, III, and rough), have been shown not to attach to the target tissue (Ishikawa and Isayama, 1987*b*). Accordingly, adherence is thought to be of major importance to the establishment of this infection (Yokomizo and Shimizu, 1979), though the mechanisms of the adherence are as yet by no means understood. On the other hand, phase-I cells in X mode are known to produce the substance(s) capable of agglutinating erythrocytes from several animal species (Nakase, 1957*c*; Bemis *et al.*, 1977; Bemis and Plotkin, 1982; Éliás *et al.*, 1982; Krüger and Horsch, 1982; Blom *et al.*, 1983; Rimler and Simmons, 1983; Spasojević-Rabrenović and Lončarević, 1984; Collings and Rutter, 1985). However, little is known about the haemagglutinating activity of phase variants and C-mode cells. The relationship between haemagglutination and attachment of the parasite to an epithelial surface is, therefore, still unclear and the pathogenic significance of haemagglutinin in bordetellosis remains to be determined.

The purpose of the present study was to examine the haemagglutinating pattern of *B. bronchiseptica* strains in each phase or mode and to try to identify the adhesin responsible for their binding to porcine nasal epithelial cells.

Materials and methods

Bacterial strains

B. bronchiseptica strains mainly used in the present study were phase-I strain A19, which was a primary isolate from a pig with atrophic rhinitis, and its substrains A19-CV200 (phase II), A19-CV300 (phase III), and A19-CV400 (rough phase). Three other phase-I strains (S1, H16, and ATCC4617) and their substrains in degraded phases were also examined. Methods for induction of phase variants as well as C-mode cells were described in detail elsewhere (Ishikawa and Isayama, 1986, 1987*b*). Cultures were maintained on Bordet-Gengou agar (Difco Laboratories, Detroit, MI, USA) containing defibrinated sheep blood 7%, and stored at 4°C.

Erythrocytes

Blood was collected into Alsever's solution from man (group O), horse, cow, sheep, goat, pig, dog, rabbit, guinea-pig, and chicken. Erythrocytes were washed three times in 0.01 M sodium phosphate-buffered saline (PBS; pH 7.0), after which the final deposit of packed cells, produced by centrifugation for 10 min at 700 *g*, was suspended in PBS to 0.5% v/v.

Haemagglutination assay

Bacteria for the assay were collected from 18-h cultures on Bordet-Gengou agar, washed twice, and suspended in PBS to a density of 10^{10} cells/ml. The assay was performed in microtitration plates with round-bottomed wells. After making two-fold serial dilutions of the bacterial suspensions, equal volumes (25 μ l) of erythrocytes were added. The plates were then shaken, sealed, and incubated for 2 h at 37°C before reading the results. The haemagglutination titre is the highest dilution that caused complete agglutination.

Enrichment and selection of BEA⁻ mutants

Spontaneous mutants that had lost the ability to produce bovine erythrocyte-agglutinin (BEA) were selected from strain A19. One colony of the strain was inoculated into 5 ml of Trypticase Soy Broth (BBL Microbiology Systems, Cockeysville, MD, USA) and propagated overnight at 37°C. The culture was added to 1 ml of packed bovine erythrocytes. After shaking for 2 h at ambient temperature to allow the bacteria to attach to the erythrocytes, the mixture was held overnight at 4°C. The BEA-negative (BEA⁻) bacteria-enriched supernate was absorbed one more time and then inoculated into fresh broth and the cycle was repeated. Portions of the enriched supernates were diluted and plated on Bordet-Gengou agar. After incubation for 2 days at 37°C, isolated colonies were screened with a rapid slide haemagglutination test to detect BEA⁻ mutants. At least nine such selective passages were required to permit the isolation

of BEA⁻ mutants. Spontaneous mutants were not detected during passages without the enrichment procedure.

Adherence assay

The adherence of bacteria was examined with epithelial cells obtained from ventral turbinate mucosae of pigs as described elsewhere (Ishikawa and Isayama, 1987*a* and *b*).

Competitive inhibition with bovine erythrocytes

Competitive inhibition of bacterial adherence was tested with bovine erythrocytes. A test mixture consisted of 0.3 ml of epithelial cells (6×10^5 cells), 0.15 ml of bacteria (6×10^7 cells), and 0.15 ml of erythrocytes (6×10^5 cells). The bacteria were pre-incubated for 30 min at 37°C with the erythrocytes. The adherence was compared with that in the control mixture lacking erythrocytes.

Bacterial agglutination with capsular antiserum

The agglutinability with capsular antiserum of bacteria in each phase or mode was determined as reported previously (Ishikawa and Isayama, 1986).

Electronmicroscopy

Bacterial suspensions in 1% ammonium acetate were negatively stained with uranyl acetate and examined for fimbriation with a Hitachi HS-9 electronmicroscope (Hitachi, Tokyo, Japan).

Results

Haemagglutinating activity

Initial studies were done to screen the bacteria in each phase or mode for their ability to agglutinate erythrocytes of various species. The characteristic patterns of haemagglutination are summarised in table I. Haemagglutinating activity tended to be strong among phase-I cells in X mode and to decrease on transition from X mode to C mode or from phase I to degraded phases, with the exception of the strong agglutination of human group O erythrocytes with variant cells in phases II, III, and rough. X-mode organisms agglutinated the erythrocytes of most species, e.g., bovine cells strongly, horse, pig, dog, rabbit, and guinea-pig cells moderately strongly, and human group O, sheep, and chicken cells weakly, but goat cells not at all. Identical patterns of haemagglutination were obtained when three other strains (S1, H16, and ATCC4617) and their variants were examined.

Table I. Haemagglutinating activity of *B. bronchiseptica* A19 and its BEA⁻ mutants

Erythrocyte species	Haemagglutination titre* of strain						
	A19 in phase (mode)					A19-712NH	A19-718NH
	I (X)	I (C)	II	III	Rough		
Human group O	4	8	256	256	256	4	4
Horse	8	<2	<2	<2	<2	8	8
Bovine	128	2	2	<2	<2	<2	<2
Sheep	2	<2	<2	<2	<2	2	2
Goat	<2	<2	<2	<2	<2	<2	<2
Pig	16	4	4	<2	<2	16	16
Dog	16	4	4	<2	<2	16	16
Rabbit	16	16	16	16	16	16	16
Guinea-pig	8	<2	<2	<2	<2	8	8
Chicken	4	<2	<2	<2	<2	4	4

* Highest dilution of bacterial suspension (10^{10} cells/ml) giving complete haemagglutination.

Thus, correlation was found between the known adhesiveness of the microbes for porcine nasal epithelium and their haemagglutinating activity with bovine erythrocytes.

It was thought that the isogenic mutants of phase-I strains which were BEA-defective would be a useful tool for attachment studies. According to the protocol described above, two BEA⁻ mutants of strain A19, designated A19-712NH and A19-718NH, were obtained.

The haemagglutination spectra of these mutants were examined. They failed to agglutinate bovine erythrocytes, whilst their behaviour with the erythrocytes of other species remained unchanged (table I).

Adhesiveness

The ability of BEA⁻ mutants to attach to porcine nasal epithelial cells was compared with that of their parent strain (table II). The phase-I parent in X mode adhered well to the epithelial cells, whereas the mutants showed feeble adherence to the same cells, as did the cells in C mode or in degraded phases.

Competitive inhibition with bovine erythrocytes

To confirm the involvement of BEA in epithelial cell adherence, X-mode cells were pre-incubated with bovine erythrocytes (table III). The erythro-

Table II. K-agglutinability and adhesiveness of *B. bronchiseptica* A19 and its BEA⁻ mutants

Strain	Phase (mode)	Agglutination titre* with K antiserum	Adhesiveness†
A19	I (X)	2560	16.3 ± 3.4
	I (C)	< 10	0.4 ± 0.1
	II	< 10	0.7 ± 0.4
	III	< 10	0.3 ± 0.2
	Rough	ND	0.1 ± 0.1
A19-712NH	I (X)	2560	0.5 ± 0.1
A19-718NH	I (X)	2560	0.9 ± 0.5

ND = not determined, because of spontaneous agglutination in PBS.

* Highest dilution of antiserum giving complete agglutination.

† Mean number of bacteria attached per epithelial cell ± SD was determined from triplicate assays.

Table III. Effect of pre-incubation of *B. bronchiseptica* A19 X-mode cells with bovine erythrocytes on their adherence to porcine nasal epithelial cells

Pre-incubation	Adherence* (percentage reduction)
None	12.4 ± 1.0
Bovine erythrocytes	1.9 ± 0.4 (85)

* Mean number of bacteria attached per epithelial cell ± SD was determined from triplicate assays.

cytes prevented adhesion of all strains examined. This may suggest that the erythrocytes inhibit adhesion by competing with the epithelial cells for the specific binding sites on the bacterial surface.

Capsulation and fimbriation

BEA⁻ mutants, as well as their parent, possessed capsules and were agglutinated by capsular antiserum (table II). No fimbriation was observed when they were examined by electronmicroscopy.

Discussion

Multiple types of haemagglutinins are likely to be expressed by *B. bronchiseptica*, and at least two types are thought to exist on the surface of X-mode organisms since mutants deficient in the production of BEA agglutinate the erythrocytes of other species.

A correlation was found between the lack of haemagglutinating activity for bovine erythrocytes and a concomitant decrease in adherence to porcine nasal epithelial cells. The organisms that could agglutinate bovine erythrocytes were strongly adherent to porcine nasal epithelial cells, whereas organisms that did not agglutinate the erythrocytes exhibited only negligible adherence. The result of the competitive inhibition tests indicated the possible involvement of BEA in the epithelial-cell adherence. Although BEA thus seems to be important in adherence, other cell-surface structures such as capsules and fimbriae, do not seem to be critical for adherence. The ability of the bacteria to adhere correlated neither with capsulation nor with fimbriation. Mutants lacking BEA still reacted with capsular antiserum. Thus, if BEA is a constituent of capsules, it is not the major epitope recognised by antibodies in the capsular antiserum. On the other hand, both the mutants and the parent were devoid of any detectable fimbriae, though the possession of fimbriae by this species has been reported (Bemis *et al.*, 1977; Bemis and Plotkin, 1982; Blom *et al.*, 1983; Bemis and Wilson, 1985; Collings and Rutter, 1985; Kielstein and Reckling, 1985; Lee *et al.*, 1986). Our results indicate that BEA is of a non-fimbrial nature and probably functions as an adhesin that mediates the attachment of *B. bronchiseptica* to porcine nasal epithelial cells. These findings may be of some value for further elucidation of the adhesion mechanisms of *B. bronchiseptica* with BEA as a likely marker for isolates of this species that have the capability to adhere to the organism's target cells on porcine nasal epithelium.

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