Journal of Industrial Microbiology (1996) 17, 53–55

Effect of hydromechanical stress on cellular antigens of *Bordetella pertussis*

ME Rodriguez, DF Hozbor and OM Yantorno

Centro de Investigación y Desarrollo de Fermentaciones Industriales (CINDEFI), Facultad de Ciencias Exactas, Universidad Nacional de La Plata, 47 y 115, (1900) La Plata, Argentina

Cells of *Bordetella pertussis* grown in a bioreactor under stirring conditions were studied to investigate the effect of shear stress on cellular-bound filamentous haemagglutinin (FHA). FHA attached to the bacterial surface, unlike extracellular FHA, was not affected at the shear levels tested. Moreover, no other cellular immunogen involved in the whole-cell protective activity seemed to be affected by hydromechanical forces.

Keywords: *Bordetella pertussis*; cellular antigens; pertussis vaccine; shear stress

Whooping cough is a disease caused by infection of the human respiratory tract by the Gram-negative bacterium *Bordetella pertussis*. Although efforts have been focused on the development of an acellular pertussis vaccine, suspensions of killed whole cells of *B. pertussis* are currently used in most countries for vaccination. One of the main *B. pertussis* protective antigens is the filamentous haemagglutinin (FHA), a 220–180 kDa cell surface protein and putative adhesin of this bacterium [5,6]. Antibodies against FHA provide protection against bacterial adherence which is likely to be a primary mechanism determining both bacterial proliferation and toxic effects in susceptible cells [11]. For this reason, this antigen should be taken into account in any acellular or cellular vaccine formulation. In a previous study we demonstrated [9] that hydromechanical forces generated in aerated-stirred bioreactors during *B. pertussis* cultivation do not affect bacterial growth but cause chain scission in extracytoplasmic FHA. At increasing shear stress (estimated as the power transmitted by the agitator and the aerator per unit of liquid volume [E]), it was possible to verify a breakdown of extracellular FHA. This is important since FHA components of molecular weight lower than 92 kDa have been reported as not capable of eliciting protective antibodies [4,7,11]. When large-scale growth of virulent *B. pertussis* is required for vaccine production, the power input to the fermentation system becomes an important parameter to be considered for process design. In this regard, knowledge about extracytoplasmic FHA breakdown by shear forces is of particular relevance to design and scale-up of the process of extracellular antigen production. However, this finding led us to a further question: is cell-bound FHA or other cellular protective antigen(s) affected by hydromechanical forces? In spite of its importance, no previous study was performed on the effect of this fermentation parameter on the quality of *B. pertussis* cellular antigens for vaccine production.

Correspondence: OM Yantorno, CINDEFI, Facultad de Ciencias Exactas, UNLP, Calles 47 y 115, (1900) La Plata, Argentina
Received 25 October 1994; accepted 13 June 1996

Materials and methods

In order to evaluate whether shear forces affect cell-bound FHA and other cellular factors involved in protection, *Bordetella pertussis* strain 8132 (Pasteur Institute Collection) was grown in a 6-L bioreactor (LKB 1601 Ultroferm, LKB, Bromma, Sweden) having a working volume of 3.0 L, operated at different levels of shear stress (Table 1), as previously reported [9]. Bacterial cells cultured for 24 h under each condition tested were centrifuged at 8000 × *g* for 15 min, washed and assayed to determine their haemagglutinin activity (HA_c) and degree of adherence to HeLa 229 (human-epithelium-like, ATCC CCL 2.1) cells. Full-length FHA protein mediates the attachment of *B. pertussis* to erythrocytes and other eukaryotic cells such as HeLa 229 [2,8,13]. Adherence of *B. pertussis* to HeLa 229 cells was assayed according to Sato *et al* [10]. Briefly, 1-day-old HeLa 229 monolayer cells were prepared in a small chamber containing 1 ml of fresh Minimum Essential Medium (MEM). One-tenth volume of a *B. pertussis* cell suspension (10^{10} ml^{-1}) was added to monolayer cells, mixed gently and allowed to settle for 2 h in a 5% CO_2 incubator at 37°C. The monolayer cells were then washed thoroughly with MEM, fixed with methanol-acetic acid

Table 1 Effect of operating conditions on haemagglutinin activity (HA_c), adherence to HeLa 229 cells and protective potency of *Bordetella pertussis* cells

Expt	N[a] (rpm)	VVM[a] (L L^{-1} min^{-1})	E (watts L^{-1})	HA_c titer	Mean adherent bacteria per HeLa cell[b]	Mean protective potency[c] (IUP ml^{-1})
1	210	1.00	0.3	64	78	7.7
2	270	0.80	6.3	64	79	7.9
3	320	0.42	11.0	64	80	7.8
4	450	0.30	32.0	64	78	7.9

[a] K_La was kept constant at 60 h^{-1}.
[b] $LSD_{0.05}$ = 17.
[c] $LSD_{0.05}$ = 0.3.

(3 : 1 ratio) for 10 min and stained with Hoechst No. 33258 solution (2′-[4-hydroxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5′-bi-1H-benzimidazole, Sigma, St Louis, MO, USA). Adherent bacteria were counted by fluorescence microscopy of randomly-selected fields showing 3–5 HeLa cells per field. The value presented for each assay is the mean of seven standardized experiments. Analysis of variance (ANOVA) was used for statistical evaluation of the data. The significance of the differences between the mean values of each condition was determined with the Least Significant Difference (LSD) test at a confidence level of 95%.

Results and discussion

For the different shear conditions, the *B. pertussis* cells showed the same ($P < 0.05$) degree of adherence to HeLa cells (Table 1). In addition, cells grown under different shear conditions had the same HA_c titer, as determined by microtiter assay using goose erythrocytes ($P < 0.05$) [1]. From these results, we conclude that cell-bound FHA is not affected by the shear forces generated in the bioreactor.

To gain further insight lysed cells of *B. pertussis* from each culture condition were separated by SDS-PAGE (10% polyacrylamide), transferred to nitrocellulose sheets, and analyzed by the Western blot (immunoblot) technique [12]. Monospecific anti-FHA antibodies (kindly provided by Nicole Guiso, Pasteur Institute, Paris, France) detected bands corresponding to higher molecular weight components of FHA in every cell sample tested. Components of FHA of molecular weight lower than 180 kDa were not observed (Figure 1). These results suggest that shear forces cause no damage to the structure of cell-bound FHA protein.

In order to determine if any other *B. pertussis* protective factors were affected by shear forces, cells grown under each culture condition (Table 1) were tested for their content of protective antigens by the active mouse protection test recommended by the World Health Organization [14]. Groups of 16 N : NIH mice each were immunized intraperitoneally with 0.5 ml of Second International Standard for Pertussis Vaccine (8 IUP ml^{-1}) or sample vaccines (prepared with cells from every culture condition studied), all diluted in 0.15 M NaCl (1 : 5, 1 : 25 and 1 : 125). Five groups, each consisting of ten non-immunized mice, were separated to be used as virulence controls. Fourteen days after immunization, the mice were challenged intracerebrally with 0.03 ml of a suspension of *B. pertussis* 18323, with approximately 200 times the LD_{50}. Control mice were injected with the challenge dose and five-fold dilutions. The animals were observed daily up to day 28 after immunization. Deaths occurring between days 17 and 28 were recorded. ED_{50} values and vaccine potencies were estimated according to the method of Worcester and Wilson [15]. Validity conditions in agreement with the requirements of the Code of Federal Regulations [3] were applied. Experiments to estimate the protective capacity of cells grown under each shear condition were performed with three replicates. Means, expressed as the IUP ml^{-1} (the dose which protected 50% of mice from lethal intracerebral challenge), were compared by LSD test with a confidence level of 95%. As shown in Table 1, increasing hydromechanical forces did not have a significant effect ($P < 0.05$) on cellular *B. pertussis* immunogenicity.

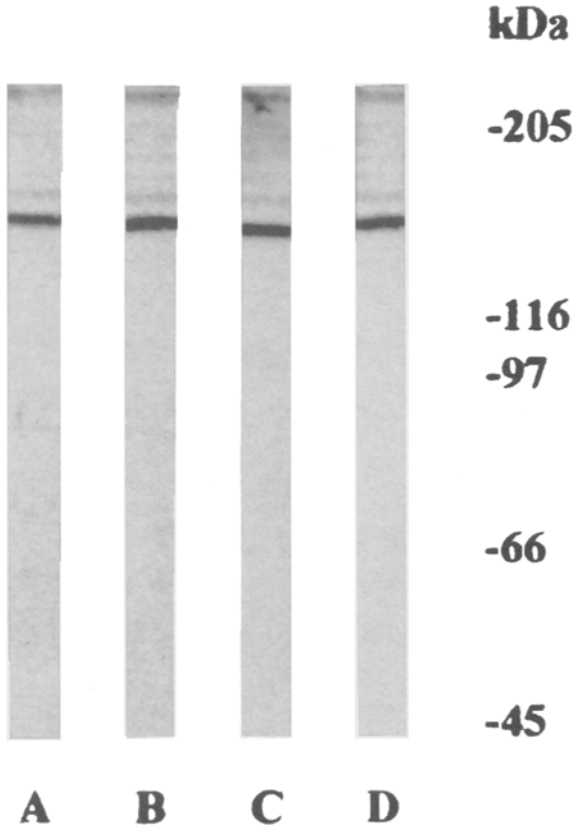

Figure 1 Western blot analysis of SDS-PAGE separation of FHA fragments in lysate from *B. pertussis* cells grown for 24 h in a 6-L fermenter, with a working volume of 2.5 L, under the following operating conditions: lane (a) N = 210 rpm, VVM = 1.00 L L^{-1} min^{-1}; lane (b) N = 270 rpm, VVM = 0.80 L L^{-1} min^{-1}; lane (c) N = 320 rpm, VVM = 0.42 L L^{-1} min^{-1}; lane (d) N = 450 rpm, VVM = 0.30 L L^{-1} min^{-1}. Migration of molecular mass markers (kDa) is indicated on the right.

In the present study we show that, in contrast to what was reported for extracellular FHA, neither cellular-bound FHA nor the protective activity of *B. pertussis* cells are significantly affected by hydromechanical forces generated during cultivation in a conventional stirred bioreactor. Thus, although any cultivation of *B. pertussis* to produce extracellular FHA must be carried out in a low-shear bioreactor, shear stress is not a factor of concern for the quality of cellular *B. pertussis* immunogens intended for whole cell pertussis vaccine production.

Acknowledgements

This work was supported by CONICET and SECYT-CAB-BIO.

References

1 Arai H and Y Sato. 1976. Separation and characterization of two distinct haemagglutinins contained in purified leukocytosis-promoting factor from *Bordetella pertussis*. Biochem Biophys Acta 444: 765.
2 Brown DR and CD Parker. 1987. Cloning of filamentous hemaggluti-

nin of *Bordetella pertussis* and its expression in *Escherichia coli*. Infect Immun 55: 154–161.
3 Code of Federal Regulations. 1988. Title 21 Part 620.4: 68–69.
4 Kimura A, KT Mountzouros, DA Relman, S Falkow and JT Cowell. 1990. *Bordetella pertussis* filamentous hemagglutinin: evaluation as a protective antigen and colonization factor in a mouse respiratory infection model. Infect Immun 58: 7–16.
5 Leininger E, CA Ewanowich, A Bhargava, M Peppler, JG Kenimer and MJ Brennan. 1992. Comparative roles of the Arg-Gly-Asp sequence present in the *Bordetella pertussis* adhesins pertactin and filamentous hemagglutinin. Infect Immun 60: 2380–2385.
6 Leininger E, P Probst, MJ Brennan and JG Kenimer. 1993. Inhibition of *Bordetella pertussis* filamentous hemagglutinin-mediated cell adherence with monoclonal antibodies. FEMS Microbiol Lett 106: 31–38.
7 Redd SC, HS Rumschlag, RJ Biellik, GN Sanden, CB Reimer and ML Cohen. 1988. Immunoblot analysis of humoral immune responses following infection with *Bordetella pertussis* or immunization with Diphtheria-Tetanus-Pertussis vaccine. J Clin Microbiol 17: 1373–1377.
8 Relman DA, M Domenighini, E Tuomanen, R Rappuoli and S Falkow. 1989. Filamentous hemagglutinin of *Bordetella pertussis*: nucleotide sequence and crucial role in adherence. Proc Natl Acad Sci USA 86: 2637–2641.
9 Rodriguez ME, AL Samo, DF Hozbor and OM Yantorno. 1993. Effect of hydromechanical forces on the production of filamentous haemagglutinin and pertussis toxin of *Bordetella pertussis*. J Ind Microbiol 12: 103–108.
10 Sato Y, K Izumiya, MA Oda and H Sato. 1979. Biological significance of *Bordetella pertussis* fimbriae or hemagglutinin: a possible role of the fimbriae or hemagglutinin for pathogenesis and antibacterial immunity. In: International Symposium on Pertussis (Manclark CR and JC Hill, eds), pp 51–57, US Department of Health, Education and Welfare Publication 79-1830. Government Printing Office, Washington, DC.
11 Thomas MG, K Redhead and HP Lambert. 1989. Human serum antibody responses to *Bordetella pertussis* infection and pertussis vaccination. J Infect Dis 159: 211–218.
12 Towbin H, T Staehelin and J Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76: 4350–4354.
13 Urisu A, JL Cowell and CR Manclark. 1986. Filamentous hemagglutinin has a major role in mediating adherence of *Bordetella pertussis* to human WiDr cells. Infect Immun 52: 695–701.
14 World Health Organization. 1989. Proposed revised requirements for diptheria toxoid, pertussis vaccine, tetanus toxoid and combined vaccines. Geneva: BS/88.1593 Rev 1: 30–45.
15 Worcester J and E Wilson. 1943. A table determining LD_{50} or 50 percent end point. Proc Natl Acad Sci USA 29: 207–212.