Acta vet. scand. 1988, *29*, 225–230.

From the Department of Anatomy and Embryology,
College of Veterinary Medicine, Helsinki, Finland.

Production and Characterization of Monoclonal Antibodies to Serotype Specific Antigens of Haemophilus Pleuropneumoniae Serotype 2

By *Armi Korvuo, Lars-Axel Lindberg* and *Jim Schröder*

Korvuo A., L.-A. Lindberg and J. Schröder: Production and characterization of monoclonal antibodies to serotype specific antigens of Haemophilus pleuropneumoniae serotype 2. Acta vet. scand. 1988, 29, 225–230. – Mouse monoclonal antibodies were produced to Haemophilus pleuropneumoniae bacteria of the most common serotype 2. 11 hybridoma cultures were recovered that produced antibodies with moderate to strong reactivity to the antigen. 10 of these antibodies were specific to isolated capsular antigens from H. pleuropneumoniae of serotype 2, while one antibody reacted with capsular antigens from bacteria of all 8 serotypes. One hybridoma producing antibodies with a titre of 1:1000 was cloned and the antibody specificity studied further. The binding of this antibody (1F3) to whole bacteria, and capsular extracts isolated at different temperatures indicate that the antibody is specific for a thermostable polysaccharide antigen present in the cellular capsule of H. pleuropneumoniae of serotype 2.

infection; pneumonia; pig.

Introduction

Haemophilus pleuropneumoniae is a pathogen, that causes a severe respiratory tract infection in pig. The disease is spread all over the world, and causes substantial economical loss (*Sebunya & Saunders* 1983). Attempts have been made to control the disease with serological diagnosis and vaccination programs. This requires extensie knowledge of H. pleuropneumoniae serotypes, since this bacterium is serotypically heterogenic with different serotypes showing a different geographical distribution. Presently 8 serotypes are known and characterized (*Nielsen & O'Connor* 1984) but at least 2 additional ones (9 and 10) are likely to exist (*Nielsen* 1985a, *Nielsen* 1985b).

The serotype specificity is determined by capsular antigens surrounding the cellular wall of H. pleuropneumoniae (*Nielsen* 1982, *Gunnarsson et al.* 1978). These can be isolated by incubation of young cultures of bacteria at 56°C in salt solution (*Nielsen* 1982). The serotype specific antigens have been characterized by rabbit antisera by agglutination, immunodiffusion or absorption tests (*Nielsen & O'Connor* 1984, *Gunnarsson et al.* 1978, *Gunnarsson et al.* 1977). Conventional antisera consist of numerous different antibody populations, that recognize the same or different antigenic determinants. Thus characterization of antigens with conventional antisera can be difficult and unreliable. Monoclonal antibodies offer clear advantages for antigen characterization and purification because of their highly specific

nature. In the present paper we describe the production and characterization of monoclonal antibodies to serotype 2 antigens of H. pleuropneumoniae. The effect of one of these antibodies in the prophylaxis of H. pleuropneumoniae infections in pigs will be tested in further experiments.

Material and methods

Bacterial strain and growth conditions

Bacterial strain 4226 of H. pleuropneumoniae serotype 2 was a gift from Dr. Ragnhild Nielsen, the State Veterinary Serum Laboratory, Copenhagen, Denmark. The bacteria were cultured on PPLO agar (Difco) enriched with 5 % horse serum, 2.5 % yeast extract and 0.1 % glucose.

Immunization protocol

Four BALB/c female mice were immunized with formalin (0.2 %) killed H. pleuropneumoniae bacteria cultured for 6 h, because in early logarithmic growth these bacteria express the polysaccharide capsule. The first inoculation was given intraperitoneally with 10^8 bacteria in Freund's incomplete adjuvant. The intraperitoneal inoculation was repeated after 3 weeks with 10^8 bacteria in 0.9 % NaCl. Blood samples were obtained and sera tested for antibodies against H. pleuropneumoniae by an enzyme linked immunosorbent assay (ELISA) described below. The mouse with the highest antibody titre was chosen for further immunization. It received a third injection of 10^8 bacteria in 0.9 % NaCl, this time intravenously. Four days after the last booster, the mouse was bled, sacrified and the spleen removed.

Cell fusion and culture

Spleen cells from the immunized mouse were fused at a ratio of 4:1 with mouse myeloma cells (NS-1) in the presence of 50 % polyethylene glycol 1500 (Baker Chemicals). Fused cells were plated in 120 wells on microtitre plates at a cell density of 5×10^5 cells per well. The hybrids were isolated in selective HAT (4×10^{-7} M aminopterin, 1×10^{-4} M hypoxanthine, 1.6×10^{-5} M thymidine) culture medium at 37°C in 7 % CO_2/93 % air humidified atmosphere.

Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 20 % horse serum, 1 mmol/l sodium pyruvate, 100 µg/ml penicillin, 100 IU/ml streptomycin and 2 mmol/l glutamine was used as culture medium. The hybridoma culture supernatants were tested for antibody production to antigens of H. pleuropneumoniae by an ELISA. The antibody producing hybridomas were expanded, and cloned two times by limiting dilution. Large quantities of antibodies were produced in ascites fluid by injecting antibody producing hybrids intraperitoneally into Pristane primed BALB/c mice.

Enzyme linked immunosorbent assay (ELISA)

Capsular antigens of H. pleuropneumoniae were extracted as described by *Nielsen* (1982). Briefly, 6 hour bacterial cultures were suspended in 2 ml cold sterile PBS. The suspension was washed once and heated in a water bath at 56°C for 30 min and subsequently centrifuged at 15000 rpm for 20 min at 4°C. The clear supernatant containing the capsular antigen was carefully recovered with a Pasteur pipette. The antigen was titrated, and an optimal dilution in 100 µl PBS was added to flat bottomed microtitre plates, and incubated over night at 4°C. The antigen solution was then flicked out, and the plates washed twice with PBS. The plates were blocked with 200 µl/well of blocking buffer consisting of PBS with 1 % BSA and 0.1 % NaN_3, and stored at 4°C until needed. If used immediately plates

were first incubated with blocking buffer at room temperature for 1 h.

Plates stored in blocking buffer were washed 3 times in buffer consisting of 0.9 % NaCl with 0.04 % Tween 20. 100 µl of diluted serum, or culture supernatant was added to wells which were then incubated at room temperature for 2 h. Unbound antibodies were removed by washing 3 times, after which 150 µl of peroxidase conjugated rabbit anti-mouse IgG (heavy and light chain specific, Cappel) diluted 1:1000 in PBS/0.1 % BSA/0.04 % Tween 20 was added to the wells, and incubated at room temperature for 1.5 h. The plates were then washed as before, and 200 µl of the enzyme substrate, 1.8 mmol/l ABTS (2. 2-azino-di-(3-ethylbenzo-thiazoline-6-sulfonate, Boehringer) in a potassium phosphate buffer with 1 mmol/l H_2O_2, pH 6, was added to the wells. The color change caused by action of enzyme on substrate was measured at 414 nm with a micro-ELISA reader (Labsystems) after the reaction had progressed to the desired degree.

Determination of serotype specificity of monoclonal antibodies

The following strains of H. pleuropneumoniae (obtained from Dr. Nielsen, Denmnark) were used for determination of serotype specificity:

Serotype 1, strain 78
„ 2, „ 78
„ 2, „ 4226
„ 3, „ 78
„ 4, „ M62
„ 5, „ K 17
„ 6, „ Femö
„ 7, „ WF 83
„ 8, „ Irland

All strains were cultured for 6 h at 37°C on PPLO agar, the capsular antigens extracted and coated on microtitre plates as described above. The serotype specificity of the monoclonal antibodies was determined by ELISA as above.

Determination of antigen specificity of monoclonal antibody 1F31F4II

Four plates of 6 h bacterial cultures were suspended each in 2 ml of cold sterile PBS, washed once and pooled together. Different antigen preparations were made from these bacterial suspensions. The bacterial suspension was divided into 7 aliquots of 1 ml each. One aliquot represented whole bacteria with intact capsular material. These bacteria were fixed to wells of microtitre plates as follows. The bacterial suspension was diluted 1:200 with PBS, and 100 µl of this dilution representing approximately 5×10^6 bacteria was added to flatbottomed microtitre plates, and the plates centrifuged for 7 min at 2500 rpm at 4°C. 150 µl of cold solution of 0.25 % glutaraldehyde in PBS was added to each well, which were then incubated for 5 min at 4°C. After this the plates were washed twice with PBS, and the plates blocked with 200 µl/well of blocking buffer. The 6 remaining aliquots were treated at different temperatures as follows. Two aliquots were kept at room temperature, 1 for 1/2 h the other for 2 h. Two further aliquots were kept in a water bath at 56°C 1 for 1/2 h and the other for 2 h. The last 2 aliquots were kept in a water bath at 100°C for 1/2 h and 2 h respectively. All aliquots were then centrifuged at 15000 rpm for 20 min. The supernatants constituting the capsular material (antigen preparation 2) were recovered, and plated onto microtitreplates as described earlier at a dilution of 1:200. The reactivity of monoclonal antibodies with different antigen preparations was tested by ELISA.

Determination of immunoglobulin class

The immunoglobulin class of our monoclo-

Table 1. Reactivity of hybrid supernatants with capsular antigens of different serotypes.

Supernatants	Serotypes								
	S1/78	S2/78	S2/4226	S3/78	S4/M62	S5/K17	S6/Femö	S7/WF83	S8/Irland
1B2	–	++	++	–	–	–	–	–	–
1B11	–	+++	+++	–	–	–	–	–	–
1C3	–	++	++	–	–	–	–	–	–
1F3	–	++++	++++	–	–	–	–	–	–
1G10	–	++	++	–	–	–	–	–	–
2B2	–	++	++	–	–	–	–	–	–
2C2	–	++	++	–	–	–	–	–	–
2C8	–	+++	+++	–	–	–	–	–	–
2E5	–	++	++	–	–	–	–	–	–
2F8	+	++	++	+	+	+	+	+	+
2G9	–	++	++	–	–	–	–	–	–

– negative
+ weakly positive
++ moderately positive
+++ strongly positive
++++ very strongly positive

nal antibodies was determined by immunodiffusion with goat-anti-mouse class and subclass specific antisera (Meloy).

Results

After fusion, the cells were plated out into 120 wells on microtitre plates, and after 2 weeks of HAT selection hybrid cell growth was observed in all wells. Antibody production was observed in 17 wells (14 %). Six of these cultures were weakly, 8 moderately, 2 strongly, and 1 very strongly positive. The 6 cultures that showed a weak antibody production were discarded from further studies.

The supernatants from the remaining 11 cultures were tested with isolated capsular antigens from H. pleuropneumoniae bacteria from all 8 serotypes. Ten of the culture supernatants were specific to antigens of H. pleuropneumoniae serotype 2 (strains 78 and 4226), 1 reacted with capsular antigens from bacteria of all 8 serotypes (Table 1). One strongly positive hybrid culture (1F3), with an antibody titre of 1:1000 as measured by ELISA, was cloned twice, and clone 1F31F4II was chosen for further studies. The titre of this antibody produced in ascites fluid was more than 1:100,000, and it was of IgG_1 subclass.

Table 2. Reactivity of monoclonal antibody 1F31F4II with whole bacteria and capsular antigens extracted at different temperatures.

Ascites dilution	Whole bacteria	CAg 1/2 h RT	CAg 2 h RT	CAg 1/2 h 56°C	CAg 2 h 56°C	CAg 1/2 h 100°C	CAg 2 h 100°C
1:1000	>2	1.91	>2	>2	>2	>2	>2
1:10000	1.60	1.68	1.87	>2	>2	>2	1.89
1:100000	0.38	0.72	0.73	0.69	0.74	0.73	0.47

Values at 414 nm by ELISA

This antibody reacted similarly with whole H. pleuropneumoniae bacteria, and with capsular extracts isolated at different temperatures (Table 2). However, at antibody dilutions of 1:10,000 and 1:100,000 the antibody binding to capsular extracts isolated at higher temperatures was slightly stronger than the binding to capsular extracts isolated at room temperature, or to whole bacteria.

Discussion

The capsular antigens of bacteria of the genera Haemophilus are polysaccharides (*Branefors-Helander et al.* 1976, *Sawata et al.* 1984). The serotype specific determinants of H. pleuropneumoniae like those of other Haemophilus strains are located in the bacterial capsule (*Nielsen* 1982, *Gunnarsson et al.* 1978, *Branefors-Helander et al.* 1976, *Sawata et al.* 1979), and are either lipopolysaccharides (LPS) or polysaccharides (PS) (*Nielsen & O'Connor* 1984, *Nielsen* 1985a, *Gunnarsson* 1979). The monoclonal antibodies produced against H. pleuropneumoniae bacteria of serotype 2 in the present study were tested against isolated capsular antigens from bacteria of all 8 serotypes, and found to be directed against serotype 2 specific antigens. Only 1 out of 11 culture supernatants crossreacted with antigens from the other serotypes.

The serotype specific antigens have been shown to be both thermolabile and thermostable (*Gunnarsson et al.* 1978, *Mittal et al.* 1983). Some species-specific antigens are also thermostable, but are located in the cellular wall, under the capsule. Our monoclonal antibody 1F31F4II is evidently directed against thermostable capsular surface antigens of H. pleuropneumoniae. This antigen is probably lipopolysaccharide or polysaccharide in nature, since antigen-antibody binding was not altered by a 2 h incubation of antigen at 100°C. Such treatment would probably have altered a protein antigen, and affected the antigen-antibody binding.

The methods used to serotype H. pleuropneumoniae bacteria, as well as to characterize serotype specific antigens have been tube agglutination tests (*Gunnarsson et al.* 1977, *Nielsen & O'Connor* 1984), slide agglutination tests (*Mittal et al.* 1982, *Nielsen & O'Connor* 1984), immunodiffusion (*Nielsen & O'Connor* 1984, *Gunnarsson et al.* 1978) immunofluorescence (*Rosendal & Boyd* 1982), ring precipitation (*Mittal et al.* 1982) indirect haemagglutination (*Nielsen & O'Connor* 1984, *Mittal et al.* 1983a) and coagglutination tests (*Mittal et al.* 1983b) all based on conventional rabbit antisera. The ELISA test described in the present paper is based on a serotype 2 specific monoclonal antibody. Such a test is especially valuable for both serotype determination, and antigen characterization in situations where immunologically crossreactive determinants exist, such as in serotypes 3, 6 and 8 of H. pleuropneumoniae (*Nielsen & O'Connor* 1984).

Beside the usefullness on monoclonal antibodies to bacterial diagnosis and antigen characterization, they might turn out to be useful for prophylactic protection of infections by passive immunization, and for antigen purification and vaccine production. These questions are adressed in a further paper.

Acknowledgements

This study was supported by funds from Monoclonal Antibodies Inc., California, USA, and by the Natural and the Medical Research Councils of the Academy of Finland. We also wish to thank Dr. Ragnhild Nielsen for her generous help.

References

Branefors-Helander P, Erbing C, Kenne L, et al.: Structural studies of the capsular antigen from Haemophilus influenzae type b. Acta Chem. Scand. B 1976, *30*, 276–277.

Gunnarsson A: Serologic studies on porcine strains of Haemophilus parahaemolyticus (pleuropneumoniae): Extraction of type-specific antigens. Amer. J. vet. Res. 1979, *40,* 469–472.

Gunnarsson A, Biberstein EL, Hurvell B: Serologic studies on porcine strains of Haemophilus parahaemolyticus (pleuropneumoniae): Agglutination reactions. Amer. J. vet. Res. 1977, *38,* 1111–1114.

Gunnarsson A, Hurvell B, Biberstein EL: Serologic studies of porcine strains of Haemophilus parahaemolyticus (pleuropneumoniae): Antigenic specificity and relationship between serotypes. Amer. J. vet. Res. 1978, *39,* 1286–1292.

Mittal KR, Higgins R, Lariviere S: Evaluation of slide agglutination and ring precipitation tests for capsular serotyping of Haemophilus pleuropneumoniae. J. Clin. Microbiol. 1982, *15,* 1019–1023.

Mittal KR, Higgins R, Lariviere S: Determination of antigenic specificity and relationship among Haemophilus pleuropneumoniae serotypes by an indirect haemagglutination test. J. Clin. Microbiol. 1983a, *17,* 787–790.

Mittal KR, Higgins R, Lariviere S: Identification and serotyping of Haemophilus pleuropneumoniae by coagglutination test. J. Clin. Microbiol. 1983b, *18,* 1351–1354.

Nielsen R: Haemophilus pleuropneumoniae infection in pigs. Thesis 1982.

Nielsen R: Serological characterization of Haemophilus pleuropneumoniae (Actinobacillus pleuropneumoniae) strains and proposal of a new serotype: serotype 9. Acta vet. scand. 1985a, *26,* 501–512.

Nielsen R: Serological characterization of Haemophilus pleuropneumoniae (Actionbacillus pleuropneumoniae) strains and proposal of a new serotype: serotype 10. Acta vet. scand. 1985b, *26,* 581–585.

Nielsen R, O'Connor PJ: Serological characterization of 8 Haemophilus pleuropneumoniae strains and proposal of a new serotype: serotype 8. Acta vet. scand. 1984, *25,* 96–106.

Rosendal S, Boyd DA: Haemophilus pleuropneumoniae serotyping. J. Clin. Microbiol. 1982, *16,* 840–843.

Sawata A, Kume K, Nakase Y: Antigenic structure and relationship between serotypes 1 and 2 of Haemophilus paragallinarum. Amer. J. vet. Res. 1979, *40,* 1450–1453.

Sawata A, Kume K, Nakai T: Relationship between anticapsular antibody and protective activity of a capsular antigen of Haemophilus paragallinarum. Jpn. J. Vet. Sci. 1984, *46,* 475–486.

Sebunya TNK, Saunders JR: Haemophilus pleuropneumoniae infection in swine: a review. J. Amer. vet. med. Assoc. 1983, *182,* 1331–1337.

Sammendrag

Produktion och karakterisering av monoklonala antikroppar mot serotypspecifika antigener av Haemophilus pleuropneumoniae, serotyp 2.

Murina monoklonala antikroppar producerades mot Haemophilus pleuropneumoniae bakteriens vanligaste serotyp 2. 11 antikroppsproducerande hybridomkulturer med moderat til stark antigenreaktivitet erhölls. 10 av dessa antikroppar var specifika mot isolerade kapselantigener från H. pleuropneumoniae, serotyp 2, medan en antigen reagerade med kapselantigener från bakterier av alla 8 serotyper. En hybridom som producerade antikroppar med en titer av 1:1000 klonades, och dess antikroppsspecifitet undersöktes ytterligare. Bindningen av denna antikropp (1F3) till hela bakterier, och till kapselextrakt isolerade vid olika temperaturer visar att antikroppen är specifik för en termostabil polysaccaridantigen i cellkapseln av H. pleuropneumoniae, serotyp 2.

(Received December 10, 1987).

Reprints may be requested from: L.-A. Lindberg, Dept. of Anatomy and Embryology, College of Veterinary Medicine, P. O. Box 6, SF-00551 Helsinki, Finland.