



Production of Monoclonal Antibodies for Avian Metapneumovirus (SHS-BR-121) Isolated in Brazil

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■ Keywords

Avian metapneumovirus, Brazil, monoclonal
antibodies, neutralizing epitopes.

■ Acknowledgments

The authors thank GF Davi, JRR dos Reis, and
JB Camargo for their valuable technical assis-
tance. L. T. C. received a grant of FAPESP (97-
13946-5).

Arrived: July / 2006
Approved: January / 2007

ABSTRACT

Avian Metapneumovirus (aMPV), also called Turkey Rhinotracheitis Virus (TRTV), is an upper respiratory tract infection of turkeys, chickens and other avian species. Five monoclonal antibodies (MAbs) were created against the Brazilian isolate (SHS-BR-121) of aMPV, MAbs 1A5B8; 1C1C4; 2C2E9 and 2A4C3 of IgG1 and MAbs 1C1F8 of IgG2a. Four MAbs (1A5B8; 1C1C4; 2C2E9 and 2A4C3) showed neutralizing activity and three (1A5B8; 1C1C4 and 2A4C3) inhibited cellular fusion *in vitro*. These MAbs were used to investigate antigenic relationship among three strains (SHS-BR-121, STG 854/88 and TRT 1439/91) of aMPV subtypes A and B using cross-neutralization test. The results confirm that the monoclonal antibodies described can be used as a valuable tool in the epizootiological and serological studies, and also for the specific diagnosis of the subtypes in the infection for Avian Metapneumovirus.

INTRODUCTION

Avian Metapneumovirus (aMPV) is member of the genus *Metapneumovirus*, family *Paramyxoviridae* (Pringle, 1998). This virus causes turkey rhinotracheitis (TRT), and it is associated with Swollen Head Syndrome (SHS) in chickens. aMPV was first reported in South Africa in the late 1970s, initially in turkeys, and subsequently also in chickens (Buys & Du Preez, 1980; Buys *et al.*, 1989). The disease is clinically characterized by apathy and swelling of the face and infra-orbital sinuses. While mortality rarely exceeds 2%, morbidity may reach 10% and in breeders, egg production is frequently affected (Cook, 2000).

It was initially believed that there was only one serotype of aMPV, within two subtypes (A and B). Four subtypes (A, B, C and D) are currently known, and present extensive differences in the attachment glycoprotein (G) gene sequences (Cook & Cavanagh, 2002, D'Arce *et al.*, 2005). In Brazil, the isolation of aMPV was accomplished by Arns & Hafez in 1995 from infected broiler breeder flocks of States of Minas Gerais and São Paulo. Serum samples from 43 flocks (broilers, broiler breeders, and layers), collected in different regions of Brazil (States of São Paulo, Minas Gerais, and Santa Catarina), were tested for presence of antibodies against the SHS-BR-121 strain. The results showed that 40.65% flocks were positive for anti-aMPV antibodies (Coswig, 1998), proving the wide distribution of this isolate in the country. In Brazil, the sequence of five aMPVs from chickens and one from turkeys were analyzed (Dani *et al.*, 1999; D'Arce *et al.*, 2005). aMPV subtypes can be differentiated by nucleotide sequence analyses (Juhász & Eaton, 1994), and using MAbs (maternal antibodies) (Collins *et al.*, 1993; Cook *et al.*, 1993). Different MAbs were produced against aMPV, demonstrating the existence of considerable antigenic variability among strains (Collins *et al.*, 1993; Cook *et al.*, 1993). In Japan, MAbs were used to investigate the antigenic



relationship among strains of aMPV. That study demonstrates that the Japanese strains present antigenic differences from British isolates (Tanaka *et al.*, 1996; Obi *et al.*, 1997). In the present study, we produced MABs against the SHS-BR-121 strain, and used them to compare the SHS-BR-121 strain with two aMPV strains isolated in Germany by cross-neutralization test.

MATERIAL AND METHODS

Virus sources

The SHS-BR-121 strain of aMPV isolated from chickens with SHS in Brazil (Arns & Hafez, 1995) was used. The other aMPV isolates were STG 854/88 and TRT 1439/91, isolated in Germany (Hafez, 1993), belonging to subtypes A and B, respectively. The strains were propagated in chicken-embryo related (CER) cell line (Ferreira *et al.*, 2003). The infected cell culture was maintained at 37° C until extensive cytopathic effect was observed. The SHS-BR-121 virus was purified by differential ultracentrifugation and by sedimentation through a 20 – 60% discontinuous sucrose gradient at 53000 X g for 90 min. The virus band was collected and used for immunization of mice and ELISA antigens. The other viruses were purified as described.

Production of monoclonal antibodies

Hybridomas secreting aMPV-specific antibodies were derived from three experiments by fusion of myeloma line cells Sp2/0 with spleen cells of BALB/c mice immunized with SHS-BR-121 according to the standard procedure described by Köhler & Milstein (1976). Five mice were immunized by intraperitoneal inoculation on days 1, 14, and 28, respectively. In the first injection, each mouse was inoculated with 15 mg purified virus, mixed with equal volume of Freund's complete adjuvant (Sigma, St. Louis, Mo, USA). The same amount of virus was mixed with an equal volume of Freund's incomplete adjuvant in the second and third injections. Twelve days after cell fusion, hybridoma colonies were screened for the presence of aMPV-specific antibodies by an indirect antigen-coating plate enzyme linked immunosorbent assay (ELISA) using microplates coated with the aMPV soluble antigen. Hybridoma colonies presenting positive reaction were transferred to wells of 24-well tissue culture plates, and retested before being submitted to limited dilutions cloning (Cook *et al.*, 1993).

Isotypes determination

The immunoglobulin subclasses secreted by the

hybridomas were determined by ELISA using a commercial kit (ImmunoPure® Monoclonal Antibody Isotyping Kit II - Pierce).

Production of antibodies in ascitic fluid

Ascitic fluid containing MABs was produced by injecting 10⁵ hybridoma cells into the peritoneal cavity of pristine-primed BALB/c mice. After 10-14 days, ascitic fluid was collected and tested for antibody titers by indirect ELISA. All biological assays were performed using MAB from ascetic fluids.

ELISA

Each of the MABs used in this study was tested by indirect ELISA on plates coated overnight with aMPV antigen purified from strain SHS-BR-121 grown in CER cultures. Individual microplates wells were coated with viral antigen diluted to give 2 mg of protein per well. The conjugate was goat anti-mouse IgG heavy-chain alkaline phosphatase, and the substrate para-Nitrophenyl Phosphate (pNPP). Incubation times were 1 h at 37 °C, and volumes per cavity were 100 µL. Serum samples with optical densities (OD) of 2 standard deviations above the ODs for the negative controls were considered positive for aMPV antibodies. Each plate contained multiple positive and negative serum samples to serve as controls.

Biological activities

MABs biological activities were examined using fusion and neutralization-inhibition tests, as described by Beller & van Wyke Coelingh (1989).

Fusion inhibition

The fusion inhibition assay was performed to identify MABs that inhibit syncytium formation *in vitro*. CER cell monolayers in 96-well plates were infected with 100 TCID₅₀ of the SHS-BR-121 strain, incubated for 1h at 37°C, the inoculum was removed, and the monolayer was washed twice with MEM to remove unadsorbed virus. Serial twofold dilutions of MABs were then added to each well. Five days later, monolayers were examined microscopically for cytopathic effects.

Viral neutralization

The test of viral neutralization was performed to analyze the ability of MABs to neutralize SHS-BR-121 strain infectivity and to prevent syncytium formation *in vitro*. For this, the procedures were described by Beeler & van Wyke Coelingh (1989). Serial twofold dilutions of MABs were mixed with 100 TCID₅₀ of strain



SHS-BR-121, and incubated for 1 hr at 37°C. Subsequently, virus-antibody mixtures were transferred to CER monolayers in 96-well plates, incubated for 5 days (37°C / 5% CO₂), and observed as described for the fusion inhibition assays.

The neutralization and fusion inhibition titers were expressed as the reciprocal of MAbs maximum dilution, which completely inhibited the infection and syncytium formation *in vitro*, respectively.

Cross-neutralization test

The cross-neutralization test with MAbs was used to analyze the antigenic relationships among three aMPV strains (SHS-BR-121, STG 854/88 and TRT 1439/91). Cross-neutralization tests with MAbs were performed using a modification of the procedure described by Collins *et al.* (1993). MAbs were tested against each sample, diluted in base 2. The cross-neutralization test was carried out as described above. The sera that did not present neutralizing activity at 1:64 dilution or below were considered negative.

RESULTS AND DISCUSSION

This paper describes the production of monoclonal antibodies against aMPV (SHS-BR-121) isolated in Brazil (Arns & Hafez, 1995) from broiler breeder flocks in the States of São Paulo and Minas Gerais. Previous studies reported close antigenic relationships among aMPV isolates. Studies of Obi *et al.* (1997) indicated similarities among different aMPV strains using conventional diagnosis techniques. The use of MAbs for the characterization of aMPV strains is helpful in antigenic relationship studies, and enable the recognition of specific epitopes; polyclonal antibodies do not have this capacity (Cook *et al.*, 1993). Therefore, we produced MAbs, and obtained five hybridoma cell lines (1A5B8; 1C1C4; 2C2E9; 1C1F8 and 2A4C3) for the antigen of SHS-BR-121 aMPV strain.

ELISA results are shown in Table 1. The cut-off point for optical densities was determined with normal serum,

diluted 1:100, with OD < 0,030.

Values presented are the mean of three repetitions.

Analyses for the antibody isotypes showed that four belonged to the IgG1 subclass (1A5B8; 1C1C4; 2C2E9 and 2A4C3) and one to IgG2a (1C1F8) (Table 1).

MAbs were also evaluated for their capacity to neutralize viral infectivity and to inhibit syncytium formation *in vitro*. The production of three clones (MAbs 1A5B8, 1C1C4 and 2A4C3) neutralized and inhibited the formation of syncytia by SHS-BR-121 aMPV, as shown in Table 1. These MAbs were efficient SHS-BR-121 inhibitors. Two MAbs (1C1F8 and 2C2E9) did not show neutralizing activity, while one MAb (1C1F8) did not show fusion inhibition activity. Our findings are similar to the results obtained for human respiratory syncytial virus (HRSV) and bovine respiratory syncytial virus (BRSV) in relation to the inhibitory activity for fusion and neutralization (Matheise *et al.*, 1995; Tanaka *et al.*, 1996). Protein F probably plays an important role in this case. It is suggested that the neutralization epitopes are the same as or very similar to the fusion-active site on protein F (Tanaka *et al.*, 1996). The two major antigens of aMPV are the F (fusion) and the G (attachment) proteins. Both form independent projections on the virus surface, which mediate adsorption and fusion of the virus envelope with the cell membranes (Juhász & Easton, 1994), and these proteins induce neutralizing antibodies (Collins *et al.*, 1993). Two MAbs obtained in this study were not able to neutralize viral infectivity, but inhibited syncytium formation; 1C1F8 and 2C2E9 inhibited possible due to reaction to F protein. The reason may be a consequence of a variation of the epitopes of those proteins (Obi *et al.*, 1997). The aMPV strain SHS-BR-121 isolated in Brazil presents differences some antigenic properties as compared to the two strains isolated in Germany. These results indicate that antigenic divergence between aMPV may occur.

In cross-neutralization tests, all of the MAbs presented neutralizing activity against the homologous virus. As the heterologous viruses TRT 1439/91 and STG

Table 1 - Properties of monoclonal antibodies created against the SHS-BR-121 strain of Avian Metapneumovirus.

MAbs	ELISA (OD) ^a	Isotype ^b Subclass	Biological activities	
			Viral Neutralization ^c	Inhibition of cell fusion
1A5B8	0,222	IgG1	256	1024
1C1C4	0,248	IgG1	128	1024
1C1F8	0,216	IgG2a	<2	<2
2C2E9	0,211	IgG1	<2	256
2A4C3	0,266	IgG1	256	128

a. Optical density (OD) of control antigen < 0,030; means of three repetitions. b. Isotype subclass of immunoglobulin was determined using a commercial kit (ImmunoPure® Monoclonal Antibody Isotyping Kit II Pierce). c. Reciprocal of dilution (log₂) that neutralized 100 TCID₅₀ of SHS-BR-121 strain.



854/88, MAbs C1F8 and 2C2E9 presented positive reaction (Table 2). Studies on antigenic relationships using conventional techniques indicated close similarities between different aMPV isolates (Gough & Collins, 1989). Antigenic variations of F and G proteins in respiratory syncytial virus (RSV) and aMPV (Beeler & van Wyke Coelingh, 1989; Collins *et al.*, 1993) were reported. These results suggest the antigenic variation amongst aMPV strains may be dependent on F and G proteins. Mab 1C1F8 reacted with aMPV subtypes A and B, possibly in a conserved region not related to neutralization. Some MAbs produced by Matheise *et al.* (1995) recognized both a bovine respiratory syncytial virus (BRSV) and human respiratory syncytial virus (HRSV) strains, demonstrating the existence of conserved epitopes in these viruses.

Table 2 - Cross-neutralization test using MAbs and different aMPV strains.

aMPV ^a Virus strain	MAb				
	1A5B8	1C1C4	1C1F8	2C2E9	2A4C3
SHS-121-BR	1024 ^b	1024	<2	1024	512
TRT 1439/91	-	512	-	128	512
STG 854/88	-	-	-	128	-

a.The different samples were tested against each monoclonal antibody. b.Titers are expressed as the reciprocal of dilution (log₂) that neutralized 100 TCID₅₀.

The relationship between neutralization epitopes and fusion sites of the F protein of aMPV should be further analyzed.

The development assays with these MAbs will constitute important tools for the analyses of the antigenic properties of aMPV, and will aid the characterization of aMPV isolates and strains.

REFERENCES

- Arns CW, Hafez HM. Isolation and identification of Avian Metapneumovirus from broiler breeder flocks in Brazil. In: Jensen MM, editor. Proceedings of the 44th Western Poultry Diseases Conference; 1995; Sacramento, California, USA; 1995. p.124-125.
- Beeler JA, van Wyke Coelingh K. Neutralization epitopes of the F glycoprotein of respiratory syncytial virus: effect of mutation upon fusion function. *Journal of Virology* 1989; 63:2941-2950.
- Buyts SB, Du Preez JH. A preliminary report on the isolation of a virus causing sinusitis in turkeys in South Africa and attempts to attenuate the virus. *Turkeys* 1980; 28:36-46.
- Buyts SB, Du Preez JH, Els HJ. Swollen head syndrome in chickens: a preliminary report on the isolation of a possible aetiological agent. *Journal of South African Veterinary Association* 1989; 60: 221-222.
- Collins MS, Gough RE, Alexander DJ. Antigenic differentiation of Avian Metapneumovirus isolates using polyclonal antisera in mouse monoclonal antibodies. *Avian Pathology* 1993; 22:469-479.
- Cook JKA, Cavanagh D. Detection and differentiation of Avian Metapneumoviruses (metapneumoviruses). *Avian Pathology* 2002; 31:117-132.
- Cook JKA. Avian Metapneumovirus infections of turkeys and chickens. *The Veterinary Journal* 2000; 160:118-125.
- Cook JKA, Jones BV, Ellis MM, Jing L, Cavanagh D. Antigenic differentiation of strains of turkey rhinotracheitis virus using monoclonal antibodies. *Avian Pathology* 1993; 22:257-273.
- Coswig LT. Presença de anticorpos contra Pneumovírus Aviário nas regiões Sul e Sudeste do Brasil [dissertação]. Pelotas (RS): Universidade Federal de Pelotas; 1998.
- D'Arce RCF, Coswig LT, Almeida RS, Trevisol IM, Monteiro MCB, Rossini LI, Di Fabio J, Hafez HM, Arns CW. Subtyping of new Brazilian avian metapneumovirus isolates from chickens and turkeys by reverse transcriptase-nested-polymerase chain reaction. *Avian Pathology* 2005; 34:133-136.
- Dani MA, Durigon EL, Arns CW. Molecular characterization of Brazilian Avian Metapneumovirus isolates: comparison between immunochemiluminescent Southern blot and nested PCR. *Journal of Virological Methods* 1999; 79:237-241.
- Ferreira HL, Pilz D, Mesquita LG, Cardoso T. Infectious bronchitis virus replication in the chicken embryo related cell line. *Avian Pathology* 2003; 32:413-417.
- Gough RE, Collins MS. Antigenic relationships of three turkey rhinotracheitis viruses. *Avian Pathology* 1989; 18:227-238.
- Hafez HM. The role of pneumovirus in the swollen head syndrome of chickens. *Review für Archiv Geflügelkunde* 1993; 57:181-185.
- Juhász K, Easton AJ. Extensive sequence variation in the attachment (G) protein gene of Avian Metapneumovirus: evidence for two distinct subtypes. *Journal of General Virology* 1994; 75:2873-2880.
- Köhler G, Milstein C. Derivation of specific anti-body producing tissue culture and tumor lines by cell fusion. *European Journal Immunology* 1976; 6:511-519.
- Matheise JP, Walravens K, Collard A, Coppe P, Letesson JJ. Antigenic analyses of the F protein of the bovine respiratory syncytial virus: identification of two distinct antigenic sites involved in fusion inhibition. *Archives of Virology* 1995; 140:993-1005.
- Obi T, Kokumai A, Takuma H, Tanaka M. Antigenic differentiation of virus of Turkey Rhinotracheitis strain using monoclonal antibodies and polyclonal antisera. *Journal Veterinary Medical Science* 1997; 59:795-799.
- Pringle CR. Virus taxonomy. *Archives of Virology* 1998; 143: 1449-1459.
- Tanaka M, Obi T, Takamura H, Kokumai N, Higashihara R, Hiramatsu K, Shimizu Y. Inhibition of cell fusion by neutralising monoclonal antibodies to F₁ protein of Turkey Rhinotracheitis Virus. *Journal Veterinary Medical Science* 1996; 58:581-582.