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Involvement of the complement system in the protection of mice from challenge with respiratory syncytial virus Long strain following passive immunization with monoclonal antibody 18A2B2

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Passive immunization of mice with 131 μg of the non-neutralizing monoclonal antibody (mAb) 18A2B2, directed against the A subgroup epitope of the G glycoprotein of respiratory syncytial virus Long strain (RSV), confers protection against viral i.n. challenge. The role of the Fc fragment of this antibody as well as the involvement of antibody-dependent cellular cytotoxicity (ADCC) and complement-mediated cytolysis towards protection was evaluated in vivo. *Passive immunization with the Fab fragment alone (618–907 μg mouse^{-1}) was unable to confer protection in mice. Furthermore, we passively immunized with the mAb 18A2B2 SCID beige mice, which are deficient in natural killer (NK) cell activity, to ascertain the role of NK cells in the protective mechanism. These mice were free of virus 5 days following viral challenge, indicating that NK cells do not contribute significantly towards the protective action of this antibody. Moreover, passively immunized BALB/c mice decomplemented with 8–10 U of cobra venom factor (CoVF) and DBA/2J mice (C5 deficient) were only partially protected. These findings suggest that in mice the alternative and classical pathways of the complement system are involved in the passive protection mechanism conferred by the non-neutralizing mAb 18A2B2. To our knowledge, it is the first description of a protective mechanism in mice that involves a non-neutralizing antibody and the complement system.*

Keywords: RSV; mAb; complement

Respiratory syncytial virus causes severe lower respiratory tract disease in infants and children[1,2]. For several years the F and G glycoproteins of RSV have been under scrutiny in the prospect of developing an effective and innocuous vaccine[1,5]. Studies involving the characterization of potential antigenic and immunogenic sites of the F and G glycoproteins revealed that some B epitopes are involved in neutralization and passive protection[3,6–11]. It was shown that the subgroup specific antigenic site is located within the amino acid region 174–188 of the glycoprotein G[8,9]. Furthermore, we previously demonstrated[10] that a synthetic peptide consisting of the amino acids 174–187 conferred protection in BALB/c mice from intranasal challenge with the RSV, Long strain. Passive immunization with mAb 18A2B2, directed against the above epitope, also mediates an antiviral effect *in vivo* but has no neutralizing activity *in vitro*[10]. These observations suggest that the Fc portion of the mAb 18A2B2 may play a critical role towards protection *in vivo* either through an ADCC response[11,12], a complement-mediated cytolysis or a complement-dependent virolysis[13–17]. In this study we examined the mechanisms involved in the protection of mice following passive immunization with mAb 18A2B2. The protective potential of the mAb was investigated in SCID mice deficient in B and T cell function, in SCID beige mice deficient in B, T, NK cells and polymorphonuclear (PMN) cells. Furthermore, complement depleted BALB/C mice and DBA/2J mice deficient in the C5 component of the complement were used to evaluate the role of the complement system in protection. Finally, IFN-γ depleted BALB/c mice allowed us to evaluate the contribution of IFN-γ towards the antiviral effect observed in BALB/c mice passively immunized with mAb 18A2B2. The results demonstrate that both pathways of the complement

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system are involved in the protection of mice passively immunized with the non-neutralizing mAb 18A2B2 directed against the A subgroup epitope of glycoprotein G of RSV, Long strain.

MATERIALS AND METHODS

Animals

BALB/c mice were purchased from Charles River Laboratory (St-Constant, Qc.) SCID and SCID Beige mice were purchased from Charles River Laboratory (Wilmington, MA) and the DBA/2J mice (C deficient strain, lacking C5) from Jackson Laboratory (Bar Harbor, ME). All mice were female, 28–40 days old.

Cells and virus

The Long strain of respiratory syncytial virus (ATCC VR-26; American Type Culture Collection, Rockville, MD) was propagated on Hep-2 cells (ATCC CCL-23) previously grown in equal parts of Eagle's minimal essential medium and medium 199 (GIBCO Laboratories, Grand Island, NY), supplemented with 50 μg ml^{-1} of gentamicyn and 5% (v/v) fetal calf serum (FCS). When infected, monolayers were incubated in serum-free medium.

Virus titration assay

Virus titrations were carried out in 24-well, flat-bottom microtiter plates on HEP-2 cells (4×10^4 cells well^{-1}). The medium from confluent cultures was removed and 200 μl from a tenfold dilution of virus was added to the wells. In the case of virus obtained from lung extracts, the solution was added as a tenfold dilution. After a 90 min adsorption period, 1.5 ml of 1% serum medium were added to each well. Plates were incubated for 5–10 days at 37°C in a humidified atmosphere containing 5% CO_2. Viral syncytia were then counted: titers were expressed as tissue culture infectious doses ($\log_{10}$ $TCID_{50}$).

mAb and Fab fragment purification

Purification of mAb 18A2B2 was carried out by standard Protein-A-Sepharose chromatography[18]. Fab fragments of mAb 18A2B2 were obtained by papain digestion. Briefly, 10 mg of purified mAb 18A2B2 were digested in 0.1 M acetate and 3 mM ethylenediaminetetraacetic acid (EDTA) buffer, pH 5.5, with 0.5 mg papain (Sigma Chemical Co., St Louis, MO) preactivated with 50 mM cysteine (Sigma) for 7 h at 37°C. The reaction mixture was then passed twice through a protein-A-Sepharose column to eliminate Fc fragments and undigested antibodies. Purified Fab fragments were dialyzed against phosphate buffer saline solution (PBS) (Gibco), pH 7.4, and stored at −20°C until use. The purity of the fragments was evaluated by standard sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under non-reducing conditions and Coomassie Blue staining[19], followed by densitometry with a GS-670 laser densitometer (Bio-Rad, Mississauga, Ontario). The activities of mAb and Fab fragments were determined by ELISA.

Passive protection assay

In passive protection studies, groups of mice were immunized intravenously (i.v.) with 131 μg of mAb 18A2B2 and were immediately challenged intranasally (i.n.) with 50 μl of $10^{7.2}$ $TCID_{50}$ RSV, Long strain. Five days following challenge the mice were killed and their lungs were tested for the presence of virus by titration assay.

Decomplementation

Complement depletion was achieved by injection of *Naja naja* cobra venom factor (CoVF) (Sigma). Each mouse received 8 doses of 1.0 or 1.25 U^{20} using the following schedule and route of inoculation, where challenge occurs at time 0: −24 h, −19 h, 0, +6 h via i.p. route and +24 h, +48 h, +72 h and +96 h post-challenge via i.v. route. At time 0 mice received 131 μg of mAb 18A2B2 (i.v.) and were challenged as described previously. In all cases described above, the animals were killed 5 days after challenge and their lungs were tested for the presence of virus by titration assay.

Depletion of IFN-γ

Depletion of IFN-γ was acheived by injection of anti-IFN-γ antibodies (CedarLane Laboratories, Horby, Ontario) via i.v. route. Each mouse received 300 μg of antibodies 2 h before challenge. In addition each mouse received 131 μg of mAb 18A2B2 i.v. 30 min before challenge. The animals were killed 5 days after challenge and their lungs were tested for the presence of virus by titration assay.

ELISA

Three hundred nanograms per well of RSV or control Hep-2 antigen purified by sucrose density step gradient centrifugation (30/50%, w/v) were adsorbed overnight, on flat-bottom microtiter plates at 4°C. The plates were washed, blocked for 30 min with PBS containing 10% FCS (Gibco Laboratories, Grand Island, NY) (v/v). MAb 18A2B2 and Fab fragments serially diluted in PBS supplemented with 10% FCS and 0.02% Tween 20, were added. After 2 h incubation at 20°C, the plates were washed and 100 μl of a 1/1000 dilution of horseradish peroxidase-labeled rabbit anti-mouse IgG (Cappel, Scarborough, Ontario) or goat anti-mouse IgG (Fab-specific) antibodies (Sigma) were added to each well. Plates were further incubated for 2 h at 20°C, washed again and the reaction developed with *O*-phenylenediamine [0.4 mg ml^{-1} in 1 M sodium citrate, pH 5.0 containing 0.03% (v/v) H_2O_2]. Results were expressed as the inverse of the last serum dilution that gave a reading above the average reading plus two standard deviations of an isotypic mAb control.

Plaque reduction assay

Neutralization assays were carried out in microtiter plates: 25 μl of mAb 18A2B2 (131 μg/100 μl) were incubated overnight in the presence or absence of 2.5 μl of mouse complement (Sigma). To the mixture was then added and mixed 25 μl of viral suspension containing 100 $TCID_{50}$ ml^{-1} of RSV, Long strain. The neutralization reaction was allowed to proceed for 4 h at 4°C. The virus–antibodies mixtures were added to plates containing Hep-2 cell sheets, incubated at 37°C for 90 min before the addition of 150 μl of 1% (w/v) methylcellulose semisolid culture medium. After an incubation period of

Table 1 Passive protection assays of mAb 18A2B2 and its Fab fragment in BALB/c mice upon i.n. challenge with RSV

	BALB/c mice with passive immunization mAb 18A2B2 (131 μg mouse^{-1})	BALB/c mice with passive immunization Fab fragments (618 μg mouse^{-1})	BALB/c mice with passive immunization Fab fragments (907 μg mouse^{-1})	BALB/c mice control group without passive immunization
	9 mice	3 mice	3 mice	5 mice
Mean viral titer[a]	<1.7±0.25	3.52±0.61	4.28±0.14	3.70±0.84
	P<0.0001	N.S.[b]	N.S.[b]	

[a]Log 10 $TCID_{50/g}$ of lungs±S.D. Mice received 131 μg of mAb 18A2B2 i.v. 30 min before i.n. challenge with 50 μl of $10^{7.2}$ $TCID_{50}$ RSV, Long strain. In the experiment using Fab fragments, each BALB/c mouse received (i.v.) a total of 618 μg or 907 μg of Fab fragments. Mice of the first group were given a total of 618 μg at time period: −30 min, 3, 6, 24 and 29 h post-infection. Mice of the second group were given a total of 907 μg at the same time period. Animals were killed 5 days following challenge and viral titers from the lungs were evaluated. [b]Not significant at the 0.05 level

Table 2 Passive protection assays of mAb 18A2B2 in SCID and SCID beige mice upon intranasal challenge with RSV

	SCID mice with passive immunization mAbs 18A2B2 (131 μg mouse^{-1})	SCID mice control group without passive immunization	SCID beige mice with passive immunization mAbs 18A2B2 (131 μg mouse^{-1})	SCID beige mice control group without passive immunization
	6 mice	6 mice	6 mice	6 mice
Mean viral titer[a]	<1.7±0	4.3±0.13	<1.7±0	4.6±0.48
	P<0.0001		*P*<0.0001	

[a]Log 10 $TCID_{50/g}$ of lungs±S.D. Mice received 131 μg of mAb 18A2B2 i.v. 30 min before i.n. challenge with 50 μl of $10^{7.2}$ $TCID_{50}$ RSV, Long strain. Animals were killed 5 days following challenge and their lungs were tested for the presence of virus by titration assay

4 days at 37°C, in a humidified atmosphere containing 5% (v/v) CO_2, the plates were read. Titers were expressed as the highest dilution that neutralized 50% of the infectious units.

RESULTS AND DISCUSSION

In order to characterize the protective mechanism conferred to mice by 131 μg of mAb 18A2B2, we passively immunized BALB/c mice with either this mAb or with its purified Fab fragment, then challenged the mice with RSV, Long strain. MAb 18A2B2 prevented replication of RSV in the lungs of treated mice (*Table 1*). However, mice receiving 618–907 μg of the Fab fragments showed no significant reduction of viral titres in the lungs (*t*-test 0.05 level) in comparison to the control mice receiving intranasal challenge only (*Table 1*). This observation is consistant with the observation made by Oldstone *et al.*[21] that Fab fragments are almost totally ineffective in the lysis of viral infected cells. Lamarre and Talbot[22] showed that the Fab fragment of a neutralizing mAb directed against the S glycoprotein of murine hepatitis virus required tenfold the amount of the whole antibody to achieve the same level of protection in mice. In our experiment, the complete absence of protection observed with the passive transfer of up to 907 μg of Fab fragments suggests that the Fc fragment of the mAb 18A2B2 plays an important role in passive protection. Unfortunately, we have not been able to evaluate the protective potential of antibody bivalency by using $F(ab')_2$ since the isotype IgG2b is degraded upon digestion with pepsin.

SCID mice have few if any lymphocytes; they are hypogammaglobulinaemic and deficient for immune functions mediated by T and B lymphocytes[23,24]. We immunized SCID mice with mAb 18A2B2 to determine whether B and T cells play a role in the protection conferred. The 6 mice receiving mAb were totally protected while the control mice had an average viral titer of $10^{4.3}$ $TCID_{50}$. Total protection observed in passively immunized SCID mice suggests that passive protection by mAb 18A2B2 is not dependent on B- or T-cell functions. Previous studies reported that mice expressing the beige mutation (bg/bg) have an NK defect which appears to lie within the lytic machinery of NK cells[25–27]. The use of beige mice has proven valuable in studying cytomegalovirus and lymphocytic choriomeningitis virus infection in the absence of functional NK cells[28,29].

Our previous observation that the presence of Fc fragments may be required for protection of mice brought forward the possibility that NK cells could be involved via an ADCC mechanism. In order to test this hypothesis we used the beige mouse model that would allow *in vivo* abrogation of NK cell activity. In our studies the total protection of the SCID beige mice passively immunized with mAb 18A2B2 (*Table 2*) suggests that NK cell lysis of virus-infected cells does not contribute significantly towards the protective action of the mAb 18A2B2.

Cobra venom factor is known for its anticomplementary effects *in vitro* and *in vivo*, particularly on the third and fifth components of the complement system[20,30]. Moreover, the role of both the alternative and classical pathways of the complement system in host recovery from viral infection has been documented. Oldstone *et al.*[15] showed that C3-dependent crosslinking of polyoma viral particles and the presence of C3b on the viral surface play a significant role in the neutralization of this virus. The importance of C3 in the clearance of polyoma–antibody complexes was shown by delayed removal of the complexes from the serum after *in vivo*

Table 3 Passive protection assays of mAb 18A2B2 in decomplemented BALB/c and DBA/2J mice upon i.n. challenge with RSV

	BALB/c mice CoVF decomplemented with passive immunization mAb 18A2B2 (131 μg mouse^{-1})	BALB/c mice control group *without* passive immunization	DBA/2J[b] mice with passive immunization mAb 18A2B2 (131 μg mouse^{-1})	DBA/2J mice control group *without* passive immunization
Mean viral titer[a]	5 mice out of 6 3.07±0.70 1 mouse out of 6 protected P=0.0002	6 mice 4.74±0.18	4 mice out of 6 2.49±0.76 2 mice out of 6 protected P<0.0001	6 mice 4.86±0.26

[a]Log 10 $TCID_{50/g}$ of lungs±S.D. [b]DBA/2J mice are deficient in C5 of the complement system. Complement depletion was achieved by injection of *Naja naja* cobra venom factor (CoVF). Each mouse received 8 doses of 1.0 or 1.25 U using the following schedule and route of inoculation, where challenge occurs at time 0: −24 h, −19 h, 0, +6 h via i.p. route and +24 h, +48 h, +72 h and +96 h post-challenge via i.v. route. At time 0 mice received 131 μg of mAb 18A2B2 (i.v.) and were challenged. Five days following challenge mice's lungs were tested for the presence of virus by titration assay

depletion of C3 by CoVF treatment. We carried out an experiment using CoVF to ascertain whether inhibition of the classical and the alternative pathway would affect the immune protection observed in mice passively immunized with mAb 18A2B2. BALB/c mice treated with CoVF had an average viral titer of $10^{3.07}$ $TCID_{50}$ in their lungs which was approx. 1000-fold higher than the average titer ($<10^{1.7}$ $TCID_{50}$) of mice passively immunized with mAb 18A2B2 (*Table 1*). However, complement-depleted mice had an average viral titer lower than mice of the control group not passively immunized nor complement-depleted ($10^{4.74}$ $TCID_{50}$). Our results show that BALB/c mice depleted of complement by CoVF treatments are not protected following viral challenge. Since the inactivator complex CoVF affects not only C3 but also C5 in mice[20], our findings strongly suggest that at least one pathway of the complement system is involved in the observed protection of mice by mAb 18A2B2. Our data are consistent with the results obtained by Hirsch *et al.*[31] who showed that 1000-fold more virus was present in the brains of complement-depleted mice. Similar observations on the role of the complement system were made by Hicks *et al.*[32] concerning complement depleted mice undergoing more severe infections with influenza viruses in comparison to normal mice.

In order to clarify the role of the classical pathway alone in the protection, we used DBA/2J mice deficient in C5 component of the complement system. *Table 3* shows that out of six DBA/2J mice passively immunized with mAb 18A2B2, only two were protected. The other four mice had a low average viral lung titer of $10^{2.49}$ $TCID_{50}$ while control mice not passively immunized with the mAb showed a high average viral titer of $10^{4.86}$ $TCID_{50}$. In comparison to mice depleted of complement by CoVF treatments, the C5-deficient mice had lower viral titers in their lungs (*t*-test, $P \leq 0.01$). These data seem to indicate that the classical pathway characterized by the action of C5 is involved to a lesser extent in the protection.

The *in vitro* neutralization tests designed to evaluate the neutralization effect of mice complement in the presence of either mAb 18A2B2 or Fab fragments did not show neutralization titers significantly different from those of the control wells that contained only viral particles and mAb 18A2B2 without complement (results not shown). These results seem to indicate that there is no viral neutralization *in vitro* when mAb 18A2B2 are incubated overnight in the presence of mouse complement. The lack of viral neutralization suggests that the antiviral mechanism of complement fixation carried out *in vivo* involving mAb and complement may require mediators not present in the tissue culture system. It is interesting to notice that Taylor *et al.*[4] produced two neutralizing mAbs, directed against the G glycoprotein of RSV, that showed complement-dependent cell lysis of fibroblasts infected cells. These results suggest that the complement system may show different mechanisms of action with respect to the type of mAb involved such as neutralizing or non-neutralizing.

It was shown that administration of anti-IFN-γ serum enhanced the mortality of mice infected with herpes simplex virus[33] and raised virus titers of lymphocytic choriomeningitis virus[34] and vaccinia virus[35] infected organs. In our study, we attempted to evaluate the effect of IFN-γ as a complementary mechanism in the protection of mice. Our results show that three mice injected with anti-IFN-γ antibodies and passively immunized with mAb 18A2B2 were totally protected as were the mice passively immunized with mAb 18A2B2 that were not IFN-γ depleted (*Table 1*). This finding suggests that the participation of IFN-γ in the observed protection with mAb 18A2B2 is unlikely. This conclusion is also supported by the fact that we were unable to detect any IFN-γ in the sera of mice vaccinated with the peptide G/174-187 (result not shown).

Our study demonstrates that the complement system is partially involved in the protection of mice passively immunized with mAb 18A2B2 and challenged with RSV, Long strain. However, depletion of the complement system appears not to be sufficient to totally abrogate the protection of mice passively immunized with mAb 18A2B2. Macrophages and monocytes known notably for their viral phagocytic properties and cytokines production could confer a complementary protection in our animal model. This hypothesis is yet to be verified.

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