27

INDUCTION OF A PROTECTIVE IMMUNE RESPONSE TO MURINE CORONAVIRUS WITH NON-INTERNAL IMAGE ANTI-IDIOTYPIC ANTIBODIES

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ABSTRACT

Neurotropic murine coronaviruses (MHV) provide an excellent animal model to study experimental modulation of the immune response to a viral pathogen with anti-idiotypic antibodies. It is known that among the various types of anti-idiotypic antibodies (anti-Id), those designated beta (β) or internal image can molecularly mimic the antigen and induce biological activities such as anti-viral protection and neutralization. We have recently shown that polyclonal non-internal image anti-idiotypic antibodies of the γ-type could induce protective anti-coronavirus immunity[1].

In the present study, a polyclonal anti-Id (Ab2) was induced against a neutralizing murine monoclonal antibody (MAb1), designated 5B170.11. Mice immunized with this affinity-purified rabbit $Ab2_{\alpha}$, a non-internal image antibody, were partially protected against lethal infection by the JHM strain of MHV. However, other polyclonal and monoclonal non-internal image Ab2 induced against another neutralizing MAb1, designated 4-11G.6, were not able to protect mice against lethal infection with the A59 strain of MHV.

These results demonstrate that anti-viral protection by altering the idiotypic network with non-internal image-bearing anti-idiotype reagents can be achieved even with some anti-Id of the α-type.

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INTRODUCTION

Anti-idiotypic antibodies are potentially involved in the regulation of the immune response to a given antigen (Ag)[2]. Moreover, anti-Id have been used to manipulate the immune response *in vivo* in several experimental systems[3,4].

Anti-Id (Ab2) are classified in three different categories based on the idiotopes they recognize on Ab1. $Ab2_{\alpha}$ recognize idiotopes far from the antigen combining site (paratope) of Ab1. $Ab2_{\gamma}$ recognize idiotopes near the paratope of Ab1 and can compete with antigen for the binding site of Ab1. $Ab2_{\beta}$ are referred to as internal image anti-Id. They have the capacity to mimic the antigen used to generate the Ab1 and can substitute for antigen in inducing an anti-antigen response because they recognize an idiotype at the level of the paratope. However, the idiotypic network is complex because some non-internal image anti-Id can also induce an immune response to antigen, which can be described as biological rather than a structural mimicry. For example, $Ab2_{\alpha}$ could induce an anti-hepatitis B surface Ag response[5] and neutralizing antibodies against HIV[6]. Also, we have described an $Ab2_{\gamma}$-induced protective immune response against MHV-A59[1]. Therefore, non-internal image Ab2 can have interesting biological properties, although we do not yet understand their mechanisms of action.

Neurotropic murine coronaviruses (MHV strains JHM and A59) provide an excellent animal model to study the manipulation of the idiotypic network and its effect on a viral infection.

In the present study, we describe the production and characterization of non-internal image Ab2s against *in vitro* neutralizing and *in vivo* protective monoclonal antibodies (MAb1) specific to MHV-A59 and MHV-JHM. MAb1 4-11G.6 recognizes a discontinuous epitope on the spike (S) glycoprotein of MHV-A59, whereas MAb1 5B170.11 recognizes a linear epitope on the homologous protein of MHV-JHM. These two MAb1 had both been characterized previously and shown to neutralize virus infectivity *in vitro* and passively protect mice *in vivo* against a lethal MHV infection[7,10]. This suggested that the S protein has biological importance in immune protection against MHV infection, which was confirmed by the demonstration that affinity-purified S glycoprotein could vaccinate against lethal coronavirus infection[7].

Administration of these Ab2s to BALB/c mice showed that some non-internal image $Ab2_{\alpha}$ could partially mimic interesting biological properties of internal image Ab2, such as *in vivo* protection against viral infection. This emphasizes the complexity of the idiotypic network and how little is known on the mechanisms of induction of a protective immune response by anti-Id.

MATERIALS AND METHODS

Animals. New-Zealand white female rabbits of 2.5 to 3 kg were purchased from *Ferme de sélection Cunipur*, Stukely Sud, Québec, Canada. Four to 5 week-old female BALB/c mice were purchased from Charles River, St-Constant, Québec, Canada.

Antibodies. The production and characterization of the hybridoma secreting mouse neutralizing MAb 4-11G.6, specific for a discontinuous epitope on the S glycoprotein of MHV-A59, has been previously described[7]. Monoclonal antibody 5B170 is specific for a continuous epitope on the S protein of MHV-JHM[8]. All antibodies including normal rabbit immunoglobulins (NRIg) were purified by standard Protein-A-Sepharose chromatography.

Polyclonal anti-Id and antibody assays. The immunization protocols for generating polyclonal anti-Id and their characterization have been described elsewhere[1], including virus

neutralization and protection assays and ELISA for detection of Ab3 against MHV-A59 in mice sera. ELISA for detection of idiotype in antiviral sera and of inhibition of binding of idiotype to antigen by anti-Id were also described previously[1].

Monoclonal anti-Id. The production of MAb was described previously[7]: BALB/c mice were immunized with 100 μg of affinity purified MAb 4-11G.6 and given two booster injections of 50 μg.

ELISA for detection of MAb2. Microtiter plates were coated with affinity purified $F(ab')_2$ MAb1 [1.25 μg/mL in phosphate buffered saline (PBS)] and incubated for 16 h at room temperature. The plates were blocked with PBS containing 10% (v/v) fetal calf serum and 0.2% (v/v) Tween-20 for 30 min at 37°C. Hybridoma culture supernatants (4 days of growth) diluted 1/2 were added to the wells and incubated for 90 min at room temperature. The plates were then washed five times with PBS containing 0.1% (v/v) Tween-20. Peroxidase-labeled goat anti-mouse Fc antibody (ICN Biologicals, Miles) was then added and incubated for 90 min at room temperature. The plates were washed five times and the reaction was developed with *O*-phenylenediamine and hydrogen peroxide. The reaction was stopped with 1N HCl and the absorbance read at 492 nm using an SLT EAR 400 AT plate reader.

Virus and cells. MHV-A59 and MHV-JHM were obtained from the American Type Culture Collection (Rockville, MD), plaque-purified twice, and passaged four times at a multiplicity of infection of 0.01 on DBT cells as described previously[9].

Plaque assays with brain homogenates of mice immunized with anti-Id. Brains were collected 5 days after virus challenge and plaque assays performed as described elsewhere[10].

Statistics. Results of *in vivo* protection assays were analyzed with the Kaplan-Meier survival curve[11]. Antiviral Ab3 antibody responses were evaluated at a 1/500 dilution and analyzed with the Mann-Whitney test[12]. Brain viral titers observed after NRIg or anti-Id treatment and the repeated experiments were first analyzed with a Manova test[13]. This test included an interaction test which in our case was shown to be significant, so the two treatments were then compared separately by a Student t-test[14].

RESULTS AND DISCUSSION

Polyclonal Anti-5B170.11

A polyclonal anti-Id 5B170.11 was purified by affinity chromatography from the serum of a rabbit immunized with MAb1 5B170.11. To determine if the anti-Id bound to the paratope of MAb1, inhibition of virus-binding by anti-Id was tested. This inhibition of attachment assay can discriminate α-type from β and γ-types anti-Id. The polyclonal anti-Id produced against MAb1 5B170.11 did not inhibit the interaction between MAb1 and antigen, consistent with it being an $Ab2_\alpha$ (Fig. 1A). The anti-Id was also tested for it capacity to inhibit the neutralizing ability of MAb1 in an inhibition of virus-neutralization assay. As much as 10 μg of anti-Id could not reduce the neutralization titer of MAb1 (data not shown), which confirmed the results of the inhibition of attachment assay.

On the basis of previous studies showing that non-internal image anti-Id could trigger an antigen-specific immune response[1,16,17] like internal image anti-Id, we examined the *in vivo* modulation capability of the rabbit polyclonal $Ab2_\alpha$ anti-5B170.11. Two groups of 6 BALB/c mice were immunized with anti-Id or NRIg three times at two-week intervals. After the third injection, the MHV-specific Ab3 response was examined by ELISA. No detectable antiviral antibodies were produced in mice immunized with $Ab2_\alpha$ (data not shown). To verify whether the $Ab2_\alpha$ could nevertheless induce a protective immune response, mice were challenged intracerebrally with 10 LD_{50} of MHV-JHM, 10 days after the last booster anti-Id injection. All mice showed clinical signs of MHV infection. Animals in control groups died

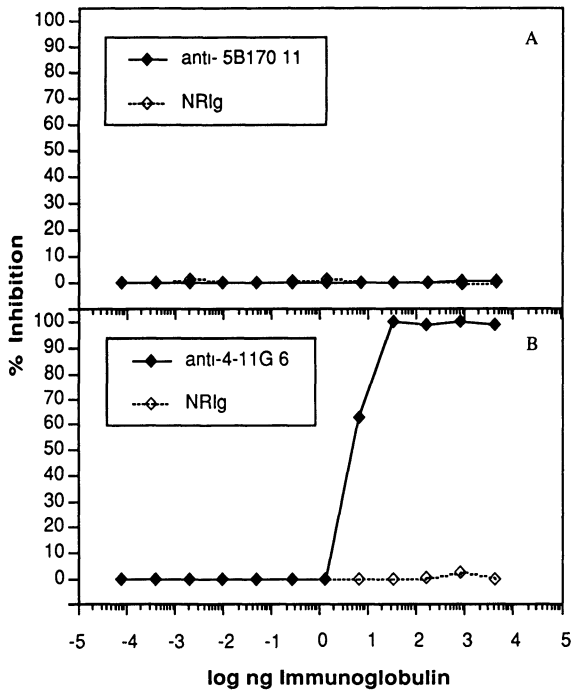

Figure 1. Inhibition of attachment assay for discrimination of α-type from β and γ-type anti-Id Microtiter plates were coated with 5 μg/mL of viral antigen Biotinylated MAb1 and dilutions of purified Ab2 or NRIg were pre-incubated together and transferred onto the viral antigen-coated plates. The residual binding of MAb1 to viral antigens was detected using peroxidase-labeled streptavidin

from MHV-JHM infection within 6 to 10 days, whereas 16% to 33% of mice in the $Ab2_{\alpha}$ group lived longer (until day 41) (Fig. 2A). In a repeat experiment some mice survived in both control and $Ab2_{\alpha}$ treated group (Fig. 2B). In the third repeat experiment, anti-viral Ab3s were detected by ELISA at a dilution of 1/500 (data not shown). The presence of specific Ab3s at this dilution was significant for a p value of 0.0014 in the Mann-Whitney test. However, these Ab3s could not neutralize viral infection *in vitro* (data not shown). No specific reactivity was observed in control groups (NRIg) or pre-immune sera. We then evaluated whether the apparent partial protection of mice correlated with reduced viral titers

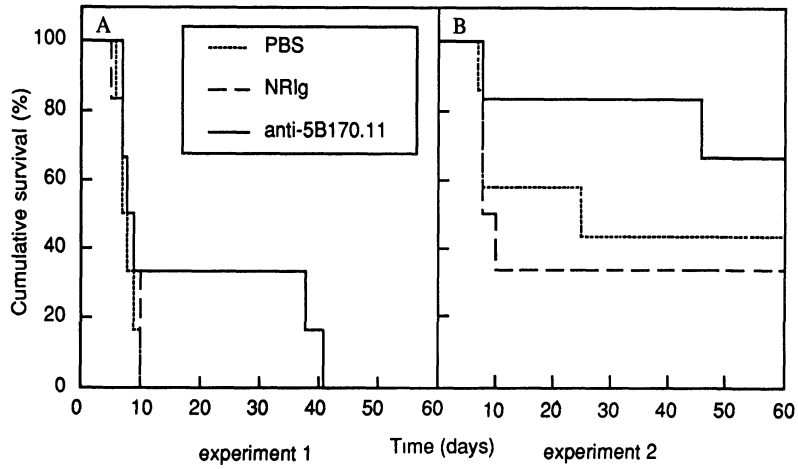

Figure 2. Survival curves of mice immunized with polyclonal Ab2 anti-5B170.11. Groups of 6 BALB/c mice were immunized with Ab2, NRIg or PBS and challenged with 10 LD_{50} of MHV-JHM. A: experiment 1; B: experiment 2

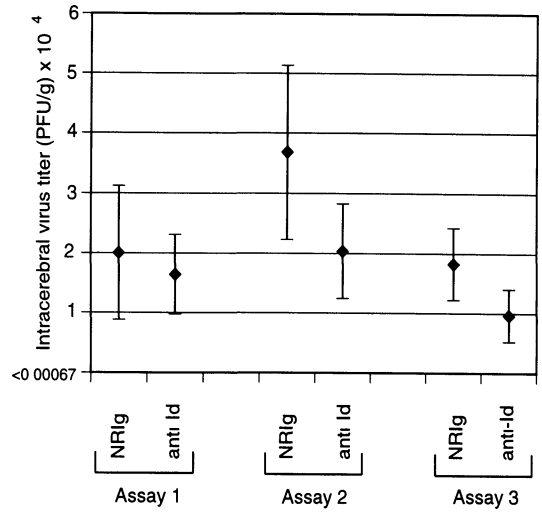

Figure 3. Brain viral titers observed after anti-Id and NRIg treatments Virus titers from brains of mice immunized with Ab2 anti-5B170 11 or NRIg were quantitated by plaque assay

in the brain (Fig 3) The first plaque assay did not show a significant reduction of viral titers but two other plaque assays from the same brain aliquots did show significant reductions in viral titers between mice treated with $Ab2_{\alpha}$ and NRIg The reduction in viral titer was significant at a p value of 0 014 with the second assay and at a p value of 0 004 for the third assay Such reduced viral titers could explain the observed apparent protection

We observed either a weak or non-existent antiviral Ab3 response (data not shown) and variable protection after treatment with polyclonal $Ab2_{\alpha}$ anti-5B170 11 (Fig 2) This suggests either a need to optimize the conditions for antiviral Ab3 induction, or an involvement of cellular protective responses The induction of specific immune responses by $Ab2_{\alpha}$ has been studied in different experimental systems[15 16 17 18 19] and the activation of the cellular component of the immune response by anti-Id was reported[20 21 22]

Further experiments are needed to understand the mechanisms involved in the induction of protective immunity by non-internal image anti-Id For example, very little is known on the interactions between anti-idiotypic antibody and immune cells

The reasons why some $Ab2_{\alpha}$ induce protection and others do not are unclear $Ab2_{\alpha}$-induced immune responses might be the result of the induction of a regulatory pathway of idiotypes Anti-Id could induce a different series of immunological reactions within an idiotypic network than those induced by the antigen

Table 1. Ab2 anti-4-11G 6

	anti-4-11G 6				
	Polyclonal	MAb2 3-2A 1	MAb2 8-11G 1	MAb2 2-10F 1	MAb2 7-11E 1
Anti-Id	γ	α	α	α	α
Ab3[a]	<100	<100	<100	<100	<100
Neutralizing Ab3[b]	<50	<50	<50	<50	<50
Protection[c]	—	—	—	—	—

[a]Highest dilution where Ab3 anti-virus is detectable by ELISA
[b]Reciprocal of the highest dilution of serum that neutralized 50% of input virus
[c]BALB/c mice immunized with Ab2 and challenged intracerebrally with MHV-A59

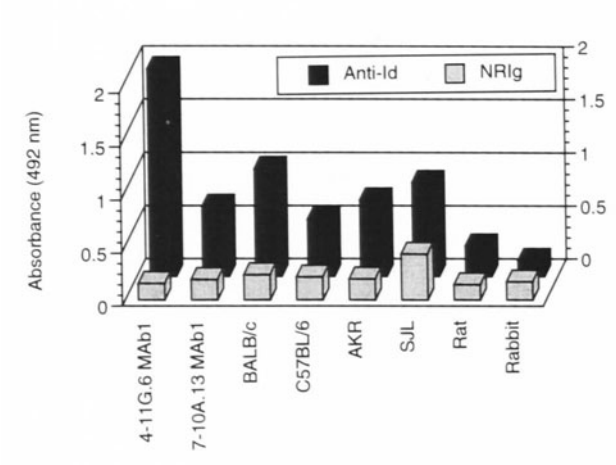

Figure 4. Detection of idiotype by Ab2. Microtiter plates were coated with 1.5 μg/mL of purified polyclonal Ab2 anti-4-11G.6 or NRIg. The binding of syngeneic (BALB/c), allogeneic (C57BL/6, AKR, SJL) and xenogeneic (rat and rabbit) anti-viral sera produced against MHV-A59 was determined by ELISA using peroxidase-labeled species-specific anti-Ig.

Polyclonal and Monoclonal Anti-4-11G.6

Polyclonal and monoclonal anti-Id were also produced in animals immunized with MAb1 4-11G.6. They are listed in Table 1.

Polyclonal Anti-4-11G.6. As a first approach towards determining whether the polyclonal anti-Id against MAb1 4B11.6 was an internal image ($Ab2_\beta$) anti-Id, its ability to bind to the paratope of MAb1 was tested. The results of the ELISA inhibition of attachment assay are shown in Fig.1B. Binding of MAb1 4-11G.6 to viral antigen could be inhibited by purified anti-Id in a dose-dependent manner. Twenty ng of anti-Id was enough to inhibit 100% of MAb1 binding to antigen, while the same amount of NRIg did not have any effect on this binding, indicating that the polyclonal anti-Id was not an $Ab2_\alpha$. The ability of the polyclonal Ab2 to abrogate the neutralization of virus infectivity was also tested. Ten μg of anti-Id could reduce the neutralization titer of MAb1 by 400-fold, whereas the same amount of NRIg did not have any effect (data not shown). These results confirmed the ELISA inhibition of attachment assay and suggested that this anti-Id binds at or near the paratope of MAb1 and was therefore an $Ab2_\beta$ or $Ab2_\gamma$. To distinguish between these two possibilities, we investigated the ability of this anti-Id to be recognized by antisera from different animal species raised against the initial antigen. An $Ab2_\beta$ should bind to all anti-MHV hyperimmune sera because of its internal image properties. As shown in Fig.4, our polyclonal anti-Id recognized a share idiotype in hyperimmune sera from BALB/c, C57BL/6, AKR and SJL mice. It also bound weakly to rat sera, but not to rabbit sera. These results demonstrated that this anti-Id could not induce antibody responses to the antigen across species barrier. Therefore, this polyclonal anti-Id must only bind near the antigen-binding site of MAb1. This gamma-type reaction was confirmed by the absence of both specific antiviral Ab3 induction and protection (Table 1).

Interestingly, we previously demonstrated that $Ab2_\gamma$ against MAb1 7-10A specific for a related epitope could vaccinate mice against infection by this coronavirus[1]. We now show that $Ab2_\gamma$ anti-4-11G.6 did not induce a protective immune response. MAbs1 7-10A and 4-11G.6 were previously shown to recognize two overlapping conformational epitopes by an ELISA competition assay[7]. Therefore, the mechanisms of protection induced by the $Ab2_\gamma$ anti-7-10A remain to be investigated.

Monoclonal Anti-4-11G.6. Anti-4-11G.6 MAb2 were also generated in BALB/c mice. They were all of IgG2b isotype and, as shown in Table 1, did not compete with antigen

for binding of MAb1, which suggests that they recognize framework idiotopes and can be classified as $Ab2_{\alpha}$ These MAb2, coupled to KLH to enhance their immunogenicity, did not induce specific antiviral Ab3 nor induced protection against MHV-A59 (Table 1) Previous studies have shown that such Ab2s could induce Ab3s in other viral systems[16 17] However, in the present work, no $MAb2_{\alpha}$ were able to induce an antiviral Ab3 response

The use of monoclonal anti-Id with interesting biological activities should help clarify the mechanisms of protection induced by non-internal image anti-Id Moreover, the production of monoclonal internal image $Ab2_{\beta}$, although potentially interesting for characterization of molecular determinants involved in viral pathogenesis, identification of cellular receptors and vaccination, is technically difficult Molecular cloning of the antibody repertoire could overcome this technical problem[23] Such studies are in progress

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