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PARATOPE- AND FRAMEWORK-RELATED CROSS-REACTIVE IDIOTOPES ON ANTI-ACETYLCHOLINE RECEPTOR ANTIBODIES[1]

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Cross-reactive idiotopes are a possible target for therapeutical interventions in autoimmune diseases. To investigate their role in the pathogenesis of experimental autoimmune myasthenia gravis (EAMG) we analyzed the Id of rat anti-AChR mAb 6, 35, 61, 65 and a control myeloma protein IR27. Anti-Id 6, 35, 61, 65 bound in a direct binding assay with various affinity to all rat anti-AChR mAb that were tested. Anti-Id IR27 recognized none of the anti-AChR mAb. The specificity of these crossreactions was confirmed by inhibition studies with anti-AChR mAb and two control rat myeloma proteins (IR27 and IR241). In addition, the Id expression on mAb D6, a mouse anti-human AChR mAb was recognized by anti-Id 6, 35, and 65. Anti-Id, except anti-Id IR27, bound to affinity purified IgG from the sera of rats with EAMG, but not to preimmune Lewis IgG. These results suggest extensive sharing of idiotopes among anti-AChR mAb, which are also present in EAMG serum. Anti-AChR mAb against the main immunogenic region (6, 35, 65) from different rat strains, shared at least one paratope-related cross-reactive idiotopes. In the view of the fact that anti-main immunogenic region antibodies might form a predominant fraction of the polyclonal response against AChR, it is conceivable that an anti-Id recognizing these antibodies could have therapeutical applications as for example a selective immune absorbent or in immunotoxin therapy.

EAMG[3] mirrors human MG. Both diseases are characterized by abnormal muscular weakness and increased fatiguebility of voluntary muscles, caused by antibodies against the nicotinic AChR (1). The antibody dependence of EAMG and MG is demonstrated by the effectiveness of passive transfer of the disease by means of specific antibodies (2, 3) and by the beneficial effects of plasmapheresis (4, 5). These antibodies play a central role in the pathogenesis, and therefore insights into the regulation of production of these autoantibodies could be of interest for specific immunosuppressive treatment.

One theory concerning the regulation of antibody production is Jerne's network theory. According to this theory, the immune system can be seen as a web of V region domains in which the connection between clones is based on Id-anti-Id interactions. Id can be defined as the collection of antigenic determinants, i.e., idiotopes, associated with the V region of an antibody. Anti-Id are antibodies with an Ag-combining site recognizing an Id. The balance between clones as well as the various subsets of cells that regulate their expression can therefore be influenced by Ag, Id and anti-Id (6). Id-anti-Id interactions may play an important regulatory role. One of the requirements for a putative regulatory Id is the expression of CRI (7). These CRI have been identified in many autoimmune diseases (8). Whether or not CRI are present in MG is a topic of debate, because the Id sharing in MG observed in two studies (9, 10) was refuted in another (11). Also, in EAMG some groups reported the presence of CRI (10, 12, 13), but others failed to find any cross-reactivity (14, 15). Only two groups have sought CRI in EAMG in the rat. One study showed negative results (14), the other was successful in finding paratope related CRI (13). Neither of them reported on framework CRI. Hence, there is no consensus regarding the existence of CRI on anti-AChR antibodies. Therefore, we have performed an idiotypic analysis of three anti-AChR mAb, directed against the extracellularly located MIR on the AChR, two mAb recognizing intracellular determinants on the AChR and two control myeloma proteins. The idiotype profile of the anti-MIR mAb is particularly pertinent, because these mAb are capable of inducing EAMG (16, 17), and about 50 to 70% of the anti-AChR antibodies in both MG and EAMG is thought to be directed to the MIR (16, 18).

We were able to identify CRI associated with the Ag-binding site, i.e., the paratope, of these anti-MIR anti-AChR mAb, as well as non-paratope related "framework" CRI on all five anti-AChR mAb.

MATERIALS AND METHODS

mAb. Hybridoma's secreting anti-AChR mAb 6 (IgG_1, Lewis), mAb 35 (IgG_1, Sprague-Dawley), mAb 61 (IgG_{2a}, Lewis), mAb 65 (IgG_1, Louvain), and mAb 155 (IgG_{2a}, Lewis) were all produced in rats (16, 19, 20). mAb 6, 35, and 65 are directed at the MIR, residing on the extracellular site of the alpha-subunit of the AChR. mAb 61 and 155 recognize intracellular epitopes. Hybridoma D6 (IgG2a, BALB/c) (kind gift from Dr. A. Vincent, John Radcliffe Hospital, Oxford, England) is probably directed at the equivalent of the MIR on the

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[2] Address correspondence and reprint requests to Dr. M. H. de Baets, Department of Immunology, University of Limburg, P.O. Box 616, 6200 MD Maastricht, The Netherlands.
[3] Abbreviations used in this paper: EAMG: experimental autoimmune myasthenia gravis; CRI, cross-reactive idiotope; MG, myasthenia gravis; AChR, acetylcholine receptor; MIR, main immunogenic region; I_{50}, concentration of inhibitor needed for 50% inhibition; NRIgG, normal rabbit IgG; H_2O-Tw, H_2O containing 0.5% Tween; HRP, horseradish peroxidase; I^{125}-d-Bungarotoxin; PBS containing 0.5% BSA and 0.5% Tween-20.

TABLE I
Characteristics of the mAb

mAb	Isotype	Strain	Binding to AChR			
			Mammalian	Electric fish	Subunit specificity	Sequence specificity
6	IgG1	Lewis	+	++	α, MIR	67–74
35	IgG1	Sprague Dawley	++	++	α, MIR	6–85
61	IgG2a	Lou	+	++	α, cytoplasm	374–394/420–437
65	IgG1	Lou	++	±	α, MIR	ND
155	IgG2a	Lewis	+	++	α, cytoplasm	372–380
D6	IgG2	BALB/c	++	−	α, MIR	ND
IR27	IgG1	Lou	−	−		
IR241	IgG1	Lou	−	−		

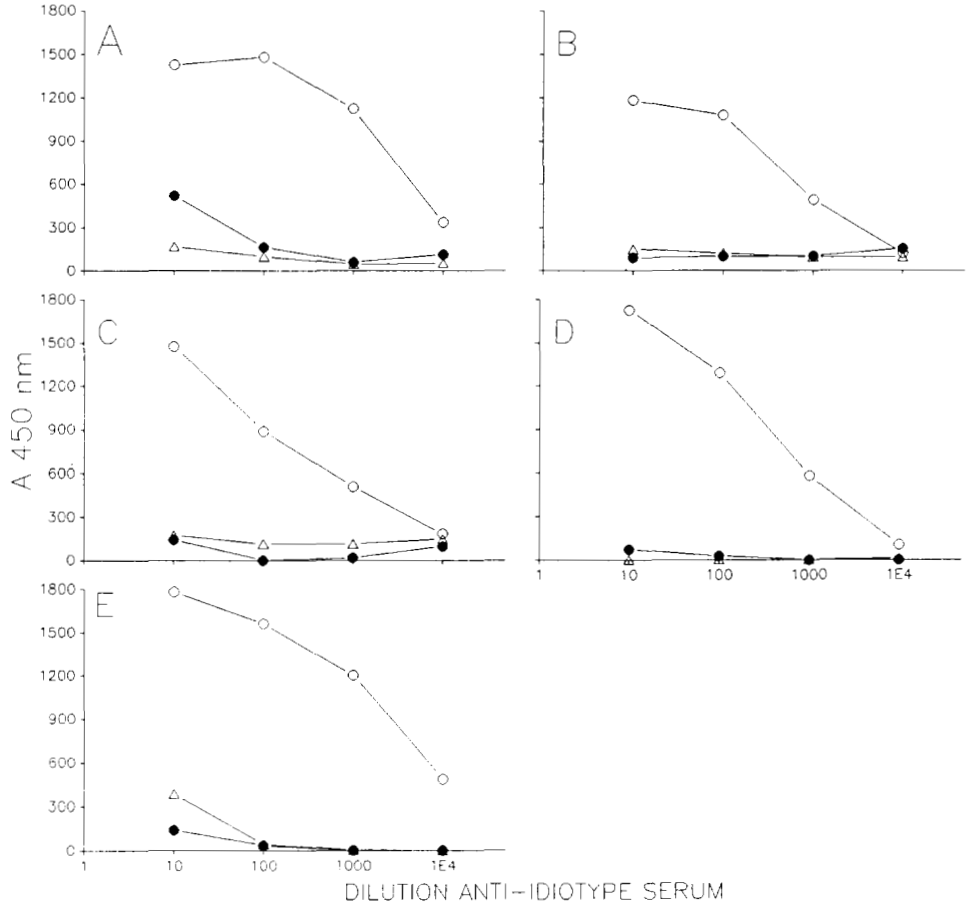


Figure 1. Absorbed anti-Id sera do not cross-react with polyclonal Lewis or Louvain IgG. Anti-Id sera were tested by an indirect anti-Id binding ELISA. After absorption by polyclonal Lewis or Louvain IgG, the anti-Id sera bound well to the homologous Id (*open circles*), but not to Lewis IgG (*filled circles*) or Louvain IgG (*open triangles*). *A*, anti-Id 6; *B*, anti-Id 35; *C*, anti-Id 61; *D*, anti-Id 65; *E*, anti-Id IR27 (1E4 = 10^4).

human rat AChR (21). Myeloma proteins IR27 and IR241 (both IgG_1, Louvain) (kind gift from Dr. H. Bazin, Experimental Immunology Unit, Université Catholique Louvain, Brussels, Belgium) were used as control rat mAb (Table I). mAb were purified by ion exchange HPLC and conjugates to HRP were prepared (mAb-HRP). MAb PM2, PM5, PM6 (mouse IgG_1, BALB/c) (kind gift of Dr. C. Verstijnen, Department of Pathology, University of Limburg, Maastricht, The Netherlands) directed against the carcinoembryogenic Ag (22,23), mAb 1.15, 9.1, 14.14 (mouse IgG_1, BALB/c) directed against human thyroglobulin (24), and mAb 8 and 204 (mouse IgG1, BALB/c) (kind gift of Department of Microbiology, Dr. C. Bruggeman, University of Limburg) directed against rat cytomegalovirus (CMV) virus were used as control mouse mAb (25).

Preparation of AChR. AChR from the main electric organ of *Torpedo californica* was purified by affinity chromatography on Naja naja siamensis coupled to CNBr-activated Sepharose-4B (26).

Anti-idiotypic antibodies. NZW rabbits were immunized with purified 100 to 200 μg anti-AChR mAb or IR27 in CFA and subsequently boosted three times with mAb in IFA at 2-wk intervals. Injections were given intradermally at multiple sites on the back. Animals were bled 1 wk after the last booster immunization. The anti-Id was absorbed at 4°C, by Lewis rat IgG (5 mg/ml) coupled to Sepharose-4B columns (10 ml). After five to eight absorptions no significant binding to polyclonal Louvain or Lewis IgG was observed by an indirect anti-Id binding ELISA. Next, the serum was affinity purified on Sepharose columns, containing the mAb used for immunization. Elution was performed with 1 N HCl, containing 0.10 M glycine, 0.15 M NaCl, pH 2.8. After dialysis against PBS the eluate was used for in vitro assays.

After absorption anti-Id were tested for specificity by indirect anti-Id binding ELISA. Polyvinyl 96-well microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) were coated with 50 μl anti-AChR mAb, polyclonal Lewis or Louvain rat IgG (5 μg/ml) for 1 h at 37°C. After washing three times with H_2O-Tw, plates were incubated with PBSA-Tw (Serva/Brunschwig chemie bv, Amsterdam, The Netherlands) for 30 min at room temperature. Serial dilutions (1/10 to 1/10,000) of anti-Id were allowed to incubate for 1 h at room temperature on a rocker. After washing again with H_2O-Tw the wells were incubated with 50 μl of goat-anti-rabbit IgG (Miles/Bayer, Mijdrecht, The Netherlands) coupled to HRP for 1 h at room temperature on a rocking platform. After washing again with H_2O-Tw the colorimetric reaction was developed by adding 100 μl of 0.1 M Na-acetate buffer, pH 5.5, containing tetramethylbenzidine (10 mg/ml) and 0.01% H_2O_2. After 10 min the reaction was stopped by adding 50 μl 4 N H_2SO_4. Reading was done at 450 nm (Titertek Twinreader, Amstelstad, Amsterdam, The Netherlands).

Cross-reactive Id assays. Direct binding of mAb-HRP to anti-Id was performed using a direct Id binding ELISA. Polyvinyl 96-well microtiter plates were coated with 50 μl affinity purified anti-Id or monoclonal anti-Id (5 μg/ml) for 1 h at 37°C. After washing three

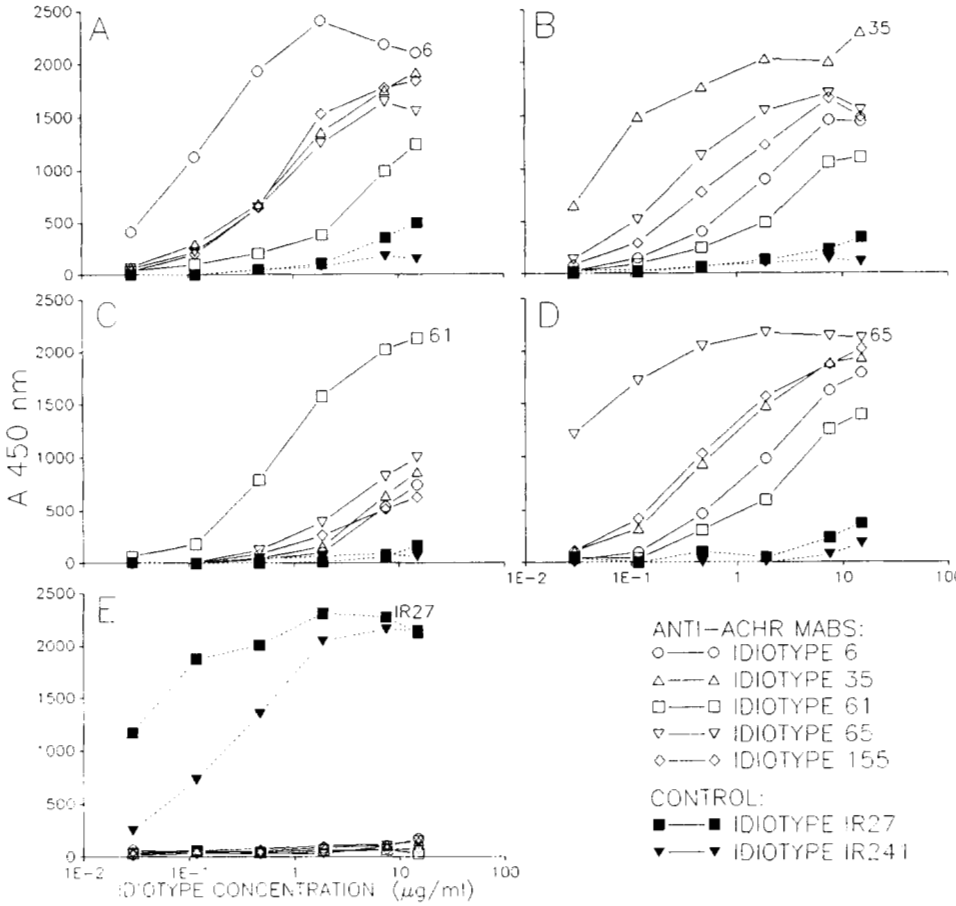


Figure 2. Affinity purified anti-Id tested for their crossreactivity, using a direct Id binding ELISA. After absorption the anti-Id were affinity purified on their corresponding mAb coupled to Sepharose, and tested in a direct Id binding ELISA. Anti-Id were coated and incubated with anti-AChR mAb coupled with HRP. Anti-Id elicited against anti-AChR mAb bind all five anti-AChR mAb. Control anti-Id IR27 binds only two myeloma proteins. *A*, anti-Id 6; *B*, anti-Id 35; *C*, anti-Id 61; *D*, anti-Id 65; *E*, anti-Id IR27 (1E-2 = 10^{-2}, 1E-1 = 10^{-1}).

times with H_2O-Tw, plates were incubated with PBSA-Tw for 30 min at room temperature. Then 50 μl of increasing amounts (0.03 to 15 μg/ml) of HPLC purified mAb, conjugated to HRP, were allowed to incubate for 1 h at room temperature on a rocker. The last part of the ELISA was performed as described above.

Cross-reacting Id were also sought using a competitive Id-binding ELISA. The inhibition of the binding of mAb to anti-Id was investigated, as described above, with little adjustments. Briefly, increasing amounts (0.003 to 10 μg/ml) of non-conjugated inhibiting mAb were allowed to incubate overnight on anti-Id coated wells (5 μg/ml). Then, 50 μl of previously determined limiting amounts of HRP-labeled mAb (range 1 to 16 μg/ml) were added without washing for 1 h, and the bound HRP activity was measured as mentioned above. The percent inhibition was calculated as follows:

(average A_{450} of duplicate wells with conjugated mAb alone) − (average A_{450} of duplicate wells in which conjugated mAb were tested in presence of putative inhibitor)/(average A_{450} of eigth wells with conjugated mAb alone) × 100

Values were expressed as the concentration needed for I_{50}.

Id-anti-Id binding specificity was investigated by inhibition ELISA using Torpedo-AChR as inhibitor. The assay was similar to the competitive Id-binding ELISA. Briefly, anti-Id was immobilized on microtiter wells as described above. HRP-labeled Id was preincubated with Torpedo-AChR (previously dialyzed against PBSA-Tw to remove cholic acid) at 0.002 to 50 μg/ml for 2 h at 4°C. The mixtures were allowed to incubate on anti-Id coated wells for 1 h. Thereafter plates were washed and the colorimetric reaction developed, as described above.

CRI on mouse mAb were analyzed by an indirect anti-Id binding ELISA. Polyvinyl 96-well microtiter plates were coated with 50 μl mAb (5 μg/ml) for 1 h at 37°C. After washing three times with H_2O-Tw, plates were then incubated with PBSA-Tw for 30 min at room temperature. Then 50 μl of affinity purified anti-Id (2.5 μg/ml) were added and allowed to incubate for 1 h at room temperature on a rocker. After washing 50 μl goat-anti-rabbit-HRP was added and allowed to incubate for 1 h at room temperature on a rocker. Plates were washed and the colorimetric reaction was developed, as described above.

Paratope-related Id assay. Paratope-related CRI were analyzed by a solid-phase Ag-binding inhibition RIA. Polyvinyl 96-wells microtiter plates (Flow laboratories Ltd/Amstelstad bv, The Netherlands) were coated with 50 μl anti-AChR mAb or affinity purified EAMG serum antibodies (5 μg/ml) for 1 h at 37°C. After washing three times with H_2O-Tw, the plates were then incubated with PBSA-Tw for 30 min at room temperature. Then increasing amounts (0.08 to 20 μg/ml) of affinity purified anti-Id were incubated overnight. Next, previously determined limiting amounts of Torpedo-AChR, labeled with I^{125}-α-BT, were added for four hours at room temperature. After washing with PBS, containing 0.5% TRITON X-100 (Sigma/Brunschwig chemie bv, Amsterdam, The Netherlands), and 0.02% NaN_3 the radioactivity bound to the wells was counted in a gamma-counter (Compugamma, Pharmacia LKB, Woerden, The Netherlands). The percentage inhibition was calculated as follows:

(average cpm of duplicate wells with Torpedo-AChR-I^{125}-α-BT alone) − (average cpm of duplicate wells in which Torpedo-AChR-I^{125}-α-BT were tested in presence of putative inhibitor)/(average cpm of duplicate wells with Torpedo-AChR-I^{125}-α-BT alone) × 100

Values were expressed as the concentration needed for I_{50}.

Affinity purification of anti-AChR antibodies. Sera of six to eight rats (1 ml/rat), obtained 6 wk after immunization with Torpedo AChR, were pooled. Serum IgG was precipitated by adding an equal volume of saturated ammonium sulfate. After dialysis against PBS this preparation was affinity purified, by overnight absorption at 4°C, on 1 mg/ml Torpedo AChR coupled Sepharose 4B columns. Elution was performed with glycine-HCl containing 1 N HCl, 0.10 M glycine and 0.15 M NaCl, pH 2.8, containing 0.9% NaCl. After dialysis against PBS the eluate was used for an indirect anti-Id-binding ELISA, similar to the assay used for the detection of CRI on rat mAb, as described above, whereby the affinity purified anti-AChR IgG was coated (5 μg/ml) on microtiter wells.

Production of monoclonal anti-Id. BALB/c mice (Charles River Wiga GmbH, Frankfurt, FRG) received s.c. injections of mAb 65 (50 μg) in PBS in an equal volume of CFA in hind-foot pads and at the base of the tail. Six booster injections at 3-day interval were given (27), the first in IFA and subsequent boosters were in PBS. Cells for fusion were obtained from draining inguinal and para-aortal lymph nodes, 3 days after the last injection. Lymph node cells were fused to the mouse tumor cell line SP2/0-Ag14 according to Köhler and Milstein (28). Supernatants were screened for anti-Id 65 mAb by an indirect anti-Id binding ELISA, similar to that described above for

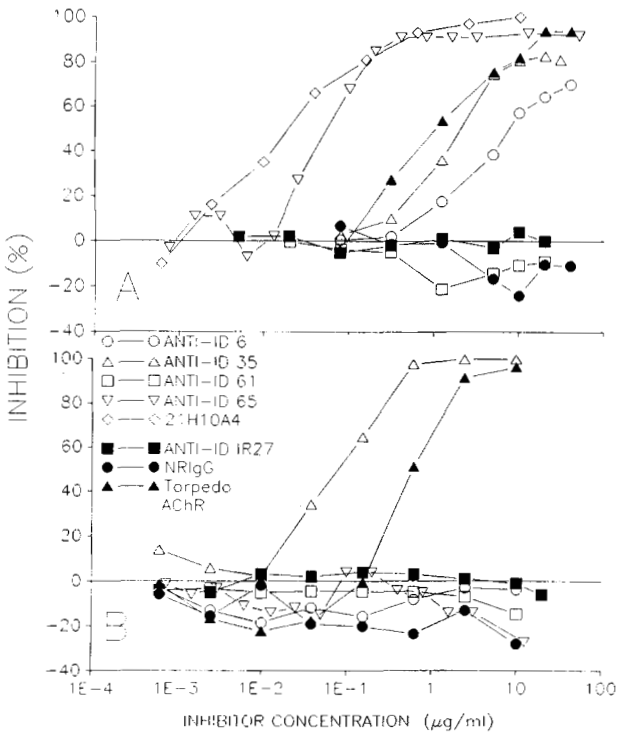


Figure 3. Anti-MIR antibodies share paratope-related CRI. Paratope-related CRI were analyzed by a solid phase antigen binding inhibition RIA. Anti-AChR mAb were coated on a microtiter plate, and incubated with increasing amounts of anti-Id, unlabeled Torpedo AChR, or normal rabbit IgG. Then ^{125}I-α-BT-Torpedo AChR was added. Binding is expressed as percentage of binding of ^{125}I-α-BT-Torpedo AChR to the anti-AChR mAb in the presence of buffer. *A*, Binding of mAb 65 to Torpedo AChR can be inhibited by polyclonal anti-Id 6, anti-Id 35, anti-Id 65, and monoclonal anti-Id 21H10A4 or Torpedo AChR. *B*, Binding of mAb 35 to Torpedo AChR can only be inhibited by polyclonal anti-Id 35 or Torpedo AChR (1E-4 = 10^{-4}, 1E-3 = 10^{-3}, 1E-2 = 10^{-2}, 1E-1 = 10^{-1}).

TABLE II

Binding anti-ACHR monoclonal[a]

	6	35	61	65	155
Inhibitor					
Anti-ID 6	0.16[b]	≫20	≫10	14	≫20
Anti-ID 35	≫10	0.06	≫10	5	≫20
Anti-ID 61	≫10	≫20	0.36	≫20	≫20
Anti-ID 65	≫30	≫50	NT[c]	0.08	≫30
Anti-ID IR27	≫20	≫20	NT	≫20	≫20
NRIgG[d]	≫30	≫50	≫10	≫20	≫30
Torpedo-ACHR[e]	1.0	0.6	1.4	0.36	0.14

[a] Anti-MIR antibodies share paratope related Id. Paratope-related CRI were analyzed by a solid-phase Ag-binding inhibition RIA. Anti-AChR mAb were coated on a microtiter plate, and incubated with increasing amounts of anti-Id, unlabeled Torpedo AChR, or normal rabbit IgG. Then I^{125}-α-BT-Torpedo AChR was incubated, and after washing radioactivity bound to the wells was counted. Inhibition is expressed as the concentration (μg/ml) needed for 50% inhibition of binding of I^{125}-α-BT-Torpedo AChR to the anti-AChR mAb. In each instance anti-Id blocks the binding of homologous Id and in addition the binding of mab 65 is blocked by anti-Id 6 and anti-Id 35, but not by anti-Id 61 or anti-Id IR27.

[b] Concentration (μg/ml) for 50% inhibition.

[c] NT, Not tested.

[d] Normal rabbit IgG (NRIgG) never showed any blocking.

[e] Unlabeled Torpedo-AChR was always able to block.

polyclonal rabbit anti-Id. Briefly, 96-well microtiter plates were coated with 50 μl Fab_2 mAb 65 (1 μg/ml). After washing with H_2O-Tw and preincubation with PBSA-Tw, supernatants were incubated overnight. After washing again with H_2O-Tw the wells were incubated with 50 μl rabbit-anti-mouse IgG (DAKO, Santa Barbara, CA) coupled to HRP for 1 h at room temperature on a rocker, and bound HRP activity was measured as described above.

RESULTS

Characteristics of anti-Id sera. Anti-Id were produced in rabbits for the analysis of the Id on mAb 6, 35, 61, 65, and 155. After extensive absorptions on rat IgG, rabbit anti-Id sera bound to the homologous mAb, but not to control Lewis or Louvain IgG in an indirect anti-Id binding ELISA (Fig. 1). Each anti-Id, raised against a given anti-AChR mAb, bound to all five anti-AChR mAb tested. The amount of heterologous Id needed to reach a given absorption in the ELISA was at least 16 times higher than the required amount of homologous Id. Control myeloma proteins IR27 or IR241 were not recognized. Anti-Id IR27 bound only to IR27 and IR241, but not to any of the anti-AChR mAb. Therefore, CRI were shared among all five anti-AChR mAb irrespective of their fine specificities, but not with the control myeloma proteins (Fig. 2).

Analysis of paratope related cross-reactive idiotopes. Next, we determined whether the idiotypic determinants were associated with the Ag-combining site. The binding of mAb 65 to Torpedo AChR could be inhibited by anti-Id 65 (I_{50} = 0.08 μg/ml), and also by higher concentrations of anti-Id 6 and anti-Id 35 (I_{50} = 14 and 5 μg/ml, respectively). The anti-Id directed against mAb 61, recognizing an intracellular epitope clearly distinct from the MIR, did not show any inhibition of mAb 65 binding to AChR. Also, addition of increasing amounts of control anti-Id IR27 or NRIgG did not result in any inhibition (Fig. 3*A*).

The binding of other anti-AChR mAb (mAb 6, 35, 61, and 155) to Torpedo-AChR could only be inhibited by the homologous anti-Id. In Figure 3*B* typical antigen binding inhibition curves are shown for mAb 35, the results of the other anti-Id are summarized in Table II. Concentrations of homologous mAb needed for 50% inhibition varied from 0.06 to 0.36 μg/ml. Neither anti-IR27 nor normal rabbit IgG could inhibit the binding to AChR of any anti-AChR mAb tested. Non-labeled Torpedo-AChR was always inhibitory. Thus, sharing of paratope related Id's was found between anti-MIR anti-AChR mAb (mAb 6, 35, and 65), but not with other anti-AChR mAb (mAb 61 and 155) or control myeloma proteins.

Analysis of framework related crossreactive idiotopes. Cross-reactions were also analyzed by competitive inhibition of Id binding, testing the ability of each mAb to inhibit homologous, as well as heterologous, Id-anti-Id interactions. These assays do not distinguish between paratope and framework related Id. It was found that homologous Id-anti-Id bindings could be inhibited, only by the homologous mAb. The results shown for anti-Id 6 (Fig. 4*A*) are representative for those found for anti-Id 35, anti-Id 61, anti-Id 65, and anti-Id IR27 (Table III). The heterologous (criss-cross) Id-anti-Id binding, using anti-AChR anti-Id, could be inhibited by all anti-AChR mAb, but not by mAb IR241 or IR27 (Fig. 4, *B, C, D, E*). Binding of anti-Id IR27 to mAb IR27 or IR241 could not be inhibited by any of the anti-AChR mAb or by Torpedo AChR (Table III). Homologous Id-anti-Id binding was not inhibited, whereas heterologous Id interactions were inhibited by the various mAb. The difference in inhibition patterns can best be explained by the fact that the heterologous Id anti-Id binding is of lower affinity than the homologous one.

Analysis of interspecies cross-reactivity. The expression of interspecies crossreactive idiotopes was determined by analysing the binding of anti-Id 6, anti-Id 35, and anti-Id 65 to mouse mAb D6, eight control mouse mAb, normal polyclonal mouse IgG, rat mAb 6, 35, 65,

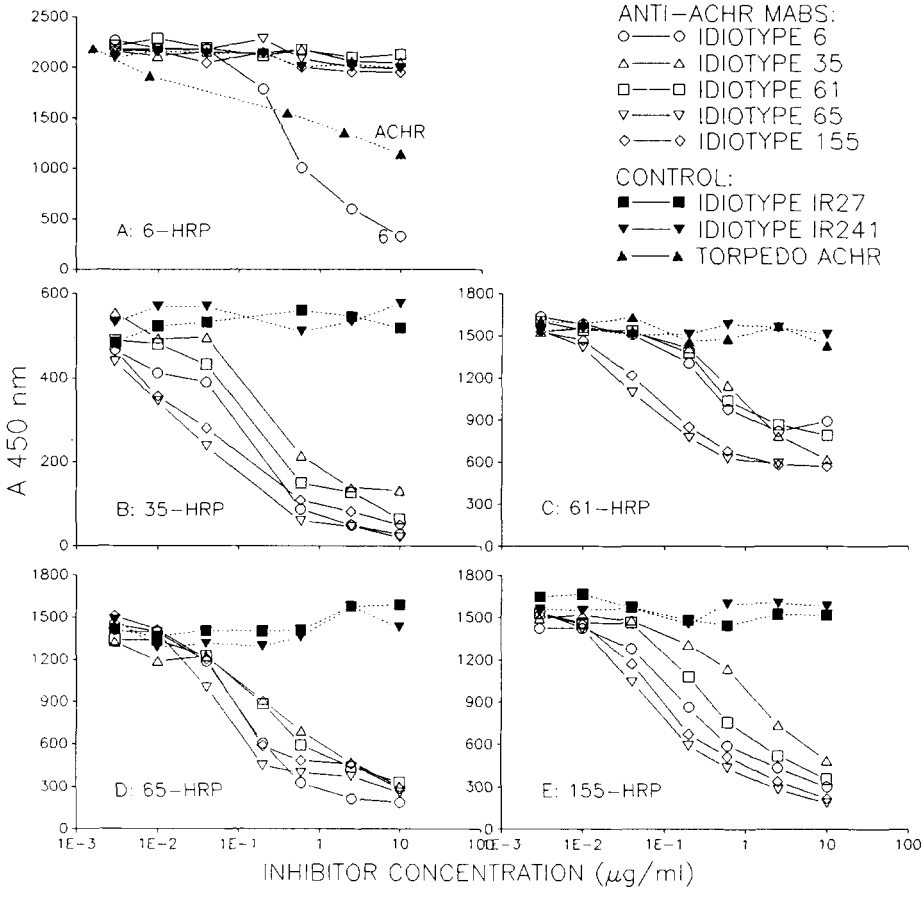


Figure 4. Framework CRI on anti-AChR mAb. Framework CRI were sought using anti-Id 6 in a competitive Id binding ELISA. Microtiter plates were coated with affinity purified anti-Id 6, and incubated overnight with increasing amounts of unlabeled anti-AChR mAb. Then anti-AChR mAb labeled with HRP were added, as indicated in the left hand corner. *A*, inhibition of binding of mAb 6-HRP to anti-Id 6; *B*, inhibition of binding of mAb 35-HRP to anti-Id 6; *C*, inhibition of binding of mAb 61-HRP to anti-Id 6; *D*, inhibition of binding of mAb 65-HRP to anti-Id 6; *E*, inhibition of binding of mAb 155-HRP to anti-Id 6 (1E-3 etc., see Fig. 3).

and normal rat IgG. mAb D6 and the rat anti-AChR mAb are directed against AChR from different species, but all bind to the phylogenetically conserved MIR. All three anti-Id recognized mAb D6, but none of the control mouse mAb, nor polyclonal BALB/c or Lewis IgG was recognized. Moreover, control anti-Id IR27 only recognized mAb IR27, but not mAb D6 or any of the control mAb (Fig. 5).

Expression of cross-reactive idiotopes in polyclonal EAMG serum. The expression of these CRI was studied on polyclonal anti-AChR antibodies present in the serum of rats with EAMG. The binding of the anti-Id to affinity purified rat anti-AChR serum antibodies was determined in an indirect Id-binding ELISA. All four anti-Id tested, directed at anti-AChR mAb, recognized serum anti AChR antibodies but not control Lewis IgG (Fig. 6). Although this indirect binding assay does not allow an exact determination of the Id concentration in the sera, an estimation can be made. It was found that, based on a parallel assay with various amounts of anti-AChR mAb, less than 0.1% of the anti-AChR antibodies in the sera were Id.[+]

The inhibition of the binding of the affinity purified rat anti-AChR serum was studied in a solid phase AChR binding inhibition RIA. None of the anti-Id was able to inhibit the binding of the EAMG serum antibodies to Torpedo AChR. Thus, CRI are expressed on a small fraction of serum anti-AChR antibodies in EAMG.

Production of monoclonal anti-Id. mAb 65, directed against the MIR of the fetal calf AChR, was used for the generation of monoclonal anti-Id in BALB/c mice. Two fusions, using lymphnode cells, resulted in the production of 14 (3% of growing hybridomas) and 2 (2%) stable anti-Id 65 clones, respectively. Of these 16 clones 2 were selected for subcloning and purification (mAb 21H10A4 and mAb 21B6G8).

The specificity of these anti-Id mAb was tested in an direct Id binding ELISA (Fig. 7). Both monoclonal anti-Id were found to be specific for mAb 65, not binding to any of the other four anti-AChR mAb or control myeloma IR27.

Next, it was investigated whether these anti-Id recognized paratope related Id on the anti-AChR mAb. It was found that anti-Id mAb 21H10A4 inhibited the binding of mAb 65 to Torpedo AChR up to 100% (I_{50}: 0.03 μg/ml), but not of the other anti-AChR mAb (Fig. 7). mAb 21B6G8 did not inhibit the Ag binding of any of the five anti-AChR mAb tested. Thus mAb 21H10A4 is directed against a private paratope-related idiotope whereas mAb 21B6G8 reacts with a private framework idiotope.

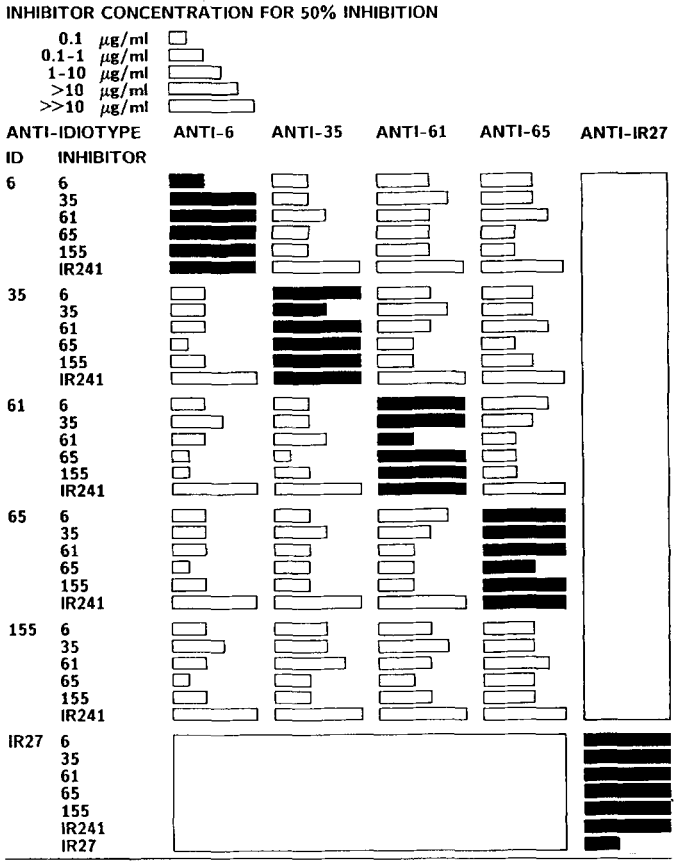
DISCUSSION

In this study the presence of CRI was investigated in a rat model for MG. Id on rat and mouse mAb against epitopes on AChR from different species were analyzed. A large number of paratope and framework related CRI were identified on anti-AChR mAb using polyclonal and monoclonal anti-Id.

It is clear, using xenogenic polyclonal anti-Id antisera, that paratope related Id were found only on mAb directed against the MIR. The MIR is formed by a set of overlapping epitopes forming an immunodominant determinant on the AChR α-subunit. It has been suggested that the majority of serum antibodies in EAMG (55 to 62%) (16) and MG (39 to 80%) (18, 29) are directed against the MIR, although another report questions the existence of the

TABLE III

Results of homologous and heterologous anti-Id cross-reactivity. CRI were sought using a competitive Id binding ELISA. Coated anti-Id are indicated at the top of each column. HRP-labeled Id used for binding is indicated at the most left hand column. Id tested for inhibition at increasing amounts (0.03 to 10 μg/ml) are indicated at the second left hand column. Values, indicated as horizontal bars, are concentrations needed for 50% inhibition, as indicated at the top: the highest dosis that was tested was 10 mg/ml, > indicates that at this concentration the inhibition was less than 50%, ≫ indicates that no inhibition was seen. Results of homologous Id-anti-Id interactions are displayed using dark bars, results of heterologous Id-anti-Id interactions are shown using open bars. For comparison, the first column is a summary of the data of Fig. 4.



MIR (30). Binding of several anti-MIR mAb has been located between amino acid 61-76 (31) and 67-76 (32) of the α-subunit. The sharing of paratope related CRI among the three anti-MIR mAb can be explained by the fact that their paratopes contain similar conformational structures, as each mAb recognizes the same epitope on the AChR and each inhibits the binding of the two mAb (33–36). These similarities in three dimensional structures of the paratope can be independent of V_H, V_L, D, or J gene families used, and the recognition by anti-Id can be based on the presence of internal image-like antibodies, which only have to resemble the Ag, i.e., the MIR, in the disposition of contact residues (35). The expression of CRI on a large number of anti-MIR mAb and the high concentration of anti-MIR antibodies in EAMG sera is in contrast with low concentrations of MIR-related CRI found in EAMG serum. Differences in affinity of the monoclonal Id's and serum anti-AChR antibodies may account for the failure to detect dominant CRI in EAMG serum using Id-anti Id binding inhibition assays.

To further define paratope-related Id of anti-MIR antibodies monoclonal anti-Id were prepared. The hybridoma

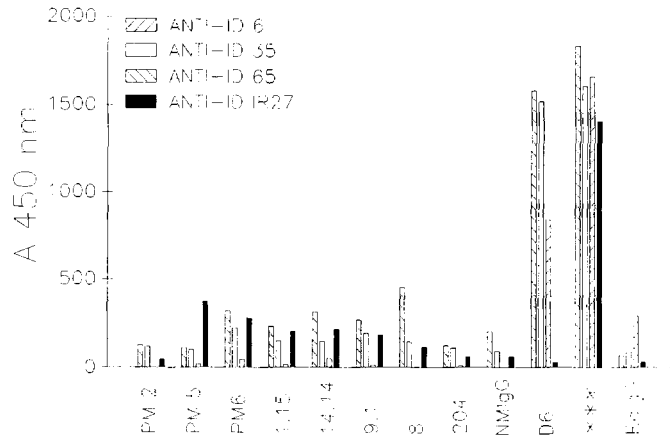


Figure 5. Anti-Id bind to CRI shared by rat and mouse. CRI on mouse mAb were analyzed by an indirect anti-Id binding ELISA. Mouse mAb directed at carcinoembryogenic Ag (PM2, PM5, PM6), thyroglobulin (1.15, 14.14, 9.1), or CMV virus (mAb 8 and 204), normal mouse IgG (*NMIgG*), Lewis rat IgG (*RaIgG*), anti-human AChR mAb D6, or several rat anti-AChR mAb were coated on microtiter plates. The plates were incubated with anti-Id, and then with goat-anti-rabbit-HRP. Affinity purified anti-Id 6, anti-Id 35, and anti-Id 65 bound to their homologous Id (i.e., respectively mAb 6, mAb 35, mAb 65, indicated by ***) and to mAb D6, but not to any other mouse mAb or polyclonal mouse or rat IgG. Control anti-Id IR27 bound only to its homologous Id (i.e., IR27, indicated by ***).

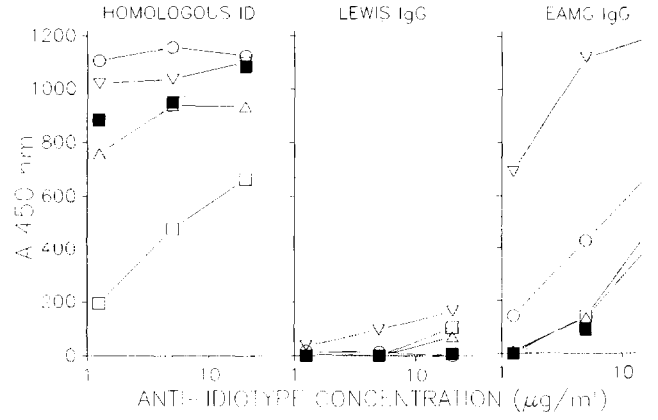


Figure 6. CRI are expressed on serum anti-AChR antibodies of rats with EAMG. Presence of CRI in EAMG serum was analyzed by an indirect anti-Id binding ELISA. Anti-AChR mAb (left-hand column), normal Lewis IgG (middle column), or affinity purified EAMG IgG (right-hand column) were coated (5 μg/ml) on microtiter plates, as indicated at the top of each column. Plates were incubated with polyclonal anti-Id, followed by goat-anti-rabbit-HRP. Anti-Id bound to their homologous Id (*left-hand column*) and to the affinity purified EAMG IgG (*right-hand column*), but not to pre-immune IgG (*middle column*); Id 6 (*open circles*), Id 35 (*open triangles*), Id 61 (*open squares*), Id 65 (*open inverse triangles*), Id IR27 (*filled squares*).

cell line (mAb 21H10A4) elicited against anti-MIR mAb 65, secreted monoclonal anti-Id binding to a paratope-related Id. However, the Id recognized by mAb 21H10A4 appeared to be a private Id. Although this demonstrated the feasibility of preparing blocking monoclonal anti-Id, additional fusions will be needed to select the appropriate monoclonal anti-Id against paratope-related CRI on anti-MIR antibodies.

Paratope-related CRI have been identified in sera of MG patients by one group (9), but not by another (11). In mice (12) and rats (13) also paratope-related CRI were detected. Negative results were reported using polyclonal anti-Id 35 to analyze paratope related Id on anti-MIR antibodies, including mAb 6, 35, and 65 (14). Their assay for the detection of paratope-related CRI was similar to ours, but they used serum instead of affinity purified anti-Id to

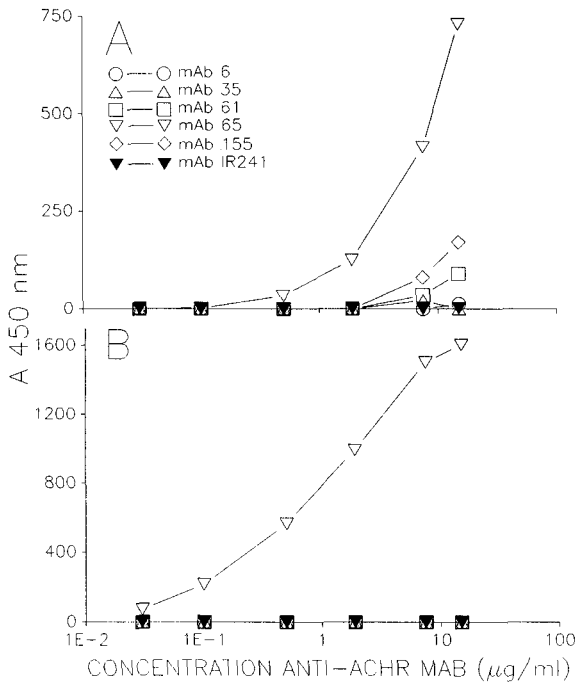


Figure 7. Anti-Id 65 mAb recognize private Id. Binding of anti-AChR mAb was tested in a direct Id binding ELISA. Microtiter plates were coated with monoclonal anti-Id 65, and incubated with anti-AChR mAb labeled with HRP. *A*, anti-Id mAb 21B6G8; *B*, anti-Id mAb 21H10A4. Both monoclonal anti-Id 65 bind only to anti-AChR mAb 65 (1E-2 etc., see Fig. 3).

inhibit the binding of the anti-AChR mAb to AChR. The use of affinity purified antibodies enabled us to test higher amounts of inhibitor, which probably explains why we were able to detect the presence of the paratope-related CRI.

In addition to paratope-related cross-reactivity we also analyzed sharing of framework Id by inhibition studies of Id-anti-Id binding. These Id-anti-Id inhibition studies do not distinguish between paratope or framework related CRI. However, knowing that the presence of paratope related CRI was restricted to the anti-MIR antibodies, additional CRI found by these assays could be attributed to framework determinants.

Only private Id were detected, when the inhibition of homologous Id-anti-Id interactions was measured (e.g., mAb 35 binding to anti-Id 35). A large cross-reactivity with very similar patterns for all five anti-Id was found, when assying the inhibition of heterologous Id-anti-Id binding (e.g., mAb 6 binding to anti-Id 35). These results can be explained by the fact that the anti-Id exhibit a much lower affinity for the heterologous Id than for their homologous Id.

For additional analysis of the framework related Id monoclonal anti-Id were prepared. Sofar, one monoclonal anti-Id was obtained recognizing a framework Id on mAb 65. Further fusions will have to clarify if it is possible to prepare monoclonal anti-Id's which show a broader cross-reactivity. Earlier studies concerning CRI provided some information about the presence of framework related CRI in MG (9, 11, 37) or in EAMG (10, 13, 14, 38). Studies in EAMG demonstrated a broad cross-reactivity between polyclonal anti-AChR antibodies of various mice strains, rat, rabbit, and even monkey. This extensive cross-reactivity most likely also included framework CRI, although this was not explicitly stated (12). Studies on mouse anti-AChR mAb the same group identified only private Id (38).

For framework CRI a different mechanism must be sought to explain cross-reactivity, when compared to paratope related CRI. These CRI were found on anti-AChR mAb against both intracellular and extracellular epitopes, clearly of different fine specificity. Examples of shared idiotypy among mAb, differing in their fine specificities have been described in several systems (36, 39–41). One explanation would be that in responses to autoantigens, such as the AChR or thyroglobulin, a restricted Id repertoire is used. Shared idiotopes among antibodies encoded by the same V_H gene family, but of different Ag specificity have been described (42). Studies of murine hybridomas indicated that three V_H families (V_HJ558, V_HQPC52, V_H7183) were primarily used in autoimmune responses in mice (40, 43).

It also has been suggested that early appearing multispecific B cells act in a cascade-like set of interactions and are responsible for the selection of later appearing Id (44, 45). Another possibility of selecting certain Id in the immune response toward an Ag is by means of regulatory idiotypes (46, 47), or anti-idiotypical T cells regulating restricted Id$^+$ cell populations (48). In light of the exciting applications for these anti-Id in Ag-specific immunotherapies, it will be worthwhile to try and select anti-Id capable of recognizing CRI in the polyclonal anti-AChR response.

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