IDIOTYPIC NETWORK CONNECTIVITY AND A POSSIBLE CAUSE OF MYASTHENIA GRAVIS

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In his theory of idiotypic networks, Niels Jerne proposed that antibody variable regions were organized into a network of complementary and interacting structures (1). The interconnection between antibodies via idiotypes (Ids) was thought to pertain not only to the development and maintenance of the B cell repertoire but to regulation of the immune response as well. Numerous studies (2–4) have now supported the notion that anti-Id antibodies contribute to the regulation of the immune response. Generally, these studies involved a single, often dominant Id, rather than a true network of Id connections. However, progress has recently been made concerning the composition of complex Id networks (5–7) and idiotypic crossregulation (6, 7).

We have discovered a profound interconnectivity between the acetylcholine receptor (AChR)[1] and α-1,3-dextran (DEX) antigen systems. Our results demonstrate a novel idiotypic relationship, whereby an anti-Id from the DEX immune response is an autoantibody against the AChR. Furthermore, because the DEX determinant is present on certain bacteria (8) (*Enterobacter cloacae* and *Serratia liquefaciens*), there may also be an idiotypic connection between antibody responses to the AChR and certain bacterial antigens. These findings may provide information about both the organization of Id networks and the initiation of the human autoimmune disease myasthenia gravis (MG), which is caused by autoantibodies against the AChR (9, 10).

Materials and Methods

Production of mAbs

mAbs were produced according to previously published methods (11). The antibodies used in these studies and some of their relevant properties are shown in Table I.

Enzyme-linked Immunoabsorbent Assays (ELISA)

Direct binding assay. This ELISA has been described elsewhere (12). For these studies, the mAbs were coated onto polyvinyl microtiter plates at a concentration of 4 μg/ml. The purified antibodies were tested for binding at a concentration of 25–30 μg/ml. In some cases, 100 μl of culture fluid supernatant were used. Plates were developed using the

[1] *Abbreviations used in this paper:* AChR, acetylcholine receptor; DEX, α-1,3-dextran; MG, myasthenia gravis.

TABLE I

Source and Specificity of mAbs

mAb	Isotype	Specificity*	Source
Sh174	γ2b, κ	AChR	BALB/c
CdB3	μ, κ	Sh174	BALB/c
CB6	μ, λ	EB3	C57Bl/6
BA6	μ, κ	EB3	BALB/c
EB3	γ1, κ	J558	A/J
J558	α, λ	DEX	BALB/c
M104	μ, λ	DEX	BALB/c
CD3	γ1, λ	J558, M104	A/J
117	μ, κ	CD3	BALB/c
MB5	γ1, κ	SRll	BALB/c
AC3	γ1, κ	SRll	BALB/c
EB5	γ1, κ	SRll	BALB/c
SR11	μ, κ	ACR24	Human
ACR24	γ1, κ	AChR	BALB/c

* The specificity of the mAbs refers to the original antigen binding detected in the initial ELISA screening of the fusion. As reported in this paper, some of these mAbs were subsequently found to also bind to other antigens.

appropriate combination of isotype-specific second antibodies labeled with alkaline phosphatase. The minimum value considered positive here was an A_{405} >0.200 above the control value obtained with the same antibody tested against BSA.

Inhibition assay. For this ELISA, DEX-BSA was coated onto microtiter plates at a concentration of 4 μg/ml. Next, varying amounts of the test solutions were added to the wells (50 μl/well), followed by the addition of 50 μl of purified SR11 or DId (final concentration 30 μg/ml). Nigerotriose is a glucose trisaccharide prepared by Dr. Johnston and was a gift from Dr. W. Schuler (Munster, Federal Republic of Germany). The initial concentration of nigerotriose used was 100 μg/ml. DEX was the same as that used to coat the plates (100 μg/ml initial concentration). EB5, the anti-Id raised against SR11, had an initial concentration of 50 μg/ml. ACR24 was used at a concentration of 100 μg/ml, as was J558. Finally, AB1, which served as a control for EB5, is a γ1, κ mAb derived from A/J mice immunized with HOPC8. This antibody was also used at an initial concentration of 50 μg/ml. The plates were developed by adding either alkaline phosphatase–coupled anti–human IgM (SR11) or anti–human IgG (DId).

Measurement of human serum antibodies. An ELISA that has been described elsewhere (13) was used to detect specific serum antibodies. Briefly, microtiter plates were coated with DEX (4 μg/ml) and blocked with 1% BSA in borate-saline buffer before addition of serum. Sera were diluted 1:40 in BSA borate-saline buffer containing 0.25% Tween 20. The plates were developed with alkaline phosphatase–coupled second antibodies as above. DEX B1355S (35% α-1,3 linkages), derived from *L. mesenteroides*, was a gift from Dr. Slodki, U.S. Department of Agriculture, Peoria, IL.

Results

The AChR-DEX Network. The studies described here led to the construction of an elaborate Id network connecting the immune responses against AChR and DEX and involving the antibodies displayed in Table I. A summary of these findings depicting the AChR-DEX network is shown in Figure 1. CdB3 is a syngeneic anti-Id mAb produced by immunizing BALB/c mice with the mAb Sh174, which precipitates rat and human muscle AChR. During the initial characterization of CdB3, it was found (D. Dwyer, unpublished observations) that this mAb did not react with a panel of 12 different antibodies, indicating its specificity for Sh174. Surprisingly, as shown in the left side of Fig. 1, CdB3 also reacts with EB3, which is a monoclonal anti-Id against the DEX-binding myeloma, J558. Although the binding of CdB3 to Sh174 (A_{405}, 0.607) and EB3 (A_{405},
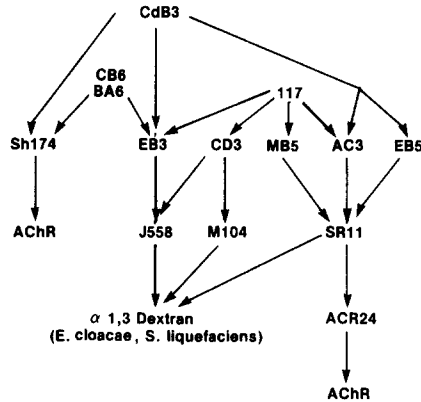
FIGURE 1.   Summary of the antibody interactions connecting the AChR and DEX immune responses. The arrows indicate a significant reaction between the antibodies as measured with an ELISA. Generally, the A_{405} values represented by the arrows were 0.400-1.200 when the plates were read 30 min after the addition of substrate.

TABLE II
Binding Specificity of Anti-Id mAbs

mAb	Absorbance (A_{405}) in ELISA with:							
	EB3 ($\gamma 1$, K)	Sh174 ($\gamma 2b$, K)	BPCl ($\gamma 2b$, K)	MB5 ($\gamma 1$, K)	AC3 ($\gamma 1$, K)	EB5 ($\gamma 1$, K)	ACR24 ($\gamma 1$, K)	CD3 ($\gamma 1$, λ)
CdB3	0.610	0.607	0.005	0.000	0.258	0.369	0.022	0.038
BA6	0.677	0.224	0.019	0.012	0.057	0.014	0.579	0.025
CB6	0.318	0.223	0.013	0.001	0.000	0.005	0.000	0.008
117	0.788	0.013	0.000	1.085	1.356	0.070	0.078	0.724

Binding was evaluated with an ELISA and the results have been expressed as the A_{405} obtained after 30 min of reaction.

0.610) is similar, these two antibodies are not identical because Sh174 does not bind to J558, and EB3 does not precipitate AChR. Two other mAbs, CB6 and BA6, derived from fusions of neonatal B cells, were selected in our laboratory on the basis of their interaction with EB3. In view of the similarities between EB3 and Sh174, CB6 and BA6 were tested against Sh174. The scheme in Fig. 1 shows that these mAbs also react with Sh174, confirming the idiotypic relationship between Sh174 and EB3.

Representative binding data from some of the key antibodies in this network are presented in Table II. Many of the antibodies in Fig. 1, including Sh174, EB3, CD3, J558, M104, SR11, and ACR24, have been described elsewhere (14, 15). From Table II it is clear that CdB3 binds to EB3, Sh174, AC3 and EB5, whereas BA6 recognizes EB3, Sh174 and ACR24. CB6 binds to EB3 and Sh174 only, and 117 binds to EB3, MB5, AC3 and CD3 (to be discussed in more detail later). These four antibodies were tested against a larger panel of antibodies that are not shown here and did not react with any others (except 117, which also bound to AB1). Because these antibodies did not react with a common epitope composed of isotypic or allotypic determinants, it can be concluded that they recognize an epitope in the variable region of these antibodies and can be considered anti-Ids. Similar crossreactive anti-Ids have been described by other investigators (5-7).

In addition, the data in Table II show that CdB3, CB6, and BA6, which are all IgM, are distinct antibodies with unique antigen-binding profiles. CdB3 and BA6 were derived from BALB/c mice whereas CB6 was obtained from a C57BL/6 mouse. Furthermore, CdB3 but not CB6 binds to EB5 and AC3 (to be discussed later), and BA6 alone binds strongly to ACR24. Interestingly, the binding of some of these anti-Ids, such as CdB3, to Id on the ELISA plate is not inhibited by soluble Id. However, these anti-Ids do bind to Id on the cell surface of the hybridoma (D. Dwyer, unpublished observations), and can regulate the relevant immune response.

The above findings prompted us to search for an idiotypic connection between the anti-AChR and anti-DEX responses using some of the human antibodies we have available. SR11 is a human mAb obtained from a myasthenic patient. As described elsewhere (15, 16) this antibody was selected because it bound specifically to ACR24, a mouse mAb directed against AChR from both *Narcine* electroplax and mammalian muscle, and not to other murine mAbs. In addition, it was shown (15) that the binding of SR11 to ACR24 was blocked by antigen (AChR), further confirming the idiotypic nature of this interaction. We also reported that serum from certain MG patients contain anti-Ids against ACR24, which are similar to SR11. These studies showed that, among the serum antibodies from some myasthenics, there is an idiotypic counterpart to the ACR24 mAb. Therefore, SR11 and an ACR24 equivalent represent an important part of the antibody repertoire expressed by patients with MG.

Previously, a panel of murine anti-Id mAbs had been produced (J. Kearney, unpublished data) which were specific for SR11. EB5, MB5, and AC3 are members of this panel of anti-Ids. In a separate study (J. Kearney, unpublished data), a murine mAb (117) derived from a primary anti-DEX response was selected for further examination because it bound to an anti-Id (CD3) in the DEX system. Thus, 117 is an anti-(anti-Id) in the DEX immune response, that has in vivo activity in suppressing the anti-DEX response, presumably by activating a set of anti-Id B cells (J. Kearney, unpublished observations). As shown in Fig. 1, the mAb 117 binds not only to CD3 but to EB3, MB5, and AC3 as well. Furthermore, CdB3, an anti-Id against Sh174, reacts with EB3, as discussed previously, and with EB5 and AC3. The binding characteristics of 117 and CdB3 provide additional support for a connection between the immune responses to DEX and AChR. All other possible combinations of antibody binding (e.g., Sh174 vs. J558, MB5 vs. AChR, etc.) were tested, and only those interactions depicted here were significant, with the exception of the BA6-ACR24 interaction, which was omitted for the sake of clarity, and the BA6-AChR interaction, which has been reported elsewhere (17). Because BA6 binds both to anti-AChR antibodies (Sh174 and ACR24) and to the AChR, it is an epibody as defined by Bona et al. (18).

The Id connections described here are largely, but not exclusively, restricted to the AChR-DEX network. There was little interaction of the antibodies from Table I with nine equivalent antibodies (Ids, anti-Ids, etc.) from the phosphorylcholine antigen system (117 bound to AB1, an anti-T15 antibody). However, some of the antibodies uppermost in our scheme (e.g., BA6 and 117) are more broadly reactive and are connected with other antigen systems.

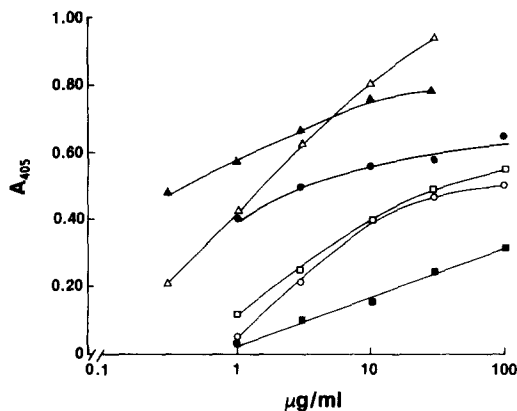The crossreactive anti-Ids, although typically of lower affinity than highly

FIGURE 2.   Binding profiles of monoclonal anti-Ids. An ELISA was used to evaluate the binding of anti-Ids at various protein concentrations. The results are presented as the A_{405} obtained with a multiscan after 15 min of reaction. The following interactions were examined: FZ1.4-ACR24 (△), EBG3-AChR (▲), 117-MB5 (●), CdB3-Sh174 (□), 117-CD3 (○), and CdB3-EB3 (■).

specific mAbs, nevertheless bind quite strongly to the Id-bearing antibody. These data are depicted in Fig. 2. Binding profiles are shown for Sh174 and 117, and for comparison, FZ1.4, which is an anti-Id specific for ACR24, and EBG3, which reacts with *Narcine* AChR. These latter two antibodies are high-affinity mAbs that were obtained by immunization with antigen. As mentioned earlier, 117 was derived from mice immunized with DEX, and yet its affinity for MB5 is very similar to that of FZ1.4. The affinity for CD3 is less, but still represents very effective binding. In contrast, CdB3 was obtained after immunization with Sh174, and the affinity of this interaction is comparable to that of the 117-CD3 interaction. The CdB3-EB3 interaction is the weakest of those depicted here, however, this anti-Id can potently suppress the immune response to DEX at standard doses of anti-Id (D. Dwyer, unpublished observations), indicating the physiological relevance of this binding.

Binding Pattern of a Human mAb against DEX.   Based on the scheme in Fig. 1, it was possible to predict that the human mAb, SR11, might bind to DEX and the results in Fig. 3 confirm this prediction. The data presented here concern the competitive inhibition of SR11 binding to DEX by various substances including the mAbs J558, EB5, and ACR24, and the trisaccharide nigerotriose. The J558 V_H is considered a representative BALB/c antibody involved in DEX binding. J558 effectively competes with SR11 for binding to DEX, suggesting that these two mAbs are binding to a similar or identical determinant. The anti-Id EB5 also effectively inhibits binding of SR11 to DEX, whereas a control mAb, AB1, does not. Surprisingly, although SR11 binds to ACR24, it is clear from Fig. 3 that ACR24 does not inhibit binding of SR11 to DEX, implying that recognition of these two molecules is accomplished by different parts of the SR11 variable region. DEX itself and nigerotriose strongly inhibit the interaction of SR11 with DEX, confirming the earlier suggestion that α-1,3-glucosidic linkages are the relevant antigenic determinant. Thus, SR11, selected for binding to ACR24, binds to DEX as well. As shown here, this recognition is probably achieved through two different sites because (*a*) ACR24 does not inhibit binding
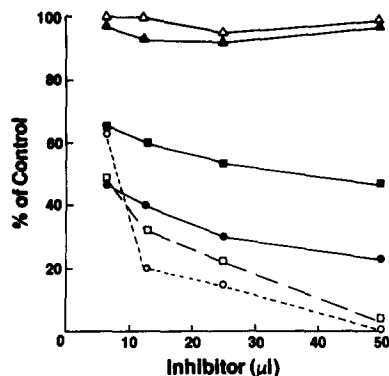
FIGURE 3. Inhibition of SR11 binding to DEX. Various substances were tested for their ability to inhibit the binding of SR11 to DEX. The results of these assays have been expressed as the percentage of a control assay where no inhibitor was added. Nigerotriose (●—●), DEX (■—■), EB5 (O— — —O), ACR24 (▲—▲), J558 (□— — —□), AB1 (△—△).

TABLE III

Purified Human Antiidiotypes against ACR24 Bind to DEX

Antigen	Addition	A$_{405}$
BSA	—	0.014
ACR24	—	0.398
DEX	—	0.740
DEX	Nigerotriose (50 μg/ml)	0.311
DEX	Nigerotriose (12.5 μg/ml)	0.478

Anti-Id was purified from the serum of a patient with MG using an ACR24 affinity column. The characterization of these antibodies has been described elsewhere (15). These data were obtained with an ELISA similar to that of Fig. 3.

to DEX, and (b) nigerotriose does not inhibit binding of SR11 to ACR24 (data not shown), although it is very effective at inhibiting DEX binding. Finally, SR11 agglutinates both E. cloacae and S. liquefaciens and reacts with these two bacteria in an ELISA (data not shown), indicating that this human mAb also recognizes DEX-like determinants on certain bacteria.

As a further indication of the degree of network connectivity, serum anti-Ids from a second myasthenic patient, that had previously been purified on an ACR24 affinity column, also bind strongly to DEX, similar to SR11. These data appear in Table III. The specificity and binding characteristics of DId have been described in detail elsewhere (15). DId binds to ACR24 and to DEX as shown, and the binding to DEX can be inhibited by nigerotriose. Thus, there is far more interconnectivity between the AChR and DEX immune responses than would occur by chance, especially considering the similarities between SR11 and DId.

Anti-DEX Antibodies in MG Patients.   If the DEX determinant is an important epitope involved in the induction of the immune response against certain bacteria and these antibodies elicit the autoimmune response in MG, then there might be antibodies against DEX in the serum of some myasthenic patients. Therefore, sera from 60 patients with MG and 40 controls were tested for antibodies against DEX. The results of this study are shown in Fig. 4. It is clear that a number of myasthenic patients (16%) have serum antibodies against DEX. Serum antibody
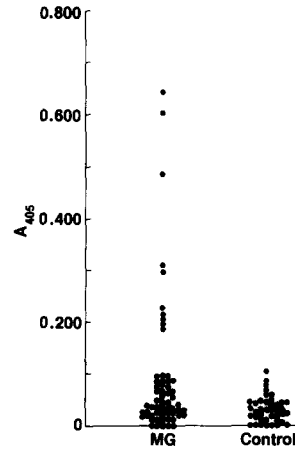
FIGURE 4. Anti-DEX antibody levels in patients with MG. Serum from myasthenic patients and nonmyasthenic controls were assayed for antibodies against DEX. The control group included patients with the suspected autoimmune diseases multiple sclerosis and Guillain-Barre syndrome. The results have been expressed as the A_{405} reading after the subtraction of background values against BSA-coated wells.

levels are considered positive if they are >3 SD above the mean of the control values (i.e., >0.106). Analysis of the immunoglobulin isotype of the anti-DEX antibodies revealed that the response is largely restricted to the IgG class (data not shown). Occasionally, low levels of IgM or IgA anti-DEX antibodies were detected.

Discussion

The data presented here illustrate two important points. The first concerns idiotypic interconnectivity between different antigen systems. Although implicit in Jerne's theory, there has been little evidence showing that the immune responses against disparate antigens are connected via Ids. The Id connections described so far (19–21) only show shared idiotypy between antibodies against different antigens, rather than the complex web of Ids constructed in Fig. 1. Furthermore, we have shown a hierarchical organization of the Id network, in agreement with earlier predictions (22, 23). Antibodies such as CdB3, 117, and BA6 are crucial links in the network, and we have termed these antibodies super organizers. Although super organizer antibodies tend to bind with a lower affinity to Ids than conventional highly specific mAbs, this binding is strong enough to elicit physiological responses (7 and D. Dwyer, unpublished results). These antibodies are probably similar to the promiscuous mAbs described by Holmberg et al. (5). The molecular basis for the recognition of shared idiotypy by super organizers is not clear. Victor-Kobrin et al. have studied similar antibodies, and they showed that antibodies bearing the crossreactive Id belong to the same V_H gene family (6). Studies to examine this issue are underway.

The second point concerns the induction of autoimmunity via the normal operation of Id networks. As Plotz (24) suggested, an anti-Id in one response may be an autoantibody against a different self antigen. In the case of MG, infection by the common opportunistic pathogens *E. cloacae* or *S. liquefaciens*

may lead to an immune response against DEX-like determinants on the bacteria. Under certain circumstances, antibodies such as SR11 and DId would be produced. These anti-DEX antibodies would then trigger an anti-Id response, typified by antibodies that resemble ACR24, however, these anti-Ids would also be autoantibodies against the AChR. The anti-AChR response would be amplified if antibodies like Sh174 were also produced via perturbations of the AChR-DEX network. Thus, a bacterial infection could initiate MG indirectly by stimulating the production of certain anti-Ids that also happen to be autoantibodies. Naturally, in addition to bacterial infection, other factors such as the appropriate immune response genes (HLA-D) and immunoglobulin genes, and T cell abnormalities are probably necessary for the induction and development of MG.

The model presented here for the initiation of autoimmunity requires that there are functional Id connections between different immune responses. Victor-Kobrin et al. (6) and Kearney and Vakil (7) have already demonstrated cross-regulation of immune responses via crossreactive anti-Ids. Preliminary data from our laboratory indicate that CdB3 can suppress the immune responses to both the AChR and DEX. Therefore, it will be important to determine whether there is a functional connectivity in the AChR-DEX network.

Summary

Extensive idiotypic connectivity has been discovered between the antibodies composing the immune responses against the acetylcholine receptor (AChR) and α-1,3-dextran. The idiotypic connections form an elaborate network linking these disparate antigen systems, and there is an hierarchical organization of the antibodies in this network. The key anti-Ids that interconnect these two responses are more crossreactive, lower-affinity antibodies. Interestingly, 15% of patients with MG, which is caused by autoantibodies against the AChR, have serum antibodies against DEX. Control sera are negative for anti-DEX antibodies. Certain anti-DEX antibodies also bind to anti-AChR antibodies via idiotypic interactions. These findings suggest a model for the initiation of autoimmunity in MG. Antibodies made in response to DEX epitopes on the surface of certain bacteria would elicit the production of anti-Ids. However, some of these anti-Ids would also be autoantibodies against the AChR. Thus, is some circumstances, autoimmunity may develop as a consequence of the normal operation of regulatory idiotypic networks.

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