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Immunology

Human T-helper lymphocytes in myasthenia gravis recognize the nicotinic receptor α subunit

(immunoregulation/autoimmune disease/immunotherapy)

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ABSTRACT **Myasthenia gravis is a human disease caused by an autoimmune response against the nicotinic acetylcholine receptor (AcChoR). Since the molecular structure of AcChoR is well known, myasthenia gravis is an excellent system for studying the recognition of a complex membrane antigen in the human immune system. Human T-helper (T_H) cell lines reactive to the AcChoR were isolated from four myasthenic patients by selection with native AcChoR from *Torpedo californica*. The selected T_H cells could efficiently recognize native and fully denatured AcChoR. The vast majority of the T_H-stimulating AcChoR epitopes were located on the denatured α subunit of AcChoR. Antibody competition experiments using a panel of rat anti-AcChoR monoclonal antibodies showed that 39–45% of the autoantibodies present in the sera of these same patients bound to the conformation-sensitive "main immunogenic region" (MIR), also located on the α subunit. However, AcChoR-induced stimulation of the T cells could not be inhibited with up to 20-fold molar excess of different rat anti-MIR monoclonal antibodies. These results suggest that the *Torpedo* AcChoR α subunit contains conformation-insensitive epitopes that play a role in the autosensitization of T_H cells and that seem to be physically separated from the MIR. The specificity of the T_H cell response may contribute to directing the B-cell response to other α-subunit determinants, such as the MIR itself.**

A core question of immunology is the elucidation at the molecular level of the mechanisms by which a complex antigen is "seen" by the two functional compartments that collaborate in antibody production—i.e., the helper/suppressor T cells and the corresponding antibody-producing B cell. Although these two cell types can be regarded as the two halves of a functional unit engineered for production of an antibody specific for a given epitope, T and B subpopulations exhibit striking differences in their reactivity with complex antigens (1). The development of monoclonal antibody (mAb) technology has provided a powerful tool for the study of antigen recognition by antibodies. On the other hand, the expectation that elucidation of the structure of T-cell receptor would, by itself, shed light on the molecular mechanism of the differences in T- and B-cell recognition has not been fulfilled. Therefore, increasing attention should be paid to understanding which parts of an antigen are actually involved in the T-cell recognition process.

The human disease myasthenia gravis (MG) is an excellent system for studying these phenomena in the human immune system. MG is a human paralysis due to an autoimmune response against the nicotinic acetylcholine receptor (AcChoR) in the neuromuscular junction, with T-dependent production of anti-AcChoR antibodies (2–4). The AcChoR is today the best characterized neurotransmitter receptor (5). It is a complex transmembrane protein formed by four subunits with a stoichiometry $\alpha_2\beta\gamma\delta$. The four subunits have highly homologous amino acid sequences, and their complete sequences are known. Models predicting their tertiary structure and transmembrane folding have been proposed (5) that allow speculations about which portions of the sequence form protruding parts of the AcChoR molecule, which could be parts of antigenic sites (6, 7). The structure of the AcChoR is highly conserved along animal evolution. AcChoRs from tissues of distant species such as the electric tissue of *Torpedo* and mammalian muscle are consistently a pseudosymmetric pentamer of homologous subunits, and subunits of corresponding molecular mass from different species are also highly homologous (5). For example, the α subunit of *Torpedo* is 80% identical to the human muscle α subunit.

The α subunit dominates the anti-AcChoR antibody response in MG. In fact, although the anti-AcChoR autoantibodies are heterogeneous in their specificity (8–11), a large fraction of them bind to a discrete area of the α subunit, known as the "main immunogenic region" (MIR) (10, 11). This predominant immunogenicity of the α subunit is somehow surprising, since all of the AcChoR subunits have similar amino acid sequence, transmembrane topology, and possibly posttranslational processing (5). The α subunit therefore does not have any unique biochemical properties to explain its immunogenic properties. Since the synthesis of anti-AcChoR antibodies is regulated by AcChoR-specific T-helper (T_H) cells (2, 12–14), it is conceivable that the determinants recognized by the T_H cells will influence and direct (15) the B-cell response toward determinants located within the MIR. It is important therefore to elucidate which areas or sequence stretches of the AcChoR molecule are recognized by T_H cells in MG.

MG offers a unique opportunity for investigating what the T cells "see" of a complex membrane antigen such as the AcChoR. It is in fact possible to obtain large amounts of purified AcChoR subunits, particularly from the electric tissue of *Torpedo*, whose AcChoR is very similar to human AcChoR, and long-term T-cell lines specific for the AcChoR can be isolated from the blood of MG patients (12, 16, 17). These T cells have the phenotype (12) and the function (16) of T_H cells, and they recognize the AcChoR in a genetically restricted fashion (17).

We report here an analysis of the subunit specificity of AcChoR-reactive human T_H cells and its relationship with the specificity of anti-AcChoR autoantibodies. In a broad perspective, the results reported here represent a first step in the elucidation of how a complex autoantigen is seen as a

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Abbreviations: AcChoR, acetylcholine receptor; mAb, monoclonal antibody; MG, myasthenia gravis; MIR, main immunogenic region; PBM, peripheral blood mononuclear cells; PHA, phytohemagglutinin; T_H, T helper; α-Butx, α-bungarotoxin.

tridimensional object by two complementary compartments of the human immune system, the T_H cells and the B cells, in a naturally occurring autoimmune state.

MATERIALS AND METHODS

T-Cell Lines. AcChoR-reactive T-cell lines were isolated in four patients who had clinical and electrophysiological signs of generalized MG (12, 16, 17). All patients had elevated titers of serum anti-AcChoR autoantibodies (see *Results*). Immunosuppressive medications had been stopped for at least 3 months at the time the blood for T-cell isolation was obtained. Peripheral blood mononuclear cells (PBM) were cultured for 3–5 days in the presence of purified *Torpedo californica* AcChoR, and the reactive lymphoblasts were isolated on discontinuous density gradients (18). The cells were further expanded and enriched by repeated stimulation cycles with interleukin 2 followed by 5 μg of native *Torpedo* AcChoR per ml plus mitomycin C-treated autologous PBM as antigen-presenting cells (12). For studying the antigenic specificity of the global anti-AcChoR T-cell response, the cells were analyzed as soon as a reasonable enrichment in AcChoR reactivity was achieved (100–400 times the reactivity of unselected PBM; ref. 12), which usually took 4–6 weeks. During this time, AcChoR used for cell stimulation was exclusively native *Torpedo* AcChoR, either membrane-bound (patients 1 and 2) or solubilized (patients 3 and 4). The T-cell proliferation assay was performed as described in ref. 12.

Purification of AcChoR and AcChoR Subunits. Membrane-bound AcChoR was purified from *Torpedo* electric tissue (19–21). Solubilized *Torpedo* AcChoR was purified by affinity chromatography using *Naja naja siamensis* α-neurotoxin as a ligand (22). The specific activities were 2–6 (membrane) and 5–7 (soluble AcChoR) nmol of α-bungarotoxin (α-Butx) binding sites per mg of protein. *Torpedo* AcChoR subunits, as well as a 90-kDa peptide that is a common contaminant of membrane-bound AcChoR preparations (19), were purified by preparative $NaDodSO_4$/polyacrylamide gel electrophoresis (PAGE) followed by electroelution, reduction (1 mM dithiothreitol, 6 min at room temperature), alkylation (3 mM iodoacetamide, 10 min at room temperature), and electrodesalting (23). The purity of the subunit preparations was assessed by gas-phase sequencing (24) and by $NaDodSO_4$/PAGE (25). Preparations of isolated subunits yielded only the expected sequences and contained only one protein band of the expected molecular mass. β-Subunit preparations were occasionally contaminated with α subunit up to a maximum of 10%. The $NaDodSO_4$ in the subunit preparations (up to 10 times the weight of the proteins) was removed by cryoprecipitation in 50 mM ammonium bicarbonate (pH 9). For use in tissue culture, the lyophilized subunits were dissolved in culture medium to a final concentration of 1 μg/ml. For the preparation of reduced and/or denatured AcChoR, purified native membrane receptor was treated with 1% $NaDodSO_4$ for 10 min at room temperature (denatured) and/or with 1 mM dithiothreitol at pH 8 for 30 min at room temperature (denatured and reduced or reduced); this was followed by 3 mM iodoacetamide for 15 min at room temperature. $NaDodSO_4$ present in denatured samples and denatured and reduced samples was eliminated by cryoprecipitation at pH 9; dithiothreitol and iodoacetamide were eliminated by dialysis against 10 mM sodium phosphate buffer at pH 7. All samples were stored at −90°C.

Anti-Human and Anti-*Torpedo* AcChoR Antibody Assays. Anti-AcChoR antibody titer of sera from myasthenic patients was assayed by immunoprecipitation (9, 26) using 0.2 nM ^{125}I-α-Butx-labeled human AcChoR or 1 nM ^{125}I-α-Butx-labeled *Torpedo* AcChoR. mAb 35 (see refs. 11, 27) was used as a positive control; normal human serum was used as a negative control.

Antibody Inhibition Assay with Rat Anti-AcChoR mAbs. Competition experiments between rat anti-AcChoR mAbs of known subunit specificity and patients' sera were performed as described in refs. 11 and 27. Inhibition of antibody binding was quantitated by the difference in specific radioactivity precipitated in the presence or absence of "protecting" mAb. Averages of percent protection were calculated for each of nine anti-MIR mAbs (nos. 35, 42, 190, 192, 195, 202, 203, 204, 207), five non-MIR anti-α-subunit mAbs (nos. 64, 152, 153, 155, 164), and five anti-β-subunit mAbs (nos. 73, 111, 118, 124, 148). One anti-δ-subunit mAb (no. 137) was also used. mAbs 35 and 42 were raised against *Electrophorus electricus* AcChoR, 64 and 73 were raised against fetal calf muscle AcChoR, 111–164 were raised against denatured subunits of *T. californica* electric organ AcChoR, and 190–207 were raised against human muscle AcChoR. All mAbs raised against non-human AcChoR cross-reacted with human muscle AcChoR. mAb 25, which is species specific for *Electrophorus* AcChoR (28), was used as a control (see refs. 10, 11, 28, 29).

RESULTS

T-Cell Reactivity with Reduced and/or Denatured AcChoR. The stimulating activity of reduced nondenatured, $NaDodSO_4$-denatured, and $NaDodSO_4$-denatured and reduced *Torpedo* AcChoR preparations was compared to that of nondenatured solubilized or membrane-bound native *Torpedo* AcChoR in the suboptimal dose range (Fig. 1). The dose–response relationship of the denatured and reduced form (treated like the isolated subunits) was comparable to

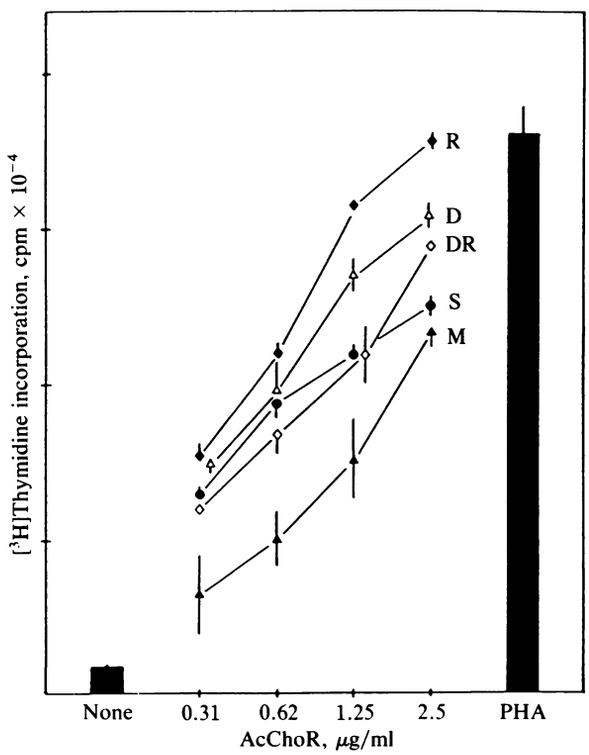

FIG. 1. Reactivity of AcChoR-specific T_H cells against reduced (R), denatured (D), denatured and reduced (DR), solubilized (S), or membrane-bound (M) *Torpedo* AcChoR. T cells were derived from the cell line used for the experiment shown in Fig. 2 and had been subjected to several additional cycles of restimulation with *Torpedo* membrane AcChoR. T-cell proliferation is expressed as [^{3}H]thymidine incorporation (mean ± SD of triplicate cultures). Black bars indicate stimulation of control cultures without antigen (None) or with mitogen [phytohemagglutinin (PHA); 1 μg/ml]. AcChoR concentrations are expressed as μg of protein per ml.

that of native solubilized AcChoR. The reduced or denatured AcChoR had higher stimulating activity than the native membrane-bound AcChoR.

AcChoR Subunit Reactivity of Autoreactive T Cells. Short-term T-cell lines were selected from four MG patients using native *Torpedo* AcChoR as stimulating antigen (membrane AcChoR in patients 1 and 2; solubilized AcChoR in patients 3 and 4). After 4–6 wk of culture these T cells were homogeneously positive for the helper/inducer phenotype CD3, CD4, in accordance with our previous results (12, 16), demonstrating that T cells selected by this procedure have uniformly the phenotype (12) and the function (16) of T_H cells. These T cells were then exposed for the first time to denatured isolated *Torpedo* AcChoR subunits. In patients 1 and 4 the proliferative response to α subunit was equal to or within the range of the response to solubilized or membrane-bound intact AcChoR; in contrast, the proliferative responses to all of the other subunits as well as the 90-kDa contaminant were within the background range (Table 1; Fig. 2). In patients 2 and 3 the T-cell response to α subunit was much higher than that to other subunits, but significant reactivity against β, γ, and δ subunits was detected in patient 2 and reactivity against β subunit was detected in patient 3 (Table 1).

Characterization of Serum Anti-AcChoR Autoantibodies. In all four patients a substantial fraction of serum autoantibodies was directed to MIR-related AcChoR determinants. The fraction of antibodies to isolated denatured subunits was negligible (Table 2). The fraction of patient anti-MIR antibodies did not increase when a mixture of anti-MIR mAbs was used in the competition assay (S.J.T., unpublished observations). We therefore conclude that the mAbs used do not "protect" all potentially immunogenic AcChoR determinants. This emphasizes the polyclonal nature of the B-cell anti-AcChoR response (8–11). The antibody cross-reactivity between *Torpedo* and human AcChoR was low, in accordance with similar earlier reports (30).

Effects of Anti-MIR mAbs on AcChoR-Reactive T Cells. In earlier experiments, we tested if the polyclonal antibodies present in the serum of myasthenic patients had any effect on the response to AcChoR of their T-cell lines. T-cell lines from two different patients, whose anti-AcChoR antibody titers were 10 and 30 nM, respectively, were tested for anti-AcChoR reactivity in the presence either of fetal calf serum or of autologous serum up to a concentration of 20%. No difference in the T-cell response to AcChoR was detected. These results confirmed earlier reports from our and other laboratories (31, 32), which demonstrated either no inhibition (32) or even an increase (31) in the T-cell response to AcChoR when cultivated in the presence of autologous serum instead of fetal calf serum (32) or universal AB serum (31). Since this lack of inhibition could be due to inability of the human anti-MIR antibodies to efficiently bind and mask this region on *Torpedo* AcChoR, we examined whether anti-AcChoR T-cell proliferation could be inhibited by anti-MIR mAbs 6, 35, and 202 (see *Materials and Methods* and refs. 11, 28, 29). AcChoR-reactive T cells from patients 1, 3, and 4 were stimulated with 25 nM soluble *Torpedo* AcChoR in the presence of either 10, 50, or 250 nM mAb. Relative stimulation ranged between 90% and 148% of AcChoR-stimulated control cultures without mAb ([^{3}H]thymidine uptake, 10–20 $\times 10^3$ cpm; data not shown). In patient 1, additional mAbs were tested (anti-MIR mAb 192; anti-α subunit/non-MIR mAbs 64, 153, 155; see *Materials and Methods*). No inhibitory effect was found at concentrations up to 250 nM. To increase further the effective concentration of mAbs in culture, we pulsed antigen-presenting cells from patient 1 with soluble *Torpedo* AcChoR before adding mAbs (500 nM) and T cells. No inhibition was observed (Table 3).

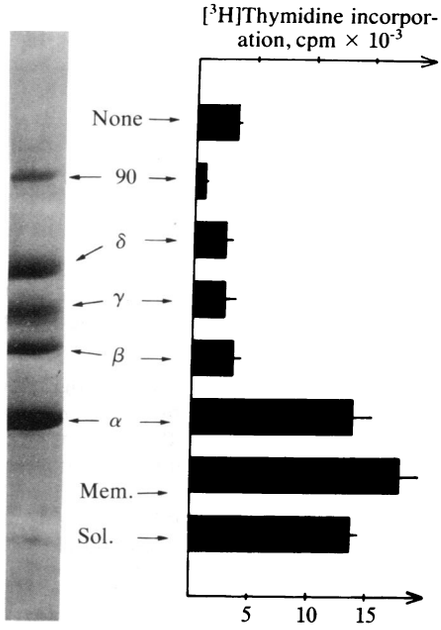

FIG. 2. Subunit specificity of AcChoR-reactive T_H cells from MG patient 1. The NaDodSO$_4$/PAGE pattern of a typical membrane preparation is shown on the left. Horizontal bars on the right indicate proliferative reactivity ([^{3}H]thymidine incorporation; mean ± SD of triplicate cultures) of selected AcChoR-reactive T cells in the absence of antigen (None) or in the presence of the 90-kDa contaminant protein (90; 1 μg/ml; ref. 22), purified *Torpedo* AcChoR subunits (α, β, γ, δ; 1 μg/ml), intact *Torpedo* membrane AcChoR (Mem.; 5 μg/ml), and *Torpedo* solubilized AcChoR (Sol.; 5 μg/ml). PHA-stimulated proliferation was 29,700 ± 1200 cpm (1 μg of PHA per ml, Wellcome).

DISCUSSION

This study is the first step of an attempt to elucidate the structure of the antigenic epitopes recognized by the human T cells that regulate the synthesis of anti-AcChoR autoantibodies in MG. We present evidence that a sizable population of T_H cells in MG can recognize epitopes on the α subunit of the nicotinic receptor, independently from their tertiary

Table 1. AcChoR subunit specificity of AcChoR-reactive T_H cells from MG patients

	[^{3}H]Thymidine incorporation, cpm × 10^{-3}							
Patient	No antigen	Soluble AcChoR, 5 μg/ml	Membrane AcChoR, 5 μg/ml	PHA, 5 μg/ml	α subunit, 1 μg/ml	β subunit, 1 μg/ml	γ subunit, 1 μg/ml	δ subunit, 1 μg/ml
1	3.5 ± 0.3	13.6 ± 0.6	17.7 ± 1.7	29.7 ± 1.2	13.7 ± 1.8	3.5 ± 0.6	2.6 ± 0.9	2.9 ± 0.7
2	3.7 ± 0.1	16.2 ± 2.1	11.2 ± 5.2	28.9 ± 4.6	25.9 ± 1.4	9.9 ± 2.3	7.8 ± 1.3	9.6 ± 2.8
3	1.8 ± 0.9	14.7 ± 0.8	14.6 ± 3.5	18.1 ± 1.7	17.5 ± 2.1	8.4 ± 0.5	3.7 ± 0.8	3.0 ± 0.3
4	2.9 ± 1.0	23.5 ± 1.1	5.5 ± 0.7	26.2 ± 1.3	9.5 ± 1.7	1.8 ± 0.2	3.0 ± 0.2	2.6 ± 0.4

Data are expressed as the mean ± SD of triplicate cultures.

Table 2. Subunit specificity of anti-AcChoR antibodies in sera of MG patients

Patient	Anti-human AcChoR titer, nM	% inhibition: Anti-MIR	Anti-α subunit, non-MIR	Anti-β subunit	Anti-δ subunit	Anti-*Torpedo* AcChoR titer, nM
1	2.1	45 ± 13	3 ± 5	5 ± 8	14 ± 8	≈0
2	9.0	39 ± 13	1 ± 2	8 ± 5	0 ± 4	0.2
3	26.0	45 ± 10	0 ± 0	2 ± 2	2 ± 5	≈0
4	33.0	40 ± 10	0 ± 1	3 ± 4	2 ± 2	0.1

Values are expressed as average % inhibition of serum binding to human muscle AcChoR (mean ± SD), when a mAb directed against the different subunit or the MIR was present in the reaction mixture.

structure. These determinants are phylogenetically conserved, since these T_H cells recognize *Torpedo* AcChoR, and they seem to be different from the area of the α subunit recognized by the majority of anti-α antibodies—i.e., the MIR.

The α subunit was recognized by the vast majority of anti-AcChoR T_H cells selected by stimulation with *Torpedo* AcChoR. The reactivity displayed by some T_H cells with subunits other than α might be due either to cross-reactivity between different subunits, since they have a relatively high extent of sequence homology (5), or to the existence of T-cell clones specific for either β, γ, or δ subunit, since the response of polyclonal T-cell populations was studied. Experiments using clones of these cross-reacting cell lines will answer this question.

Since the human T_H cells react to native or fully denatured AcChoR with comparable efficiency, their recognizing ability does not require any tridimensional tertiary structure of the epitopes. This finding is strikingly different from the conformation dependency of antigen recognition displayed by most anti-AcChoR antibodies (10, 11, 28, 29) and confirms what was found in experimental systems such as myoglobin (33), lysozyme (34), and insulin (35) recognition by mouse T cells, where T cells elicited by immunization with a native protein frequently can react equally well when challenged with either the native or denatured forms of its antigen or its peptide fragments (reviewed in ref. 1). The molecular mechanism(s) of this cross-reactivity are not known. A possible explanation is the requirement of macrophage processing for T-cell activation and the fact that macrophages seem to present denatured antigens more effectively than native antigens (36, 37). The observation that human AcChoR-specific T cells react to denatured *Torpedo* AcChoR has important practical consequences since it indicates that it may be possible to use AcChoR fragments or synthetic peptides to further define the T-cell-stimulating determinants and, perhaps, eventually for therapeutic purposes to inactivate or delete the autoimmune T_H cells (38).

It is difficult to ascertain how closely these *in vitro*-selected T-cell populations reflect the *in vivo* repertoire of autoimmune T cells. Ideally, one would like to use human AcChoR for the selection of T cells from MG patients, but limitations in the availability of purified human receptor have made their use in bulk cultures impossible. Evidence that *Torpedo* AcChoR is a reasonable substitute for human AcChoR comes from preliminary results (not reported here) obtained using PBM from patient 2. When these unselected cells were stimulated in microculture using small amounts of purified human AcChoR, the extent of cross-reactivity between *Torpedo* and human AcChoR was high, indicating that *Torpedo* and human AcChoR share a number of T-cell-stimulating epitopes that are important *in vivo*. In comparison, antibody cross-reactivity between *Torpedo* and human AcChoR is low (ref. 30 and Table 2).

Although the MIR recognized by anti-AcChoR antibodies and the epitopes recognized by the T_H cells are both on the α subunit, the lack of inhibition exerted by "masking" the MIR epitopes with anti-MIR mAbs or with antibodies present in autologous serum indicates that the two sets of epitopes are physically separated. We cannot presently exclude that these negative results could be due to inability of the mAb to bind to processed MIR with sufficient affinity. However, in favor of the possibility that they reflect a real topographical separation between the MIR and the T-cell epitopes is the fact that they are consistent with the notion that in the human immune system, like in experimental systems (39–41), the determinants recognized by T cells may be different from those recognized by the antibodies in the same individuals. A mAb of known specificity able to reduce or inhibit the T-cell response will be a valuable tool for definition of the fine T-cell specificity because that will indicate recognition of the same determinants (39–41).

It has been convincingly proposed that the antigenic sites utilized by antigen-specific T_H cells of a particular individual can influence the specificity of the subsequent antibody response to that antigen (1, 15, 42). It is therefore likely that also in myasthenic patients the antigenic determinants recognized by the T_H cells may focus the B-cell response toward the MIR. Therefore, the explanation of the dominance of the MIR in the anti-AcChoR autoantibody response should be searched in the T-cell compartment. To answer this question will require definition of the epitopes recognized by the T cells, identification of the HLA class II restriction elements that associate with the epitope(s) recognized by the T_H cells (17, 43), and localization of the epitopes on the AcChoR structure to understand their spatial relationship with the MIR. Identification of the minimal antigenic peptide recognized by a specific T cell, together with the associated major

Table 3. AcChoR-induced T-cell proliferation (patient 1) in the presence of different anti-MIR mAbs

No AcChoR/ no mAb	AcChoR/ no mAb	AcChoR/ mAb 6	AcChoR/ mAb 25	AcChoR/ mAb 35	AcChoR/ mAb 202
1.8 ± 0.1	30.8 ± 0.5	30.3 ± 2.0	35.3 ± 3.3	34.9 ± 1.9	31.0 ± 0.9

Mitomycin C-treated PBM (2×10^5) were incubated with 25 nM soluble *Torpedo* AcChoR (or medium as a control). After 6 hr, the culture supernatants were removed, and mAbs were added to a final concentration of 500 nM (expressed in mol of ^{125}I-labeled α-Butx binding sites bound per liter of mAb solution). AcChoR-reactive autologous T cells (1×10^4) were added after 30 min. Values are expressed as [^{3}H]thymidine incorporation (cpm $\times 10^{-3}$, mean ± SD).

histocompatibility complex (MHC) molecule, may provide the means to create a physical picture of the antigen–MHC molecular complex bound by the T-cell receptor.

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