Peptide analogs to pathogenic epitopes of the human acetylcholine receptor α subunit as potential modulators of myasthenia gravis

(antigens/peptide analogs/peptide ligand/human acetylcholine receptor/immunotherapeutic strategies)

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ABSTRACT Myasthenia gravis is an autoimmune disease in which T cells specific to epitopes of the autoantigen, the human acetylcholine receptor, play a role. We identified two peptides, p195–212 and p259–271, from the α subunit of the receptor, which bound to major histocompatibility complex (MHC) class II molecules on antigen-presenting cells (APCs) from peripheral blood lymphocytes of myasthenia gravis patients and stimulated lymphocytes of >80% of the patients. We have prepared analogs of these myasthenogenic peptides and tested their ability to bind to MHC class II determinants and to interfere specifically with T-cell stimulation. We first determined relative binding efficiency of the myasthenogenic peptides and their analogs to APCs of patients. We found that single substituted analogs of p195–212 (Ala-207) and p259–271 (Lys-262) could bind to human MHC molecules on APCs as efficiently as the original peptides. Moreover, dual analogs containing the two single substituted analogs in one stretch (either sequentially, Ala-207/Lys-262, or reciprocally, Lys-262/Ala-207) could also bind to APCs of patients, including those that failed to bind one of the single substituted analogs. The single substituted analogs significantly inhibited T-cell stimulation induced by their respective myasthenogenic peptides in >95% of the patients. The dual analogs were capable of inhibiting stimulation induced by either of the peptides: They inhibited the response to p195–212 and p259–271 in >95% and >90% of the patients, respectively. Thus, the dual analogs are good candidates for inhibition of T-cell responses of myasthenia gravis patients and might have therapeutic potential.

Myasthenia gravis (MG) is an autoimmune disorder, the symptoms of which are known to be caused by autoantibody response to the nicotinic acetylcholine receptor (AChR; reviewed in ref. 1). Nevertheless, T cells play a role in the pathogenesis of MG (2, 3), and treatment of patients with anti-CD4 antibodies can ameliorate the disease (4). The higher frequency of certain histocompatibility antigens (HLA-B8, DR3) in MG patients (5) suggests a defect of immunoregulation that might be expressed at the level of antigen-major histocompatibility complex (MHC)-T-cell interactions.

Previous reports from our laboratory demonstrated that peripheral blood lymphocytes (PBLs) of 73% and 56% of MG patients proliferated in response to peptides p195–212 and p259–271, respectively. These peptides represent sequences of the human AChR α subunit (hAChRa; ref. 6). The response to these peptides was both significantly greater in MG patients than in healthy controls and correlated with HLA-DR11 and HLA-DR3, respectively, indicating that certain peptides of AChR are particularly immunogenic for certain HLA-DR types (6). Furthermore, using a technique previously developed in our laboratory (7), we have shown that the myasthenogenic peptides were bound to HLA-DR and DQ determinants on the surface of antigen presenting cells (APCs) of MG patients and that binding was correlated with the proliferative potential of their lymphocytes (8).

Several immunotherapeutic strategies, including the use of immunosuppressive therapy (e.g., corticosteroids, azathioprine, and cyclosporine), plasmapheresis and intravenous immunoglobulin have been used for treatment of MG (reviewed in ref. 1). A problem inherent in these approaches is that the therapeutic effect is nonspecific and therefore may interfere with immune responses to other antigens. A more specific approach would be to use altered peptide ligands of the myasthenogenic peptides in an attempt to preserve and even improve their MHC binding, while distorting their interaction with the T-cell antigen receptor so that they will antagonize the original antigen.

To fulfill the requirements of (i) efficient binding to MHC determinant and (ii) engagement with the myasthenogenic specific T-cell receptors (TCRs) without concomitant activation of the T cells, we applied only minor substitutions to the original peptides: Single substituted analogs of the myasthenogenic peptides p195–212 and p259–271 were synthesized and characterized in our laboratory. Using a murine model, we previously showed that the analog of p195–212 (Ala-207) and that of p259–271 (Lys-262) were able to modulate T-cell responses both in vitro and in vivo. Thus, the analogs were found to inhibit proliferative responses of cells of p195–212 and p259–271-specific T-cell lines, respectively. Furthermore, i.v. or i.p. administration of these analogs to mice primed with the respective myasthenogenic peptide was shown to inhibit proliferative responses of the lymph node cells to the immunogens (9).

In the present study, we show that the single substituted analogs Ala-207 and Lys-262, can bind to human MHC molecules as efficiently as the original peptides. Moreover, dual analogs containing the two single substituted analogs in one stretch (in either a sequential or a reciprocal order) could also bind to APCs of patients, including to those that failed to bind one of the single substituted analogs. The single substituted analogs significantly inhibited stimulation of PBLs of MG patients induced by their respective myasthenogenic peptide. Moreover, the dual analogs inhibited T-cell stimulation induced by either of the peptides.

Abbreviations: AChR, acetylcholine receptor; hAChRa, human AChR α subunit; APCs, antigen-presenting cells; MG, myasthenia gravis; PBLs, peripheral blood lymphocytes; TCR, T-cell receptor; MHC, major histocompatibility complex; IL-2, interleukin 2; SI, stimulation index; MFI, mean fluorescence intensity.

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MATERIALS AND METHODS

MG Patients. Fifty-five patients with MG (36 females, 19 males), examined at the Department of Neurology (Hadassah University Hospital, Jerusalem) participated in this study after giving informed consent. The patients' ages ranged from 21 to 72 yr and duration of the disease ranged from 1 to 38 yr. The diagnosis was based on clinical, electrodiagnostic, and pharmacological criteria (6). Fourteen patients underwent thymectomy; thymus was found in three. Fifty-seven percent of the patients received immunosuppressive medications (azathioprine and/or corticosteroids). Forty-two patients were HLA typed. They were found to have a higher frequency of HLA-B8 (26% in MG patients vs. 9% in healthy Israeli population; P < 0.001, χ² test) and HLA-DR3 (24% vs. 11%; P < 0.02), as reported for other populations of MG patients (5). In addition, the patients had a lower frequency of HLA-DR1 (2% vs. 20%; P < 0.005) than in the population of healthy controls.

Myasthenogenic Peptides and Their Analogs. The synthetic peptides p195–212 (DTPYLDITYHVFMQRPLVL) and p259–271 (VIivelipstssavdtpyl) of the hACHrε were synthesized and characterized as described (6) with an automated synthesizer (Applied Biosystems model 430A) using the company's protocol for r-butyloxy carbonyl (r-Boc) strategy.

Single amino acid substituted analogs of p195–212 (Ala-207) and p259–271 (Lys-262; ref. 9) were synthesized as described above.

Dual peptide analogs were constructed by sequential synthesis of the combined two single amino acid substituted myasthenogenic analogs starting either with the p195–212 analog (Ala-207/Lys-262, DTPYLDITYHVFMQRPLVLIV- VKLIPSSAVDTPYLDITYHVFMQRPLVL) or with the p259–271 analog (Lys-262/Ala-207, VIivelipstssavdtpyl) (reciprocal dual analog). Synthesis was performed by the solid-phase method using a multiple peptide synthesizer (AMS 422, Abimed-Technik, Germany) while protecting the side-chain groups following the company's protocols for fluor oren-9-yl-methoxycarbonyl (Fmoc) strategy.

Crude peptides were purified to homogeneity by semi-preparative HPLC on a Lichrosorb RP-8 column (7 μm; 250 × 10 mm; Merck) using a linear gradient of acetonitrile in double distilled water containing 0.1% trifluoroacetic acid. Purity of peptides was ascertained by analytical HPLC (Lichrosorb RP-18; 5 μm; 250 × 4 mm; Merck) using the above gradient and by amino acid analysis.

Proliferation Assays of PBLs. PBLs of MG patients were collected from the Ficoll medium interface following Ficoll/Hypaque density centrifugation (Pharmacia) as described (6) and cultured in 96-well microtiter plates (Becton Haemek, Israel) with various concentrations of p195–212 or p259–271 (total, 200 μl). After incubation for 6 days, 0.5 μCi of [methyl-3H]thymidine (5 Ci/mmol; 1 Ci = 37 GBq; Nuclear Research Center, Negev, Israel) was added. Sixteen hours later, cells were harvested onto filter paper and radioactivity was counted (6).

Interleukin 2 (IL-2) Production Assays. For detection of IL-2 production and secretion by the PBLs of MG patients, 50 μl of cell-free supernatants were removed, after 24 hr incubation, from each well of the cultures that were set for measuring the proliferative activity of the cells, and were pooled. The supernatants (duplicate 50-μl aliquots) were incubated with the IL-2 detector line CLTL (5 × 10⁶ cells per well) in 96-well microtiter plates for 24 hr, followed by the addition of [H]thymidine (10). Results of proliferation and IL-2 production are expressed as stimulation indices (SIs; mean cpm ratio between wells in which PBLs were incubated in the presence and absence of a myasthenogenic peptide). Mean cpm values were based on triplicate wells. SEM values did not exceed 10% of the mean.

Inhibition Studies. Single (25–100 μM) and dual (10–50 μM) analogs were added to the microtiter plates together with the myasthenogenic peptides or with the T-cell mitogen phytohemagglutinin for the whole incubation period. Inhibitory activity of the analogs on the stimulatory potential (presented as SI) of the myasthenogenic peptide was calculated according to the following formula:

\[
\text{SI} = \frac{1 - \text{inhibitor}}{1 - \text{SI without inhibitor}} \times 100.
\]

Biotinylation of Peptides. N-terminal biotinylation of peptide p195–212 and the analogs Ala-207 and Ala-207/Lys-206 was performed with excess biotin-N-hydroxysuccinimide (Sigma). N-terminal biotinylation of p259–271, Lys-262 and Lys-262/Ala-207 was performed with excess bovinelecidoproteate N-hydroxysuccinimide ester (Sigma) as described (8).

Direct Binding of Biotinylated Peptides to APCs of Human Subjects. APCs of patients with the autoimmune disease MG were collected from the Ficoll fraction following Ficoll/Hypaque density centrifugation as described (8). The cells in this fraction were mainly monocytes and granulocytes with <10% lymphocytes, as determined by direct staining with fluorescein isothiocyanate-conjugated anti-CD14, anti-CD3, and anti-B220 (Sigma), and were used as APCs in the binding experiments.

The APCs were washed twice with a solution of phosphate-buffered saline (PBS; pH 7.4) containing 0.1% bovine serum albumin [BSA; high purity grade 6; Amresco (Euclid, OH); referred to as PBS/BSA] and were incubated (1 × 10⁶ cells per sample) for 20 hr (37°C) with the biotinylated peptides or with PBS/BSA alone, followed by incubation with phycocerythin-conjugated streptavidin (Jackson ImmunoResearch), biotinylated anti-streptavidin (1:60; Vector Laboratories), and an additional incubation with phycocerythin-streptavidin, all at 4°C for 30 min. After each incubation, the cells were washed twice at 4°C with the above solution. Thereafter, the cells were analyzed by flow cytometry using the FACSsort cytometer and CELLQUEST software (Becton Dickinson). In each analysis, minimally 3000 cells were examined. Binding data are presented as mean fluorescence intensity (MFI) of the cells in the presence or absence of the biotinylated peptides and as the ratio of these values (binding index).

Calculation of K₄₅-Like Values. Reciprocals of net MFI values of the binding signals were plotted versus reciprocals of the peptide concentrations [D]. These double reciprocal conversions fit to the following equation:

\[
\frac{1}{\text{MFI}} = \frac{K_9}{\text{MaxSignal}} \times \frac{1}{[D]} + \frac{1}{\text{MaxSignal}}.
\]

Linear regression of the plots for each peptide were then calculated using the STATVIEW II software for Macintosh. K₄₅-like values were rescued from the above equation according to the parameters of each regression line.

HLA Examination. PBLs were isolated from heparinized blood samples and separated into T-cell- and B-cell-enriched fractions (8). The T and B cells were serologically tested for HLA class I (A, B, and C) and class II (DR and DQ) antigens, respectively, by the standard complement-dependent microcytotoxicity technique. In addition, DNA samples of the patients were obtained via salting out and were amplified by PCR for the second exon of DRB1 and DQB1 genes. Donor blocking and hybridization were carried out according to the 11th International Histocompatibility Workshop protocols (8). HLA-DR and DQ frequencies of the MG patients were compared to HLA frequencies of 1153 healthy Jewish bone marrow donors that were typed similarly at the Tissue Typing Unit (Hadassah University Hospital).
Statistical Analyses. Significance of differences was examined by Student’s t test, χ² test, or Fisher’s exact test. A P value of <0.05 was considered significant.

RESULTS

Stimulation of PBLs of MG Patients by Peptides p195–212 and p259–271 of the hAChRα. To test for the possible inhibitory effect of single amino acid substituted analogs of the myasthenogenic peptides p195–212 and p259–271 of the hAChRα, we first had to test the ability of PBLs of MG patients that participated in the study to respond to these T-cell epitopes. Therefore, PBLs of 55 MG patients were incubated with p195–212 or p259–271 (5–50 μM) and their proliferative responses and IL-2 secretion were determined. Patients whose PBLs responded in at least one of the assays with a SI ≥2 were defined as responders. As shown in Fig. 1, 38 patients (69%) responded to p195–212 and 31 (57%) responded to p259–271. PBLs of only 10 patients (18%) were not stimulated by either of the peptides. Thus, lymphocytes of 82% of MG patients responded to at least one myasthenogenic peptide. These percentages of responses are very similar to the results obtained in a previous study (6), in which 20/25 (80%) patients responded to either (or both) of the above peptides.

Relative Binding Efficiency of Myasthenogenic Peptides and Their Analogs. Single amino acid substituted analogs of p195–212 (Ala-207) and p259–271 (Lys-262) that were shown in our laboratory to inhibit, respectively, p195–212- and p259–271-specific responses in a mouse model (9) were used in this study. To this end, bovine was covalently attached to the N termini of the myasthenogenic peptides, single substituted analogs, and dual peptide analogs, built of the two single substituted analogs sequentially (Ala-207/Lys-262) or in the reciprocal order (Lys-262/Ala-207), and their binding to the APCs of MG patients was measured. Fig. 2 shows the extent of binding observed with each of the peptides to APCs of four representative patients. In these patients, analog Ala-207 bound to a similar or higher extent than the myasthenogenic peptide p195–212, whereas binding of analog Lys-262, although a little less efficient than that of p259–271, was not significantly different from the latter. In addition, the reciprocal dual analog Lys-262/Ala-207, showed preferential binding to APCs of these patients in comparison to the myasthenogenic peptides, p195–212 (38%; 3-fold higher binding) or p259–271 (35%; 13-fold higher) and to the Ala-207/Lys-262 dual analog (50%; 4-fold higher). Moreover, it was at least as efficient as each of the single substituted analogs, except in the case of patient B-ME.

Overall, analog Ala-207 bound APCs of MG patients at least as well as p195–212 (binding index ± SEM; 28.0 ± 7.1 and 36.5 ± 7.4 for p195–212 and Ala-207, respectively; n = 33; P = 0.12 by paired Student’s t test), and the binding of analog Lys-262 did not differ significantly from the binding of p259–271 (7.8 ± 2.4 and 6.6 ± 1.8, for p259–271 and Lys-262, respectively; n = 28; P = 0.2). In addition, the reciprocal dual analog, Lys-262/Ala-207, was a better binder than Ala-207/Lys-262 dual analog (34.1 ± 9.1 and 21.8 ± 8.4; n = 22; P < 0.05). Moreover, the dual analogs were able to bind also to APCs of patients that failed to bind one of the myasthenogenic peptides or their single substituted analogs (e.g., Lys-262/Ala-207 bound to APCs of patient N.A. with binding index = 8.1, whereas no binding signal was observed with p259–271).

We have further measured the relative binding efficiencies (Kd-like values) of the analogs to APCs of several MG patients. To this end, APCs of the patients were incubated with a log concentration range of the myasthenogenic peptides and their analogs, and the binding signals were plotted as a function of peptide concentration. Double-reciprocal conversion of the plots was performed, from which a linear regression curve was derived. Artificial equilibrium constants (Kd-like) were calculated from the regression line for each peptide and each individual was tested. Table 1 summarizes the Kd-like values of the myasthenogenic peptides and their analogs in three representative MG patients. The apparent binding affinity of Ala-207 was higher than the affinity of the myasthenogenic peptide p195–212, whereas analog Lys-262 had either a 2- to 5-fold weaker (patients N.S. and H.G.) or higher (patient R.F.) affinity than the myasthenogenic peptide p259–271 to APCs. The reciprocal dual analog Lys-262/Ala-207 had a higher affinity of binding to APCs of MG patients in comparison to the Ala-207/Lys-262 dual analog and an equal or better binding efficiency compared to the myasthenogenic peptides.

In Vitro Inhibition of p195–212 and p259–271 Induced Stimulation of PBLs of MG Patients. The analogs were then tested for their effect on PBLs of the MG patients. The single analog Ala-207 did not trigger PBLs of any of the patients, including patients that were responders to p195–212, either to proliferate or to secrete IL-2 at the concentration range tested (25–100 μM). The single analog Lys-262 (25–100 μM) and the dual reciprocal analog, Lys-262/Ala-207 (10–50 μM), stimulated PBLs of only one patient (SI = 2.3 and 2.4, respectively), whereas the Ala-207/Lys-262 dual analog (10–50 μM) stimulated PBLs of 8/44 patients tested (18%; P < 0.05 vs. Lys-262/Ala-207, Fisher’s exact test). These results suggest a significantly lower percentage in comparison to the responses observed with the myasthenogenic peptides (figure 1 and ref. 6). Thus, the single and dual analogs bind appreciably to APCs of MG patients without inducing significant stimulatory activity of their PBLs and therefore may be candidate inhibitors of the stimulatory activity induced by the myasthenogenic peptides.

We tested the ability of the two single (Ala-207 and Lys-262) and the two dual (Ala-207/Lys-262 and Lys-262/Ala-207) analogs to inhibit the proliferative responses and IL-2 secretion of PBLs of MG patients triggered by the myasthenogenic T-cell epitopes p195–212 and p259–271. Table 2 summarizes the inhibitory capacity of the single and dual analogs on p195–212-induced stimulation of PBLs of MG patients. Analog Ala-207 inhibited the stimulatory activity of p195–212 in 36 MG patients of 37 responders that were tested in inhibition studies. For example, the mean SI of responders for proliferation of PBLs to p195–212 was 3.1 and 1.5 in the absence and presence of analog Ala-207, respectively (n = 25; P < 0.0001, paired Student’s t test). The dual analogs inhibited the responses of PBLs of 34 of 35 responders tested to p195–212 (Table 2). For example, the mean SI of responders for proliferation of PBLs to p195–212 was 1.2 and 1.1 in the presence...
of Ala-207/Lys-262 and Lys-262/Ala-207, respectively (n = 24; P < 0.0001 vs. p195-212 only).

Table 3 summarizes the inhibitory capacity of the single and dual analogs on p259–271-induced stimulation of PBLs of MG patients. Analogs Lys-262 inhibited the stimulatory activity of p259–271 in 27 MG patients of 28 responders that were tested in the inhibition studies. For example, the mean SI of responders for proliferation of PBL to p259–271 was 3.2 and 1.3 in the absence and presence of analog Lys-262, respectively (n = 20; P < 0.0001). The dual analogs Ala-207/Lys-262 and Lys-262/Ala-207 inhibited the responses of PBLs of 24 and 23 of 25 responders tested to p259–271, respectively (Table 3). For example, the mean SI of responders for proliferation of PBL to p259–271 was 1.4 and 1.2 in the presence of Ala-207/Lys-262 and Lys-262/Ala-207, respectively (n = 19; P < 0.0001 vs. p259–271 only). Thus, the dual peptide analogs Ala-207/Lys-262 and Lys-262/Ala-207 could inhibit significantly the responses to both myasthenogenic peptides, p195–212 and p259–271. It is noteworthy that the latter did not inhibit significantly phytohemagglutinin-induced stimulation of the cells (data not shown), suggesting that their inhibitory activity is restricted.

**DISCUSSION**

This study demonstrates a specific approach for the treatment of myasthenia gravis based on synthetic peptides that bind to MHC class II gene products but do not activate the T cells involved in MG-related autoimmune responses. To this end, single substituted analogs of two myasthenogenic peptides that...
The MHC-bound antagonists may inhibit antigen-dependent T-cell activation by interfering with early intracellular events required for effector function, such as \( \zeta \) chain and ZAP70 phosphorylation, Ca\(^{2+} \) influx, and inositol phosphate turnover (17, 18). Alternatively, some of the TCR antagonists may transduce a differential signal upon interaction with the TCR and render the T-cell anergic (19). Moreover, TCR antagonists have been shown to block T-cell activation at concentrations 1000- to 10,000-fold less than required to competitively inhibit peptide binding to MHC class II determinants (20).

The feasibility of using TCR antagonists as modulators of autoimmune diseases was tested by some groups in experimental models: A pool of two single substituted analogs of peptide 139–151 of myelin proteolipid protein was shown to inhibit induction of experimental allergic encephalomyelitis (EAE). The TCR antagonist pool was \( ~ 10 \)-fold more potent than each analog alone (21). Alternatively, one analog with two different substitutions also was capable of blocking the induction of EAE and preventing clinical disease progression if administered at the first signs of disease (22). In both cases, a small number of analogs could block the response, even when a diverse TCR repertoire was used (21, 22). In agreement, no single peptide analog could completely inhibit all clones or primed lymph node cells of C57BL/6 mice immunized with peptide 146–162 of the Torpedo californica AChRa, suggesting that a combination of several antagonists may be required for maximal inhibition of the response (23), as demonstrated in the present paper.

Currently accepted treatments for MG involve agents that are both nonspecific and have multiple adverse side effects. Therefore, the use of synthetic peptides based on sequences of the human AChR that are aimed at inhibiting specific antigen responses without harming all other immune responses is of utmost importance. Thus, the dual analogs are good candidates for inhibition of T-cell responses of MG patients and might have therapeutic potential.

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