Muscle Responds to an Antibody Reactive with the Acetylcholine Receptor by Up-Regulating Monocyte Chemoattractant Protein 1: A Chemokine with the Potential to Influence the Severity and Course of Experimental Myasthenia Gravis

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Muscle Responds to an Antibody Reactive with the Acetylcholine Receptor by Up-Regulating Monocyte Chemoattractant Protein 1: A Chemokine with the Potential to Influence the Severity and Course of Experimental Myasthenia Gravis

Sara Reyes-Reyna, Timothy Stegall, and Keith A. Krolick

Autoantibodies with reactivity against the postjunctional muscle receptor for acetylcholine receptor are able to interfere with contractile function of skeletal muscles and cause the symptoms of myasthenia gravis (MG) in humans, as well as in experimental animal models of MG. In the study described below using a rat model of MG, it was observed that exposure to acetylcholine receptor-reactive Abs also induced increased levels of chemokine (i.e., monocyte chemoattractant protein 1) production by skeletal muscle cells. This was true of both cultured rat myocytes exposed in vitro and rat muscle exposed in vivo following passive Ab transfer. Increased monocyte chemoattractant protein 1 production may explain the increased trafficking of leukocytes through muscle following Ab transfer described in this and other reports. These observations may also be relevant to the induction of disease symptoms in experimental animal models of MG, since numerous reports from this and other laboratories indicate that the cytokine environment provided by leukocytes trafficking through muscle may play a pivotal role in disease progression. The Journal of Immunology, 2002, 169: 1579–1586.

Muscle to an Antibody Reactive with the Acetylcholine Receptor by Up-Regulating Monocyte Chemoattractant Protein 1: A Chemokine with the Potential to Influence the Severity and Course of Experimental Myasthenia Gravis

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yasthenia gravis (MG) is a neuromuscular disease caused by a T cell-dependent autoantibody response against the postjunctional muscle receptor for acetylcholine receptor (AChR). Symptoms of MG include weakness and rapid-onset fatigue. Although anti-AChR Abs appear to be the cause of neuromuscular dysfunction, direct correlations between the titers of circulating autoantibody and the severity of disease symptoms have been difficult to demonstrate (3–6). One explanation for these observations, and the hypothesis that drove the study reported below, proposes that muscle is not a passive participant in the development of neuromuscular disease symptoms in MG or experimental MG (EAMG) and, in fact, plays a very important active role by producing immunomodulating factors that can influence the eventual pathological impact of the immune system. For example, studies recently reported from this laboratory demonstrated that a cultured rat myocyte line (LE1), when exposed to the cytokine IFN-γ, produced substantial levels of the chemokine monocyte chemoattractant protein 1 (7). Since IFN-γ has been reported to exacerbate symptoms of EAMG (8–14), increased MCP-1 production, followed by increased trafficking and activation of leukocytes in skeletal muscle was considered as a potential influence on disease progression. The focus of the earlier study was in vitro myocyte activation by IFN-γ. However, the study described below was designed to assess, both in vitro and in vivo, MCP-1 production by muscle from Lewis rats following exposure to Abs reactive with the AChR. Results indicated that AChR-reactive Abs were able to cause increased production of MCP-1 both by cultured LE1 myocytes and by skeletal muscle in Lewis rats.

Materials and Methods

Rats

Inbred female Lewis rats (8–10 wk old) were purchased from Harlan Laboratories (Indianapolis, IN) and housed under the guidelines set up by the University of Texas Health Science Center, San Antonio (San Antonio, TX) Institutional Animal Care and Use Committee.

Antibodies

The following Abs were used in this study: goat anti-MCP-1 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-MCP-1 (Chemicon International, Temecula, CA), rabbit anti-goat Ig (Vector Laboratories, Burlingame, CA), and mouse mAbs against rat CD4⁺ cells (W3/25) and macrophages (ED-1) (Serotec, Oxford, U.K.).

mAb35, first described by Tzartos et al. (15), is an AChR-reactive IgG1 rat Ab and a potent inducer of symptoms of EAMG (15, 16). mAb35 was prepared in this laboratory from the culture fluids of the hybridoma obtained from the American Type Culture Collection (ATCC 175). Subclones of the mAb35 hybridoma were maintained in serum-free medium-hybrido
doma medium (Life Technologies, Grand Island, NY) and produced up to 25 μg/ml specific Ab. mAb35 was purified on hydroxyapatite columns (Sigma-Aldrich, St. Louis, MO) (17). Purity of mAb35 was assessed by SDS-PAGE and binding against AChR was assessed by ELISA (see below). Purified mAb35 was stored frozen as 1-mg/ml stock solutions in PBS. In some experiments, F(ab’₂)₃ were required. These fragments were prepared by digestion using immobilized pepsin (Pierce, Rockford, IL) at pH 4.0 following the protocol suggested by the manufacturer. Complete digestion was confirmed by PAGE.
Polyclonal serum-derived rat anti-AChR Abs against AChR were prepared as in previous studies using AChR purified from the electroplax tissue of *Toxoilon californica* (Pacific Biomarine, Venice, CA). Rats were immunized against AChR by intraperitoneal injection of 50 μg AChR emulsified in CFA, followed 1 mo later by another injection of AChR in saline. Serum concentrations of specific anti-AChR Ab generally reached levels of 300–400 μg/ml. Anti-AChR Abs were affinity purified from serum by adsorption to, and high salt elution from, Sepharose 4B columns coupled with purified AChR as in other studies (18).

α100–116 synthetic peptide

This AChR peptide represents an immunodominant T cell epitope in Lewis rats (19–21). It is composed of a 17-aa region of the α subunit of the *T. californica* AChR with the sequence [100]YAIVHMTKLLLDYTGKI[116]. Although capable of priming helper T cells that can drive disease-causing Ab responses, it does not itself stimulate the production of Ab that reacts with native AChR or the ability to cause disease (20). The peptide was prepared by Dr. R. Cook of the Protein Chemistry Core Facility at the Baylor College of Medicine (Houston, TX). Following preparation, the peptide was purified by reversed-phase fast protein liquid chromatography and a working concentration determined by mass spectroscopy.

LE1 myocyte line

As described previously (22), the Lewis rat myocyte cell line (LE1) used in the studies described below was derived from the extensor digitorum longus (EDL) muscle (found in past studies to be highly dysfunctional in EAMG-induced Lewis rats (18)). It was cloned by limited dilution culture techniques and was selected from among several clones produced based on its ease of maintenance in culture and for the stability of its phenotype. LE1 myocytes constitutively express neural cell adhesion molecule, AChR, and low levels of ICAM-1. Upon activation with appropriate cytokines, LE1 cells can be induced to express class II MHC molecules (i.e., RT1), IL-15, NO, and a group of chemokines (7, 14, 22, 23). In addition, the highly proliferative myoblasts that were initially recovered from muscle can be induced to differentiate and fuse into a more mature form, the nonproliferative multinucleated myotube. Myotube formation is accompanied by a marked increase in the expression of myosin H chain and surface AChR compared with the less mature myoblasts. It was the mature myotube form that was used in the studies described below.

Myocyte activation in vitro

LE1 cells (myotube form) were exposed for up to 72 h (appropriate times determined in pilot studies) to various doses of Abs to assess peak responsiveness. Responses measured in this study were the changes in the production of MCP-1 mRNA assessed by RT-PCR, as well as for secreted MCP-1 by ELISA.

RT-PCR for expression of MCP-1 and FcγR mRNA

As described previously (7), cellular RNA was TRIzol extracted from myocytes cultured in 24-well plates and reverse transcribed using Superscript II (Life Technologies). PCR was then performed by specific amplification of MCP-1 or FcγR cDNA sequences using primer pairs complementary to exon sequences. The housekeeping gene mRNA GAPDH provided the positive control. Those sequences are as follows: GAPDH: sense 5'-CAT TGT ATC GTG TGT GGA TCT GAC ATG C-3', antisense 5'-CCC TGT TCG TGT TCG AGC ATT TGT-3'; 254 bp (24); rat MCP-1: 5'-GTT AAT GCC CCA CCA TCC TGC TGC-3', antisense 5'-GTC ATG TCA CTI GTT GTG CCA CAA-3', 211 bp (25); rat FcγRII: sense 5'-TGG TGC ATC CAT AGC TAC AA-3', antisense 5'-TGG TGC ATC CAT AGC TAC AA-3'; 200 bp (26); rat FcγRIII: sense 5'-TGG TGC TTT GTC TGC TGC AGC ACC TGC-3', antisense 5'-GCC TGA CAC CTC AAT GTG ATT CTT CTC CCA-3', 203 bp (27); rat FcγRI: sense 5'-TGG TGC TTT GTC TGC TGC AGC ACC TGC-3', antisense 5'-GCC TGA CAC CTC AAT GTG ATT CTT CTC CCA-3', 203 bp (27). starts at 95°C for 30 min. Annealing and polymerization temperatures for the generation of PCR products were 56 and 72°C, respectively, in the presence of 1 mM Mg²⁺.

PCR products were separated on 2% agarose gels stained with ethidium bromide. Gels were scanned and data stored using an Inotech Alpha Imager 2000 (Wohlen, Switzerland) according to the manufacturer’s instructions. Data management and semiquantification on this system can be performed by comparing ratios of PCR signals found in the linear range of detection and normalized to signals coming from mRNA encoding for GAPDH. Identifications of all PCR products were based on the predicted size of the product (in relation to the primers chosen and the cDNA expected) and by direct sequencing of the product. Oligonucleotide synthesis and sequencing was performed in the Center for Advanced DNA Technologies, housed in the Department of Microbiology (University of Texas Health Science Center, San Antonio, TX), and directed by Dr. B. Wicken.

ELISAs

For Abs to AChR. Tests for serum Ab reactive with AChR were performed by ELISA using protocols similar to those previously published (18). Ninety-six-well flat-bottom plates (ICN Pharmaceuticals, Costa Mesa, CA) were coated with 50 μl of a solution containing 20 μg/ml AChR at 4°C overnight. The following morning, the AChR solution was replaced with 50 μl of 1% BSA (immunohistochemistry grade BSA; Santa Cruz Biotechnology) for 2 h at room temperature. The BSA was then replaced with serial 1/5 dilutions of serum samples placed in triplicate wells for 2 h. Plates were washed five times with PBS followed by the addition of goat anti-Ig conjugated with alkaline phosphatase. After 30 min, the plates were washed five times with PBS. Fifty microliters of p-nitrophenyl phosphate substrate (Sigma-Aldrich) was added to each well and incubated at room temperature. Absorbances were read on a Dynatech plate reader (Dynatech Laboratories, Chantilly, VA) at 10-min intervals. All assays utilized positive and negative control sera with known reactivities.

For expression of MCP-1 protein.

Verification of protein expression by cells as noted as RT-PCR positive for MCP-1 message was performed by the capture ELISA described previously (7). ELISA plates (ICN Pharmaceuticals) were coated with polyclonal rabbit Ab (Ab1) with reactivity for MCP-1 (PeproTech, Rocky Hill, NJ). The coated wells were then blocked with 1% BSA, washed, and incubated with the test samples (culture fluids of activated cells), followed by incubation with a secondary polyclonal Ab (Ab2) of goat origin, also with reactivity for MCP-1. Following another washing step, the wells were incubated with a third Ab (Ab3), biotin tagged and of rabbit origin, with reactivity for goat Ig (i.e., with reactivity against Ab2). Finally, incubation with alkaline phosphatase-coupled ExtrAvidin (Sigma-Aldrich) followed by appropriate substrate allowed the detection of MCP-1 by monitoring subsequent colorimetric changes.

Passive Ab transfer

Following a protocol optimized in previous studies of disease induction (16), transfers of either mAb35 (50 μg) or polyclonal rat Ab (100–200 μg) were performed by i.v. injection, followed at various time points by immunohistochemical determinations of MCP-1 expression by muscle. It is of note that in previous studies, peak disease intensity was observed 24–48 h following transfer of Ab-reactive Abs.

Adaptive cell transfer

Induction of adoptive anti-AChR Ab responses followed the protocol described in past studies (20, 28). Lymph node cells (LNC) obtained and pooled from AChR-immunized Lewis rats were transferred (5–10 × 10⁶ cells/recipient, i.v.) into groups of 2–4 sublethally (400 rad) irradiated syngeneic recipient rats. Injection (i.v.) of LNC recipients with AChR in saline 24 h later resulted in serum titers of anti-AChR Abs in recipient rats that were detectable 3–5 days later, leveling off by ~2–3 wk. It is of note that i.v. injection of soluble AChR (in saline) is not immunogenic in immunologically naïve irradiated rats. Thus, as described previously, anti-AChR immune responses and subsequent induction of AChR-dependent neuromuscular disease observed in adoptive recipients are due to responses transferred immune cells. Furthermore, transfer of LNC from immunized donors resulted in no production of Ab reactive with the native AChR.

Immunohistochemistry for detection of muscle-derived MCP-1

EDL muscles (found in past studies to be highly dysfunctional in EAMG-induced Lewis rats (18)) from pairs of Lewis rats were evaluated in each experiment. Fixation and staining protocols followed were those described in the report of a previous study (7). EDL muscles from each hind leg of anesthetized rats were surgically extracted, snap frozen, and stored at ~80°C. Ten-micrometer sections were cut on a Thermo Shandon SME cryotome (Pittsburgh, PA), allowed to air dry, and were fixed onto microscope slides in cold acetone (−20°C). Before incubation with primary Ab, sections were exposed to 1% nonimmune serum as a blocker. The blocking serum used corresponded to the species from which the secondary Ab originated. Thus, muscle sections were incubated with primary goat anti-MCP-1 or mouse anti-CD4 or mouse anti-macrophage Abs at predetermined optimal concentrations for 30–60 min. Following a washing step, species-appropriate biotinylated secondary Ab, supplied in avidin-biotin complex kits from Vector Laboratories, were used at optimal concentrations to probe the muscle sections for 30 min. Tests of MCP-1 production were performed in the presence of 1% saponin in order that probing Ab could more effectively

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were tested in comparison to the intact Ab. Before using pepsin-
digestion of MCP-1 in LE1 cells, F(ab')2 of the AChR-reactive Ab
reactivity of the observed up-regulation and to further assess the speci-
fic expression of the unstimulated cultures (Fig. 3). Thus, to consider the
identity of the PCR product was verified by 1) its predicted
size of slightly >200 bp; 2) its comigration with a product gener-
cated from cells known to produce this chemokine, namely, LPS-
activated Lewis rat spleen cells; and 3) direct sequence analysis. Semi-
quantifications of the inductions of MCP-1 messages were per-
formed once the conditions were determined that guaranteed that
the signals monitored were produced in the linear range of the
relationship between numbers of cycles of PCR amplification (32
cycles for MCP-1, 22 cycles for GAPDH), and the resulting signal
intensity from bands of ethidium bromide-stained cDNA.

Although a low level of MCP-1 mRNA was often detectable
before activation, following exposure of LE1 myocytes to either
polyclonal (Fig. 1) or monoclonal (Fig. 2) rat anti-AChR Abs, a
substantially increased expression of mRNA encoding sequences
for MCP-1 was observed. In multiple experiments, increased lev-
els of MCP-1 mRNA were observed within 6–12 h that were de-
pendent on the dose of Ab used, and became less pronounced at later
point time points. Furthermore, in conjunction with the above stud-
ies of mRNA expression, culture fluids from mAb35-activated
LE1 myotubes were assayed by ELISA for the presence of se-
creted MCP-1. As shown in Table I, rapidly increased levels of
MCP-1 protein were detected in culture fluids from mAb35-activat-
ed LE1 cells that reflected the increased levels of MCP-1-encoding sequences of
mRNA extracted from LE1 cells.

**Optimal production of MCP-1 by LE1 cells stimulated by exposure to polyclonal anti-AChR Ab may depend on a myocyte FcyR.**

During the execution of the experiments presented in the previous
section, it was noted that the “negative” control stimulus, nonim-
mune rat Ig, consistently induced MCP-1 mRNA that exceeded
that of the unstimulated cultures (Fig. 3). Thus, to consider the
characteristic of nonimmune rat Ig that was providing such a stim-
ulus and to further assess the specificity of the observed up-regu-
ulation of MCP-1 in LE1 cells, F(ab')2 of the AChR-reactive Ab
were tested in comparison to the intact Ab. Before using pepsin-
digested Ab as a stimulus, retention of its Ag-binding activity was
confirmed, and adjustment of molar concentrations were made so
that concentrations and binding activity of the digested Ab were
comparable to those of the intact Ab. It was observed in several
experiments that the F(ab')2 Ab provided a less effective stimulus
of MCP-1 up-regulation in LE1 cells than was provided by the
intact Ab (Fig. 3). That is, when compared with fold inductions of
MCP-1 message using undigested Ab, fold inductions using the
pepsin-digested Ab were reduced by ~30–70%. Furthermore, as

![FIGURE 1.](Image 362x127 to 483x308) Polyclonal Lewis rat anti-AChR Ab stimulates the up-regula-
tion of mRNA encoding for MCP-1 in both LE1 myoblasts (upper panel) and myotubes (lower panel). mRNA for MCP-1 and for GAPDH
were detected by RT-PCR of extracted total RNA from the cultured myo-
cytes. RT-PCR products were generated from unactivated or Ab-activated
myocytes and were visualized in the lanes marked as − or +, respectively. Myocyte activation involved 12- and 72-h exposures (as indicated) to 10
µg/ml affinity-purified anti-AChR Ab. RT-PCR products were also eval-
uated that were derived from RNA extracted from activated spleen cells
(shown in lane C). All PCR products were visualized by ethidium bromide
staining.

![FIGURE 2.](Image 374x560 to 471x734) Monoclonal Lewis rat anti-AChR Ab (mAb35) stimulates the production of MCP-1 mRNA by LE1 myocytes. Myocytes were incu-
bated with 25 µg/ml mAb35 or control Ab for 24 h. Upper panel, Ethidium
bromide-stained gel of RT-PCR products using primers specific for MCP-1
and GAPDH. Lower panel, Semiquantification of mRNA increases were
performed as a function of mAb35 dose. Following RT-PCR and agarose
gel electrophoresis of PCR products, bands were scanned for ethidium
bromide staining intensity. Ratios of MCP-1 message to GAPDH message
were determined as described in Materials and Methods and in Ref. 7.
Table I. MCP-1 production by LE1 myotubes stimulated with mAb35

<table>
<thead>
<tr>
<th>Hours of Stimulation</th>
<th>+/- mAb35*</th>
<th>MCP-1 in Culture Fluids** (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>-</td>
<td>Undetectable</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.53 ± 0.07</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td>Undetectable</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2.32 ± 0.04</td>
</tr>
<tr>
<td>24</td>
<td>-</td>
<td>Undetectable</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2.4 ± 0.65</td>
</tr>
<tr>
<td>48</td>
<td>-</td>
<td>Undetectable</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.77 ± 0.58</td>
</tr>
</tbody>
</table>

*LE1 myotubes were incubated with 25 μg/ml Ab for the times indicated.
**MCP-1 levels were determined from triplicate cultures by ELISA as described in Materials and Methods. Readings of OD resulting from the testing of fresh culture medium mixed with known quantities of pure MCP-1 (7).

shown in Fig. 3, MCP-1 induction appeared to occur with delayed kinetics. These results suggest that the Fc portion of the Ab may influence the effectiveness of inducing chemokine production by the AChR-reactive Ab. Interestingly, when tested for the possibility that an FcR was involved in this phenomenon, it was observed that LE1 cells constitutively produce mRNA for rat FcγRIII (Fig. 4). No FcγRIII mRNA was detected.

Increased production of MCP-1 can be detected in vivo in the muscles of Lewis rats following the administration of AChR-reactive Abs

The question was asked whether Ab with reactivity against the AChR could, in the absence of an active immune response, stimulate muscle to produce MCP-1. Therefore, both polyclonal rat anti-AChR Abs, as well as AChR-reactive mAb35, were transferred i.v. into Lewis rats. Immunohistochemical staining (example shown in Fig. 5, A–C) demonstrated that transfer of AChR-reactive polyclonal Abs, but not Abs reactive with an irrelevant Ag such as keyhole limpet hemocyanin (KLH), resulted in increased production of MCP-1 in the EDL muscle (summarized in Table II). Ab probing for the presence of MCP-1 indicated that >70% of muscle sections examined, coming from rats that received AChR-reactive Ab 6 h earlier, were transiently positive for this chemokine. By 24 h, the fraction of positively stained sections had returned to baseline.

When a similar study was performed using mAb35, similar immunohistochemical staining results were obtained (example shown in Fig. 5, D and E). Thus, as summarized in Fig. 6, upper panel, a rapid increase in the frequency of MCP-1+ muscle sections of ~3-fold over constitutive levels was observed following administration of mAb35, but not following the administration of an irrelevant isotype-matched control mAb. Frequencies of MCP-1+ sections increased with the same rapid kinetics as was observed for the polyclonal anti-AChR Ab (Fig. 6, lower panel); however, the return to pretransfer levels appeared to occur more slowly following mAb35 administration.

Finally, in experiments in which rats that received the F(ab')2 form of mAb35, increased expression of MCP-1 was noted (example shown in Fig. 5E). However, the frequency of muscle sections that demonstrated MCP-1 production tended (no statistical significance could be demonstrated) to be lower than those obtained following the transfer of undigested mAb35, and the effects did not last as long (Fig. 7).

Transfer of mAb35 resulted in increased trafficking of leukocytes into muscle

The significance of chemokine production by muscle in EAMG-induced rats may be found in the ability to recruit leukocytes. Therefore, frequencies of macrophages and T lymphocytes were determined following administration of mAb35. Immunohistochemical staining for cells that express the rat monocyte/macrophage marker, ED1, as well as for T cells bearing the markers CD4 and CD8, revealed that rapid influxes of significant, although not dense, infiltrates could be found in EDL muscles following transfer of mAb35 Ab. As summarized in Fig. 8, within 6 h following mAb35 transfer, there were signs of increasing numbers of both macrophages and CD4+ T cells (reflected by the increased proportion of muscle sections in which leukocytes were observed). During the next 24 h or so, additional increases were observed reaching ~10-fold over the usual 2% of sections demonstrated by muscles from either untransferred rats or rats transferred with non-immune Ig. In all groups examined, the infiltrating cells detected were either macrophages or CD4+ T cells; neither CD8+ T cells nor NK cells were detected.
Increased production of muscle MCP-1 was observed following adoptive transfer of AChR-immunized LNC.

To obtain results under conditions that more closely resembled active immunization (the most effective way to induce symptoms of EAMG), while avoiding the complications of nonspecific activating effects of CFA, Lewis rat LNC from AChR-immunized donors were transferred and Ag challenged in immunologically naive recipient rats. EDL muscles from LNC recipients were then examined for increased production of MCP-1. Thus, rats that received LNC from AChR-immunized donors were challenged with AChR (i.v.) as in previously reported studies of EAMG (20, 21, 29). Approximately 3 days following Ag challenge, just as titers of AChR-reactive Abs became detectable, EDL muscles of LNC recipients showed increased MCP-1 expression. Maximum MCP-1 expression was observed at ~7 days after Ag challenge (Table III).

To further assess the importance of Ab production on the induction of MCP-1 following LNC transfer, LNC, obtained from donor rats immunized with the α100–116 synthetic peptide, were transferred into immunologically naive recipient rats, which were then challenged with the same peptide. As described in a past report (20), activation of LNC with reactivity against this immunodominant T cell epitope results in prominent helper activity, but

<table>
<thead>
<tr>
<th>Hours Postchallenge</th>
<th>Ab&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MCP-1 Fraction of Total Sections&lt;sup&gt;b&lt;/sup&gt; (%)</th>
<th>$\chi^2$</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
<td>37/145 (25.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Anti-AChR</td>
<td>59/80 (73.8)</td>
<td>31.6</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>6</td>
<td>Anti-KLH</td>
<td>37/116 (31.9)</td>
<td>1.28</td>
<td>NS</td>
</tr>
<tr>
<td>24</td>
<td>Anti-AChR</td>
<td>22/137 (16.0)</td>
<td>5.64</td>
<td>NS</td>
</tr>
<tr>
<td>24</td>
<td>Anti-KLH</td>
<td>24/104 (23.1)</td>
<td>0.25</td>
<td>NS</td>
</tr>
<tr>
<td>48</td>
<td>Anti-AChR</td>
<td>27/153 (17.6)</td>
<td>3.55</td>
<td>NS</td>
</tr>
<tr>
<td>48</td>
<td>Anti-KLH</td>
<td>32/183 (17.5)</td>
<td>3.66</td>
<td>NS</td>
</tr>
</tbody>
</table>

<sup>a</sup> One hundred micrograms of affinity-purified Lewis rat Ab, reactive with either AChR or KLH as indicated, was transferred i.v. into pairs of Lewis rats. Following the number of hours indicated after transfer, EDL muscles were harvested for sectioning and staining for MCP-1. Results from one of two experiments are shown.

<sup>b</sup> Values are shown for numbers of sections scoring positive for MCP-1 over the total number of sections evaluated. The percent positive is shown in parentheses.

<sup>c</sup> Values of $\chi^2 \geq 5.99$ represent ≥95% confidence level (2 df) with regard to a significant difference from muscles of nontransferred rats.

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**FIGURE 5.** Immunohistochemical staining demonstrates that injection of AChR-reactive Abs results in increased MCP-1 production in the EDL muscles of Lewis rats. EDL, muscle sections were obtained either from rats following transfer of AChR-reactive polyclonal rat Ab (A–C) or monoclonal (mAb35) Ab (D–F). Determinations of muscle MCP-1 were performed 6 h after Ab transfer by immunohistochemistry using primary goat Abs followed by biotinylated rabbit anti-goat Ig and HRP-conjugated streptavidin as described in Materials and Methods (original magnification, ×600). A. Muscle from a recipient of polyclonal anti-AChR Ab was stained using a goat anti-MCP-1 Ab; note the brown MCP-1 staining compared with B. B. Muscle from a recipient of anti-KLH Ab was stained using a goat anti-MCP-1 Ab; only the blue counterstaining is observed. C. Muscle from a recipient of anti-AChR Ab was stained using a nonimmune goat Ig control Ab. D. Muscle from a recipient of mAb35 Ab was stained using a goat anti-MCP-1 Ab. E. Muscle from a recipient of mAb35 F(ab’)2 Ab was stained using a goat anti-MCP-1 Ab. F. Muscle from a recipient of H-ras control Ab was stained using a goat anti-MCP-1 Ab.

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**FIGURE 6.** Transfer into Lewis rats of either polyclonal or monoclonal AChR-reactive Abs results in increased frequencies of EDL muscle sections positive for MCP-1 production. Following the number of hours indicated after transfer of 50 μg of mAb35 or control Abs (upper panel) or 100 μg of affinity-purified rat anti-AChR serum Abs and control Abs (lower panel), EDL muscles were harvested for sectioning and staining for MCP-1<sup>+</sup> muscle sections. Statistical analyses were performed comparing the staining of muscles from untransferred vs Ab-transferred rats. When compared with frequencies of MCP-1<sup>+</sup> muscle sections for a group of rats before transfer of any Ab (dashed horizontal lines in each panel), bars labeled with an asterisk indicate frequencies of muscle sections from Ab-transferred rats with $\chi^2$ values of >5.99 (∼95% confidence level (2 df)) and p values of <0.05.
no Ab with reactivity against the native AChR unless in the presence of B cells primed against the native AChR. As shown in Fig. 9, α100–116 peptide challenge of recipients that had received LNC from donors immunized with the α100–116 peptide resulted neither in the production of Ab with reactivity against the native AChR nor in increased MCP-1 production associated with the EDL muscles. This contrasts with the result observed in previous studies in which it was observed that IL-15 is produced following the transfer of peptide-reactive T cells (29). IL-15 was, as predicted, detected in the same muscles that scored negative for MCP-1 production (data not shown). AChR-reactive Ab was also detected in recipients of AChR-primed LNC following peptide challenge, although with substantially lower titers than in those recipients challenged with the native Ag. Nonetheless, recipients challenged with the α100–116 synthetic peptide also demonstrated increased production of muscle-derived MCP-1. Overall, there was a clear association between increased MCP-1 production and the presence of detectable levels of serum Ab reactive with the AChR.

### Discussion

EAMG, like human MG, is an autoantibody-mediated neuromuscular disease that is not generally characterized as being associated with heavy muscle infiltrates of macrophages, neutrophils, or the Th1 subset of CD4+ T cells. However, it has been noted by a number of investigators that various cytokines, some such as IFN-γ derived from inflammatory subsets of cells (8–13), are apparently able to influence the course of the disease. Studies performed in our laboratory have considered the possibility that cytokines, IFN-γ for one, may play important disease-determining roles that go beyond that of immune system modulators. That is, the cellular targets of IFN-γ effects may not be restricted to cells in the immune system and may include, e.g., skeletal muscle itself. Consistent with this possibility, and of interest to the EAMG model under

#### Table III. Production of MCP-1 by skeletal muscle 7 days following adoptive transfer of AChR-reactive LNC

<table>
<thead>
<tr>
<th>Priming Ag/Challenge Ag</th>
<th>Fraction of MCP-1+ Muscle Sections (%)</th>
<th>χ²</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>No transfer</td>
<td>78/305 (25.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AChR/AChR</td>
<td>123/132 (93.2)</td>
<td>179.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>AChR/PBS</td>
<td>19/90 (21.1)</td>
<td>0.76</td>
<td>NS</td>
</tr>
<tr>
<td>OVA/OVA</td>
<td>34/172 (19.8)</td>
<td>0.83</td>
<td>NS</td>
</tr>
</tbody>
</table>

a A total of 10^7 LNC were transferred into immunologically naive recipient rats. After 7 days, EDL muscles were obtained, sectioned, and evaluated for numbers of sections staining positive for MCP-1. Results from one of two experiments are shown.
b Priming Ag = Ag used to immunize donor rats. Challenge Ag = Ag used to immunize recipient rats.
c Muscle sections were stained for the detection of MCP-1. Values are shown for numbers of sections scoring positive over the total number of sections evaluated. The percent positive is shown in parentheses.
d Values of χ² ≥ 5.99 represent ≥95% confidence level (2 df) with regard to a significant difference from muscles of nontransferred rats.

#### FIGURE 7. F(ab’)2 of mAb35 are less effective at stimulating the production of MCP-1 protein by muscle following transfer into Lewis rats. Following the number of hours indicated after transfer of 50 μg of mAb35 (■) or the F(ab’)2 of mAb35 (□), EDL muscles were harvested for sectioning and staining for MCP-1+ muscle sections. Statistical analyses were performed comparing the staining of muscles from rats receiving mAb35 or the F(ab’)2 of mAb35 compared with frequencies of MCP-1+ muscle sections for a group of rats before transfer of any Ab (dashed horizontal line in each panel). Bars labeled with an asterisk indicate frequencies of muscle sections from Ab-transferred rats with χ² values of >5.99 (>95% confidence level (2 df) and p values of <0.05).

#### FIGURE 8. Both macrophages and T cells are recruited to skeletal muscle in rats following transfer of mAb35 Ab. Following the number of hours indicated after transfer of 50 μg of mAb35, EDL muscles were harvested for sectioning and staining for percent CD4+ muscle sections (W3/25+ cells, ■) or percent macrophage+ muscle sections (ED1+ cells, ●). As indicated, each of the analyses yielded results by 48 h posttransfer with p values of <0.001.

#### FIGURE 9. Increased MCP-1 production is observed in rats that received LNC from donor rats previously immunized with AChR. A total of 10^7 LNC was transferred from donor rats into pairs of immunologically naive recipient rats. The donor rats were immunized (primed), and the recipients challenged, with the Ags indicated. After the number of days indicated, EDL muscles were obtained from the LNC recipients, sectioned, and evaluated for numbers of sections staining positive for MCP-1. Values shown indicate the percents of sections staining positive. When compared with the percent of MCP-1+ muscle sections for a group of rats before transfer of any Ab (dashed horizontal line), bars labeled with an asterisk indicate percents of muscle sections from cell-transferred rats with χ² values of >5.99 (>95% confidence level (2 df) and p values of <0.05. Results from one of two experiments are shown.
study here, various IFN-γ-induced, immunologically relevant surface
and secreted products of cultured skeletal muscle cells have been studied
in this and other laboratories (7, 14, 22, 23, 30–36).

To promote the effective exchange of immunologically important
factors (such as cytokines) between leukocytes and skeletal muscle,
leukocytes might have to traffic through the muscle to deliver and/or pick up various signals. Thus, the present study
focused on the production of one particular muscle-derived leuko-
cyte chemoattractant (i.e., MCP-1). MCP-1 was chosen as the focus
of this investigation because previously published results (7) from this laboratory indicated that although a number mRNAs for
other chemokines can be induced in the LE1 muscle cell line (e.g.,
RANTES and IFN-γ-inducible protein 10), MCP-1 mRNA ap-
ppeared to be most prominent at all developmental stages displayed
by the cell line. Additional studies are in progress intended to
follow-up. Moreover, the goal of the study described above, in contrast to the previous investigation that was concerned primarily
with the activation of myocytes by cytokines, was to determine the
ability of AChR-reactive Ab to stimulate myocytes to produce im-
munologically relevant factors. More specifically, it was our con-
tention that, although weakness and rapid fatigue characteristic of
MG are generally attributed to the direct effects of anti-AChR Abs
on AChR function, it was of importance to ask whether AChR-
reactive Abs may also have other less direct immunopathological
influences on disease progression and severity such as the ability to
stimulate the production of the leukocyte-recruiting chemokine
MCP-1. AChR-reactive Abs were therefore tested, in the absence
of an active immunization, as a stimulus of MCP-1 up-regulation
in vitro using LE1 myocytes, as well as in vivo following their i.v.
administration to rats. Furthermore, although mAb35 is an easily
obtainable Ab that demonstrates very consistent disease-inducing
activities, making it suitable for passive transfer studies, poly-
clonal serum Abs obtained from AChR-immunized rats were also
tested in this system because they contain multiple AChR specific-
ities that more closely represent the Abs that participate in the
actual disease (such as those produced during active immunization
studies). The comparison between the monoclonal and polyclonal
sources of AChR-reactive Abs allows insights into the likely
pathological relevance of the single-specificity mAb (raised by
specificity-biased hybridoma techniques).

In vitro, mAb35 was clearly able to stimulate increased produc-
tion of both MCP-1-associated mRNA and, ultimately the secreted
MCP-1 protein itself. The effect appeared to have a nonspecific
component associated with it, in that nonimmune rat Ig could also
demonstrate the ability to increase the production of MCP-1 in
LE1 cells. Although evidence is circumstantial, some of this effect
may be due to interactions with the cell through an FcγR, since 1)
proteolytic removal of the Fc of the AChR Ab decreased its ability
to activate the myocytes into MCP-1 production, and 2) a mRNA
that encodes for the rat FcγRIII (but not FcγRII) was found to be
constitutively expressed by LE1 cells.

In vivo, up-regulated MCP-1 production by skeletal muscle was
noted in association with the presence of circulating Abs reactive
with the AChR. This was observed following the passive transfer
of either polyclonal or monoclonal AChR-reactive Abs, as well as
following the active production of AChR-reactive Ab by adop-
tively transferred lymphocytes obtained from AChR-immunized
rats. In all cases, exposure of muscle to control Abs of irrelevant
binding specificities did not cause the inductive effect.

It is important to note that in the studies described above, rats
were exposed to circulating AChR-reactive Abs, but in the absence
of immunizations requiring inflammatory adjuvants. This may be
an important variable in light of the results reported by Li et al.
(37) that lead to the conclusion that MCP-1 production is not a
factor in the progression of EAMG in Lewis rats. This may indi-
cate that rats immunized in the presence of adjuvants may dem-
onstrate a variety of different activities than would a rat given a
soluble dose of AChR-reactive Ab (or even Ab produced by LNC
that are activated in the absence of adjuvant, such as in the adop-
tive transfer study described above). Direct and/or indirect adju-
vant influences have the potential for up-regulating or down-reg-
ulating the activities of a variety of cell types, with the net result
being an influence over the activities of the muscle itself.

With regard to the Ab transfer studies, removal of the Fc region
of the transferred Abs did not eliminate the ability to induce in-
creased MCP-1 production, although the efficiency of the induc-
tion appeared to be somewhat compromised. Speculating, perhaps
the binding of AChR by Ab provides a signal leading to up-regu-
lated MCP-1 production, but that once bound to muscle the Ab is
capable of binding to a second muscle membrane molecule (e.g.,
an FcR). This secondary binding may provide an additional signal
that results in more effective triggering of MCP-1 production.
On the other hand, the altered response (decreased only slightly) re-
sulting from the elimination of the Fc region of the Ab might have
occurred due to altered Fc-dependent patterns of circulation. There
is no direct evidence for these explanations at this time.

With regard to the adoptive transfer studies, evidence was pro-
vided pointing to the AChR-reactive Ab as the principle stimulus
of muscle MCP-1 production and not other non-Ab activities asso-
ciated with the immune response (i.e., cytokine-producing cells).
That is, transfer into immunologically naive rats of LNC respon-
sive to the AChR peptide known to activate AChR-reactive T cells
(i.e., the immunodominant α100–116 T cell epitope), followed by
challenge with the same peptide, resulted in no observed increases
in muscle MCP-1 production. No Ab reactive with the native
AChR was detected in these rats. Thus, cytokines provided by
transferred T cells were not sufficient in themselves to stimulate
muscle to produce MCP-1. This contrasts with previous findings
in which transfer and trafficking of T cells appeared to result in the
induction of IL-15 by muscle (29). Transfer of α100–116-reactive
LNC in the present study, in fact, did result in the predicted up-
regulation of IL-15.

The conclusion drawn from these two scenarios was that AChR-
reactive Ab alone can mediate an increased production of MCP-1
by skeletal muscle. This conclusion includes the corollary that the
presence of cytokine-producing T cells cannot themselves induce
MCP-1 production. This result appears to contradict predictions
derived from earlier in vitro results (7) in which the LE1 myocyte
line could be triggered to produce MCP-1 by IFN-γ. This observa-
tion may only point to the dangers of attempting to translate in
vitro results directly into in vivo predictions. However, the inability
to demonstrate increased MCP-1 production in EDL muscles
containing trafficking leukocytes may have a kinetics explanation.
That is, it may also be noteworthy that previous studies indicated
that IL-4 is able to stimulate the production of the IFN-γ-inducing
cytokine IL-15 in LE1 cells (14), that some of the leukocytes that
move through skeletal muscle of EAMG-induced rats are IL-4 pro-
ducers (29), and that anti-AChR Ab, as well as purified α100–
116-reactive T cells, are both able to induce IL-15 production by
muscle in vivo (29). Thus, one might speculate that the full effects
of potential IFN-γ producers recruited into muscle due to MCP-1
production may only be felt once trafficking T cells were activated
into IFN-γ production by IL-15.

Therefore, the overall conclusion of this study, and other studies
from this laboratory (7, 14, 22, 23, 29), is that muscle is likely not
a passive participant in the development of disease symptoms in
EAMG and, in fact, may play a very important active role by
producing immunomodulating factors. Although not directly
proven at this time, various muscle-derived factors (e.g., muscle-derived cytokines, chemokines and/or cell membrane molecules) may influence the eventual immunopathological impact of the immune system on muscle that can further influence the immune response against the muscle.

Acknowledgments

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References