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

*J Immunol* 2002; 169:1579-1586; doi: 10.4049/jimmunol.169.3.1579

http://www.jimmunol.org/content/169/3/1579
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.

Myasthenia gravis (MG) is a neuromuscular disease caused by a T cell-dependent autoimmune 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. 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. Since IFN-γ has been reported to exacerbate symptoms of EAMG, 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-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-hybridoma 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 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.

Copyright © 2002 by The American Association of Immunologists, Inc.
Polyclonal serum-derived rat anti-AChR Abs against AChR were prepared as in previous studies using AChR purified from the electroplax tissue of Torpedo californica (Pacific Biomorphic, Venice, CA). Rats were immunized against AChR by initial i.c. 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 limiting 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 FcyR 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 FcyR cDNA sequences using primer pairs complementary to exonic sequences. The housekeeping gene mRNA GAPDH provided the positive control. Those sequences are as follows: GAPDH: sense 5′-CAT TGT ATC CGT TGT GGA TCT GAT CTT C-3′, antisense 5′-CCC TGT TGC TGT AGC ATT GTT GCC CCA CTC ACC TGC TGC-3′; antisense 5′-CTG ATC CTT GTT GGT CCA-3′, antisense 5′-CCG TGA CAC CTC AAT GTG ATT CTT TCT C-3′; sense 5′-GCC AAC CCA CTC ACC TGC TGC-3′, antisense 5′-CTG ATC CTT GTT GGT CCA-3′, antisense 5′-CTG ATC CTT GTT GGT CCA-3′, antisense 5′-CTG ATC CTT GTT GGT CCA-3′, antisense 5′-CTG ATC CTT GTT GGT CCA-3′, antisense 5′-CTG ATC CTT GTT GGT CCA-3′, antisense 5′-CTG ATC CTT GTT GGT CCA-3′, antisense 5′-CTG ATC CTT GTT GGT CCA-3′, antisense 5′-CTG ATC CTT GTT GGT CCA-3′, antisense 5′-CTG ATC CTT GTT GGT CCA-3′, antisense 5′-CTG ATC CTT GTT GGT CCA-3′, antisense 5′-CTG ATC CTT GTT GGT CCA-3′, antisense 5′-CTG ATC CTT GTT GGT CCA-3′, antisense 5′-CTG ATC CTT GTT GGT CCA-3′, antisense 5′-CTG ATC CTT GTT GGT CCA-3′, antisense 5′-CTG ATC CTT GTT GGT CCA-3′, antisense 5′-CTG ATC CTT GTT GGT CCA-3′, antisense 5′-CTG ATC CTT GTT GGT CCA-3′, antisense 5′-CTG ATC CTT GTT GGT CCA-3′, antisense 5′-CTG ATC CTT GTT GGT CCA-3′, antisense 5′-CTG ATC CTT GTT GGT CCA-3′. 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. Identification of all PCR products were based on both 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. Wickes.

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-rat 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 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 5% BSA, washed, and incubated with the test samples (culture fluids of activated cells), followed by incubation with a second 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 AChR-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 3–7 days later, leveling off by 2–3 wk. It is of note that single i.v. injection of soluble AChR (in saline) is not immunogenic in immunologically naive 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 by 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 cryostome (Pittsburgh, PA), allowed to air dry, and were fixed onto microscope slides in cold acetone. Before incubation with primary Abs, 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 Abs, 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
reach its cytoplasmic target. Next, washed sections were incubated for 30 min with avidin-biotin complexes conjugated with HRP. Specimens, counterbound with HRP-tagged ExtrAvidin (Sigma-Aldrich), were exposed to the diaminobenzidine substrate, and counterstained with Gill's hematoxylin (Fisher Scientific, Pittsburgh, PA). Tissue sections were evaluated by light microscopy for the presence of the brown precipitate characteristic of positive staining. Representative stained muscle sections were then photographed. Adjacent tissue sections were evaluated for nonspecific staining using nonimmune species-appropriate primary Abs. Of note, no endogenous peroxidase was observed in the muscle specimens and therefore created no background staining difficulties.

**Statistical analyses**

Statistical comparisons of frequencies of positively stained muscle sections obtained from Ab-transferred rats vs rats receiving no Ab were performed by \( \chi^2 \) analyses and Student’s \( t \) tests.

**Results**

Previous in vitro investigations have indicated that Lewis rat myocytes have the potential to produce, among other things, a family of chemokines in response to certain cytokines (7). The results reported below were obtained from experiments designed to determine 1) whether increased production of MCP-1 by myocytes could also be induced in vitro in response to Ab reactive with the AChR, and 2) the relative role played by anti-AChR Ab vs cytokine-producing cells with regard to MCP-1 expression by muscle in vivo.

**Anti-AChR Ab is able to stimulate increased in vitro production of MCP-1 in LE1 myocytes**

Levels of MCP-1-encoding mRNA were monitored by RT-PCR. The identity of the PCR product was verified by 1) its predicted size of slightly >200 bp; 2) its comigration with a product generated 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 performed 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 levels of MCP-1 mRNA were observed within 6–12 h that were dependent on the dose of Ab used, and became less pronounced at later time points. Furthermore, in conjunction with the above studies of mRNA expression, culture fluids from mAb35-activated LE1 myotubes were assayed by ELISA for the presence of secreted MCP-1. As shown in Table I, rapidly increased levels of MCP-1 protein were detected in culture fluids of 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 FcγR**

During the execution of the experiments presented in the previous section, it was noted that the “negative” control stimulus, nonimmune 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 stimulus and to further assess the specificity of the observed up-regulation 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](image1.png)  
**FIGURE 1.** Polyclonal Lewis rat anti-AChR Ab stimulates the up-regulation 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 myocytes. 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 \( \mu \)g/ml affinity-purified anti-AChR Ab. RT-PCR products were also evaluated 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](image2.png)  
**FIGURE 2.** Monoclonal Lewis rat anti-AChR Ab (mAb35) stimulates the production of MCP-1 mRNA by LE1 myocytes. Myocytes were incubated with 25 \( \mu \)g/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 diluted culture fluids were converted to concentration (nanograms per milliliter) by comparison with standard curves generated 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 Ab. 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’)** 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, 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 nonimmune 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>Table II. Production of MCP-1 by skeletal muscle of Lewis rats following passive transfer of Abs reactive with either AChR or KLH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hours Postchallenge</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>24</td>
</tr>
<tr>
<td>24</td>
</tr>
<tr>
<td>48</td>
</tr>
<tr>
<td>48</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 χ<sup>2</sup> ≥5.99 represent ≥95% confidence level (2 df) with regard to a significant difference from muscles of nontransferred rats.
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 results 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

![FIGURE 7.](image-url)

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 lines 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.

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 total of 10⁷ 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.

FIGURE 9. Increased MCP-1 production is observed in rats that received LNC from donor rats previously immunized with AChR. A total of 10⁷ LNC were 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 scoring 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.

![FIGURE 8.](image-url)

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+ sections (ED1+ cells, ○). As indicated, each of the analyses yielded results by 48 h posttransfer with p values of <0.001.
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, leukocyte 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 leukocyte 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 appeared 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 immunologically relevant factors. More specifically, it was our contention 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, polyclonal serum Abs obtained from AChR-immunized rats were also tested in this system because they contain multiple AChR specificities 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 production 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 adoptively 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 indicate that rats immunized in the presence of adjuvants may demonstrate 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 adoptive transfer study described above). Direct and/or indirect adjuvant influences have the potential for up-regulating or down-regulating 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 increased MCP-1 production, although the efficiency of the induction appeared to be somewhat compromised. Speculating, perhaps the binding of AChR by Ab provides a signal leading to up-regulated 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) resulting 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 provided pointing to the AChR-reactive Ab as the principle stimulus of muscle MCP-1 production and not other non-Ab activities associated with the immune response (i.e., cytokine-producing cells). That is, transfer into immunologically naive rats of LNC responsive 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 observation 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 producers (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
We thank Irma Gonzales for her technical support and Dr. Y. Mulhern for many helpful discussions.

References