effective to ameliorate ALS pathogenesis induced by the mutant SOD1 protein.

Although the precise pathologic role of $O_2^{\bullet-}$ in motor neuron degeneration remains to be fully clarified, the present study is consistent with the possible involvement of nonneuronal cells in mitochondrial-derived, superoxide-induced injury in motor neurons. Thus, a rational therapeutic strategy that delivers antioxidants to surrounding astrocytes or microglia may significantly help motor neurons survive oxidative stress.

Acknowledgments

The authors thank Drs. K. Amano and K. Yamakawa (RIKEN Brain Science Institute) for FISH analysis of VAChT-Cre.Slow mice; Drs. K. Endo and M. Ichikawa (Tokyo Metropolitan Institute for Neuroscience; TMIN) for valuable advice on electron microscopy; Drs. K. Kohyama, Y. Matsumoto, and T. Uchihara (TMIN) for muscle biopsy; and Dr. S. E. Craven for critical reading of the manuscript. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and from the Japanese Ministry of Health, Labor and Welfare, Research on Psychiatric and Neurological Diseases and Mental Health.

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Life Sciences 80 (2007) 2206–2209
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Ubiquitous expression of acetylcholine and its biological functions in life forms without nervous systems

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Received 26 September 2006; accepted 26 January 2007

Abstract

Using a radioimmunoassay (RIA) with high specificity and sensitivity (1 pg/tube) for acetylcholine (ACh), we have been able to measure the ACh content in samples from the bacteria, archaea and eucarya domains of the universal phylogenetic tree. We found detectable levels of ACh to be ubiquitous in bacteria (e.g., *Bacillus subtilis*), archaea (e.g., *Thermococcus kodakaraensis* KOD1), fungi (e.g., shiitake mushroom and yeast), plants (e.g., bamboo shoot and fern) and animals (e.g., bloodworm and lugworm). The levels varied considerably, however, with the highest ACh content detected in the top portion of bamboo shoot (2.9 μmol/g), which contained about 80 times that found in rat brain. In addition, using the method of Fonnum, various levels of ACh-synthesizing activity also were detected, a fraction of which was catalyzed by a choline acetyltransferase (ChAT)-like enzyme (sensitive to bromoACh, a selective ChAT inhibitor) in *T. kodakaraensis* KOD1 (15%), bamboo shoot (91%) and shiitake mushroom (51%), bloodworm (91%) and lugworm (81%). Taken together, these findings demonstrate the ubiquitous expression of ACh and ACh-synthesizing activity among life forms without nervous systems, and support the notion that ACh has been expressed and may be active as a local mediator and modulator of physiological functions since the early beginning of life.

Keywords: Acetylcholine; Archaea; Bacteria; Choline acetyltransferase; Eucarya; Radioimmunoassay

Introduction

Although acetylcholine (ACh) is generally known as an important neurotransmitter in the central and peripheral nervous systems of vertebrates and insects, ACh and ACh-synthesizing activity have also been detected in life forms without nervous systems, such as plants, fungi and even bacteria (Horiuchi et al., 2003). Moreover, ACh has been detected in a number of non-neuronal cells in mammalian species, including lymphocytes (see reviews by Kawashima and Fujii, 2000, 2003, 2004); mucocutaneous epithelial keratinocytes (see a review by Grando, 1997); gastrointestinal, respiratory and urogenital epithelial cells (see reviews by Wessler et al., 1998, 2001); and vascular endothelial cells (see a review by Kirkpatrick et al., 2001). These findings suggest that ACh has been expressed from the beginning of life and that, in addition to its role as a neurotransmitter, it serves as a local mediator regulating various physiological functions (Grando et al., 2003; Horiuchi et al., 2003; Wessler et al., 1998).

Wheelis et al. (1992) proposed on the basis of structural features in the small subunit of ribosomal RNA to divide the life on earth into three distinct domains, bacteria, archaea and eucarya. In the present study, we evaluated the ACh content and ACh-synthesizing activity of several representative species

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0024-3205/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.lfs.2007.01.059

from each domain and discussed the potential functions of ACh in life forms without nervous systems.

Materials and methods

Materials

Bacteria: gram-positive bacteria, *Bacillus subtilis* PCI 219. Archaea: thermococcales, *Thermococcus kodakaraensis* KOD1; halophiles, *Haloferax volcanii*. Eucarya: animals (insects), bloodworm (*Chironomus*, larval form); animals (annelids), lugworm (*Marphysa sanguinea*); fungi (Basidiomycota), shiitake mushroom (*Lentinus edodes*, stem portion); fungi (Ascomyta), yeast (*Saccharomyces servisiae*); plants (Anthophyta), bamboo shoot (*Phyllostachys bumbusoides*, top portion); plants (Pterophyta), fern (*Gelichenia gluaca*).

Methods

The ACh content in samples was determined using an RIA as described elsewhere (Horiuchi et al., 2003; Kawashima et al., 1980; Yamada et al., 2005).

ACh-synthesizing activity was determined using a modification of the method of Fonnum (1975) in the presence or absence of 100 μM bromoACh (BrACh), a specific inhibitor of choline acetyltransferase (ChAT) (Tuček, 1982). The ChAT-like activity was defined by subtracting the activity found in the presence of BrACh from that found in the absence of BrACh, and expressed as a percentage of the total ACh-synthesizing activity (Horiuchi et al., 2003; Yamada et al., 2005).

Results

The ACh content

ACh was detected in all of the samples tested from the bacteria, archaea and eucarya domains (Fig. 1). Less ACh was detected in samples of archaea than in samples of eucarya, and bamboo shoot (plants) expressed the highest levels of ACh (about 3 μmol/g). The animals tested (bloodworm and lugworm) expressed ACh at levels comparable to those seen in fungi (shiitake mushrooms and yeast) and plants (fern).

ACh-synthesizing activity

Variable levels of ACh-synthesizing activity were detected in all of the samples tested (Fig. 1), though the activity was not necessarily correlated with the respective ACh contents. For example, samples from shiitake mushroom, yeast and fern showed rather low ACh-synthesizing activities, though their

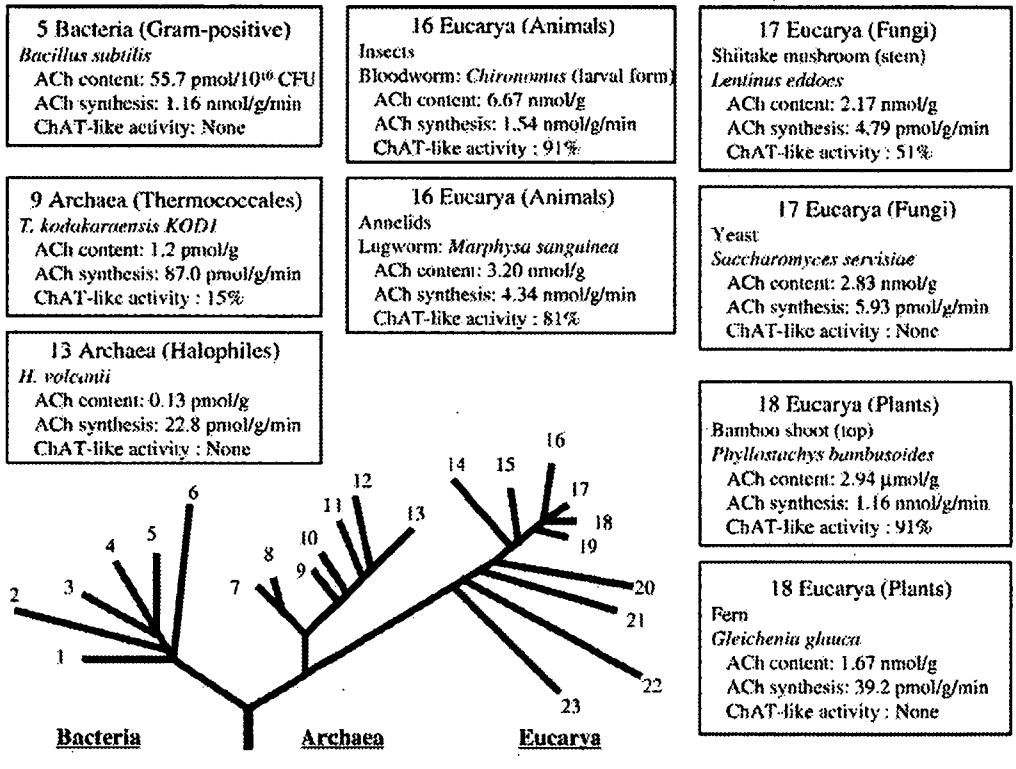

Fig. 1. Expression of acetylcholine (ACh) and ACh-synthesizing activity in representative life forms and the rooted universal phylogenetic tree adopted from Wheelis et al. (1992). Bacteria: 1, thermotogales; 2, flavobacteria and relatives; 3, cyanobacteria; 4, purple bacteria; 5, gram-positive bacteria; and 6, green non-sulfur bacteria. Archaea — kingdom Crenarchaeota: 7, the genus *Pyrodictium*; and 8, the genus *Thermoproteus*; and archaea — kingdom Eurycarhaeota: 9, Thermococcales; 10, Methanococcales; 11, Methanobacteriales; 12, Methanomicrobailes; and 13, extreme halophiles. Eurycarya: 14, entamoebae; 15, slim molds; 16, animals; 17, fungi; 18, plants; 19, ciliates; 20, flagellates; 21, trichomonads; 22, microsporidia; 23, diplomonads. ACh, acetylcholine; CFU, colony forming unit; ChAT, choline acetyltransferase.

ACh contents were quite high. And despite the very high levels of ACh found in bamboo shoot, the observed ACh-synthesizing activity was about the same as in the animals tested (bloodworm and lugworm).

High levels of ChAT-like activity (sensitive to BrACh, a specific ChAT inhibitor) were detected in samples from animals (Fig. 1). In plants, the rapidly growing top portion of bamboo shoot also expressed a high level of ChAT-like activity, but the fern showed no ChAT-like activity at all. In fungi, about 50% of ACh-synthesizing activity in the stem of the shiitake mushroom was ascribed to ChAT-like activity, while no ChAT-like activity was detected in yeast. In archaea, *T. kodakaraensis* KOD1 expressed a small amount of ChAT-like activity.

Discussion

Our present findings, as well as those summarized in our earlier reports (Horiuchi et al., 2003; Yamada et al., 2005), all demonstrate that ACh and the capacity to synthesize ACh are expressed ubiquitously among earth's life forms and suggest that ACh is an evolutionarily old compound, acting as a local mediator and neurotransmitter to regulate various physiological functions.

Although the functions of ACh in life forms without a nervous system are not yet fully understood, it has been suggested that in plants ACh is involved in the regulation of differentiation, water homeostasis and photosynthesis (Wessler et al., 2001). Moreover, on the basis of the asymmetric expression of acetylcholinesterase (AChE) activity in gravi-stimulated maize and rice seedlings and the cloning of AChE from maize (Sagane et al., 2005), Momonoki (1997) and Momonoki et al. (2000) proposed that ACh contributes to the growth of plants by facilitating transport of water and electrolytes. It is also noteworthy that Katsukawa and Shishido (2005) reported that, except in hymenium, there is a higher density of RNA expression in the stipe (stem) of the shiitake mushroom than in the pileus (cap). That the ACh content and the ACh-synthesizing activity are also both higher in the stipe than in the pileus of the shiitake mushroom (Horiuchi et al., 2003) supports the notion that ACh is expressed in activated cells.

Not surprisingly, ACh-synthesizing activity in samples from animals was mostly inhibited by BrACh, suggesting that ACh is mainly synthesized by ChAT in life forms with a nervous system. Nevertheless, Fujii et al. (1995) demonstrated that human T cells express mRNA encoding the same ChAT found in the central nervous system, revealing for the first time that non-neuronal T cells also have the ability to synthesize ACh using ChAT. Furthermore, the specific enhancement of ChAT-catalyzed ACh synthesis seen in phytohemagglutinin-stimulated T cells (Fujii et al., 1996) lent additional support to the idea that ChAT is involved in the ACh synthesis seen in activated non-neuronal cells. Although the specific enzyme catalyzing ACh synthesis in samples from plants and fungi remains to be determined, ChAT-like activity was detected in samples from the rapidly growing top portion of bamboo shoot and the stem of shiitake mushroom, suggesting that ChAT expression also is associated with the growth of plants and fungi. In the flagellated archaeon *T. kodakaraensis* KOD1, weak ChAT-like activity was detected in a sample harvested at the stage of exponential cell growth, suggesting that ACh may be involved in regulating cell growth and/or motility in unicellular organisms.

In summary, ACh and the capacity to synthesize ACh are expressed ubiquitously among life forms without nervous systems. In some organisms, moreover, ChAT-like activity appears to be induced under specific conditions, such as during periods of rapid growth or in response to stressful stimuli. However, the physiological functions of ACh in life forms without nervous systems remain to be clarified.

Acknowledgements

The authors are grateful to Dr. Masako Seki, Dr. Toyoshige Endo, Dr. Takashi Kato, Dr. Tamotsu Kanai, Dr. Taku Amo, Dr. Hiroshi Nishimasu, Dr. Takayoshi Wakagi, and Dr. Hirofumi Shoun for their help and advice.

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Immune system expression of SLURP-1 and SLURP-2, two endogenous nicotinic acetylcholine receptor ligands

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Received 26 September 2006; accepted 26 December 2006

Abstract

A novel transduction pathway via which apoptosis of keratinocytes is regulated through nicotinic acetylcholine (ACh) receptors (nAChRs) has emerged in studies of secreted mammalian Ly6/urokinase plasminogen-type activator receptor-related protein-1 and-2 (SLURP-1 and SLURP-2, respectively). SLURP-1 reportedly binds to α7 nAChRs and enhances the amplitude of macroscopic currents induced by ACh, leading to facilitation of apoptosis, whereas SLURP-2 binds to α3 nAChRs and prevents apoptosis. These observations prompted us to test whether SLURPs are expressed in immune cells and are involved in the regulation of immune function. We initially used reverse transcription-polymerase chain reaction analysis to characterize the expression profiles of SLURP mRNAs in several murine tissues and organs. Although SLURP-1 mRNA was not expressed in the pancreas, all other tissues and organs tested, including spleen and thymus, expressed both SLURP-1 and SLURP-2 mRNAs. Expression of both mRNAs also was detected in T and B cells, bone marrow-derived dendritic cells (DCs) and macrophages. Moreover, as in keratinocytes, stimulation of MOLT-3 human leukemic T cells with recombinant human SLURP-1 evoked intracellular Ca^{2+} signaling. These results suggest that both SLURP-1 and SLURP-2 are expressed in various immune cells and organs, and that not only ACh but also SLURPs may be involved in regulating lymphocyte function via nAChR-mediated pathways.

Keywords: Dendritic cells; Lymphocytes; Macrophages; Nicotinic acetylcholine receptor; SLURP-1; SLURP-2

Introduction

Acetylcholine (ACh) is well known as the neurotransmitter in the cholinergic nervous system; however, we have shown that ACh also is synthesized by choline acetyltransferase in T-lymphocytes (Fujii et al., 1995). In addition, it is now well established that most immune cells express both muscarinic and nicotinic ACh receptors (mAChRs and nAChRs, respectively), via which their function is regulated (see reviews by Kawashima and Fujii, 2000, 2003, 2004). In this context, it was of particular interest to us that a novel transduction pathway via which regulation of keratinocyte function is mediated by nAChRs has emerged in studies of secreted mammalian Ly6/urokinase-type plasminogen activator receptor-related protein-1 and -2 (SLURP-1 and SLURP-2, respectively). Chimienti et al. (2003) showed that SLURP-1 has the ability to potentiate the signal transduction via human α7 nAChRs induced by ACh, most likely by functioning as an endogenous allosteric ligand of α7 nAChRs. Mutation of SLURP-1 gene has been implicated in the pathogenesis of Mal de Meleda, which is characterized by hyperproliferative epithelium (Fischer et al., 2001). In addition, SLURP-1 binds to the conventional ligand binding site on keratinocyte nAChRs and exhibits a proapoptotic effect (Arredondo et al., 2005). SLURP-2, which shares substantial protein sequence homology with SLURP-1, also has been identified in human epidermal and oral keratinocytes (Tsuji et al., 2003; Arredondo et al., 2006). SLURP-2 competes more effectively with epibatidine than nicotine for binding to α3 nAChRs, thereby delaying keratinocyte differentiation and preventing apoptosis (Arredondo et al., 2006). These observations prompted us to test whether SLURPs are expressed in immune cells and are involved in the regulation of immune function.

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doi:10.1016/j.lfs.2006.12.028

Materials and methods

Animals

C57BL/6 mice were purchased from Japan SLC. The mice were then sacrificed by cervical dislocation according to guidelines of our institutional Ethics Committee, and the desired tissues, organs and immune cells were harvested.

Preparation of mouse leukocyte subsets

Mononuclear leukocytes (MNLs) were prepared from surgically isolated spleen or thymus using Lympholyte®-M (CEDARLANE Laboratories, Ontario, Canada) according to the manufacturer's instructions. T and B-lymphocytes were enriched from the splenocyte suspensions by positive selection using a magnetic cell sorting system and anti-CD4- or anti-CD45R-coated microbeads (Miltenyi Biotec), respectively.

Isolation of peritoneal macrophages

Thioglycollate-elicited peritoneal macrophages were obtained from mice using the method of Edelson and Cohn (1976). Mice were injected intraperitoneally with 3% Brewer thioglycollate medium containing 0.3 mM thioglycollate (3 ml/mouse) (Difco, Detroit, MI). Four days later, cells were harvested by lavage with cold DPBS. Peritoneal cells were then recovered by centrifugation and resuspended in RPMI containing 10% FBS and plated into appropriate wells. The cells were allowed to adhere for 2 h, after which they were washed free of nonadherent cells.

Preparation of dendritic cells

The procedure of Inaba et al. (1992) was used to prepare dendritic cells (DCs) from mouse bone marrow cultures. Briefly, bone marrow was flushed from femurs and tibias and then depleted of erythrocytes using ammonium chloride. Bone marrow cells were then plated at a density of 1×10^6/ml in RPMI 1640 supplemented with 10% FBS, penicillin-streptomycin and 200 U/ml mouse GM-CSF and incubated for 6 days. Non-adherent cells were then collected, and the DCs were purified using a magnetic cell sorting system with anti-CD11c-coated microbeads (Miltenyi Biotec).

Cell culture and stimulation

All immune cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 50 U/ml of penicillin and 50 U/

Mouse SLURP-1

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  1 atgaccttcgctgggccatgtggctgctgctcttggcagcctggagcatgggctatggt  60
  1  M  T  L  R  W  A  M  W  L  L  L  L  A  A  W  S  M  G  Y  G   20

 61 gaggccttccgatgctatacctgtgagcagcccacggccattaactcatgcaagaatatt 120
 21  E  A  F  R  C  Y  T  C  E  Q  P  T  A  I  N  S  C  K  N  I   40

121 gctcagtgcaagatggaagacacagcctgtaagactgtactggagacagtggaagcagtcg 180
 41  A  Q  C  K  M  E  D  T  A  C  K  T  V  L  E  T  V  E  A  A   60

181 ttcccttcaaccacagtcccatggtgacccgctcctgctccagctcgtgtctggccacc 240
 61  F  P  F  N  H  S  P  M  V  T  R  S  C  S  S  S  C  L  A  T   80

241 gaccctgatggcattggcgttgcccatcctgtcttctgttgcttccgtgacctctgcaac 300
 81  D  P  D  G  I  G  V  A  H  P  V  F  C  C  F  R  D  L  C  N  100

321 tcagggtttccaggcttcgtggcaggcctctag                            333
101  S  G  F  P  G  F  V  A  G  L  *                             110
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Mouse SLURP-2

```
  1 atgaggcttcccttctggttccttctggccgtggtcttgagcatggagctagctgtaaca  60
  1  M  R  L  P  F  W  F  L  L  A  V  V  L  S  M  E  L  A  V  T   20

 61 cagggcctgcaatgccacctgtgcaagggatttggaggatgctctcgcccgtctagctgc 120
 21  Q  G  L  Q  C  H  L  C  K  G  F  G  G  C  S  R  P  S  S  C   40

121 ccatggagctccacccactgtgtcatcattgccacccgttctcccatcagctttacagat 180
 41  P  W  S  S  T  H  C  V  I  I  A  T  R  S  P  I  S  F  T  D   60

181 ctgcctctggtgacgaagatgtgctacagtggctgtcctgatgtctccagcttgggctta 240
 61  L  P  L  V  T  K  M  C  Y  S  G  C  P  D  V  S  S  L  G  L   80

241 ggtcctcatgtatccatcgcctgctgccagtccaatctctgcaacagggactga        294
 81  G  P  H  V  S  I  A  C  C  Q  S  N  L  C  N  R  D  *         97
```

Fig. 1. Nucleotide and deduced amino acid sequences of mouse SLURP-1 and SLURP-2. Arrowheads indicate the predicted signal peptide cleavage sites. The vertical bars correspond to potential exonic junctions. The primers used for RT-PCR are marked with arrows indicating the direction of amplification. These nucleotide sequences in this figure will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers NM020519 (SLURP-1) and AB272582 (SLURP-2).

ml streptomycin at 37 °C under an atmosphere of 95% air/5% CO_2. MNLs were stimulated with 3 μg/ml concanavalin A (ConA; SIGMA); DCs and macrophages were stimulated with 1 μg/ml lipopolysaccaride (LPS; SIGMA) for 24 h.

RNA preparation and RT-PCR analysis

Total RNA was obtained from each murine tissue and immune cell type and stored at −80 °C until use. The specific sense and anti-sense primers used for RT-PCR analysis of SLURP-1, SLURP-2, IL-2, TNF-α and GAPDH expression were custom synthesized by SIGMA Genosys (see Fig. 1 and refer to Kawashima et al. in the same issue). Semiquantitative RT-PCR analysis was carried out as previously described (Fujii et al., 2003).

Construction and expression of human SLURP-1

The coding region of human SLURP-1 was cloned using standard RT-PCR techniques. The cDNA was then subcloned into the pFLAG-CMV3 vector (SIGMA) to introduce a leader and an N-terminal FLAG epitope tag (DYKDDDK) before the

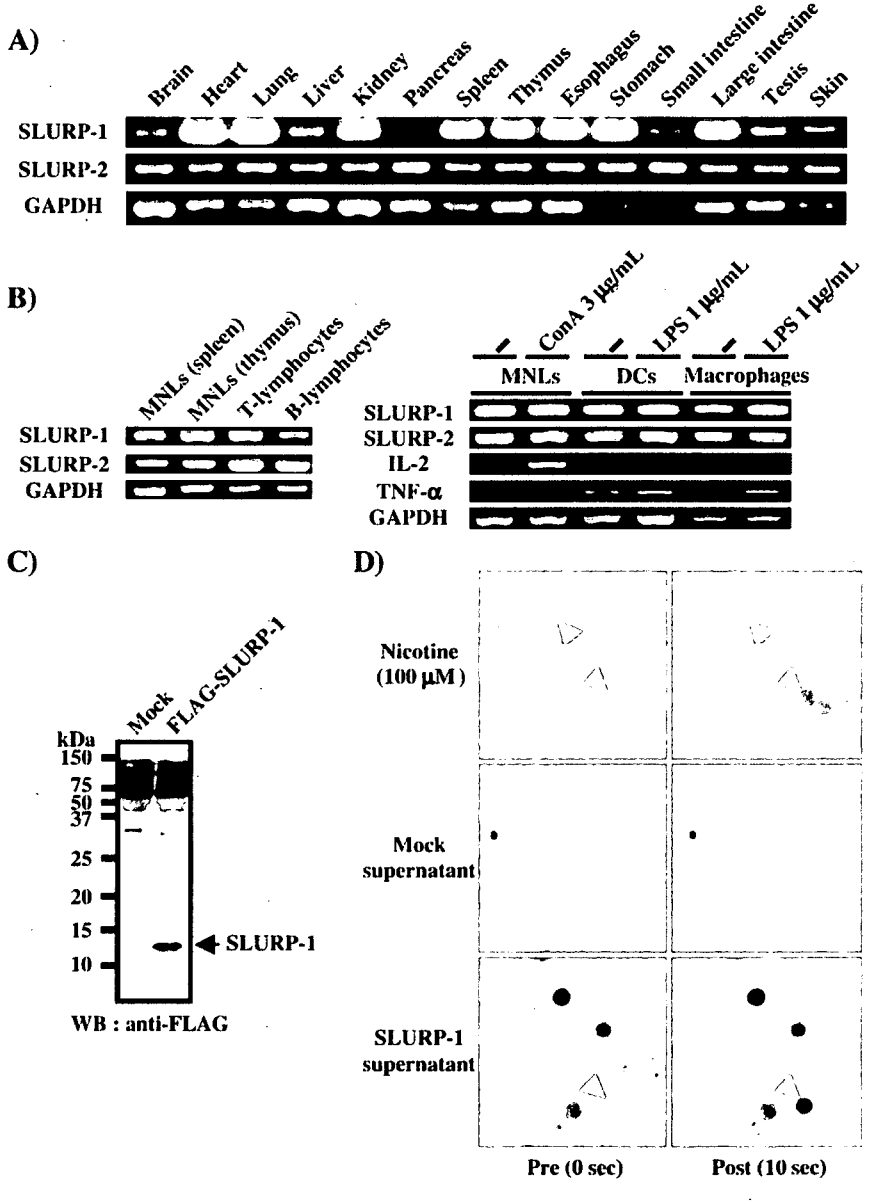

Fig. 2. Expression profiles of SLURP-1 and SLURP-2 mRNAs. (A, B) The primers described in Fig. 1 were used for amplification of SLURP-1 and SLURP-2. The primers used for amplification of IL-2, TNF-α and GAPDH are described elsewhere in the same issue (Kawashima et al.). GAPDH served as an internal control, while IL-2 and TNF-α served as positive controls for the effects of ConA or LPS, respectively. (C) Culture supernatant from mock or SLURP-1-transfected COS7 cells were immunoblotted with anti-FLAG antibody. (D) Images of fluo-3-loaded MOLT-3 cells showing SLUPR-1-evoked changes in intracellular Ca^{2+}. Cells were stimulated with supernatant conditioned by cells transfected with SLURP-1 or empty vector (Mock), or with 100 μM nicotine, which served as a positive control. The images shown were obtained immediately before and after 10 s of stimulation. The arrowheads indicate cells preferentially responding to nicotine or SLURP-1.

start of the mature polypeptide. Proper construction of the plasmid was verified by DNA sequencing.

Plasmid encoding SLURP-1 was transfected into COS7 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions, after which the cells were incubated for 24 h. The supernatants conditioned by the transfectants were then applied to MOLT-3 human leukemic T cells, and SLURP-1-evoked Ca^{2+} responses were evaluated as previously described (Fujii and Kawashima, 2000).

Results and discussion

RT-PCR analysis revealed the expression of SLURP-1 and SLURP-2 mRNAs in several murine tissues including spleen and thymus (Fig. 2A). In addition, macrophages, DCs, and T and B cells also expressed both SLURP-1 and SLURP-2 mRNAs (Fig. 2B). SLURP-2 expression is reportedly up-regulated threefold in psoriatic lesional skin, as compared with nonlesional skin (Tsuji et al., 2003). In our experiment, by contrast, we observed no change in expression of SLURP-1 or SLURP-2 mRNA when MNLs were treated with ConA or macrophages and DCs were treated with LPS. We cannot rule out an effect on the expression of SLURP proteins, however.

Using *Xenopus* oocytes expressing recombinant nAChR α7 subunit, Chimienti et al. (2003) was able to show that SLURP-1 increases the amplitude of ACh-evoked macroscopic membrane currents. Because SLURP-1 alone had no effect on current amplitudes, those investigators concluded SLURP-1 is a positive allosteric effector of α7 nAChRs. By contrast, we observed that, by itself, recombinant human SLURP-1 evoked intracellular Ca^{2+} signaling in MOLT-3 cells (Fig. 2D). This apparent discrepancy might reflect differences in the method of assay or its sensitivity. Collectively, these results, along with the recent findings by Arredondo et al. (2005, 2006), suggest that SLURPs expressed in various immune cells and organs are able to directly bind to nAChRs and are involved in regulation of lymphocyte function through nAChR-mediated pathways.

Acknowledgement

The authors are grateful to Dr. Takeshi Fujii for his critical discussion. This study was supported in part by the grant from Smoking Research Foundation (K, K).

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Available online at www.sciencedirect.com
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Life Sciences 80 (2007) 2314–2319
Life Sciences
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Expression and function of genes encoding cholinergic components in murine immune cells

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Received 26 September 2006; accepted 28 February 2007

Abstract

It is now evident that acetylcholine (ACh) synthesized by choline acetyltransferase (ChAT) and released from T cells during antigen presentation binds to muscarinic and nicotinic ACh receptors (mAChRs and nAChRs, respectively) on T and B cells or dendritic cells, leading to modulation of their function. In the present study, we used reverse transcription-polymerase chain reaction (RT-PCR) to investigate whether mononuclear leukocytes (MNLs), bone marrow-derived dendritic cells (DCs) and macrophages from C57BL/6J mice express components of the cholinergic system. Expression of ChAT mRNA was detected in MNLs activated with ConA and DCs stimulated with LPS, but not in resting MNLs and DCs or in resting and stimulated macrophages. MNLs, DCs and macrophages all expressed mRNAs encoding the five mAChR subtypes (M_1–M_5) and the nAChR α2, α5, α6, α7, α10 and β2 subunits. Expression of VIP mRNA was detected in MNLs and macrophages, but not in DCs. MNLs, DCs and macrophages all expressed VIP receptor-1 (VPAC1) and -2 (VPAC2) mRNAs, as well as mRNAs encoding secreted mammalian Ly-6/urokinase-type plasminogen activator receptor-related protein (SLURP)-1 and SLURP-2, two endogenous nAChR ligands. These results suggest that the lymphocytic cholinergic system is activated by ACh via mAChR- and nAChR-mediated pathways during antigen presentation between T cells and DCs or macrophages, leading to modulation of immune cell function. Moreover, VIP released from both postganglionic cholinergic neurons and immune cells may play a role in the cholinergic anti-inflammatory reflex, acting via VPAC1 and VPAC2 on immune cells.

Keywords: Acetylcholine; Acetylcholinesterase; Dendritic cells; Choline acetyltransferase; Macrophage; Mononuclear leukocytes; Muscarinic acetylcholine receptor; Nicotinic acetylcholine receptor; SLURP-1; SLURP-2; VIP; VPAC1; VPAC2

Introduction

Activation of human T cells by phytohemagglutinin (PHA) via the T cell receptor (TCR)/CD3 complex or with anti-CD11a monoclonal antibody enhances synthesis of acetylcholine (ACh) and expression of choline acetyltransferase (ChAT) and M_5 muscarinic ACh receptors (mAChRs) (see reviews by Kawashima and Fujii, 2000, 2003, 2004; Fujii et al., 1996, 2002, 2003a,b, 2006). In addition, both T and B cells express various mAChR and nicotinic ACh receptor (nAChR) subtypes (Sato et al., 1999; Kawashima and Fujii, 2000, 2003, 2004). Muscarinic stimulation of T and B cells evokes oscillating intracellular Ca^{2+}-signaling and up-regulation of *c-fos* expression (Fujii and Kawashima, 2000a,b), whereas nicotine evokes a rapid and transient Ca^{2+}-signal that is abolished by α-bungarotoxin, suggesting involvement of the nAChR α7 subunit in nicotinic Ca^{2+}-signaling (Kimura et al., 2003; Kawashima and Fujii, 2004). Moreover, Zimring et al. (2005) showed that M_1 mAChRs are involved in an early stage of $CD8^+$ cytolytic T cell development, and Skok et al. (2005) suggested that nAChRs are involved in the regulation of antibody synthesis in B cells. Taken together, these findings suggest that ACh synthesized and released from T cells during antigen presentation and T cell adhesion to vascular endothelial cells or keratinocytes acts on mAChRs and nAChRs in an autocrine and/or paracrine fashion to regulate immune cell function (Kawashima and Fujii, 2004).

Co-localization and co-release of vasoactive intestinal polypeptide (VIP) with ACh is consistent with the notion that VIP is expressed in a population of peripheral parasympathetic

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0024-3205/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.lfs.2007.02.036

cholinergic neurons (Lundberg et al., 1981). By acting on its receptors, VPAC1 and VPAC2, and activating adenylate cyclase activity, VIP reportedly exerts a variety of immunomodulatory and anti-inflammatory effects (see a review by Delgado et al., 2003). In addition, VIP selectively potentiates ACh- and nicotine-evoked membrane currents in rat cholinergic neurons by increasing the affinity of the agonist for its receptor (Liu et al., 2000). This suggests that VIP may be involved in the regulation of immune cell function, acting by itself and/or via facilitation of nAChR-mediated cholinergic transmission.

Arredondo et al. (2005, 2006) showed that secreted mammalian Ly-6/urokinase-type plasminogen activator receptor-related protein (SLURP)-1 and SLURP-2, two endogenous nAChR ligands, are expressed in mucocutaneous keratinocytes. While SLURP-1 activates the nAChR α7 subunit and promotes apoptosis, SLURP-2 activates the α3 subtype and prevents apoptosis. It would be of interest to know whether SLURP peptides are also expressed in immune cells.

To evaluate the biological significance of the various lymphocytic cholinergic components and their related peptides in the regulation of immune cell function, we used reverse transcription-polymerase chain reaction (RT-PCR) to investigate which of the cholinergic and peptidergic components are expressed by splenic mononuclear leukocytes (MNLs), bone marrow-derived dendritic cells (DCs) or macrophages from C57BL/6J mice.

Materials and methods

Preparation of MNLs, DCs and macrophages

MNLs

C57BL/6J mice were sacrificed by cervical dislocation and the spleen was dissected out. To isolate MNLs, single-cell suspensions of the spleen prepared by passage through a nylon mesh were layered on Lympholyte®-M (CEDARLANE Laboratories, Ontario, Canada) and centrifuged at 1400 ×*g* for 20 min at 20 °C. Cell surface expression of antigens was identified by monoclonal antibody staining of freshly isolated MNLs, followed by flow cytometry using a FACSCalibur (Becton-Dickinson, Palo Alto, CA, USA) with analysis using CellQuest software (Becton-Dickinson). MNLs used for further studies consisted of 95% lymphocytes (6% $CD4^+$ and 9% $CD8^+$ T cells; 80% $CD45R^+$ B cells) and 5% monocytes ($CD80^+$ cells).

Portions of MNLs were then incubated for 24 h in the presence or absence of 3 μg/mL concanavalin A (Con A).

DCs

The procedure of Inaba et al. (1992) was used for generation of DCs from mouse bone marrow cultures. Briefly, bone marrow was flushed from femurs and tibias and then depleted of erythrocytes using ammonium chloride. The remaining bone

Table 1
Sequences of primers used for PCR study

Name	Forward sequence (5′→3′)	Reverse sequence (5′→3′)	Length (bp)
mAChR			
M_1	GCAGCAGCTCAGAGAGGTCACAG	GCCTTTGCCGCCTCGGTCTCG	308
M_2	TGTCAGCAATGCCTCCGTTATG	GCCTTGCCATTCTGGATCTTG	480
M_3	GGTGTGATGATTGGTCTGGCTTG	AGAAGCAGAGTTTTCCAGGGAG	498
M_4	TCAAGAGCCCTCTGATGAAGCC	AGATTGTCCGAGTCACTTTGCG	477
M_5	GCTGACCTCCAAGGTTCCGATTC	CCGTCAGCTTTTACCACCAATC	485
nAChR			
α2	CGGGTGCCCCGGTGGCTGATGA	GAGGTGACAGCAGGATCTCACTAG	295
α3	GCTGTGCTTCGGTGGTGGTCAGC	GCCGGAGGGATCCAAGTCACTTC	502
α4	GAATGTCACCTCCATCCGCATC	CCGGCAGTTGTCTTTGACCAC	790
α5	GGGTTCGTCCTGTGGAACACCTGA	GGTCCTCTAGGATTATATCG	432
α6	GTGTTCGTGCTGAACATA	CTACCTCGTTTGTTTCATTGT	394
α7	ATCTGGGCATTGCCAGTATC	TCCCATGAGATCCCATTCTC	199
α9	CCTTACCCAGACGTCACCTTCACTC	AACACCATAGCGAAGAAAATCCACA	737
α10	AATGTGACCCTGGAGGTGAC	GTAGGCGTCTGTCCACTCCTG	105
β2	CTCCAACTCTATGGCGCTGCT	GAGCGGAACTTCATGGTGCAG	513
β3	CTCCTCAGACATTTGTTCCAAGG	AATGAGGTCGACCATGGT	459
β4	TCTGGTTGCCTGACATCGTG	GGGTTCACAAAGTACATGGA	859
ChAT	GCAGTACTACAGACTCTTCTCATC	TCCAAGACAAAGAACTGGTTGC	135
AChE	GCAGCAATATGTGAGCCTGA	GGTCGAACTGGTTCTTCCAG	197
VIP	ATCAAACGACACTCTGATGC	GCCTTATGTAACACAGCAGC	599
VPAC1	TGTCCCTTGCCAGCCTCC	CCGCTCAGAGAAGAAGGA	302
VPAC2	GTGCTGGTCAAGGACAGCGT	AGGCGAGTTGCTGTCATG	247
SLURP-1	CAGGGTTTCCAGGCTTCGTG	AGCTTGGTGGACAGTGAGTG	167
SLURP-2	ATGAGGCTTCCCTTCTGGTTC	TCAGTCCCTGTTGCAGAGATTG	294
IL-2	GATGAACTTGGACCTCTGCG	AACATTTTTGAGCCCTTGGG	396
TNF-α	AGAAGGGGGACCAACTCAGC	TTGAGTCCTTGATGGTGGTG	329
GAPDH	CGTATTGGGCGCCTGGTCACCAG	GACCTTGCCCACAGCCTTGGCAGC	624

PCR, polymerase chain reaction; bp, base pairs; mAChR, muscarinic acetylcholine (ACh) receptor; nAChR, nicotinic ACh receptor; ChAT, choline acetyltransferase; AChE, acetylcholinesterase; VIP, vasoactive intestinal polypeptide; VPAC, VIP receptor; SLURP, secreted mammalian Ly-6/urokinase-type plasminogen activator receptor-related protein; IL-2, interleukin-2; TNF-α, tissue necrosis factor-α; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

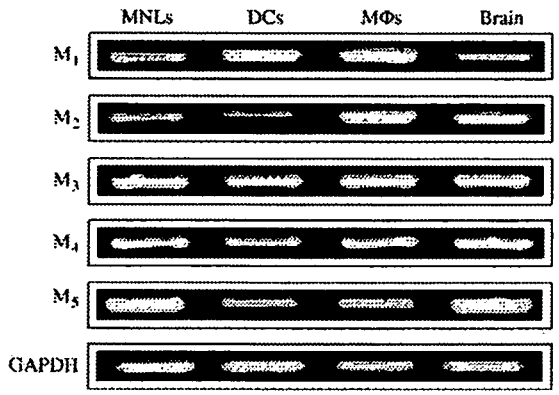

Fig. 1. RT-PCR analysis of the expression of mRNAs encoding the M_1–M_5 mAChR subtypes and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in mononuclear leukocytes (MNLs), bone marrow-derived dendritic cells (DCs), macrophages (MΦs) and brain from C57BL/6J mice.

marrow cells were plated at a density of 1×10^6/mL in RPMI 1640 supplemented with 10% FCS, penicillin–streptomycin and 200 U/mL mouse GM-CSF and maintained for 6 days. Nonadherent cells were then collected, and the DCs were purified using a magnetic cell sorting system and anti-CD11b-coated microbeads (Miltenyi Biotec). Portions of the isolated DCs were then incubated for 24 h in the presence or absence of 1 μg/mL lipopolysaccharide (LPS).

Peritoneal macrophages

Thioglycollate-elicited peritoneal macrophages were obtained using the method of Edelson and Cohn (1976). Mice were injected intraperitoneally with 3 mL of 3% Brewer thioglycollate medium containing 0.3 mM thioglycollate (Difco, Detroit, MI, USA). Four days later, cells were harvested by lavage with cold DPBS. Peritoneal cells were recovered by centrifugation and resuspended in RPMI containing 10% FBS, plated into appropriate wells and allowed to adhere for 2 h, then washed free of nonadherent cells. Portions of macrophages were then incubated for 24 h in the presence or absence of 1 μg/mL LPS.

Extraction of total RNA

Total RNA was extracted from MNLs, DCs, macrophages and brain, which served as a positive control, using RNA Bee™ (TelTest, Friendswood, TX, USA) as described previously (Fujii et al., 1998). RNA concentration and quality were assessed based on 260 nm/280 nm absorbance ratios.

First strand cDNA synthesis and RT-PCR analysis

First-strand cDNA was prepared using 1 μg of total RNA, 50 pmol of random primer $p(dN)_6$ and SuperScript™ II (Invitrogen, Carlsbad, CA, USA), as described elsewhere (Fujii et al., 1998). Using a DNA Thermal Cycler (Perkin-Elmer Cetus, CT, USA), 2 μL of the cDNA obtained was amplified in a 50 μL reaction volume containing 25 pmol of the respective forward and reverse primers and AmpliTaq DNA polymerase. For the mAChR M_1–M_5 subtypes, nAChR α2–α10 and β2–β4 subunits, ChAT, AChE, VIP, VPAC1, VPAC2, SLURP-1 and SLURP-2, the amplification protocol entailed 40 cycles of denaturation at 95–98 °C for 30 s, annealing at 50–58 °C for 30 s and extension at 72 °C for 30 s to 1 min. As an internal control for the efficiency of cDNA amplification, we also amplified glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA using the primers shown in Table 1. Amplification entailed 20 cycles of 95 °C for 1 min, 55 °C for 1.5 min and 72 °C for 1.5 min, followed by a 15-min final extension at 72 °C. The PCR products present in 10 μL of the reaction mixture were then separated according to size on 2.5% agarose gels and visualized by ethidium bromide staining. The resultant fluorescent bands were digitized and quantified using a Luminous Imager (Aisin Cosmos R&D, Aichi, Japan).

The forward and reverse oligonucleotide primers used for RT-PCR analysis in the present study (Table 1) were custom synthesized at SIGMA Genosys (Hokkaido, Japan). All other reagents used were of reagent grade and purchased from commercial sources (e.g., Wako Pure Chemicals, Osaka, Japan).

Results

mAChRs

mRNAs encoding all five mAChR subtypes were expressed in MNLs, DCs and macrophages from C57BL/6J mouse (Fig. 1).

nAChRs

mRNAs encoding the nAChR α2, α5, α6, α7, α10 and β2 subunits were expressed in all three immune cell types (Table 2), whereas those encoding the α3 and β3 subunits were not detected in any of the immune cells. Variable results were observed for mRNAs encoding the α4, α9 and β4 subunits.

ChAT

Significant expression of ChAT mRNA was detected in rat brain, which served as a positive control. By contrast, resting MNLs, DCs

Table 2
Expression for nAChR subunits in immune cells from C57BL/6J mice

Subunit	MNLs	DCs	MΦs
α2	+	+	+
α3	−	−	−
α4	−	−	+
α5	+	+	+
α6	+	+	+
α7	+	+	+
α9	+	−	−
α10	+	+	+
β2	+	+	+
β3	−	−	−
β4	+	+	+

nAChR, nicotinic acetylcholine receptor; +, positive expression; −, negative expression; MNLs, mononuclear leukocytes; DCs, bone marrow-derived dendritic cells; MΦs, macrophages.

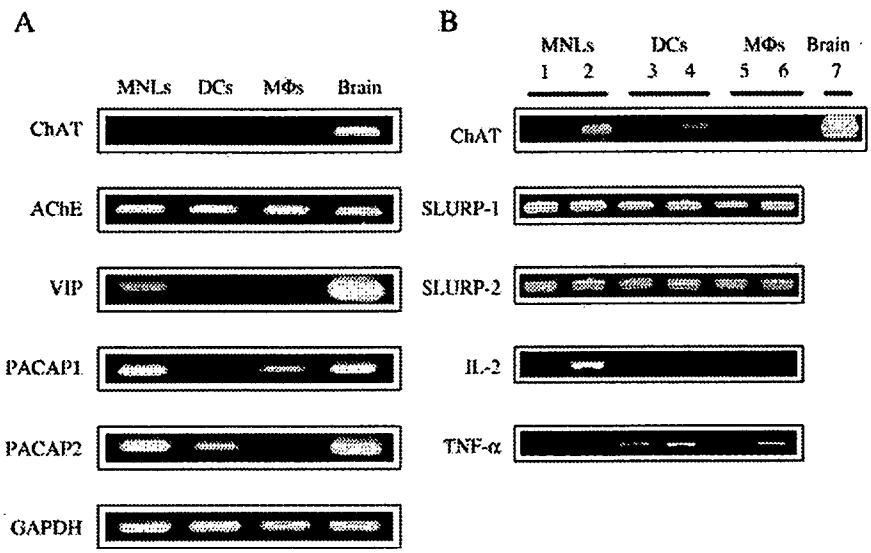

Fig. 2. RT-PCR analysis of the expression of mRNAs encoding the cholinergic components and related peptides in mononuclear leukocytes (MNLs), bone marrow-derived dendritic cells (DCs), macrophages (MΦs) and brain from C57BL/6J mice: A, resting conditions; B, resting conditions (lanes 1, 3 and 5), and activated conditions with 3 μg/mL ConA (lane 2) and 1 μg/mL LPS (lanes 4 and 6). ChAT, choline acetyltransferase; AChE, acetylcholinesterase; VIP, vasoactive intestinal polypeptide; VPAC, VIP receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SLURP, secreted mammalian Ly-6/urokinase-type plasminogen activator receptor-related protein; IL-2, interleukin-2; TNF-α, tissue necrosis factor-α; Con A, concanavalin A; LPS, lipopolysaccharide.

and macrophages did not express ChAT mRNA (Fig. 2A). Upon stimulation with 3 μg/mL Con A, however, expression of ChAT mRNA was induced in MNLs (Fig. 2B), and stimulation with 1 μg/mL LPS induced ChAT mRNA expression in DCs. No ChAT mRNA was detected in either resting or stimulated macrophages. The efficacy of Con A or LPS was confirmed by assessing expression of IL-2 and TNF-α mRNA, respectively.

AChE

AChE mRNA was expressed in MNLs, DCs and macrophages as well as in the brain (Fig. 2A).

VIP and VIP receptors

VIP mRNA was expressed in MNLs, macrophages and brain, but not in DCs (Fig. 2A). VPAC1 and VPAC2 mRNAs were detected in the brain, MNLs, DCs and macrophages, though the levels of their expression varied among the cell types (Fig. 2A).

SLURP

Expression of SLURP-1 and SLURP-2 mRNA was detected in MNLs, DCs and macrophages; however, neither Con A nor LPS had any effect on the levels of their expression (Fig. 2B).

Discussion

Under our experimental conditions, mRNAs encoding all five mAChR subtypes (M_1–M_5) were expressed in MNLs, DCs and macrophages from C57BL/6J mice. mAChRs have been reported to play a role in the function of $CD8^+$ T cells (Zimring et al., 2005), and their stimulation is known to evoke Ca^{2+}-signaling and to facilitate *c-fos* expression in both T and B cells (Fujii and Kawashima, 2000a,b). Beyond that, however, little is known about the immunological significance of mAChRs on lymphocytes.

We also found that mRNAs encoding the nAChR α2, α5, α6, α7, α10 and β2 subunits were expressed in all three immune cell types tested. Kuo et al. (2002) previously observed that the α3, α5, α7, β2 and β4 subunits were expressed in immature T cells from C57BL/6J mice, while the α2, α5 and α7 subunits were expressed in peripheral T cells. This suggests that the expression pattern of nAChR subunits in immune cells may vary depending upon the cells' stage of development and the stimulus given during development. Skok et al. (2005) used radioligand binding assays and ELISAs to show that the nAChR α4, α7 and β2 subunits are expressed on B cells from C57BL/6J mice. They also observed that mice lacking the nAChR α4, α7 and β2 subunits exhibit stronger immune responses to protein antigens than wild-type mice. Consistent with that finding, we observed in a preliminary experiment that two weeks after immunization with ovalbumin (OVA), mice lacking the nAChR α7 subunit produced higher levels of anti-OVA IgG_1 antibody than wild-type mice (Fujii et al., in preparation). Apparently, nAChRs play a key role in the regulation of antibody synthesis.

In the mammalian periphery, ACh is synthesized by both ChAT and carnitine acetyltransferase (CarAT) (Tuček, 1988), though PHA enhances only ChAT activity in MOLT-3 cells, a human leukemic T cell line (Fujii et al., 1996). The results of the present study suggest that both DCs and T cells have the capacity to synthesize ACh using ChAT. That ChAT expression was detected in splenic MNLs and DCs only after stimulation with Con A and LPS, respectively, suggesting that activation of T cells via the TCR/CD3 complex or stimulation of DCs via the Toll-like receptor is involved in regulating lymphocytic cholinergic

activity. The fact that ChAT expression was not detected in resting splenic MNLs can be ascribed, at least in part, to the low content of T cells (15%), especially $CD4^+$ T cells (6%), playing a major role in ACh synthesis by ChAT (Fujii et al., 2003b). By contrast, our findings indicate that neither resting nor activated macrophages have the ability to synthesize ACh using ChAT. These findings support the notion that the lymphocytic cholinergic system is activated during antigen presentation between T cells and DCs or macrophages, and that ACh released from T cells and DCs acts on the cells' own mAChRs and nAChRs to modulate their function.

Our finding that MNLs, DCs and macrophages all express AChE suggests that ACh is normally present in the vicinity of these immune cells. It is plausible that AChE acts to prevent unnecessary signal propagation by ACh that leaks from the immunological synapses during antigen presentation and during cell-to-cell adhesion of T cells to DCs or macrophages.

In rats, the half-life ($T_{1/2}$) of VIP in the circulation is about 0.6 min, and it is cleared from the lung with a $T_{1/2}$ of about 3 min (Refai et al., 1999). Expression of VIP and its receptors in immune cells suggests that VIP released from MNLs and macrophages, as well as that released from postganglionic cholinergic neurons, acts via VPAC1 and VPAC2 to modulate immune function and anti-inflammatory responses (Delgado et al., 2003). Furthermore, VIP increases the affinity of nAChRs for their agonists, thereby potentiating ACh-evoked whole-cell currents in rat cholinergic neurons at low agonist concentrations (Liu et al., 2000). This suggests that the nAChR-mediated effects of ACh released locally from T cells and DCs during antigen presentation could be potentiated in the presence of VIP. Since a $T_{1/2}$ for ACh in tissue or the circulation is far shorter than 1 s, VIP released from cholinergic nerve endings rather than ACh would be expected to play the major role in anti-inflammatory effects observed after vagal nerve stimulation (Tracey, 2002).

The biological significance of the endogenous nAChR ligands SLURP-1 and SLURP-2 has been studied extensively in keratinocytes by Arredondo et al. (2005, 2006). In keratinocytes, SLURP-1 and SLURP-2 bind to the nAChR $\alpha7$ and $\alpha3$ subunits, respectively, thereby facilitating or preventing apoptosis, respectively. Our findings demonstrate that mRNAs encoding both SLURP-1 and SLURP-2 are expressed in MNLs, DCs and macrophages. However, because activation of these cells by Con A or LPS did not affect the level of transcription, further studies will be needed to confirm the expression of SLURP-1 and SLURP-2 and their biological significance in immune cells.

In summary, transcripts encoding mAChRs, nAChR, AChE, VIP, VPAC1, VPAC2, SLURP-1 and SLURP-2 are all expressed in murine MNLs, DCs and macrophages, though the level of that expression varied among the cell types. Immunological stimulation induced ChAT gene expression in MNLs and DCs, but not in macrophages.

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Journal of Neuroimmunology 188 (2007) 80–85

Journal of Neuroimmunology

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Diminished antigen-specific IgG_1 and interleukin-6 production and acetylcholinesterase expression in combined M_1 and M_5 muscarinic acetylcholine receptor knockout mice

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Received 20 February 2007; received in revised form 22 May 2007; accepted 23 May 2007

Abstract

Immunological activation of T cells enhances synthesis of acetylcholine (ACh) and transcription of choline acetyltransferase (ChAT), M_5 muscarinic ACh receptor (mAChR) and acetylcholinesterase (AChE). Stimulation of mAChRs on T and B cells causes oscillating Ca^{2+}-signaling and up-regulation of *c-fos* expression; moreover, M_1 mAChRs play a crucial role in the differentiation of $CD8^+$ T cells into cytolytic T lymphocytes. Collectively, these findings suggest that immune cell function is regulated by its own cholinergic system. Bearing that in mind, we tested whether immune function can be regulated *via* mAChR-mediated pathways by immunizing combined M_1 and M_5 mAChR knockout (M_1/M_5 KO) and wild-type (WT) C57BL/6JJcl mice with ovalbumin (OVA) and measuring serum IgG_1 and IgM 1 wk later. We found that serum levels of total and anti-OVA-specific IgG_1 were significantly lower in M_1/M_5 KO than WT mice, though there was no difference in serum levels of total and anti-OVA-specific IgM between the two genotypes. Secretion of interleukin (IL)-6 from activated spleen cells was significantly reduced in M_1/M_5 KO mice, whereas there was no significant change in gamma interferon secretion. Expression of AChE mRNA was significantly reduced in activated spleen cells from M_1/M_5 KO mice. These results suggest that M_1 and/or M_5 mAChRs are involved in regulating cytokine (*e.g.*, IL-6) production, leading to modulation of antibody class switching from IgM to IgG_1, but are not involved in the initial generation of the antibody response. They also support the notion that a non-neuronal cholinergic system is involved in regulating immune cell function.

Keywords: Acetylcholine; Class switching; Cytokine; IgG_1; Muscarinic receptor; Non-neuronal cholinergic system

1. Introduction

T and B cells express both muscarinic and nicotinic acetylcholine (ACh) receptors (mAChRs and nAChRs, respectively), stimulation of which elicits various functional and biochemical effects (see reviews by Kawashima and Fujii, 2000, 2003a,b, 2004; Fujii and Kawashima, 2001a). Moreover, Fujii et al. (1995, 1996) confirmed that T cells have the ability to synthesize ACh and to express the same choline acetyltransferase (ChAT) that is expressed in brain. Expression of other cholinergic components, including the high affinity choline transporter and acetylcholinesterase (AChE), also has been detected in T cells and mononuclear leukocytes (MNLs) from both humans and mice (Kawashima and Fujii, 2003a,b, 2004; Kawashima et al., 2007). Taken together, these findings support the notion that lymphocytes express their own cholinergic system which regulates immune cell activity.

Five distinct mAChR subtypes (M_1–M_5) have been identified based on molecular cloning (Bonner et al., 1987, 1988; Alexander and Peters, 1999). Of those, M_1, M_3 and M_5

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doi:10.1016/j.jneuroim.2007.05.017

mAChRs are coupled to $G_{q/11}$, which upon stimulation mediates activation of phospholipase C activity, resulting in increases in the intracellular free Ca^{2+} concentration. M_2 and M_4 mAChRs are coupled to $G_{i/o}$, which upon stimulation mediates inhibition of adenylyl cyclase activity and thus cAMP formation (Hulme et al., 1990). While the levels of mAChR expression may vary among the subtypes, lymphocytes from both humans and mice express all five subtypes of mAChR mRNA and protein (see reviews by Kawashima and Fujii, 2000, 2003a,b, 2004; Fujii and Kawashima, 2001a; Kawashima et al., 2007).

Activation of T cells *via* T cell receptor/CD3-mediated pathways using phytohemagglutinin (PHA) or concanavalin A (ConA), or *via* CD11a-mediated pathways using antibody, enhances ACh release as well as expression of ChAT and M_5 mAChR (Fujii et al., 1996, 2002, 2003a,b, 2006; Kawashima and Fujii, 2004; Kawashima et al., 2007). Furthermore, B cell activation by *Staphylococcus aureus* COW I up-regulates expression of M_5 mAChR mRNA without affecting expression of M_3 or M_4 mAChR mRNA (Fujii et al., 2003a). This suggests that immunological activation of T or B cells facilitates lymphocytic cholinergic transmission through augmented ACh synthesis and selective up-regulation of M_5 mAChR expression, from which it could be inferred that signal transduction *via* M_5 mAChRs is involved in regulating immune function. To date, however, no specific function has ever been attributed a particular mAChR subtype. On the other hand, Zimring et al. (2005) recently reported that $CD8^+$ T cells from M_1 mAChR knockout mice show a defect in their differentiation into cytolytic T lymphocytes when stimulated *in vitro*. By contrast, no defect was observed in mice with targeted deletion of either M_3 or M_5 mAChRs or a combined deletion of M_2 and M_4 mAChRs.

To further investigate the involvement of a non-neuronal cholinergic system in the regulation of immune function, we examined the production of antigen-specific serum IgM and IgG_1 in combined M_1 and M_5 mAChR knockout (M_1/M_5 KO) mice 1 wk after immunization with ovalbumin (OVA). In addition, to investigate the role of mAChRs in antigen-specific cytokine production, we compared antigen-specific secretion of gamma interferon (IFN-) and interleukin (IL)-6 cytokine from OVA-activated spleen cells isolated from M_1/M_5 KO and wild-type mice.

2. Materials and methods

2.1. Animals

M_1/M_5 KO mice were generated by crossing M_1 KO (Ohno-Shosaku et al., 2003) and M_5 KO mice (Nakamura et al., 2004), both of which were backcrossed with C57BL/6JJcl mice (CLEA Japan, Tokyo, Japan) for at least 10 generations. Four male and 5 female 10-wk-old M_1/M_5 KO and 6 male and 4 female wild-type C57BL/6JJcl mice were used for study.

2.2. Immunization and bleeding

Mice were immunized by intraperitoneal injection of 100 g of OVA (Sigma, St. Louis, MO, USA) mixed with 5×10^8 CFUs of *Bordetella pertussis* (LSL, Tokyo, Japan) and Alu-Gel-S (Serva, Heidelberg, Germany). One wk after the immunization, blood samples were obtained by cardiac puncture under deep anesthesia with ether. Serum was separated by centrifugation and stored in a deep freezer until assayed.

2.3. Determination of serum IgG_1 and IgM

2.3.1. Total IgG_1 and anti-OVA specific IgG_1

Total serum IgG_1 levels were quantified by ELISA using 96-well microtiter plates coated with goat anti-mouse IgG_1 (Cat # A90-105A, Bethyl, Montgomery, TX, USA) and goat anti-mouse IgG_1 conjugated with horseradish peroxidase (Cat # A90-105P, Bethyl, Montgomery, TX, USA). Mouse monoclonal anti-OVA-specific IgG_1 (HYB 099-01, Antibodyshop, Gentofte, Denmark) was used as the standard.

Serum levels of anti-OVA-specific IgG_1 were determined by ELISA using 96-well microtiter plates coated with OVA and goat anti-mouse IgG_1 conjugated with horseradish peroxidase (Cat # A90-105P, Bethyl, Montgomery, TX, USA). Mouse monoclonal anti-OVA-specific IgG_1 (HYB 099-01, Antibodyshop, Gentofte, Denmark) served as the standard.

2.3.2. Total IgM and anti-OVA-specific IgM

Serum concentrations of total IgM and anti-OVA-specific IgM were respectively determined using a commercially available Mouse IgM ELISA Quantitation Kit (Bethyl, Montgomery, TX, USA) and a Mouse Anti-Ovalbumin IgM ELISA Kit (Cat. # 600-170-OGM, Alpha Diagnostic, San Antonio, TX, USA) according to manufacturer's instructions.

2.4. Secretion of IFN- and IL-6 and expression of ChAT and AChE mRNAs in spleen cells

2.4.1. Preparation of spleen cells

Mice were sacrificed by deep anesthesia with ether, after which the spleen was dissected out. Suspensions of single spleen cells were prepared by passing the cells through a nylon mesh, after which contaminating erythrocytes were removed by treatment with ammonium chloride lysis buffer. Cell surface expression of antigens was identified by monoclonal antibody staining, followed by flow cytometry using a FACSCalibur (Becton-Dickinson, Palo Alto, CA, USA) with analysis using CellQuest software (Becton-Dickinson). Spleen cells thus prepared consisted of 90% lymphocytes (30% $CD4^+$ and 15% $CD8^+$ T cells; 45% $CD45^+$ B cells) and 10% monocytes ($CD14^+$ cells).

2.4.2. Determination of IFN- and IL-6 secretion in spleen cells

Spleen cell samples were suspended in a 10% fetus bovine serum/RPMI 1640 culture medium (2×10^6 cells/mL) and then incubated with 100 g/mL OVA at 37 °C in a CO_2 incubator. The conditioned media were collected after 24 h or 48 h of culture to assess secretion of IL-6 or IFN- , respectively. IL-6 and IFN- levels were determined using commercially available ELISA kits (IFN- , BD Sciences, San Diego, CA, USA; IL-6, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.4.3. Expression of AChE and ChAT mRNAs in spleen cells

2.4.3.1. Extraction of total RNA from spleen cells. After incubating spleen cells for 48 h in the presence of 100 g/mL OVA, total RNA was extracted from the cells using RNA Bee™ (TelTest, Friendswood, TX, USA) as described previously (Fujii et al., 1998). RNA concentration and quality were assessed based on 260 nm/280 nm absorbance ratios.

2.4.3.2. First strand cDNA synthesis. First-strand cDNA was prepared using 1 g of total RNA, 50 pmol of random primer $p(dN)_6$ and SuperScript™ II (Invitrogen, Carlsbad, CA, USA) in a 20- L reaction volume, as described elsewhere (Fujii et al., 1998).

2.4.3.3. Conventional polymerase chain reaction (PCR) analysis. Using a DNA Thermal Cycler (Perkin-Elmer Cetus, CT, USA), 2 L of cDNA was amplified in a 50- L reaction volume containing 25 pmol of the respective forward and reverse primers and AmpliTaq DNA polymerase (Takara, Shiga, Japan). The following sense and antisense oligonucleotide primers were custom synthesized at Sigma Genosys (Hokkaido, Japan) and used for PCR analysis of ChAT and AChE:

5 -GCAGTACTACAGACTCTTCTCATC-3 (ChAT sense),
5 -TCCAAGACAAAGAACTGGTTGC-3 (ChAT antisense),
5 -GCAGCAATATGTGAGCCTGA-3 (AChE sense), and
5 -GGTCGAACTGGTTCTTCCAG-3 (AChE anti-sense).

For ChAT and AChE, the amplification protocol entailed 40 cycles of denaturation at 95 °C for 30 s, annealing at 53–57 °C for 30 s and extension at 72 °C for 30 s. The PCR products present in 10 L of the reaction mixture were then separated according to size on 2% agarose gels and visualized by ethidium bromide staining. The sizes of the PCR products were estimated from the migration of DNA markers run concurrently.

All other reagents used were of reagent grade and purchased from commercial sources (*e.g.*, Wako Pure Chemicals, Osaka, Japan).

2.4.3.4. Real time PCR analysis. After our conventional reverse transcription (RT)-PCR analysis confirmed the expression of AChE mRNA (see Results), we used real time PCR to determine the relative levels of AChE mRNA expression in M_1/M_5 KO and wild-type mice. The following sense and antisense oligonucleotide primers for the mouse gene encoding AChE were designed with assistance of Sigma Genosys (Hokkaido, Japan):

5 -CGGAGGCTCTCATCAATACTGG-3 (AChE sense) and
5 -GGGACCCCGTAAACCAGAAAG-3 (AChE antisense).

The PCR was carried out using an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and the TaqMan Universal Master Mix reagent (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's protocol. Briefly, 1 L of the diluted cDNA sample produced from 1 g of total RNA was added to 25 L of the PCR master mix. To correct for minor variations in mRNA extraction and reverse transcription, the gene expression values were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), after which the ChAT/GAPDH ratios obtained from M_1/M_5 KO mice were expressed as percentages of the mean value obtained from wild-type mice.

2.5. Statistical analysis

All values are expressed as means±SEM. Statistical comparisons between the M_1/M_5 mAChR deficient and wild-type mice were carried out using an unpaired *t*-test. Values of $P<0.05$ were considered significant. In addition, linear regression analyses were performed to investigate whether there were any correlations among the respective immunoglobulin levels, cytokine secretion and/or expression of AChE mRNA.

3. Results

3.1. Serum IgG_1 and IgM

In order to investigate the IgG_1 and IgM responses in M_1/M_5 KO mice, we measured both total serum and antigen-specific IgG_1 and IgM concentrations one week after immunizing the mice with OVA.

3.1.1. Total serum IgG_1 and IgM

We found that total serum IgG_1 was significantly lower in M_1/M_5 KO mice than in wild-type mice ($P=0.043$) (Fig. 1A). By contrast, total serum IgM was about the same in M_1/M_5 KO and wild-type mice (Fig. 1B).

3.1.2. Anti-OVA-specific serum IgG_1 and IgM

Serum levels of anti-OVA-specific IgG_1 were significantly lower in M_1/M_5 KO than wild-type mice ($P=0.016$) (Fig. 2A), whereas no significant difference was observed in the serum levels of anti-OVA-specific IgM between the two genotypes (Fig. 2B). Apparently, M_1/M_5 KO mice have a specific defect affecting the IgG_1 response; the IgM response appears to be normal in this genotype.

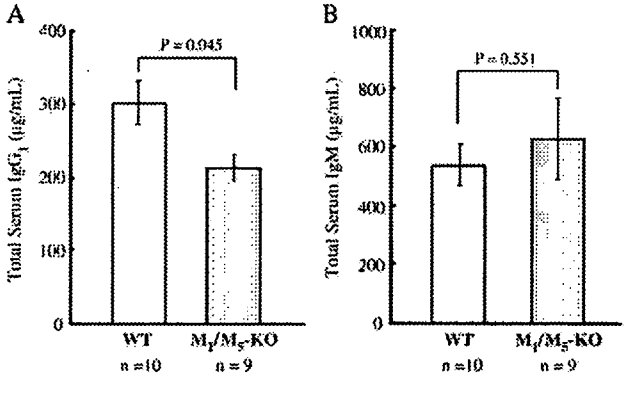

Fig. 1. Serum concentrations of total IgG_1 (A) and total IgM (B) one wk after immunizing M_1/M_5 KO and wild type (WT) C57BL/6JJcl mice with OVA. The bars represent means±SEM.

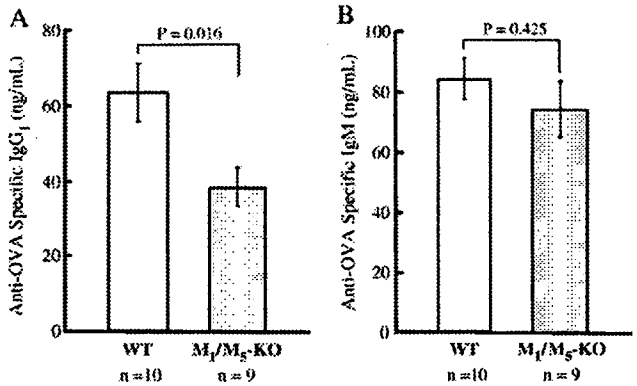

Fig. 2. Serum concentrations of anti-OVA-specific IgG_1 (A) and anti-OVA-specific IgM (B) immunizing M_1/M_5 KO and wild type (WT) C57BL/6JJcl mice with OVA. The bars represent means±SEM.

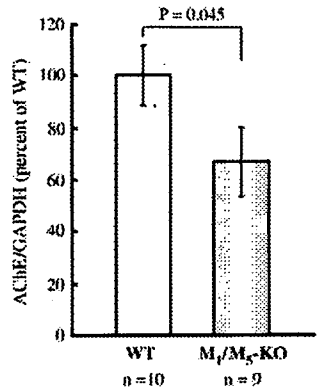

Fig. 4. Real time PCR analysis of AChE mRNA expression in spleen cells from M_1/M_5 KO and wild type (WT) C57BL/6JJcl mice. Spleen cells were cultured with 100 g/mL OVA for 48 h. The bars represent means±SEM for the levels of AChE mRNA normalized to the levels GAPDH mRNA and expressed a percentage of the mean levels in wild-type cells.

3.2. IFN- and IL-6 secretion from activated spleen cells

To assess the possibility that the modified IgG_1 response is related to cytokine production in M_1/M_5 KO mice, we measured IFN- and IL-6 production in antigen-stimulated spleen cells after 48 and 24 h of culture, respectively. Although OVA-activated spleen cells from M_1/M_5 KO mice tended to secrete less IFN- than spleen cells from wild-type nice, the difference was not statistically significant because of large inter-individual variation (Fig. 3A). On the other hand, spleen cells from M_1/M_5 KO mice secreted significantly less IL-6 than those from wild-type mice ($P=0.039$) (Fig. 3B), which suggests that disruption of the IgG_1 response is related to diminished IL-6 production by spleen cells.

3.3. Expression of ChAT and AChE mRNAs in OVA-activated spleen cells

Using conventional RT-PCR, we detected expression of AChE mRNA in spleen cells from both M_1/M_5 KO and wild-type mice, but no expression of ChAT mRNA was detected in either genotype (data not shown). Using real time PCR, we determined that the level of AChE mRNA expression was significantly lower in M_1/M_5 KO than in wild-type mice ($P=0.045$) (Fig. 4), suggesting that expression of AChE is regulated to some extent by M_1/M_5 mAChR signaling.

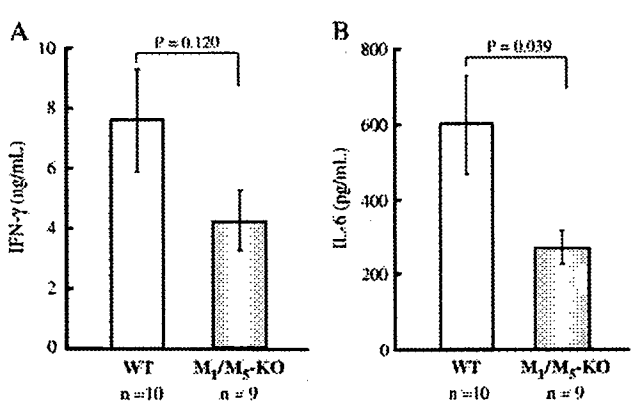

Fig. 3. Secretion of IFN- (A) and IL-6 (B) in spleen cells from M_1/M_5 KO and wild type (WT) C57BL/6JJcl mice. Spleen cells were cultured with 100 g/mL OVA for 48 (A) or 24 (B) h. The bars represent means±SEM.

3.4. Correlations between the measurements

Linear regression analyses revealed that IL-6 secretion from spleen cells correlated significantly with serum levels of anti-OVA-specific IgG_1 and with IFN- secretion, suggesting that cytokine production in immune cells is regulated, at least in part, by signaling *via* M_1 and/or M_5 mAChR-mediated pathways [$r=0.565$ ($P=0.012$) and $r=0.570$ ($P=0.011$)] (Table 1). In addition, we also detected a weak but significant correlation between serum levels of total IgM and anti-OVA-specific IgM ($r=0.473$, $P=0.041$).

Table 1
Results of linear correlation of analyses between A and B

A	B	*P* value	*r*
Anti-OVA-specific IgG_1	Total IgG_1	0.214	–
Anti-OVA-specific IgG_1	Anti-OVA specific IgM	0.355	–
Anti-OVA-specific IgG_1	IFN-	0.246	–
Anti-OVA-specific IgG_1	IL-6	0.012	0.565
Anti-OVA-specific IgG_1	AChE	0.722	–
Anti-OVA-specific IgM	Total IgM	0.041	0.473
Anti-OVA-specific IgM	IFN-	0.082	–
Anti-OVA-specific IgM	IL-6	0.216	–
Anti-OVA-specific IgM	AChE	0.102	–
Total IgG_1	Total IgM	0.450	–
Total IgG_1	IFN-	0.448	–
Total IgG_1	IL-6	0.907	–
Total IgG_1	AChE	0.781	–
Total IgM	IFN-	0.055	–
Total IgM	IL-6	0.360	–
Total IgM	AChE	0.452	–
IFN-	IL-6	0.011	0.570
IFN-	AChE	0.056	–
IL-6	AChE	0.302	–

$N=19$. *r*, correlation coefficient; OVA, ovalbumin; IFN- , gamma interferon; IL-6, interleukin-6; AChE, acetylcholinesterase.