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Experimental Autoimmune Myasthenia Gravis and CD5$^+$ B-lymphocyte Expression

Myasthenia gravis is one of the typical organ specific autoimmune disease and the CD5$^+$ B-lymphocytes are known to be associated with the secretion of autoimmune antibodies. The authors performed the study to establish an animal model of experimental autoimmune myasthenia gravis (EAMG) by immunizing the nicotinic acetylcholine receptor (AChR) and to understand CD5$^+$ B-lymphocyte changes in peripheral blood of EAMGs. Lewis rats weighing 150-200 g were injected subcutaneously three times with 50 μg AChR purified from the electric organ of *Torpedo marmorata* and Freund's adjuvant. The EAMG induction was assessed by evaluating clinical manifestations. The CD5$^+$ B-lymphocyte was double stained using monoclonal PE conjugated anti-CD5$^+$ and FITC conjugated anti-rat CD45R antibodies and calculated using a fluorescence-activated cell sorter (FACS). In three out of ten Lewis rats injected with purified AChR, the EAMG models were established. The animals showed definite clinical weakness responded to neostigmine; they had difficulty in climbing the slope, or easily fell down from a vertical cage. The range of CD5$^+$ B-lymphocytes of peripheral blood in the EAMG models was 10.2%-17.5%, which was higher than in controls. In conclusion, the EAMG models were successfully established and the CD5$^+$ B-lymphocyte expression in peripheral blood increased in EAMGs. This provided indirect evidence of the autoimmune pathomechanism of human myasthenia gravis.

Key Words : *Myasthenia gravis; Disease model, animal; Autoimmune diseases; Antigen, CD5; Acetylcholine receptors*

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INTRODUCTION

Myasthenia gravis is one of the most organ-specific autoimmune neurological diseases. According to studies, it occurs as the result of functional or structural impairment in neuromuscular transmission caused by attacks by autoimmune antibodies on nicotinic acetylcholine receptors (AChR) (1, 2, 3). However it is not well understood what is the starting point of autoimmune AChR antibody development in the body and what is the exact mechanism of neurophysiological transmission impairment by the antibodies in the neuromuscular junction (4, 5).

It has been hypothesized that autoimmune anti-AChR antibody development may proceed in one of two ways: either autoimmune anti-AChR antibody is continuously produced as the result of loss of normal control due to primary defect in certain B-lymphocytes, or B-lymphocytes capable of secreting anti-AChR antibody, which has been anergic since the initial stage of ontogenesis become active because they are sensitized to autoantigens or cross-reactable outside antigens (6).

Increases in both anti-AChR antibody titer and CD5$^+$ B-lymphocyte expression in the peripheral blood of patients with myasthenia gravis have been accepted as an indirect evidence of the autoimmune pathogenetic mechanism of myasthenia gravis (7); increased CD5$^+$ B-lymphocyte expression indicates that B-lymphocyte activation is able to produce anti-AChR antibody and such a phenomenon is also found in typical autoimmune diseases including B-cell leukemia, rheumatoid arthritis, and Sjögren's syndrome (8, 9).

The purpose of this study was to investigate changes in the CD5$^+$ B-lymphocyte expression in peripheral blood of rats with experimental autoimmune myasthenia gravis (EAMG). We first tried to induce animal models of myasthenia gravis by injecting purified AChR from electric organs of *Torpedo*

marmorata into Lewis rats in which the clinical and electrophysiological properties of myasthenia gravis were similar to those seen in humans. Then the frequency of $CD5^+$ B-lymphocyte expression in peripheral blood of these EAMG rats was measured.

MATERIALS AND METHODS

Purification of nicotinic AChR

The purified nicotinic AChR used in this experiment had been purified as follows. Ground electric organ of *Torpedo marmorata* (*Narke japonica*) was dissolved in 1% Triton X-100 20 mM phosphate buffer (100 mM Nacl, PH 7.4, 0.02% sodium azide, 0.1 mM PMSF). After centrifugation at 100,000 *g* for 15 min the supernatant was applied to a CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) affinity chromatography containing cobratoxin (0.2 mg/mL/resin) at 4℃, overnight (10). After washing the Sepharose column at least four times with 1% Triton X-100 phosphate buffer, AChR was eluted slowly for at least 2 hr with 1M calbacol and 1% Triton X-100 phosphate buffer (PH 7.5) at 4℃. The eluted receptor was dialyzed overnight against 500 mL of 0.02 M NaH_2PO_4/Na_2HPO_4 (pH 7.4), 0.01 mM NaCl, 1% Triton X-100 and during the following 24 hr the dialysis buffer was replaced three times. Purified AChR was then condensed to the required concentration.

Experimental animal model of myasthenia gravis

50 μg of purified AChR mixed with Freund's adjuvant was injected subcutaneously into female Lewis rats aged 8-12 weeks (body weight 150-200 g). Control rats were the same or similar in all respects, except that they received only Freund's adjuvant. The second injection with the same amount was done one week later. If there was no clinical evidence of myasthenia gravis four weeks later, one more injection was performed (6, 11, 12).

Muscle weakness in injected animals was regarded as evidence of experimental autoimmune myasthenia gravis. Daily body weight, any changes of activities, and response to neostigmine were checked (13), and if there was no evidence of muscle weakness, as indicated by changes in these parameters, the duration of holding the horizontal bar was measured. When clinical features of EAMG were evident in injected rats, these animals were sacrificed and the $CD5^+$ B-lymphocytes expression in peripheral blood was measured.

Measurement of $CD5^+$ B-lymphocytes

Using Histopaque-1077 (Sigma, St Louis, MO, U.S.A.), monocytes were separated from the venous blood of experimental animals. 1×10^6 mononuclear blood cells were dispersed in 200 mL volume and stained with antibodies labelled by immunofluorescence. In every experimental procedures, 0.04% sodium azide (Sigma, St Louis, MO, U.S.A.), 5% heat inactivated fetal calf serum and phosphate buffered saline containing Ca^{2+} and Mg^{2+} were used to minimize the risk of cell capping and infection by mycoplasma or viruses.

For grouping lymphocytes, double staining using PE conjugated anti-$CD5^+$ (Serotec, Oxford, U.K.) and FITC conjugated anti-rat CD45R (Pharmingen, San Diago, Ca, U.S.A.) monoclonal antibodies were used; these react specifically with $CD5^+$ antigen and B-cells, respectively. After staining and washing, the number and proportion of lymphocytes reacting with PE conjugated anti-$CD5^+$ and FITC conjugated anti-rat CD45R, respectively, were calculated and analyzed, using FACScan (Becton-Dickinson, San Jose, Ca, U.S.A.) projected by air cooled argon razor. 530 μm and 575 μm filters for PE and FITC were used and the results were automatically recorded in the chart (7).

RESULTS

Induction of experimental autoimmune myasthenia gravis (EAMG)

Myasthenia gravis developed in three out of ten Lewis rats treated with AChR subcutaneously, but in the other seven there was no evidence of myasthenia gravis until 15 weeks after the first injection.

In one rat (No. 2), definite muscle weakness developed 5 weeks later, and in the other two rats (Nos. 3, 4), decreased food intake, loss of body weight and slowness of movement were noticed 6 weeks later. Clinical weakness in thee EAMGs responded variably to neostigmine 0.1 mg. These rats were easily pulled backward by the tail without resistance and fell easily when holding a vertical bar of their cage (Fig. 1).

Proportions of $CD5^+$ B-lymphocyte expression in peripheral blood

The $CD5^+$ B-lymphocytes were measured in peripheral blood samples of three established EAMGs (Nos. 2, 3, 4), two rats which did not develop clinical weakness by the injection of AChR (Nos 1, 5), and two control rats which received only Freund's adjuvant (Nos. 6, 7) (Table 1). In rats with EAMG, the proportion of $CD5^+$ B-lymphocytes among total lymphocytes increased; in rat number 2, this was 17.5%; in number 3, 10.2%; and in number 4, 12.5%.

The $CD5^+$ B-lymphocyte proportions in rats without muscle weakness (Nos. 1, 5) were 2.46% and 1.06%, respectively. In control rats (Nos. 6, 7), the $CD5^+$ B-lym-

Fig. 1. Clinical manifestations of experimental autoimmuine myasthenic gravis (EAMG). The experimental Lewis rat (No. 2), which was injected three times with 50 μg AChR showed clinical weakness, low body weight and decreased activity and easily fell down from the vertical cage (B). However, a control Lewis rat (No. 7) did not show clinical weakness and decreased activity (A).

Table 1. Experimental autoimmuine myasthenia gravis (EAMG) and CD5+ expression

Lewis rats	Amounts of AChR injection	Clinical weakness	Time of sacrifice after AChR injection (weeks)	CD_{5}^{+} B-lymphocyte in peripheral blood(%)
Experimental groups				
1	50 μg×3 SC	None	15	2.46
2	50 μg×3 SC	5th wk	6	17.5
3	50 μg×3 SC	6th wk	7	10.2
4	50 μg×3 SC	6th wk	6	12.5
5	50 μg×3 SC	None	7	1.06
Control groups				
6	—	None	6	0.0
7	—	None	6	0.2

None; Clinical weakness did not develop after the injection of AChR 50 μg & Freund's adjuvant (Nos. 1, 5) or only Freund's adjuvant (Nos. 6, 7).
SC; Subcutaneously injected.

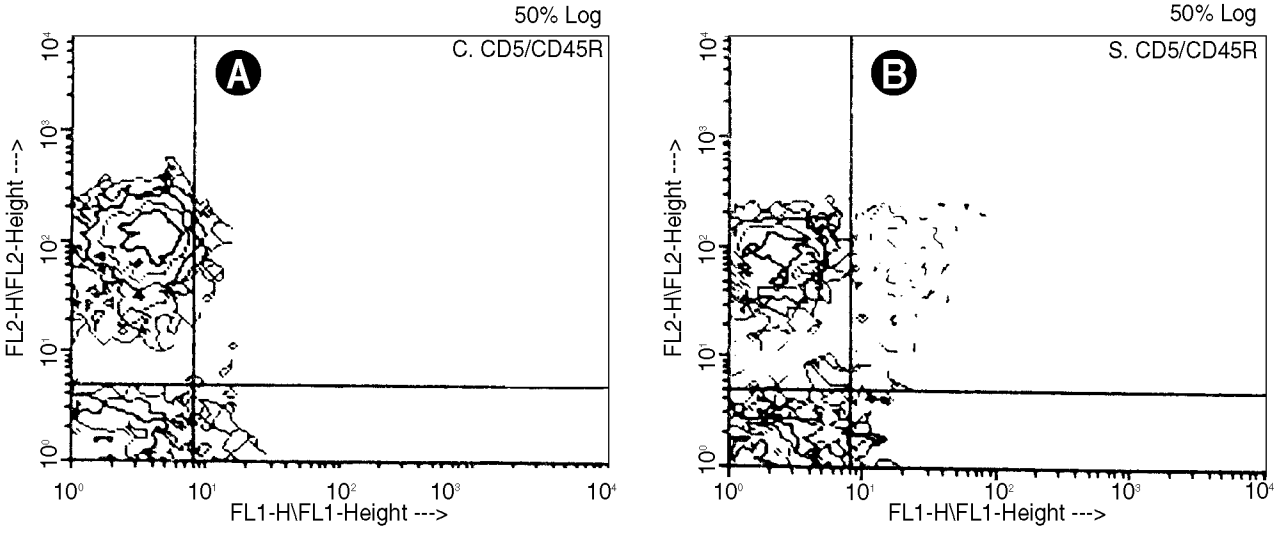

Fig. 2. The CD5+ B-lymphocyte expression in peripheral blood by fluorescence activated cell sorter (FACS). The frequency of CD5+ B-lymphocytes was 0.2% in normal control (No. 7) (A), and 17.5% in the EAMG rat (No. 2) which showed clinical manifestations similar to those of human myasthenia gravis after three injections of 50 μg AChR (B).

phocyte was not detected (0.0%, 0.2%).

DISCUSSION

Myasthenia gravis (MG) is one of the most common neuromuscular junction disorders and characterized by voluntary muscle weakness and fatigue. Its clinical features vary; there may be mild symptoms such as ptosis and diplopia, or more severe ones such as upper extremity weakness, dysarthria and respiratory failure. Characteristically, the severity of these features fluctuates; clinical symptoms improve with rest or sleep and are aggravated by the repetitive use of involved muscles (5, 14).

Studies have shown that MG is caused by functional and structural abnormalities of AChR resulting from an attack by autoimmune AChR antibody to AChR on postsynaptic membrane of neuromuscular junction. AChR attacked by autoimmune AChR antibody is unable to effectively transmit the action potential of peripheral nerves to muscle fibers, causing muscle weakness to develop (3, 5, 15).

Since most patients with MG have thymic abnormalities, it was previously thought that the causes of MG were closely associated with the autoimmune mechanism (16). In 1939 Blalock reported that muscle biopsy of patients with MG revealed severe lymphocyte infiltration (17) and the phenomenon of MG improved by thymectomy (17, 18, 19). Moreover, many autoimmuine diseases, such as hypothyroidism, hyperthyroidism, rheumatism, and diabetes mellitus, tend to be more frequent in patients with MG, and this also suggests that an autoimmune mechanism is involved in the pathogenesis of MG (3, 20, 21, 22).

Induction of the EAMG animal models by the injection of AChR or by passive transfer provided direct evidence that autoimmune mechanism is involved in the development of MG. It was reported that EAMG could be induced in mammals, birds, and reptiles by injection of purified AChRs from electric organ or human muscles with complete Freund's adjuvant. In the early 1970s Patrick and Lindstrom first reported that an animal model with clinical features very similar to those found in human MG was induced by injecting AChR extracted from the electric eel into rabbits (13). The rabbits showed initial weakness between 7 and 15 after AChR injection, which was remitted spontaneously after 2-3 days. The second episode of weakness between 26 and 35 days and were slowly progressive. However they did not show typical features of symptom flucuation as in human MG. In EAMG rabbit models, increased circulating antibodies to eel AChR and response to neostigmine were demonstrated (12, 13).

In our experiment, muscle weakness was observed in three out of ten Lewis rats. Clinical weakness was found in 4-6 weeks after the injection of AChR purified from *Torpedo marmarata*, but no fluctuation in muscle strength was noted in all three (13). They had difficulty in climbing a slope, easily fell from a vertical cage, which responded variably to neostigmine. All three EAMGs also showed body weight below average and poor eating (12, 13). The above features meant that three EAMGs were induced definitely by the AChR injection.

Passive transfer, another method of EAMG induction, has been reported. The sera of patients with MG is injected into animals to cause the animal model of myasthenia gravis (23, 24). Drachman et al. reported that in 94% of patients with MG induced characteristic signs of EAMG in the animals involved (18). According to the report, the injection of purified IgG was more efficient than the serum and the decreased amplitude of miniature end plate potential (MEPP) was characteristic.

Tzartos and Lindstrom demonstrated that anti-AChR antibody was polyclonal and its binding sites were different (25). Most anti-AChR antibody bound to "major immunogenic region (MIR)" in the extracellular portion of the α-subunit of AChR (27); only a small proportion bound to other sites of this subunit (25, 26, 27). It has been shown that only anti-AChR antibody binding to MIR plays a major role in the pathogenesis of MG and is capable of cross-linking and modulating AChR and the passive transfer of EAMG.

$CD5^+$ is a surface marker of lymphocytes, and is abundant on the surface of T lymphocytes (7, 8, 9). Lymphocytes stained by anti-$CD5^+$ antibodies are, therefore, mostly T lymphocytes and only a few B-lymphocytes are known to react with anti-$CD5^+$ antibodies. $CD5^+$ B-lymphocytes are reported to secrete autoimmune antibodies and are increased in representative autoimmuine diseases such as B-cell chronic lymphocytic leukemia and rheumatoid arthritis (8). In humans, $CD5^+$ B-lymphocytes account for 2-3% of total lymphocytes and 20-30% of B lymphocytes, while in Lewis rats, the proportions vary.

In our study (Table 1), the number of $CD5^+$ B-lymphocytes were markedly higher in rats with clinical muscle weakness (Nos. 2, 3, 4) than in either those injected with AChR but without muscle weakness (Nos. 1, 5) or normal controls (Nos. 6, 7). In rats injected with AChR but without muscle weakness, $CD5^+$ B-lymphocytes were very low (2.46% and 1.06%, respectively), and did not show any increased number, compared with those in normal controls.

These findings are consistent with the results by Ragheb and Lisak who reported increased $CD5^+$ lymphocytes in the peripheral blood of patients with MG (9) and suggest that the $CD5^+$ B-lymphocyte would be associated with the secretion of some autoimmune antibodies in EAMGs.

T as well as B lymphocytes are known to play a very important role in the pathogenesis of MG. For example, in cultures of lymphocytes from peripheral blood of patients with

MG induced by AChR from electric eels lymphocyte proliferation was observed and this phenomenon has been interpreted as an evidence supporting the previous sensitization of these lymphocytes to AChR (21, 28). In addition, histologic findings have indicated that in the thymus of patients with MG, T-lymphocytes are decreased and B-lymphocytes are markedly increased and these B-lymphocytes are located mainly in the medulla of thymus (3). Interrelationships between T and B lymphocytes are quite complex and all such cells are thought to be involved in both humoral and cellular mechanisms in MG (3). From the immunological standpoint, MG could be caused by humoral immune reaction provoked by abnormal T-cell regulation.

By injecting AChR extracted from electric eels into Lewis rats, the authors of this report successfully induced EAMG in a form similar to that of MG in humans; in these rats, increased $CD5^+$ B-lymphocyte expression was observed. These findings suggest that an autoimmune pathogenetic mechanism is involved in human MG.

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