Effect of Myasthenia Serum and Anti- acetylcholine Receptor Monoclonal Antibody on the Distributions of T-cell Subsets in Myasthenia Patients and Normal Subjects*

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SUMMARY The distribution of OKT4 $^+$ helper and OKT8 $^+$ suppressor T-cells was studied in 41 myasthenia gravis (MG) patients and 20 normal subjects. When a purified T-cell population was studied, the mean percentage of OKT4 $^+$ cells of MG patients was slightly lower than that of normal subjects ($41.6 \pm 3.9\%$ vs $43.5 \pm 4.7\%$, $p = 0.04$); the mean percentage of OKT8 $^+$ cells in the former was much lower when compared with that in the latter ($29.2 \pm 4.5\%$ vs $33.8 \pm 6.2\%$, $p < 0.001$), resulting in a significantly higher ratio of OKT4 $^+$/OKT8 $^+$ cells in MG patients ($1.45 \pm 0.22\%$ vs $1.32 \pm 0.20\%$, $p < 0.02$). Neither the age of onset of disease nor sex had any effect on the distribution of T-cell subsets. The mean percentages of OKT4 $^+$ and OKT8 $^+$ cells of MG patients and normal subjects were decreased after incubation with MG sera, although only the decrease in OKT4 $^+$ cells reached a statistically significant level, but no such phenomenon was noted after incubation with normal sera. Furthermore, the percentages of OKT4 $^+$ and OKT8 $^+$ cells of normal subjects were also decreased after incubation with an anti-acetylcholine receptor (AChR) monoclonal antibody, BK73G. It is therefore concluded that the presence of anti-AChR antibody may account for the immunoregulatory aberration manifested by the loss of suppressor T-cells and the increased helper/suppressor cell ratio in MG patients.

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Among the various auto-antibodies that have been found in the sera of myasthenia gravis (MG) patients,[1-3] the anti-acetylcholine receptor (AChR) antibodies are considered to play a significant pathogenic role.[4] Many investigators have reported evidence suggesting that the abnormal auto-antibodies may result from abnormal cell-mediated immunity.[3,5-7] The changes of T-cell subpopulations in MG patients determined by enumeration of Tμ and Tγ cells, according to the method of Moretta *et al*,[8] have been studied by many investigators; however, the results did not show any consistent findings.[9-12] These discrepancies may have been due to differences in sampling time, separation technique of lymphocytes, the reagents used for enumeration of T-cell subsets and clinical stage.

Study of human T-cell functions had been hampered largely by the lack of defined T-cell differentiation antigens like Ly system in mice;[13] however, this problem has been overcome by the hybridoma technique.[14] By using this technique, a series of human T-cell differentiation antigens, which possess particular immunobiological functions, was developed.[15,16] The detection of these antigens has been considered more specific and more reproducible than the detection of T-cell surface receptors. In this paper we

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used monoclonal antibodies directed against T-cell surface antigens to study the distribution of helper and suppressor T-cells in MG patients. Moreover, we also tried to explore the role of anti-AChR antibody in the occurrence of altered T-cell function in MG patients by using anti-AChR monoclonal antibodies.

MATERIALS AND METHODS

Patients and control subjects

Forty-one MG patients (17 males and 24 females, ranging in age from 7 to 60 years), who had been diagnosed and treated at the Department of Neurology, National Taiwan University Hospital, and 20 normal controls (10 males and 10 females, ranging in age from 18 to 33 years) were included in this study. According to Osserman's criteria, 22 patients belonged to group I, 11 to group IIa, and eight to group IIb. Twenty-four patients had undergone anticholinesterase therapy only, nine had received steroids, and eight had been treated with a combination of anticholinesterases and steroids. Thymectomy had been performed on nine patients; among them, three had thymic hyperplasia and four had thymoma. The intervals between thymectomy and immunological studies ranged from one month to 84 months, with a mean of 32 months.

Isolation of mononuclear cells and T lymphocytes

The peripheral blood mononuclear cells (MNCs) were isolated by the method of Boyum.[17] Pure lymphocyte suspensions were prepared by depleting monocytes from MNCs by the method of plastic petri dish adherence,[18] and T cells were purified by E-rosetting followed by Ficoll/Hypaque centrifugation performed twice.[19] Over 97 per cent of the T cells prepared this way were E-rosette positive. The T cells were finally resuspended in suspension medium (RPMI-1640 supplemented with 5% heat-inactivated foetal calf serum and 25 mM HEPES buffer) at a concentration of $5x10^6$ cells/ml.

Enumeration of T-cell subpopulations by indirect immunofluorescence

Monoclonal antibodies, anti-OKT4 and anti-OKT8, were purchased from Orthoclone Pharmaceutical Corp. (Raritan, N.J., U.S.A.). Fluorescein-conjugated goat IgG anti-mouse IgG was obtained from Pel-Freez Biologicals (Rogers, Arkansas, U.S.A.). Enumeration of T-cell subpopulations was done according to the instructions included in the monoclonal antibody packages. Five microlitres of reconstituted monoclonal antibody solution were added to the purified T-cell suspensions at a concentration of $5x10^6$ cells/ml in suspension medium. The mixture was incubated in ice water for 30 minutes and agitated every 10 minutes. The cells were then washed twice with 2 ml of wash medium (phosphate buffered saline, pH 7.2) and resuspended in 0.1 ml of wash medium. One-tenth ml of fluorescein-conjugated goat anti-mouse IgG (1 to 1 dilution) was added to the cell suspension; it was incubated in ice water for 30 minutes and agitated every 10 minutes. Finally the cells were washed twice with 2 ml of wash medium, resuspended in 0.05 ml of mounting medium (phosphate buffered saline, pH 7.2, containing 30% glycerol), and the fluorescent cells counted with the aid of an Olympus fluorescence microscope. Background staining was determined by reaction of the cell suspension with a 1:1 dilution of fluorescein-labelled goat anti-mouse IgG after incubation with bovine serum albumin at a concentration of 1 mg/ml in RPMI-1640 (the medium used in lyophilising anti-OKT antibodies). Uniformly, less than 5 per cent of the cells appeared fluorescent in the reagent control.

Incubation of cells with serum from either MG patients or normal subjects

Two hundred and fifty microlitres of T-lymphocyte suspension and an equal volume of serum were mixed and incubated in a 37°C water bath for 30 minutes, washed three times, then resuspended in suspension medium at a concentration of $5x10^6$ cells/ml. Then, the $OKT4^+$ and $OKT8^+$ cells were enumerated.

Incubation wih monoclonal anti-AChR antibodies

Four monoclonal antibodies, BK73G, BK56I, BK38I and D547, were used in this study. BK73G and BK56I are anti-AChR antibodies that bind to human end-plates and induce experimental MG in rats; BK38I is an anti-AChR antibody that does not bind to mammalian receptors, and D547 is a monoclonal antibody directed against mammalian estrogen receptors (a gift of Dr. G. Greene).[20] Each monoclonal antibody was given a code number that was not identified until the entire study was completed. Two hundred and fifty microlitres of T-lymphocyte suspension were mixed with an equal volume of the monoclonal anti-AChR antibody. The mixtures were incubated in a 37°C water bath for 30 minutes, washed three times, resuspended in suspension medium at a concentration of $5x10^6$ cells/ml, then $OKT4^+$ and $OKT8^+$ cells were enumerated.

Statistics

The Student's t-test and paired t-test were used for statistical analysis.

RESULT

Table 1 and Figure 1 show the distribution of T-cell subsets among the purified T-cell populations from MG patients and normal subjects. The mean percentage of $OKT4^+$ cells of MG patients was slightly

Table 1 Distributions of $OKT4^+$ cells and $OKT8^+$ cells and $OKT4^+/OKT8^+$ ratios among purified T cells in MG patients and normal subjects.

	$OKT4^+$ (%)	$OKT8^+$ (%)	T4/T8 ratio
MG patients (n=41)	41.6 ± 3.9*[c]	29.2 ± 4.5[a]	1.45 ± 0.22[b]
Group[d] I (n=22)	41.2 ± 3.9	28.3 ± 4.6[a]	1.49 ± 0.3[b]
Group IIa (n=11)	41.2 ± 4.3	29.4 ± 2.3[b]	1.44 ± 0.13
Group IIb (n=8)	41.6 ± 3.5	31.5 ± 5.5	1.35 ± 0.21
Normal subjects (n=20)	43.5 ± 4.7	33.8 ± 6.2	1.32 ± 0.2

*Mean ± SD.
a : $p < 0.001$ compared with normal subjects.
b : $p < 0.02$ compared with normal subjects.
c : $p = 0.04$ compared with normal subjects.
d : Clinical subtypes classified by Osserman's criteria.

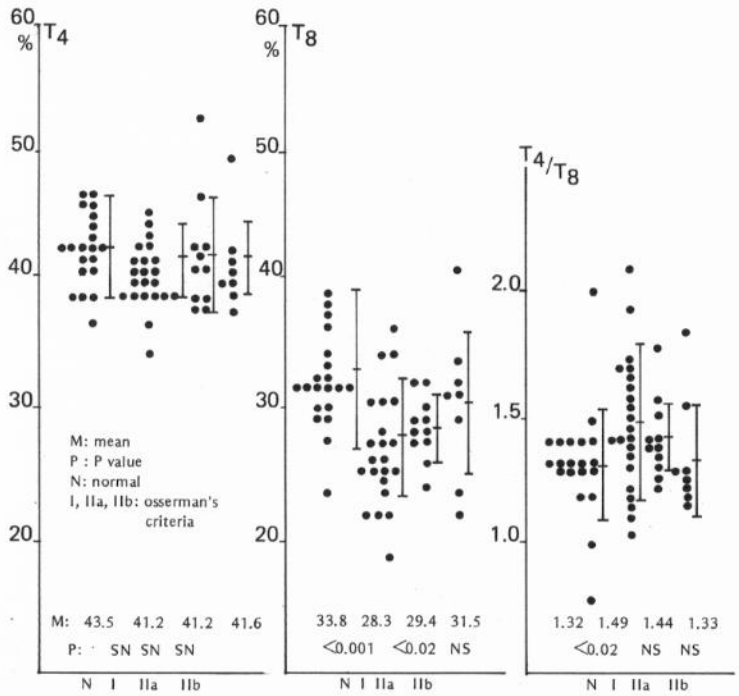

Fig. 1 Relationship between T-cell subpopulations and clinical subtypes. P values were obtained by comparing data of patients with those of normal subjects (Table 1), using the Student's t-test.

Table 2 Distribution of $OKT4^+$ cells and $OKT8^+$ cells and $OKT4^+/OKT8^+$ ratio in MG patients receiving various modes of treatment.

	$OKT4^+$ cells (%)	$OKT8^+$ cells (%)	T4/T8 ratio
Steroid treated group (n = 17)	41.1 ± 2.9*	29.4 ± 5.7[a]	1.45 ± 0.27
Anti-cholinesterase treated group (n = 24)	41.9 ± 4.6	29.0 ± 3.2[b]	1.45 ± 0.17[a]
Thymectomy group (n = 9)	40.2 ± 1.6	29.7 ± 3.7	1.37 ± 0.19

*Mean ± SD.
a : $p < 0.02$ compared with normal subjects.
b : $p < 0.01$ compared with normal subjects.

lower than that of normal subjects (41.6 ± 3.9% vs 43.5 ± 4.7%, $p = 0.04$), whereas that of $OKT8^+$ cells in the former was significantly lower than that in the latter (29.2 ± 4.5% vs 33.8 ± 6.2%, $p < 0.001$), resulting in the increased $OKT4^+/OKT8^+$ ratio in MG patients (1.45 ± 0.22 vs 1.32 ± 0.2, $p < 0.02$). When the MG patients were divided into three clinical subtypes according to Osserman's criteria, the same tendency was noted in group I and group IIa.

The mean percentages and the ratio of $OKT4^+/OKT8^+$ in steroid-treated, non-steroid treated and thymectomy patients are shown in Table 2. There was no significant difference in mean percentages in $OKT4^+$ cells and $OKT8^+$ cells among these groups. The patients were also compared according to the age of onset and sex (Table 3 and Figure. 2). The myasthenic symptoms occurred before 30 years of age in most female patients; whereas males usually developed symptoms after 30 years of age. All four patient groups had low $OKT8^+$ ($p < 0.05$) percentages. All had increased $OKT4^+/OKT8^+$ ratios ($p < 0.03$), except the early-onset female group. In addition, the late-onset group of females had significantly lower $OKT8^+$ percentages than did the early-onset group of females ($p < 0.05$).

The mean percentages of $OKT4^+$ and $OKT8^+$ cells before and after incubation with sera of MG patients and normal subjects are shown in Table 4 and Figure. 3. After incubation with MG sera, the percentages of $OKT4^+$ and $OKT8^+$ cells were decreased in MG patients and normal subjects, but only the decrease in $OKT4^+$ cells reached a statistically significant level ($p < 0.001$). In contrast, no such phenomenon was found when cells were incubated with normal sera. The $OKT4^+/OKT8^+$ ratio was not significantly changed in either MG patients or normal subjects after incubation with normal or MG sera. The mean percentages of $OKT4^+$

Table 3 Effects of sex and age of onset of disease on distribution of $OKT4^+$ cells, $OKT8^+$ cells and $OKT4^+/OKT8^+$ ratios in MG patients.

Age of onset	$OKT4^+$ cells (%)	$OKT8^+$ cells (%)	T4/T8 ratio
Males after 30 (n = 11)	41.1 ± 5.7*	29.4 ± 2.4[a]	1.47 ± 0.21[b]
before 30 (n = 6)	42.3 ± 4.4	28.5 ± 6.8[c]	1.54 ± 0.29[b]
Females after 30 (n = 6)	41.5 ± 2.6	26.5 ± 3.3[d,e]	1.58 ± 0.14[d]
before 30 (n = 18)	40.4 ± 2.1[a]	29.7 ± 4.1[a]	1.38 ± 0.15

*Mean ± SD.
a : $p < 0.02$ compared with normal subjects.
b : $p < 0.03$ compared with normal subjects.
c : $p < 0.05$ compared with normal subjects.
d : $p < 0.01$ compared with normal subjects.
e : $p < 0.05$ compared with females with onset before age 30.

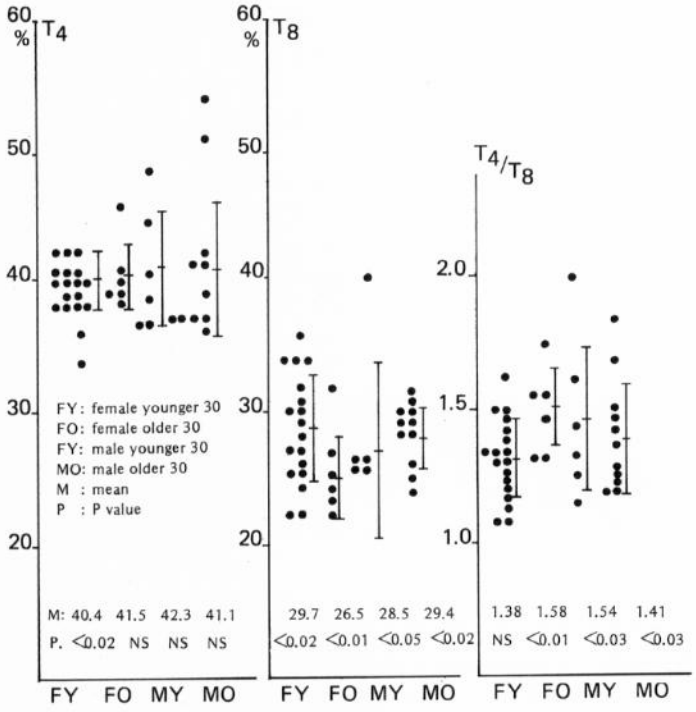

Fig. 2 Relationship between T-cell subpopulations and sex and age of onset. P values were obtained by comparing data of patients with those of normal subjects (Table 1), using the Student's t-test.

and $OKT8^+$ cells before and after incubation with monoclonal anti-AChR antibodies are shown in Table 5 and Figure 4. The percentages of $OKT4^+$ and $OKT8^+$ cells were decreased only when they were incubated with monoclonal antibody BK73G ($p<0.001$); no such change was noted when they were incubated with other monoclonal antibodies.

DISCUSSION

The availability of monoclonal antibodies against T cells has made it possible to enumerate T-cell subpopulations in various diseases. In this study, we intended to clarify the immunopathogenesis of MG by using monoclonal antibodies to enumerate the helper T cells ($OKT4^+$ cells) and suppressor T cells ($OKT8^+$ cells) in such patients. There was no difference in the mean percentage of $OKT4^+$ cells between MG patients and normal subjects. In contrast, our previous study showed an increase of $T\mu$ cells (helper T-cells) in MG patients[12] by using the method of Moretta *et al.*[8] The reason for this discrepancy may be due to the different methods used to count the helper T cells, because $OKT4^+$ cells represent the T cells carrying the differentiation antigen of OKT4 while $T\mu$ cells represent the T cells bearing receptors for Fc IgM.

Loss of suppressor T-cell function in MG patients has been reported by several investigators.[21,22] In this study, the decrease in $OKT8^+$ cells is in agreement with our previous report[12] and the results obtained by others.[23,24]

The ratio of $OKT4^+/OKT8^+$ has been used as a parameter of T-cell regulation in cellular immunity. An increase in this ratio, which may be due to either an increase in helper T cells or a decrease in suppressor T cells, has been found in a large proportion of patients with autoimmune disease.[25] In this study, we also found an increased $OKT4^+/OKT8^+$ ratio in MG patients (group I and group IIa), which was due to a decrease in $OKT8^+$ suppressor cells. The normal value for group IIb may be due to the small number of cases studied in this series.

The heterogeneity of MG in terms of immunological status has been reported by Sagar *et al*[7] and Compston *et al.*[26] The latter divided MG into three clinical types, i.e., patients with thymoma, late onset without thymoma and early onset without thymoma. In each clinical type, a characteristic immunological status was demonstrated. Sagar *et al*[7] also reported that the

Table 4 Distribution of $OKT4^+$ cells and $OKT8^+$ cells and their ratios before and after incubation with normal and MG sera.

	$OKT4^+$ cells (%)	$OKT8^+$ cells (%)	T4/T8 ratio
MG patients			
normal serum	40.9 ± 6.4 (before)*	29.2 ± 5.4 (before)	1.43 ± 0.18 (before)
(n = 13)	41.9 ± 2.9 (after)	29.5 ± 4.5 (after)	1.44 ± 0.20 (after)
autologous	41.2 ± 4.6 (before)	29.2 ± 4.6 (before)	1.43 ± 0.19 (before)
serum (n=39)	36.9 ± 5.2[a] (after)	27.5 ± 5.3[b] (after)	1.38 ± 0.29 (after)
Normal subjects			
Normal serum	43.9 ± 5.2 (before)	34.1 ± 6.8 (before)	1.33 ± 0.24 (before)
(n=16)	40.9 ± 5.7 (after)	35.0 ± 6.4 (after)	1.20 ± 0.30 (after)
MG serum	44.0 ± 5.2 (before)	32.7 ± 4.6 (before)	1.36 ± 0.20 (before)
(n=15)	37.3 ± 3.0[a] (after)	29.8 ± 4.4[c] (after)	1.29 ± 0.20 (after)

*Mean ± SD. The comparisons were done by paired t-test.
a : $p < 0.001$ compared with before.
b : $p = 0.067$ compared with before.
c : $p = 0.044$ compared with before.

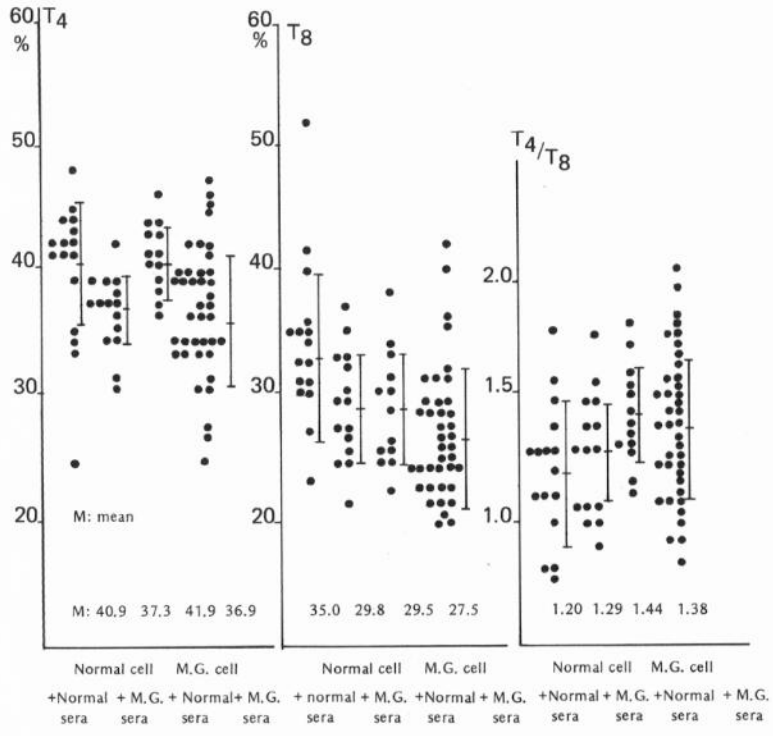

Fig. 3 The effect of patients' sera on the expression of OKT4 and OKT8 antigens on T-cells.

early-onset group produced a wide range of auto-antibodies but without significant change in cellular immunity, whereas the late-onset group, which produced only a few auto-antibodies, had impaired cellular immunity. Our findings that there is a significant difference in $OKT8^+$ cells between the early-onset and late-onset female groups ($p < 0.05$) and that the lowest percentage of $OKT8^+$ cells occurs in the late-onset female group (Table 3) are compatible with the results of Sagar *et al.*[7]

In systemic lupus erythematosus, Koike *et al*,[27] Sakane *et al*[28] and Strelkauskas *et al*[29] demonstrated a circulating cytotoxic auto-antibody directed against the suppressor T-cell subset which resulted in the loss of suppressor cells. A similar serum factor may also play an important role in the pathogenesis of MG. Richman *et al*[30,31] demonstrated a nicotinic AChR on T cells that appears to affect suppressor cell function. They proposed that an auto-immune response may be directed against lymphocytic nicotinic AChR in MG with resultant injury to that receptor. Mischak and Dau[32] also mentioned that the anti-AChR antibody may react with the receptor on lymphocytes and produce, or maintain, significant immunoregulatory alterations such as suppressor cell dysfunction. Shore *et al*[22] also demonstrated that a serum factor capable of inhibiting E-rosette formation was present in the IgG fraction in MG children. Our previous study[12] showed that MG patients' sera could block the Fc receptors on T cells, especially those on Tγ cells. Again, in this study we demonstrated that both $OKT4^+$ and $OKT8^+$ cells decreased after incubation with MG sera. Furthermore, an anti-AChR monoclonal antibody (BK73G), which had been shown to be capable of inducing experimental MG,[20] was found to be able to block the expression of $OKT4^+$ and $OKT8^+$ antigens on T cells. Taken together, all these lines of evidence strongly suggest that an anti-AChR antibody-like factor is present in MG patients and the binding of such a factor to the ACh receptors may alter the regulatory T-cell function which is ofen seen in MG patients.

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Table 5 Distribution of T-cell subpopulations of normal subjects before and after incubation with different monoclonal antibodies.

	$OKT4^+$ cells (%)	$OKT8^+$ cells (%)	T4/T8 ratio
Normals + Medium (n = 16)	43.9 ± 5.0*	33.0 ± 4.6	1.35 ± 0.20
Normals + BK73G (n = 16)	35.6 ± 7.3[a,b]	28.9 ± 4.9[a]	1.28 ± 0.35
Normals + BK561 (n = 16)	40.4 ± 4.8	34.0 ± 5.5	1.23 ± 0.31
Normals + BK381 (n = 16)	39.6 ± 5.7	33.1 ± 4.5	1.23 ± 0.27
Normals + D547 (n = 16)	42.2 ± 3.9	33.9 ± 4.6	1.26 ± 0.25

*Mean ± SD. The comparisons were done by paired t-test.
BK73G and BK56I are monoclonal antibodies against the human ACh receptor. BK38I is an antibody that does not bind to mammalian ACh receptors.
a : $p < 0.01$ compared with D547 (monoclonal antibody against the mammalian estrogen receptor).
b : $p < 0.001$ compared with normal subjects (no antibody added).

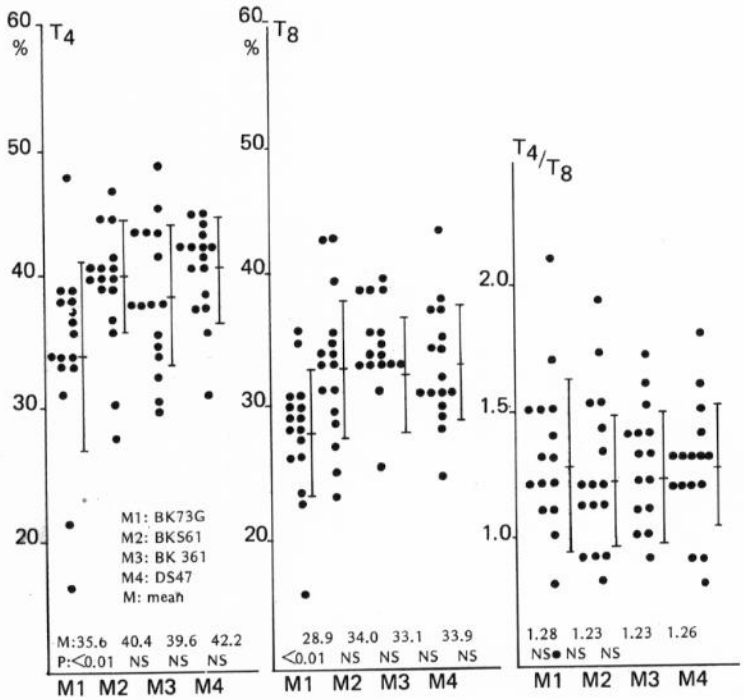

Fig. 4 The effect of anti-acetylcholine receptor monoclonal antibodies on the expression of OKT4 and OKT8 antigens on T cells. P values were calculated by the paired t-test.

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