

Stimulatory Roles of Muscarinic Acetylcholine Receptors on T Cell Antigen Receptor/CD3 Complex-Mediated Interleukin-2 Production in Human Peripheral Blood Lymphocytes

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SUMMARY

It is known that there are some bidirectional interactions between the nervous and the immune systems via neurotransmitters and cytokines. To clarify whether any neurotransmitters modulate lymphocyte functions, we examined the effects of oxotremorine-M (Oxo-M) on interleukin-2 (IL-2) production in human peripheral blood lymphocytes by using enzyme-linked immunosorbent assays, Northern blot analyses, reverse transcriptase-polymerase chain reaction, and fluorescence-activated cell sorter. Pretreatment of cells with Oxo-M (10 nM to 10 μ M) for 4–24 hr enhanced phytohemagglutinin (PHA)-induced IL-2 mRNA expression and markedly increased IL-2 production

compared with those induced by PHA alone. Oxo-M alone did not affect IL-2 mRNA expression and IL-2 production. In CD3-positive T cells, pretreatment with Oxo-M for 24 hr enhanced PHA-induced IL-2 production. Furthermore, pretreatment with Oxo-M enhanced PHA-induced mRNA expression of the α and β subunits of IL-2 receptors and DNA synthesis. Cytometric analysis showed Oxo-M treatment did not up-regulate expression of cell surface molecules such as CD3, CD2, CD4, CD8, and IL-2 receptors. These results suggest that activation of muscarinic receptors enhances T cell antigen receptor/CD3-induced IL-2 production.

Several studies suggest that the nervous system regulates immune functions. Electron microscopic studies demonstrate that the vagus nerves exist in lymphoid tissues such as the spleen and thymus and that nerve terminals form synaptic contacts with lymphocytes (1). In addition, lymphocytes possess various neurotransmitters and neuropeptide receptors, such as β -adrenoceptors, muscarinic ACh receptors, 5-hydroxytryptamine₁ receptors, and type B cholecystokinin receptors. It is known that the β -adrenoceptor/G_s/adenylyl cyclase system is involved in the inhibitory regulation of IL-2 production and thus proliferation of T cells (2). Despite several studies on the functional roles of the β -adrenergic system, details of the biochemical mechanisms of the role of the muscarinic ACh system in the immune system (3) have not been elucidated. Muscarinic ACh receptors mediate changes in cGMP levels in T cells (4). Pharmacologically distinguish-

able forms of the muscarinic ACh receptors occur in different tissues. They have been classified as M1, M2, and M3 subtypes on the basis of the effectiveness of antagonists (i.e., pirenzepine, AF-DX 116, and 4-DAMP). With molecular cloning techniques and by analyzing cDNA sequences, the muscarinic receptor subtype genes have been characterized and named m1 to m5 (5). Recently, we demonstrated that the muscarinic subtype of ACh receptors is expressed in a human Jurkat leukemic helper T cell line (JP111) and that activation of these receptors induces PLC activation (6).

Activation of the multicomponent TCR/CD3 complexes by antigen, lectins, or anti-CD3 mAb activated multiple signal transduction pathways, such as the rapid PLC-mediated hydrolysis of phosphatidylinositol biphosphate into two secondary messengers, inositol-1,4,5-triphosphate and diacylglycerol (7), resulting in an increase in $[Ca^{2+}]_i$ and the activation of PKC. Another rapid event that follows TCR/CD3 activation is the phosphorylation of the tyrosine residues of several proteins, including the TCR ζ subunit and

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ABBREVIATIONS: ACh, acetylcholine; TCR, T cell antigen receptor; mAb, monoclonal antibody; PLC, phospholipase C; PKC, protein kinase C; IL-2, interleukin-2; CsA, cyclosporin A; Oxo-M, oxotremorine-M; PHA, phytohemagglutinin; TPA, 12-O-tetradecanoylphorbol-13-acetate; DiOC₆, 3,3'-dihexyloxycarbocyanine iodide; TdR, [*methyl*-³H]thymidine (thymine deoxyriboside); hPBL, human peripheral blood lymphocytes; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorter; RT, reverse transcription; PCR, polymerase chain reaction; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; FCS, fetal calf serum; SSC, standard saline citrate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; AM, acetoxymethyl ester.

PLC- γ 1 (8). The downstream target of these activated tyrosine kinases is possibly involved in p21^{ras} activation (9).

IL-2 is the first in the series of lymphocytotropic hormones, and it has a pivotal role in the generation and regulation of immune responses. Activation of TCR/CD3 complexes induces IL-2 production in T cells, and some immunosuppressants, such as CsA and FK-506, inhibit this IL-2 production (10). CsA- and FK-506-binding proteins identified as immunophilins (cyclophilins and FKBP, respectively) (11) are targets for Ca²⁺/calmodulin-dependent phosphatase 2B, also known as calcineurin (12). However, the detailed mechanisms of IL-2 production and regulation are not clear.

In this study, we found that muscarinic ACh receptors exist in both CD4- and CD8-positive lymphocytes and that activation of the receptors enhances TCR/CD3 complex-induced mRNA expression of IL-2 and IL-2 receptor subunits, production of IL-2, and cell proliferation.

Experimental Procedures

Materials. FK-506 (tacrolimus) and CsA were kindly donated by Fujisawa Pharmaceutical (Osaka, Japan) and Sandoz (Basel, Switzerland) respectively. Pirenzepine and AF-DX 116 were donated by Nippon Boehringer Ingelheim (Hyogo, Japan) RPMI-1640 and FCS were from GIBCO BRL (Gaithersburg, MD). Ficoll-Paque was from Pharmacia LKB (St. Quentin, France); Oxo-M and 4-DAMP were from Research Biochemical (Natick, MA). Fura-2/AM was from Dojin Laboratories (Kumamoto, Japan). PHA, TPA, and DiOC₆ were from Sigma Chemical (Poole, Dorset, UK). Mouse mAb of anti-human CD3 (NU-T3), CD4 (NU-TH1), and CD8 (NU-TS/C) were from Nichirei (Tokyo, Japan). cDNA probes of IL-2 were from Oncor (Gaithersburg, MD). IL-2 receptor α and β subunits were from Oncogene Science (Cambridge, UK). IL-2 ELISA kit was purchased from DuPont-New England Nuclear (Boston, MA). The 3'-terminal labeling and multi-prime labeling kits, [α -³²P]ddATP (110 TBq/mmol), [α -³²P]dCTP (110 TBq/mmol), and TdR (1.56 TBq/mmol) were purchased from Amersham International (Buckinghamshire, UK).

Preparation of hPBL. hPBL were extracted from the venous blood of healthy volunteers by dextran sedimentation followed by Ficoll-Paque gradient centrifugation. hPBL populations were T cell enriched by the removal of adherent cells. hPBL were cultured in RPMI-1640 medium supplemented with 5% heat-inactivated FCS in a humidified atmosphere of 10% CO₂ and air at 2×10^6 cells/ml. Trypan blue dye staining showed that the viability of hPBL was >98%.

Positive panning separation of CD3-positive T cells from hPBL. CD3-positive T cells were separated from hPBL by positive selection using a panning technique (13). Briefly, polystyrene T-25 culture flasks covalently coated with anti-CD3 monoclonal antibodies were generously provided by AIS (Menlo Park, CA). Before panning, the antibody-coated flasks were washed three times with a PBS/Ca²⁺, Mg²⁺-free medium. Approximately 5×10^7 fresh hPBL were incubated at room temperature with 4 ml of PBS/Ca²⁺, Mg²⁺-free medium containing 0.5% human γ -globulin (Wako Pure Chemicals, Osaka, Japan) and then were introduced into the anti-CD3 mAb coated T-25 flasks. After 1 hr of incubation at room temperature, nonattached cells were removed by pipetting, and 10 ml of the "release medium" (10% FCS RPMI 1640) was added. After 72 hr of culture in a humidified atmosphere of 5% CO₂ at 37°, adherent cells were removed from the flasks by pipetting. These cells were then replated at $0.5\text{--}1 \times 10^6$ cells/ml in a fresh RPMI 1640 medium.

³²P-Labeling of cDNA probes. cDNA probes for the IL-2 receptor α and β subunits were labeled using a 3'-end labeling kit with [α -³²P]ddATP, and cDNA probe of IL-2 was labeled using the multi-prime labeling kit with [α -³²P]dCTP.

RNA isolation and northern blot analysis. Total cellular RNA was isolated by 4 M guanidine thiocyanate extraction, as described by

Chomczynski and Sacchi (14). hPBL or Jurkat cells were washed twice in ice-cold PBS before lysis. RNA samples were quantified spectrophotometrically by absorbance at 260 and 280 nm. Approximately 20 μ g of each total RNA sample was analyzed by electrophoresis on 1% (w/v) agarose-6% (v/v) formaldehyde gels. For Northern blot analysis, the nitrocellulose filters were prehybridized for 5 hr at 40° in 50% deionized formamide, 4 \times SSC (0.5 M NaCl, 0.05 M sodium citrate, pH 7.0), 50 mM sodium phosphate, pH 6.5, 5 \times Denhardt's solution, and 100 μ g/ml heat-denatured salmon sperm DNA. RNA: cDNA hybridizations were performed in 50% deionized formamide, 4 \times SSC, 50 mM sodium phosphate, pH 6.5, 5 \times Denhardt's solution, 100 μ g/ml heat-denatured salmon sperm DNA, and $\sim 2 \times 10^7$ cpm of labeled probe at 42° for 18 hr. Filters were washed three times in 2 \times SSC/0.1% sodium dodecyl sulfate at 37° (for cDNA probe of IL-2) for 30 min. Filters were dried, and autoradiography was performed by exposure of the filter to Kodak X-Omat AR film at -80° for 1-7 days.

RT-PCR analysis. Twenty-one-nucleotide functional PCR primers of human m1-5 muscarinic receptor were designed (15). Primer sequences, corresponding base sites (coding initiation site = 1), size of the PCR product, and sequence number (GenBank) are as follows: m1, GAAGAAGAGGAAGAGGACGAA as upper, bases 826-847, CAGGAGAGGGGACTATCAGCA as lower, bases 1378-1399, PCR product 573 bp, sequence number X15263; m2, GGGTCCTCTCTTTCATCCTCT as upper, bases 443-464, TCCTGGGTTATTTCATCATCT as lower, bases 891-912, PCR product 469 bp, sequence number X15264; m3, AGCCAAACGAACAACAAAGAG as upper, bases 531-552, TTGAAGGACAGAGGTAGAGTG as lower, bases 1356-1377, PCR product 846 bp, sequence number X15266; m4, CGCTATGAGACGGTGGAAATG as upper, bases 76-98, CGTCTTGGCTTTCTTCTCCTT as lower, bases 703-724, PCR product 648 bp, sequence number X15265; and m5, GGAAACAGAGAAGCGAACCAA as upper, bases 654-675, AGCACAACCAATAGCCCAAGT as lower, bases 1433-1454, PCR product 800 bp, sequence number M80333 A computer program showed that all the primers were 100% homologous to their target sequences and had no obvious homologies with any genes recorded in the GenBank. Total RNA (1 μ g) was incubated at 37° for 60 min with a mixture of 100 units of RT, 1 \times first-strand buffer, 10 mM dithiothreitol, 0.5 mM concentration of each dNTP, and 50 units of RNase inhibitor in a final volume of 20 μ l. After incubation, the reaction mixture was further incubated for 10 min at 70° to inactivate RT. The control templates [human fetal brain MATCHMAKER cDNA library (Clontech, Palo Alto, CA)] were digested with *Eco*RI and extracted. An aliquot (2 μ l) of RT products or control templates was mixed with 1 munits of TUB DNA polymerase, 200 nM each of sense and antisense primers in a buffer containing 1 \times TUB buffer and 0.2 mM concentration of each dNTP in final volume of 20 μ l. The mixture was overlaid with 30 μ l of liquid paraffin to prevent evaporation and then amplified. PCR was started at 94° for 1 min followed by 40 cycles of annealing at 53° for 1 min and extension at 72° for 1.5 min except for the final cycle, in which extension time at 72° was prolonged for an additional 7 min before cooling at 4°. PCR products were resolved by electrophoresis in a 1.5% agarose gel in 1 \times Tris/borate/EDTA buffer (8.9 mM Tris, 8.9 mM borate, 2 mM EDTA). The gel was stained with ethidium bromide and photographed.

Assay of IL-2 production. The amount of IL-2 produced by cultured T cells was determined using an ELISA kit for human IL-2 (DuPont-New England Nuclear). In brief, cell-free culture supernatants were obtained by centrifugation at 500 $\times g$ for 5 min. Samples were assayed according to the manufacturer's instructions and analyzed on an automated ELISA plate reader (MTP-120, Corona Electric). Data are expressed as IU (1 IU/ml = 0.2 ng/ml).

Measurement of DNA synthesis. [³H]TdR incorporation was used as an estimate of the rate of DNA synthesis. Cells were cultured for the times indicated, and [³H]TdR (500 nM, 74 kBq/ml) was added 12 hr before the end of culture. [³H]TdR incorporation was terminated by filtration through a glass-fiber filter (Whatman GF/C), and

cells were counted for their radioactivity. Data are expressed as dpm of [^3H]TdR incorporated into 4×10^5 cells.

Measurement of $[\text{Ca}^{2+}]_i$. hPBL or Jurkat cells at 2×10^6 cells/ml in RPMI-1640 medium containing 0.3% BSA were incubated with $5 \mu\text{M}$ Fura-2/AM at 37° for 20 min. The suspension was diluted 10-fold in RPMI-1640 medium and incubated at 37° for 30 min. The suspension was centrifuged at $300 \times g$ for 5 min to wash out any free dye and resuspended in Tyrode-HEPES buffer at 2×10^6 cells/ml. Intracellular Fura-2 fluorescence was monitored in cell suspensions of 1×10^6 cells with a fluorescence spectrophotometer (Hitachi F-2000) in a temperature-controlled cuvette while being stirred at 37° . The fluorescence intensity at 510 nm was monitored when excited at 340/380 nm. The equation $[\text{Ca}^{2+}]_i = K_d(R - R_{\min})/(R_{\max} - R) \times F_{\min}(340 \text{ nm})/F_{\max}(380 \text{ nm})$ was used, in which R equals the ratio in the fluorescence intensity of Fura-2-loaded cells at 340/380 nm; F_{\min} is the fluorescence intensity of the Ca^{2+} -free dye at 340 nm, determined in a 10 mM EGTA solution of Triton X-100-lysed cells; and F_{\max} is the fluorescence intensity of the Ca^{2+} -saturated dye at 380 nm, determined in a Triton X-100-lysed cell suspensions containing 1 mM CaCl_2 . The limiting ratios, R_{\min} (Ca^{2+} -free dye) and R_{\max} (Ca^{2+} -saturated dye), were determined in Triton X-100-lysed cell suspensions using EGTA or Ca^{2+} buffers, respectively. The change in the ratio on stimulation (R) was then used to calculate the change in $[\text{Ca}^{2+}]_i$. The published K_d value for the Fura-2/ Ca^{2+} interaction (224 nm) was used for these calculations (16).

Flow cytometric analysis of cell surface of CD3, CD2, CD4, CD8, and IL-2 receptor α subunit molecules. hPBL cells (1×10^6) were incubated in PBS with $1 \mu\text{g}$ of murine mAb of CD3, CD2, CD4, CD8, or IL-2 receptor α subunit for 30 min at 4° and then stained with $1.25 \mu\text{g}$ of fluorescein isothiocyanate-labeled secondary antibody for 30 min at 4° . The stained cells were analyzed with a flow cytometer (EPICS-CS, Coulter Electronics) to assess the surface expression of CD3, CD2, CD4, CD8, or IL-2 receptor α subunit molecules.

Subset analysis of hPBL by FACS. Approximately 1×10^7 hPBL cells/ml in PBS were reacted with NU-TH/I (anti-CD4 mAb, conjugated to phycoerythrin) or NU-TS/C (anti-CD8 mAb, conjugated to phycoerythrin) and then loaded with a lipophilic cationic membrane potential probe, DiOC₆. The dye was dissolved in dimethylsulfoxide and stored frozen as a 1 mM stock solution. Before stimulation, cells at a concentration of 1×10^6 cells/ml were incubated with 50 nM of DiOC₆ in PBS for 15 min at 4° to allow equilibration of the probe. During any series of experiments, the interval between dye addition to a sample and introduction of the sample into the flow cytometer was the same. The fluorescence distributions for phycoerythrin and DiOC₆ were obtained using the FACS system (EPICS-CS). The green (530–570 nm) fluorescence of DiOC₆ and the orange fluorescence of phycoerythrin in individual cells were measured at excitation wavelengths of 488 nm from an argon ion laser. Fluorescence parameters were collected using the liner mode to calculate mean fluorescence intensity. Data were collected for 1×10^4 cells/sample. In a cell suspension equilibrated with a labeled lipophilic cation, hyperpolarization of the cell membrane leads to further uptake of the indicator by cells, and depolarization leads to release of indicator from cells into the buffer. At the dye concentration used in these experiments, the fluorescence of individual cells was increased monotonically with the amount of intracellular DiOC₆. Thus, the fluorescence of an individual cell is increased by hyperpolarization and decreased by depolarization of the membranes.

Results

Muscarinic agonist-induced IL-2 production in hPBL. Incubation of hPBL with $1 \mu\text{M}$ ACh (in the presence of $100 \mu\text{M}$ physostigmine, a cholinesterase inhibitor), $1 \mu\text{M}$ Oxo-M, or $1 \mu\text{M}$ nicotine alone for 24 hr did not induce IL-2 production (Table 1). However, pretreatment with $1 \mu\text{M}$ ACh (in the presence of $100 \mu\text{M}$ physostigmine) or $1 \mu\text{M}$ Oxo-M for

24 hr enhanced PHA-induced IL-2 production, but nicotine pretreatment had no effect (Table 1). Anti-CD3 mAb ($1 \mu\text{g}/\text{ml}$)-induced IL-2 production was also enhanced by Oxo-M (62%), and it was similar to PHA-induced IL-2 production (69%). Using CD3-positive T cells prepared by a positive panning selection from hPBL, we examined the effects of Oxo-M on PHA ($10 \mu\text{g}/\text{ml}$)-induced IL-2 production. We obtained similar results to those with hPBL. Incubation with PHA ($10 \mu\text{g}/\text{ml}$) and $1 \mu\text{M}$ Oxo-M for 24 hr enhanced IL-2 production by 69% in hPBL and by 76% in CD3-positive cells, but $1 \mu\text{M}$ Oxo-M alone for 24 hr did not affect IL-2 production. In addition, PHA-induced IL-2 production was enhanced by pretreatment with Oxo-M at the concentration range of 10 nM to $10 \mu\text{M}$ during 24-hr incubation (data not shown). Although the addition of just $0.1 \mu\text{M}$ Oxo-M did not enhance PHA-induced IL-2 production, pretreatment for >1 hr did enhance it (Fig. 1A). Furthermore, although IL-2 production induced by PHA alone was observed 12 hr after treatment, by pretreating with Oxo-M for 24 hr, PHA-induced IL-2 production began earlier (after 3 hr) and doubled its production after 12–24 hr (Fig. 1B). When hPBL were pretreated with Oxo-M for 24 hr, PHA alone produced IL-2 at low concentrations ($1\text{--}3 \mu\text{g}/\text{ml}$) (Fig. 2).

Effects of FK-506 and CsA on IL-2 production from hPBL. FK-506 and CsA, two immunosuppressant drugs, are known to inhibit the production of several cytokines, such as IL-2 (10). Therefore, we examined the effects of FK-506 and CsA on PHA-induced IL-2 production and the Oxo-M-induced enhancement of IL-2 production in hPBL. Although IL-2 production was not induced by 10 nM TPA or 500 nM ionomycin alone, on treatment with both TPA (pretreatment for 1 hr) and ionomycin, IL-2 production was markedly induced (Fig. 3A). This IL-2 production was almost completely inhibited ($\sim 95\%$ inhibition) by 10 nM FK-506 and 100 nM CsA (Fig. 3). However, FK-506 and CsA caused significant but less potent inhibition in IL-2 production induced by PHA alone than in that by TPA plus ionomycin ($\sim 50\%$ inhibition, Fig. 3A). In addition, FK-506 and CsA significantly inhibited IL-2 production by PHA pretreated with Oxo-M for 24 hr (Fig. 4), but the IL-2 amounts inhibited by FK-506 and CsA were not largely altered between PHA alone and Oxo-M plus PHA (Fig. 4). Pretreatment with 10 nM TPA for 1 hr also enhanced PHA-induced IL-2 production, and the enhancement was

TABLE 1
Influences of cholinergic agonists on PHA-induced IL-2 production from hPBL

After hPBL were incubated with several cholinergic agonists ($1 \mu\text{M}$ ACh in the presence of $100 \mu\text{M}$ physostigmine, $1 \mu\text{M}$ Oxo-M, $1 \mu\text{M}$ nicotine) for 24 hr, $10 \mu\text{g}/\text{ml}$ PHA or the vehicle (control) was added to the culture medium and further incubated for 24 hr, the IL-2 content in the medium was measured by ELISA for human IL-2, as described in Experimental Procedures. CD3-positive T cells prepared from hPBL by a positive panning selection method, as described in Experimental Procedures, were also pretreated with $1 \mu\text{M}$ Oxo-M for 24 hr. PHA ($10 \mu\text{g}/\text{ml}$) was added into the culture medium and further incubated for 24 hr, and the IL-2 content in the medium was measured. PHA-induced IL-2 production and Oxo-M-induced enhancement were found in all four experiments.

Treatment	Control	<i>n</i>	Plus PHA	<i>n</i>
	IU/ml		IU/ml	
None	3.1 ± 0.3	4	31.4 ± 2.9	4
ACh	3.5 ± 0.5	4	48.8 ± 4.8^a	4
Oxo-M	3.2 ± 0.4	4	53.1 ± 6.4^a	4
Nicotine	3.0 ± 0.4	4	28.5 ± 3.2	4

^a $p < 0.05$ vs. none.

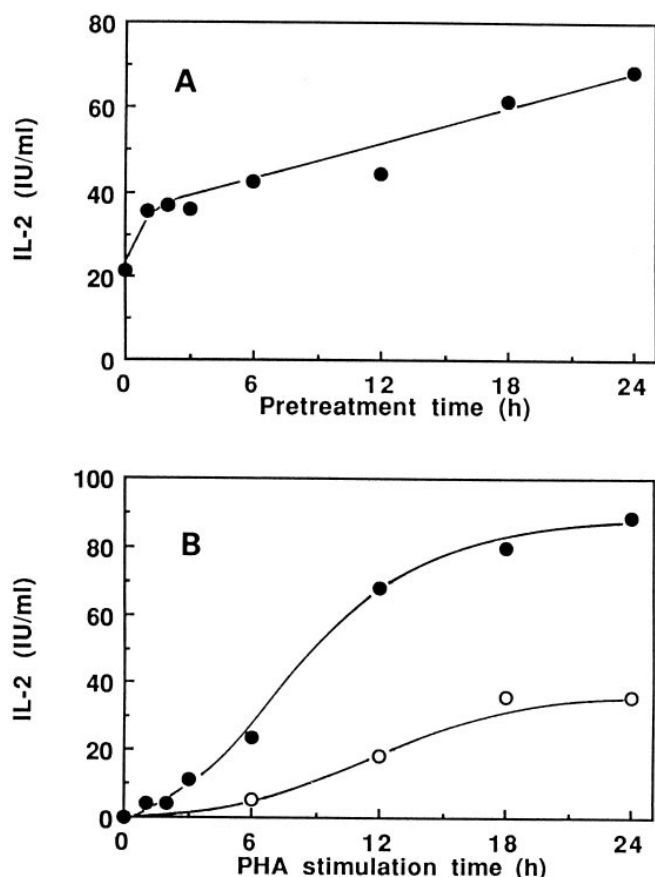


Fig. 1. Effect of Oxo-M on PHA-induced IL-2 production in hPBL. A, After hPBL were preincubated with 0.1 μ M Oxo-M for 0–24 hr, 10 μ g/ml PHA was added and incubated for another 24 hr; subsequently, the amount of IL-2 was measured. B, After hPBL were pretreated with vehicle (\circ) or 0.1 μ M Oxo-M (\bullet) for 24 hr, 10 μ g/ml PHA was added and incubated for an additional 0–24 hr. Typical data of four independent experiments are shown. Points, mean of four samples.

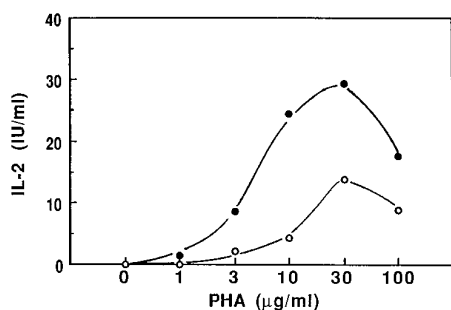


Fig. 2. Oxo-M enhanced PHA-induced IL-2 production. After hPBL were pretreated with vehicle (\circ) or 0.1 μ M Oxo-M (\bullet) for 24 hr, PHA at 0–100 μ g/ml was added, and they were further incubated for 24 hr. Points, mean of four samples.

higher with Oxo-M pretreatment (Fig. 3B). IL-2 production induced by TPA pretreatment plus PHA was not completely inhibited by FK-506 and CsA (~70% inhibition) (Fig. 3A). In the case of anti-CD3 mAb (NU-T3), similar results were obtained (data not shown).

Expression of IL-2 and IL-2 receptor subtype mRNAs in hPBL. Because pretreatment with Oxo-M enhanced PHA-induced IL-2 production, we examined whether the expression of IL-2 mRNA is also stimulated. Reed *et al.* (17) reported that stimulation by PHA alone induces IL-2 mRNA

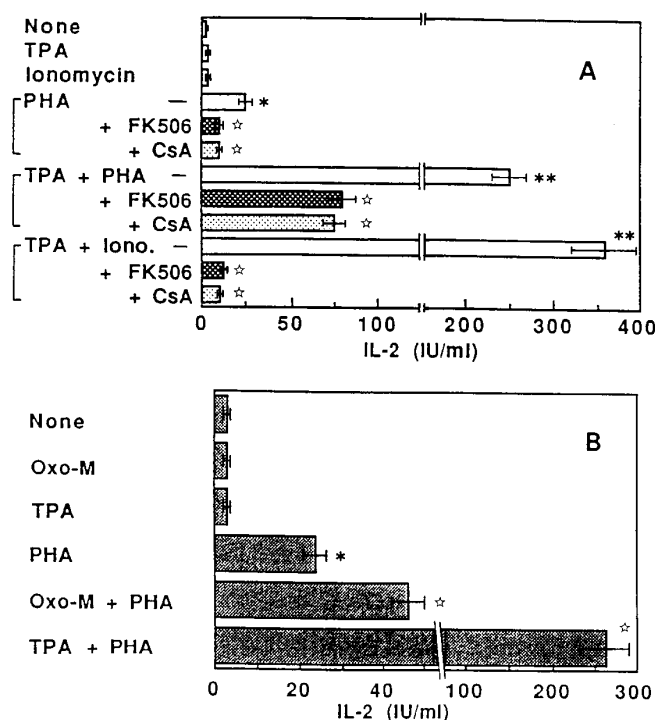


Fig. 3. Effect of TPA, ionomycin, FK-506, or CsA on IL-2 production in hPBL. A, Effects of FK-506 and CsA, TPA plus PHA, and TPA plus ionomycin (iono.) on PHA-induced IL-2 production. After hPBL were preincubated in the presence or absence of 10 nM FK-506, 100 nM CsA for 2 hr, or 10 nM TPA for 1 hr, 10 μ g/ml PHA or 500 nM ionomycin was added, and they were incubated for an additional 24 hr. *, $p < 0.01$, **, $p < 0.001$ versus the value of None; *, $p < 0.05$ versus the value of each stimulation such as PHA, TPA + PHA, or TPA + ionomycin. B, Effects of Oxo-M, TPA, and PHA on IL-2 production. After hPBL were pretreated in the presence or absence of 10 nM TPA (for 1 hr) or 0.1 μ M Oxo-M (for 24 hr), 10 μ g/ml PHA was added, and they were incubated for an additional 24 hr. Points, mean of four samples. *, $p < 0.01$ versus None; *, $p < 0.05$ versus the value of PHA treatment.

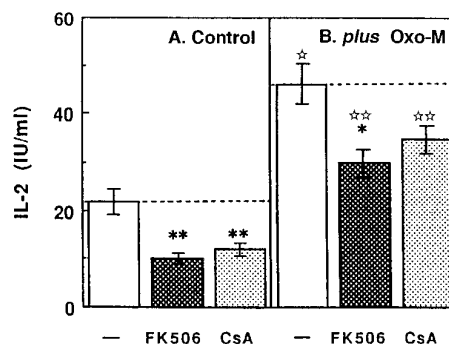


Fig. 4. Effects of FK-506 and CsA on PHA-induced IL-2 production and the Oxo-M-induced enhancement of IL-2 production. After hPBL were preincubated in the presence or absence of 10 nM FK-506 or 100 nM CsA for 2 hr, hPBL were treated in the absence (A) or presence (B) of 0.1 μ M Oxo-M for 24 hr; then, 10 μ g/ml PHA was added, and they were incubated for an additional 24 hr. Subsequently, the amount of IL-2 produced was measured in the medium. Points, mean of four samples. *, $p < 0.05$, **, $p < 0.01$ versus the value of each stimulation such as PHA or TPA + Oxo-M; *, $p < 0.05$, **, $p < 0.001$ versus the value in the absence of Oxo-M.

expression 6–14 hr into the incubation; this is followed by the expression of IL-2 receptor mRNA (α subunit) 14–24 hr into the incubation. In this study, IL-2 mRNA expression (~1 kb) (18) was detected by stimulation with both Oxo-M and PHA

(pretreatment with Oxo-M for 24 hr plus PHA for 2 hr) (Fig. 5A, lane 4). However, PHA alone did not induce the IL-2 mRNA expression after 2 hr (Fig. 5A, lane 3). Activation by PHA alone for just 12 hr induced the expression of IL-2 mRNA (Fig. 5B, lane 3), and pretreatment with Oxo-M for 24 hr plus PHA enhanced the expression (Fig. 5B, lane 4). Furthermore PHA-induced expression of α subunit mRNA (~ 3.5 and ~ 1.5 kb) (19, 20) and the mRNA of the β subunit (~ 4 kb) of IL-2 receptors were also enhanced by pretreatment of hPBL with Oxo-M within 18 hr (Fig. 6). Two bands corresponding to 3.5 and 1.5 kb were observed via gel electrophoresis (Fig. 6A). Both mRNAs are known to be capable of encoding functional IL-2 receptors (19, 20).

Muscarinic agonist-enhanced DNA synthesis by activation of the TCR/CD3 complex in hPBL. We further examined whether cholinergic agonists affected proliferation of lymphocytes. When incubated alone, $0.1 \mu\text{M}$ ACh (in the presence of $10 \mu\text{M}$ physostigmine), $0.1 \mu\text{M}$ Oxo-M, and $0.1 \mu\text{M}$ nicotine did not induce [^3H]TdR incorporation (Table 2). However, PHA-induced [^3H]TdR incorporation during incubation for 24 hr (Table 2) and 48 hr (Table 2) was enhanced by pretreatment with ACh or Oxo-M for 24 hr but was not enhanced by nicotine pretreatment. Thus, activation of muscarinic receptors also seems to modulate the activation of the TCR/CD3 complex and, therefore, proliferation of human lymphocytes.

Effects of Oxo-M treatment on expression of CD3, CD2, CD4, CD8, and IL-2 receptor α subunits on hPBL and T cells. To assess whether Oxo-M pretreatment up-regulates the expression of cell surface molecules involved in the PHA response in T cells, flow cytometric analysis was performed. hPBL treated with or without $0.1 \mu\text{M}$ Oxo-M for 24 hr showed almost equal fluorescence shifts with regard to CD3 and CD2 molecules (Fig. 7A). Oxo-M did not up-regulate CD4, CD8, or IL-2 receptor α subunit molecules. Similar results were obtained in CD3-positive T cells (Fig. 7B). Thus,

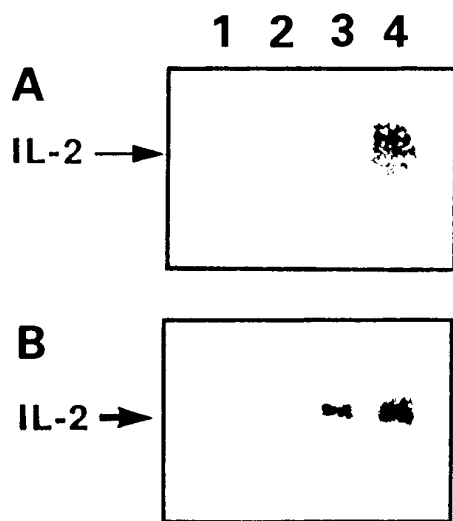


Fig. 5. Oxo-M enhanced PHA-induced IL-2 mRNA expression. hPBL were treated with vehicle/vehicle (lane 1), vehicle/Oxo-M (lane 2), vehicle/PHA (lane 3), and Oxo-M/PHA (lane 4). hPBL were first incubated with vehicle (lanes 1–3) or $1 \mu\text{M}$ Oxo-M (lane 4) for 3 hr. Subsequently, hPBL were incubated with vehicle (lane 1), $1 \mu\text{M}$ Oxo-M (lane 2), or $10 \mu\text{g/ml}$ PHA (lanes 3 and 4); then, PHA was added, and they were incubated for 2 hr (A) or 12 hr (B). IL-2 mRNA was determined at the ~ 1 -kb mark. Another experiment showed similar results.

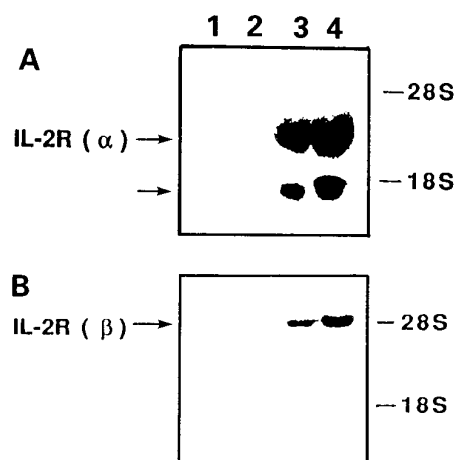


Fig. 6. Stimulatory effects of Oxo-M on the mRNA of the α and β subunits of IL-2 receptors. hPBL were treated with vehicle/vehicle (lane 1), vehicle/Oxo-M (lane 2), vehicle/PHA (lane 3), and Oxo-M/PHA (lane 4). hPBL were first incubated with vehicle (lanes 1–3) or $1 \mu\text{M}$ Oxo-M (lane 4) for 6 hr. Subsequently, vehicle (lane 1), $1 \mu\text{M}$ Oxo-M (lane 2), or $10 \mu\text{g/ml}$ PHA (lanes 3 and 4) was added, and they were incubated for an additional 18 hr. The mRNAs of the IL-2 receptor subunits were determined at $\sim 3.5/1.5$ kb (α subunit) and ~ 4 kb (β subunit). The positions of the 28S (~ 4.8 kb) and 18S (~ 2 kb) rRNA bands are indicated. Another experiment showed similar results. IL-2R (α), α subunit of the IL-2 receptor. IL-2R (β), β subunit of the IL-2 receptor.

we suggest that Oxo-M does not up-regulate the expression of the cell surface molecules such as CD3, CD2, CD4, CD8, and IL-2 receptor α subunit in T cells.

Expression of muscarinic receptor mRNA in hPBL. To investigate which subtypes of muscarinic receptor mRNA are expressed in hPBL, RT-PCR analyses were carried out on total RNA extracted from hPBL using human m1–5 muscarinic receptor functional PCR primer. Muscarinic receptor m1 and m2 mRNAs were detected (Fig. 8).

Effects of Oxo-M and PHA on $[\text{Ca}^{2+}]_i$ in hPBL. Incubation with $10 \mu\text{g/ml}$ PHA induced a marked and sustained increase in $[\text{Ca}^{2+}]_i$ in hPBL (Fig. 9B). Anti-CD3 mAb (NU-T3) also induced increased in $[\text{Ca}^{2+}]_i$, similar in magnitude to PHA-induced $[\text{Ca}^{2+}]_i$ (21). Oxo-M at a higher concentration ($10 \mu\text{M}$) hardly induced any increase in $[\text{Ca}^{2+}]_i$ in hPBL (Fig. 9A), although IL-2 production was enhanced by Oxo-M at low concentrations (>10 nM). A high concentration of Oxo-M ($>1 \mu\text{M}$) is required for increased $[\text{Ca}^{2+}]_i$ in Jurkat cells (6). These results suggest that increases in $[\text{Ca}^{2+}]_i$ are not involved in the Oxo-M-induced enhancement of IL-2 production.

A subset of hPBL that express muscarinic receptors. It has been reported that lectins or ACh induced depolarization of hPBL (22, 23). Thus, we examined which subset of T cells, CD4- or CD8-positive cells, are depolarized in response to Oxo-M using the lipophilic dye DiOC₆ labeling method. The mean fluorescence intensity, using DiOC₆-loaded cells, was decreased by Oxo-M treatment from 222.3 to 85.7 ($\Delta 136.6$) in CD4-positive cells, suggesting depolarization of CD4-positive cells by Oxo-M. In contrast, the mean fluorescence intensity was altered from 252.8 to 181.3 ($\Delta 71.5$) in CD8-positive cells. The value of change (136.6) in CD4 cells was almost twice more than that (71.5) in CD8 cells. Therefore, muscarinic receptors may exist in larger numbers in CD4 cells than in CD8 cells, although both cell types can express muscarinic receptors (Fig. 10).

TABLE 2

Effects of pretreatment with cholinergic agonists on [³H]TdR incorporation

Treatment	Control (24 hr)		plus PHA (24 hr)		plus PHA (48 hr)	
	Mean ± S.E. (dpm/cells)	n	Mean ± S.E. (dpm/cells)	n	Mean ± S.E. (dpm/cells)	n
None	1218 ± 113	4	4021 ± 325	4	53002 ± 2521	4
ACh	1537 ± 186	4	7065 ± 544**	4	62701 ± 2604*	4
Oxo-M	1450 ± 132	4	7855 ± 735**	4	64446 ± 2804*	4
Nicotine	1037 ± 89	4	3591 ± 347	4	51223 ± 2358	4

hPBL was pretreated with vehicle, 0.1 μ M ACh (in the presence of 10 μ M physostigmine), 0.1 μ M Oxo-M or 10 μ M nicotine for 24 hr. 10 μ g/ml PHA was added and the mixtures were further incubated for 24 hr and for 48 hr. Subsequently, hPBL thus treated was labeled by adding 0.5 μ M [³H]TdR (74 kBq/ml) for 12 hr and [³H]TdR incorporation was determined as described under "Experimental Procedures".

Significance, * $p < 0.05$, ** $p < 0.01$ vs none.

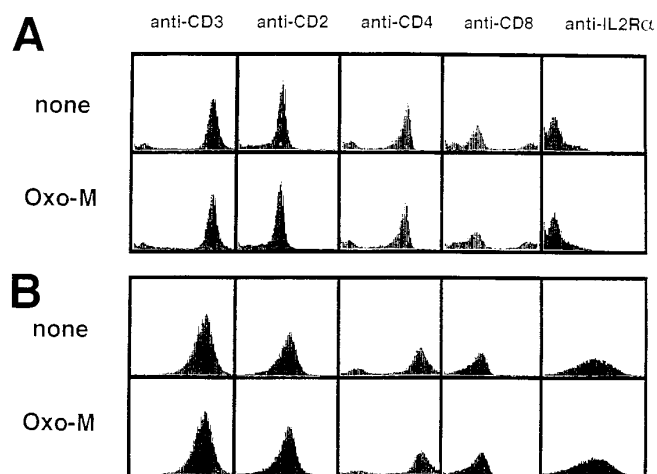


Fig. 7. Effects of Oxo-M treatment on the expression of CD3, CD2, CD4, CD8, and the α subunit of the IL-2 receptor in hPBL and T cells. hPBL (A) and T cells (B) were treated with or without 100 μ M Oxo-M; then, their fluorescence intensities were analyzed by flow cytometry using anti-CD3 mAb, anti-CD2 mAb, anti-CD4 mAb, anti-CD8 mAb, and anti-IL-2 receptor α subunit mAb. IL2R α , α subunit of the IL-2 receptor.

Discussion

Previously, Oxo-M, the nonselective muscarinic receptor agonist, was found to induce activation of PLC through mechanisms different than those of PHA in Jurkat cells (6). Here, we examined the effects of Oxo-M on IL-2 production in hPBL.

We have shown that pretreatment of hPBL with Oxo-M or ACh, but not nicotine, enhanced PHA-induced IL-2 production (Table 1). PHA-induced IL-2 production was enhanced when hPBL were pretreated >1 hr with Oxo-M (Fig. 1). Furthermore, pretreatment with Oxo-M for 24 hr doubled PHA-induced IL-2 production (Figs. 1B and 2). These findings suggest that there are muscarinic receptors on hPBL and that stimulation of these receptors enhanced IL-2 production.

FK-506 and CsA are known to be immunosuppressive drugs that inhibit IL-2 production (10). These drugs form complexes with immunophilins (FKBPs and cyclophilins) and block Ca^{2+} pathways. Ca^{2+} pathway in IL-2 production may act *via* calcineurin by stimulating the translocation of transcriptional factors such as NF-AT from the cytoplasm into the nucleus. In this study, the inhibitory effect of FK-506 on IL-2 production induced by PHA was lower than that induced by the combination of TPA and ionomycin (Fig. 3A). This suggests that other pathways are involved in PHA-

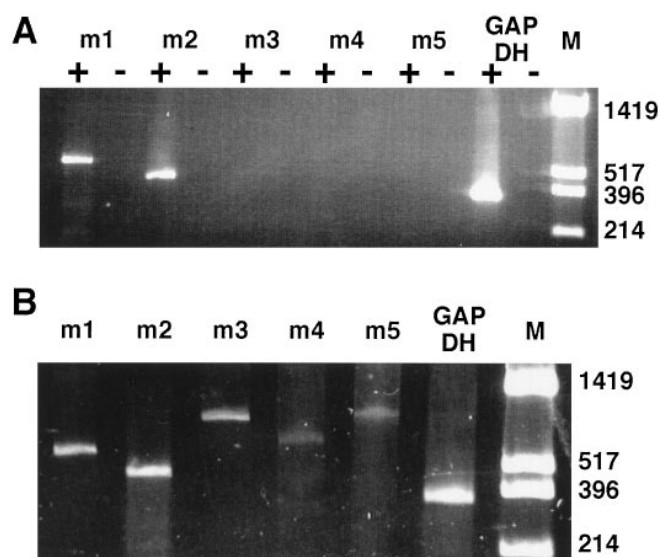


Fig. 8. Expression of muscarinic receptor mRNA in hPBL. A, RT-PCR was used to amplify signals from isolated mRNA of hPBL. Right, sizes (bp) of the *Hinf*I cut pUC19 fragments in the last lane are indicated. For each set of subtype-specific primers, mRNA was run in the presence (+) and absence (–) of RT. Blank lanes in the absence of RT, absence of contamination from cellular DNA or amplified products. B, Control (human fetal brain cDNA library). Primer sequences and sizes of the PCR products are described in Experimental Procedures.

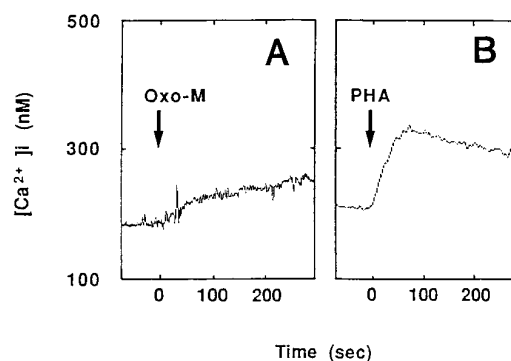


Fig. 9. Effects of Oxo-M and PHA on the $[Ca^{2+}]_i$ in hPBL. Time course of $[Ca^{2+}]_i$ rise was examined after the addition of 10 μ M Oxo-M (A) or 10 μ g/ml PHA (B). hPBL were preloaded with 5 μ M Fura-2/AM for 20 min and then washed. From the fluorescence intensity of Fura-2, $[Ca^{2+}]_i$ was determined using an Hitachi F-2000 spectrophotometer. Although a PHA-induced $[Ca^{2+}]_i$ rise was observed in all five independent experiments, an Oxo-M-induced rise was not observed in some experiments.

induced IL-2 production. Furthermore, FK-506 and CsA inhibited IL-2 production of Oxo-M-pretreated PHA stimulation. The degree of inhibition by FK-506 and CsA was not

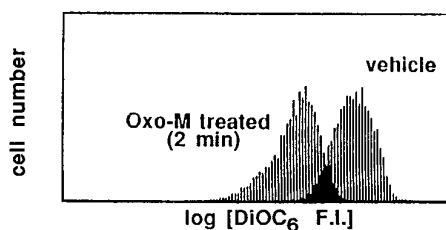


Fig. 10. FACS analysis of Oxo-M-induced depolarization of hPBL using anti-CD4 and anti-CD8 antibodies. After hPBL were preincubated with phycoerythrin-conjugated anti-CD4 antibodies or anti-CD8 antibodies for 30 min at 4°, the prelabeled hPBL were preloaded with 50 nM DiOC₆ for 15 min at 4° and then washed. The hPBL were then incubated with 0.1 μM Oxo-M for 2 min, and FACS analysis was performed. Fluorescence intensity of DiOC₆ was markedly decreased by treatment with Oxo-M. The mean of the fluorescence intensity in CD4-positive cells had decreased from 222.3 to 85.7 (Δ136.6) in the Oxo-M treated cells; the mean fluorescence intensity in CD8-positive cells ranged from 252.8 to 181.3 (Δ71.5). Typical data are shown. Another experiment showed similar results.

different between PHA alone and Oxo-M plus PHA (Fig. 4). This indicates that Oxo-M-induced enhancement of IL-2 production may not be due to $[Ca^{2+}]_i$ elevation alone.

We examined the expression of IL-2 mRNA and IL-2 receptor mRNA. These mRNAs were also enhanced Oxo-M pretreatment and PHA stimulation (Figs. 5 and 6). However, 2 hr after PHA stimulation, Oxo-M pretreatment induced IL-2 mRNA, but PHA stimulation alone did not (Fig. 5). This suggests that Oxo-M pretreatment induces IL-2 mRNA more rapidly. IL-2 receptor α subunit mRNA is also induced through the T cell activation (24), perhaps in parallel to the IL-2 mRNA activation.

Pretreatment with Oxo-M for 24 hr resulted in enhancement of DNA synthesis (Table 2), which was parallel to the IL-2 production. This DNA synthesis may be caused by Oxo-M directly or via IL-2-mediated autocrine system.

To determine whether Oxo-M increases expression of the surface molecules CD3, CD2, CD4, CD8, and IL-2 receptor α subunit involved in PHA responses, we examined the FACS analysis using each mAb but found no change in their expression (Fig. 7). Therefore, stimulatory effects of Oxo-M on IL-2 production and IL-2 receptor formation could be due to the intracellular signaling mechanism.

Muscarinic receptor subtype genes have been classified as m1 to m5 on the basis of cDNA cloning (5). RT-PCR analysis revealed that m1 and m2 receptors were expressed in hPBL (Fig. 8). m1 receptors, known to couple with G_q protein, which activates PLC-β (25), were expressed more than m2 receptors (Fig. 8A). The signals from PLC-β may lead to activation of PKC through inositol-1,4,5-triphosphate and diacylglycerol and enhance IL-2 production. The PKC family is heterogeneous [e.g., classic PKC group (α, βI, βII, γ), new PKC group (δ, ε, η, θ), and atypical PKC group (ζ, λ)], and PKC subspecies have different intracellular localization in particular cell types (26). These studies indicate that PLC and PKC activated by muscarinic receptors are different species from those activated by TCR/CD3. Some studies showed that cAMP inhibits IL-2 production in the T cell line (27–29). m2 receptors are known to couple with G_i proteins, which inhibit cAMP accumulation. Therefore, signal transduction via m2 receptors may inhibit the cAMP accumulation, followed by disinhibition of IL-2 production. Thus, m1 and m2 receptors interact to enhance IL-2 production.

In this study, Oxo-M alone caused a slight elevation of $[Ca^{2+}]_i$ in hPBL (Fig. 9A), but this elevation of $[Ca^{2+}]_i$ was lower than that induced by PHA. $[Ca^{2+}]_i$ may not be essential in muscarinic receptor-enhanced IL-2 production in hPBL.

Based on the FACS analysis, CD4- and CD8-positive cells express muscarinic receptors. These receptors are more abundant in CD4 cells than in CD8 cells (Fig. 10).

The results presented here show that m1 and m2 muscarinic receptors were functionally expressed in human T cells. They caused enhancement in TCR/CD3-mediated IL-2 and IL-2 receptor formation through G proteins, PLC and via FK-506- and CsA-insensitive pathways. CD4 cells are known to release IL-2 protein when the cells are activated. We showed that pretreatment with Oxo-M enhanced IL-2 production and that these muscarinic receptors were mainly expressed in CD4 cells.

In Alzheimer's disease, cholinergic activity is low in the central nervous system. Muscarinic receptor expression in hPBL of patients is markedly decreased, although the receptor/ACh affinity is not changed (30). The activation of T cells has been shown to decrease with increasing age in several immunodysfunction diseases (31), such as severe combined immunodeficiency or acquired immunodeficiency syndrome. These findings suggest that a selective muscarinic receptor agonist may be therapeutically useful for the treatment of several diseases that result from dysfunction of TCR/CD3 complexes in T cells. It is important to further clarify the functional roles and the intracellular mechanism of the neuroimmune interaction.

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