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Effect of MX-68 on airway inflammation and hyperresponsiveness in mice and guinea-pigs

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Abstract

MX-68 is a newly synthesized antifolate, which is a derivative of methotrexate (MTX). In this paper, the effect of MX-68 on allergic airway responses in mice and guinea-pigs was studied. In the first experiment, antigen-induced airway inflammation and airway hyperresponsiveness (AHR) to acetylcholine in mice were examined and compared with the effects of classical antifolate methotrexate and prednisolone. Mice were sensitized with ovalbumin as an antigen and challenged with ovalbumin inhalation three times. After the last inhalation, AHR and airway inflammation were observed. An increase in Th2 cytokines (IL-4 and IL-5) and a decrease in a Th1 cytokine (IFN-γ) in the bronchoalveolar lavage fluid (BALF), as well as an elevation of the immunoglobulin level in serum, were observed in sensitized mice. Oral administration of MX-68 had no effect on changes of body weight, but prednisolone reduced body weight during the experiment. The antigen-induced AHR and increases in the number of eosinophils and lymphocytes in BALF were significantly inhibited by MX-68. MX-68 interfered with the elevation of IL-4 and IL-5 levels in BALF, but had no effect on the decrease in IFN-γ. Moreover, MX-68 significantly inhibited the elevation of serum IgE and IgG levels. In the guinea-pig model for bronchial asthma, biphasic increases in airway resistance (immediate asthmatic response, IAR, and late asthmatic response, LAR), as well as accumulated inflammatory cells in BALF, were observed after repeated antigen challenge. These asthmatic responses and inflammatory signs were significantly decreased by administration of MX-68. These results suggest that MX-68 obviously has an anti-inflammatory effect in an animal model of asthma and would be useful clinically for the treatment of bronchial asthma.

Introduction

Methotrexate is a novel immunosuppressor and has recently been used for the treatment of rheumatoid arthritis (Furst & Kremer 1988; Weinblatt & Maier 1990). Many investigations demonstrate the dramatic efficacy of methotrexate on rheumatoid arthritis (Hu et al 1988; Crostein et al 1991; Sperling et al 1992; Firestein et al 1994; Smith & Sly 1996; Aggarwal & Misra 2003). Methotrexate is now one of the first-line drugs in the therapy for rheumatoid arthritis. In addition to its application in rheumatoid arthritis, methotrexate has been evaluated as a remedy for other inflammatory diseases. In this series of investigations, Gonokami et al (1995) reported that methotrexate inhibits the accumulation of lymphocytes and eosinophils in lung tissue and suppresses the late asthmatic response (LAR) in a biphasic mouse model. Some reports showed that low-dose methotrexate significantly spared steroid usage in steroid-dependent patients with asthma (Dyer et al 1991; Stewart et al 1994; Marin 1997). Furthermore, it was revealed that the production of nitric oxide (NO), which played a role in asthma by enhancing airway inflammation, was inhibited by methotrexate in murine lung epithelial cells in-vitro (Robbins et al 1998). These data suggest the usefulness of methotrexate for the treatment not only of rheumatoid arthritis, but also other allergic diseases such as asthma.

However, even at a low dose, the long-term administration of methotrexate caused gastrointestinal toxicity, bone marrow suppression and fever, as well as hepatic and pulmonary fibrosis (Weinblatt 1985; Phillips et al 1992). These side effects are mainly dependent on its long storage in the cells. Methotrexate inhibits dihydrofolate

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reductase (DHFR) and antagonizes folic acid metabolism. After methotrexate is polyglutamated by folypolyglutamate synthetase (FPGS), it is stored intracellularly and shows inhibitory activity not only against DHFR but also against other enzymes related to folic acid metabolism (Baugh et al 1973; Allegra et al 1984; Baggott et al 1986). This is one of the reasons for the side effects of methotrexate.

MX-68 (*N*-(1-((2,4-diamino-6-pteridinyl) methyl)-3,4-dihydro-2*H*-1,4-benzothiazine-7-carbonyl)- L-2-aminoadipic acid), a new antifolate, is chemically designed not to undergo polyglutamation and to retain an affinity for DHFR (Matsuoka et al 1997; Matsuoka & Mihara 1998). As a result, MX-68 becomes a more selective inhibitor to DHFR and is less toxic than methotrexate.

Recently, several reports have appeared on the usefulness of MX-68 and describe the inhibition of the development of collagen-induced arthritis (CIA) in mice (Mihara et al 1996) and autoimmune kidney disease in lupus mice (Mihara et al 1997a). Moreover, MX-68 suppresses immunoglobulin production in-vivo and in-vitro (Mihara et al 1997b). These results indicate that MX-68 also has an immunosuppressive property.

Previously, we reported the effect of some immunosuppressors on antigen-induced airway inflammation and airway hyperresponsiveness (AHR) in animal models. As a result, FK-506 showed a sufficient anti-asthmatic effect, but cyclophosphamide and rapamycin did not inhibit AHR to acetylcholine (Nagai et al 1995a, 1997).

In this study, for further research on immunosuppressive drugs, we examined the effect of MX-68 on AHR to acetylcholine and airway inflammation in mice and guinea-pigs.

Materials and Methods

Animals

Male BALB/c mice and male Hartley guinea-pigs were housed in an air-conditioned room at $22 \pm 1\,^{\circ}C$ and a relative humidity of $60 \pm 5\%$, fed a standard laboratory diet and allowed free access to water. The experiments in this study were carried out according to the Guidelines for the Care and Use of Laboratory Animals of Gifu Pharmaceutical University.

Materials

Ovalbumin (Seikagaku Kogyo, Tokyo, Japan), acetylcholine chloride (Nacalai Tesque, Kyoto, Japan), pentobarbital sodium (Abbott Laboratories, Chicago, IL), methotrexate (Sigma Chemical Co., St Louis, MO), prednisolone acetate (Shionogi, Osaka, Japan) and pancuronium bromide (Sigma) were purchased commercially.

Experiments in mice

Sensitization and challenge

Mice were actively sensitized by intraperitoneal injections of 50 μg of ovalbumin with 1 mg of aluminium hydroxide on days 0 and 12. Starting on day 22, they were exposed to ovalbumin (1% in saline solution) for 30 min 3 times every 4 days, using an ultrasonic nebulizer. Drugs were administered orally for 10 consecutive days from the day before the first challenge. On days 22, 26 and 30, the drugs were administered 1 h before the challenge.

Measurement of airway responsiveness

For all groups 24 h after last challenge, measurement of airway responsiveness in mice was carried out on day 31, according to a modified method of Konzett & Rössler (1940). Briefly, mice were anaesthetized with pentobarbital sodium ($60\,mg\,kg^{-1}$, i.p.), and the trachea and jugular vein were cannulated. To suppress spontaneous respiration, pancuronium bromide ($60\,mg\,kg^{-1}$, i.v.) was injected. The mice were ventilated (0.6 mL/mouse, 60 strokes/min) and bronchoconstriction was measured while increasing the doses of acetylcholine (from 31.25 to 2000 $\mu g\,mL^{-1}$). The changes in bronchoconstriction were represented as a percentage of maximal overflow volume obtained by clamping the tracheal cannula. The area under the curve (AUC) calculated from the results of the dose–response curve for acetylcholine was expressed as the magnitude of airway responsiveness to acetylcholine.

Determination of the number of cells in bronchoalveolar lavage fluid (BALF)

After measurement of airway responsiveness, BALF of mice was obtained according to a modified method reported by Tanaka et al (1998). Briefly, the trachea was cannulated and the air lumen was washed 4 times with 0.5 mL phosphate-buffered saline (PBS) (−) containing 0.1% bovine serum albumin (BSA) and 0.05 mM ethylenediamine tetraacetic acid (EDTA). By repeating this wash 3 times (total volume 1.5 mL) BALF was obtained. The collected BALF was centrifuged (150 *g*) at 4 °C for 10 min. Cell pellets were re-suspended in the same buffer (0.5 mL). The BALF was stained with Turk solution (Wako Pure Chemical Industries Ltd, Osaka, Japan) and the total number of cells in BALF was counted under the microscope. Next, the smear prepared with Cytospin II (Shandon) was stained with Diff Quick solution (Kokusaishiyaku, Kobe, Japan) and the number of macrophages, neutorophils, eosinophils and lymphocytes was counted in at least 300 cells under the microscope. The obtained results were expressed as total cells in BALF.

Measurement of cytokines in BALF

Cytokines (IL-4, IL-5 and IFN-γ) in BALF were measured with a commercial enzyme-linked immunosorbent assay (ELISA) kit (Endogen, Cambridge, MA).

Determination of immunoglobulin concentration

Antigen-specific IgE, total IgE and total IgG concentrations in mice serum were measured with ELISA as described below.

The antigen (ovalbumin)-specific IgE concentration was measured by the method described by Nagai et al (1997). Briefly, a 96-well immuno plate (Nunc Immunoplate I) was

coated with 100 μL/well of monoclonal anti-mouse IgE antibody (Serotec, Oxford, UK) at 5 μg mL^{-1} in PBS (−) and incubated overnight at 4 °C. After the plates were washed five times with 0.1% Tween-PBS (−) (washing buffer), 1% BSA (Wako)–PBS (−) was added and then the plates were incubated for 1 h at room temperature. After washing five times with washing buffer, 100 μL of the serum sample were added and the sample was incubated for 1 h at room temperature. To make the calculation curve, sequentially diluted monoclonal anti-ovalbumin IgE was used as the standard. After washing five times with washing buffer, 100 μL of diluted biotin-labelled ovalbumin were added and the material was incubated for 1 h at room temperature. After washing five times with washing buffer, 100 μL of diluted peroxidase-conjugated streptavidin (Dako, Denmark) were added and the solution was incubated for 1 h at room temperature. After washing five times with washing buffer, 100 μL of the substrate solution (0.1 M citric acid, 0.2 M $NaHPO_4$, *o*-phenylenediamine, 0.012% H_2O_2) were added and the material was incubated for 30 min at room temperature in the dark. To stop the reaction, 50 μL of 2 M H_2SO_4 were added and the plates were read at 492 nm.

The total IgE concentration in serum was measured by the method described by Nagai et al (1997). Briefly, a 96-well immuno plate (Nunc Immunoplate I) was coated with 100 μL/well of monoclonal anti-mouse IgE antibody (Serotec) at 5 μg mL^{-1} in PBS (−) and incubated overnight at 4 °C. After the plates were washed three times with washing buffer, 200 μL of 1% BSA–PBS were added and the plates were incubated for 1 h at room temperature.

After washing five times with washing buffer, 100 μL of serum sample were added and the sample was incubated for 1 h at room temperature. To make the calculation curve, sequentially diluted monoclonal anti-dinitrophenol IgE (Sigma) was used as the standard. After washing five times with washing buffer, 100 μL of diluted peroxidase-labelled polyclonal anti-mouse IgE polyclonal antibody (Nordic Immunological Lab., Tilburg, Netherlands) were added and the material was incubated for 1 h at room temperature. After washing five times with washing buffer, 100 μL of substrate solution were added and the material was incubated for 30 min at room temperature in the dark. To stop the reaction, 50 μL of 2 M H_2SO_4 were added and then the plates were read at 492 nm.

The total IgG concentration in serum was measured by the method described by Nagai et al (1997). Briefly, a 96-well immuno plate (Nunc Immunoplate I) was coated with 100 μL/well of monoclonal anti-mouse IgG antibody (Cappel, Cooper Biochemical Inc., Malvern, PA) at 5 μg mL^{-1} in PBS (−) and incubated overnight at 4 °C. After washing the plate three times with washing buffer, 200 μL of 1% BSA–PBS were added and the material was incubated for 1 h at room temperature. After washing as described above, 100 μL of serum sample were added and the sample was incubated for 2 h at room temperature. To make the calculation curve, sequentially diluted monoclonal mouse IgG (Miles Scientific, Naperville, IL) was used as the standard. After washing extensively, 100 μL of diluted goat anti-mouse IgG peroxidase (Cappel, Organon Teknika Co. West Chester, PA) were added and the material was incubated for 1 h at room temperature. After washing, 100 μL of substrate solution were added and the solution was incubated for 30 min at room temperature in the dark. To stop the reaction, 50 μL of 2 M H_2SO_4 were added and the plates were read at 492 nm.

Experiments in guinea-pigs

Sensitization and challenge

Guinea-pigs were actively sensitized by intraperitoneal injection of ovalbumin (0.5 and 1 mg/head on days 0 and 2) twice according to the method described previously (Nagai et al 1995b; Takeda et al 1997). On day 22, the guinea-pig was placed in a chamber connected to a Devilbiss Pulmo Aide nebulizer and it inhaled a 0.1% ovalbumin spray via a plastic face chamber on the snout for 1 min. To measure the late-phase increase in respiratory resistance (Rrs), actively sensitized guinea-pigs were repeatedly exposed to antigen following treatment on day 22. One-minute inhalations of ovalbumin (0.2, 0.4, 0.5 and 1%) were conducted on days 24, 27, 31 and 36 by using an ultrasonic nebulizer. On day 38, the guinea-pigs were exposed to 1% ovalbumin for 1 min, and the dual (i.e. immediate and late asthmatic responses; IAR and LAR) increase in Rrs was measured. Drugs were administered intraperitoneally 1 h before the challenge.

Measurement of airway responsiveness

The increase in Rrs in guinea-pigs was measured by using the modified method of Mead (1960) described by Yamauchi et al (1984). In brief, each guinea-pig was positioned inside the chamber of a body plethysmograph with the head outside of the chamber. The respiratory air flow from the face mask at the snout and the oscillating pressure in the body chamber at 2 cmH_2O with 30 Hz sine-wave pressure were recorded with a differential pressure transducer. These signals were displayed simultaneously on an X-Y oscilloscope and recorded on a polygraph. Rrs was calculated using the following formula: Rrs = recorded amplitude of pressure inside the chamber/recorded amplitude of flow. In this study, the results were expressed as the mean ± standard error of mean (s.e.m.) of the percent change in the Rrs values of individual guinea-pigs.

For all groups 24 h after last challenge, AHR was determined by measuring Rrs in response to doubling the concentration of acetylcholine (0.5–4 mg mL^{-1}) on day 39. The method of measuring Rrs was the same as that described above. The results were expressed as the mean ± s.e.m. of the provocative concentration of acetylcholine (mg mL^{-1}) required to produce a 50% increase in Rrs (PC50) determined from each dose–response curve to acetylcholine.

Measurement of the number of cells in BALF

After measurement of airway responsiveness, guinea-pigs were sacrificed by intraperitoneal injection of urethane (5 g kg^{-1}). The trachea was cannulated, and the airway lumen was washed twice with 5 mL of saline containing 0.1% BSA and 10 μg mL^{-1} of indometacin (to prevent artificial generation of cyclooxygenase products) warmed to 37 °C. BALF from each guinea-pig was pooled in a plastic

tube cooled in ice and then centrifuged (150 *g*) at 4 °C for 10 min. The cell pellets were resuspended in physiological saline (2 mL). The total number of leucocytes was counted after Turk staining, and the differentiated cell-type counts (× 500) were made from a smear prepared with the cytocentrifuge and stained according to May-Giemsa. The results are expressed as the total cell counts in BALF.

Statistics

All data are represented as mean ± s.e.m. To analyse these data, after judging whether a normal distribution or not using F-test, Student's *t*-test was used in the case of normal distribution, otherwise the Mann-Whitney test was performed between non-sensitized-ovalbumin (NS-OA), or non-sensitized-saline (NS-Sal), and sensitized-ovalbumin (S-OA) groups. Dunnett's multiple comparison test was performed between S-OA and drug-treated groups. *P* values of less than 0.05 were considered significant.

Results

Changes of body weight in mice

The body weight of mice administered MX-68 and methotrexate did not change, but prednisolone caused a significant decrease of body weight after administration for 10 days (Figure 1).

Effect of MX-68 on antigen-induced AHR to acetylcholine in mice

In contrast to unsensitized mice, the dose–response curves for acetylcholine were shifted to the left by repeated antigen provocation in sensitized mice. A significant increase in the AUC was observed in sensitized mice. MX-68 at doses of 0.1–1 mg kg^{-1} significantly inhibited the increase in the AUC in a dose-related fashion; methotrexate at a dose of 0.3 mg kg^{-1} and prednisolone at a dose of 5 mg kg^{-1} also inhibited the AHR (Figure 2).

Effect of MX-68 on antigen-induced airway inflammation in mice

To evaluate antigen-induced airway inflammation in mice, the accumulation of inflammatory cells in BALF was studied. The number of total cells, macrophages, eosinophils and lymphocytes in BALF increased after challenge. MX-68 inhibited the increase in the number of total inflammatory cells, eosinophils and lymphocytes, but not macrophages and neutrophils. Even at a low dose, MX-68 tended to decrease the number of eosinophils and lymphocytes. Methotrexate reduced the increase in total cells and prednisolone significantly reduced the increase in total cells, macrophages and eosinophils (Figure 3).

Effect of MX-68 on cytokine production in BALF of mice

The levels of IL-4, IL-5 and IFN-γ in BALF are shown in Figure 4. The levels of IL-4 and IL-5 were increased and that of IFN-γ was significantly decreased after the three antigen provocations. MX-68 clearly inhibited the increase in the levels of IL-4 and IL-5 in a dose-dependent manner but did not affect the decrease in the level of IFN-γ. Similarly, methotrexate and prednisolone inhibited the increase in the levels of IL-4 and IL-5.

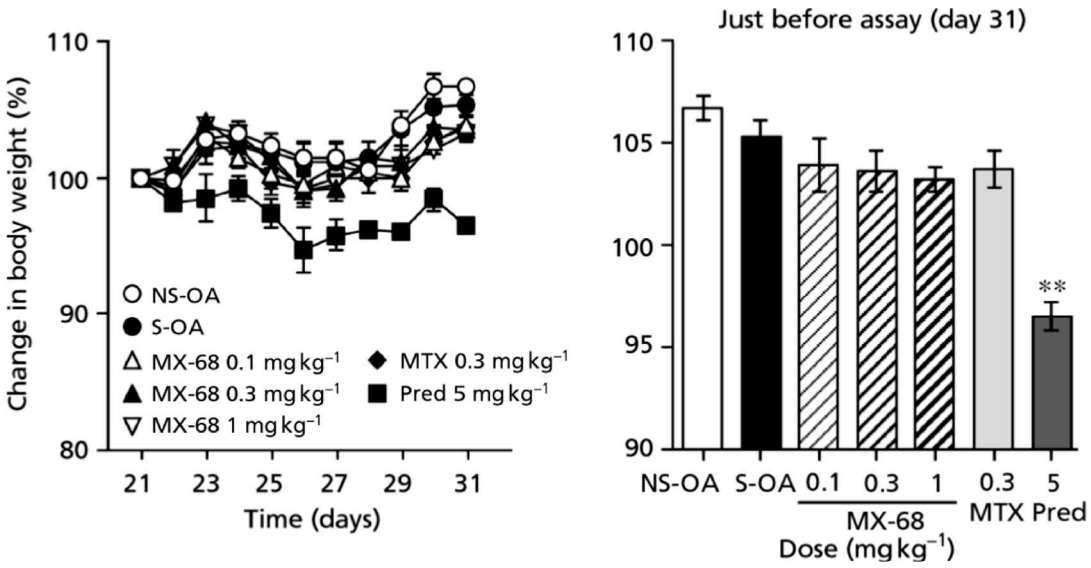

Figure 1 Effect of MX-68 on changes in body weight before and after administration for 10 days in BALB/c mice. Results are represented as the means ± s.e.m. of 6–7 mice. NS, non-sensitized; S, sensitized; OA, ovalbumin inhalation; MTX, methotrexate; Pred, prednisolone. $^{**}P < 0.01$ vs S-OA (Dunnett's multiple comparison test).

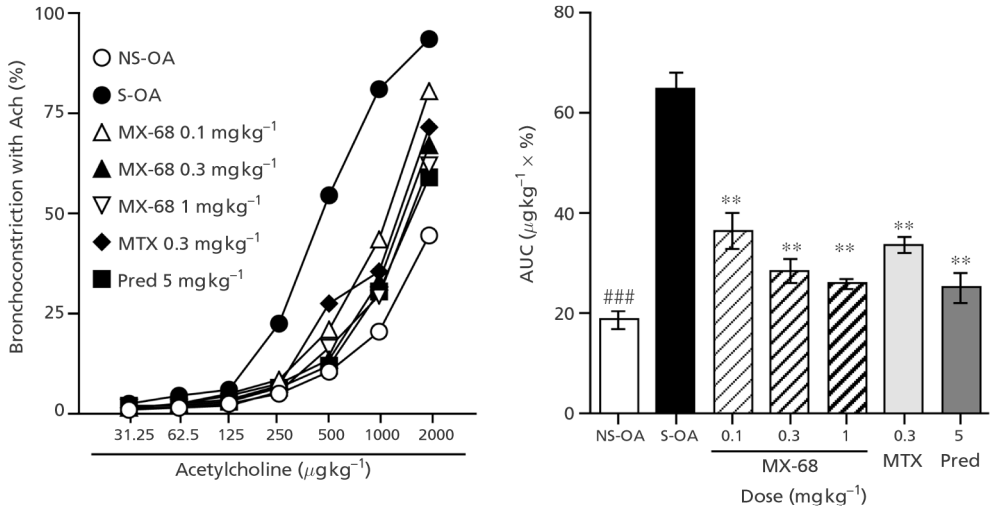

Figure 2 Effect of MX-68 on antigen-induced airway hyperresponsiveness to acetylcholine (Ach) after repeated antigen challenge in BALB/c mice. Bronchoconstriction was measured using the method of Konzett & Rössler (1940). Results are represented as the means (or means ± s.e.m.) of 6 or 7 mice. AUC, area under the curve (range: 31.25–2000 $\mu g\,kg^{-1}$); NS, non-sensitized; S, sensitized; OA, ovalbumin inhalation; MTX, methotrexate; Pred, prednisolone. ###$P < 0.001$ vs S-OA (Student's t-test); **$P < 0.01$ vs S-OA (Dunnett's multiple comparison test).

Effect of MX-68 on immunoglobulin level in serum of mice

Figure 5 shows the immunoglobulin level in serum. The levels of antigen-specific IgE, total IgE and IgG were increased after the antigen challenge. MX-68 at a dose of $1\,mg\,kg^{-1}$ and prednisolone at a dose of $5\,mg\,kg^{-1}$ significantly inhibited the elevation of serum immunoglobulin levels and MX-68 at low doses tended to decrease each immunoglobulin level. Methotrexate at a dose of $0.3\,mg\,kg^{-1}$ significantly inhibited the increase in the levels of antigen-specific IgE and IgG and showed a tendency to inhibit the increase in the level of total IgE.

Effect of MX-68 on antigen-induced AHR to acetylcholine in guinea-pigs

In guinea-pigs, Rrs was increased and antigen-induced IAR and LAR occurred at 1 min and 4 h after the last inhalation of antigen. MX-68 at a dose of $1\,mg\,kg^{-1}$ significantly inhibited both reactions (Figure 6). Particularly in LAR, Rrs recovered to the baseline level with MX-68.

The PC50 value to acetylcholine decreased significantly after the last challenge and an AHR to acetylcholine was observed in guinea-pigs. MX-68 at a dose of $1\,mg\,kg^{-1}$ significantly inhibited the antigen-induced AHR (Figure 6).

Effect of MX-68 on antigen-induced airway inflammation in guinea-pigs

In guinea-pigs, the number of eosinophils and lymphocytes in BALF increased after the antigen challenge. MX-68 at a dose of $1\,mg\,kg^{-1}$ significantly inhibited the increase in the number of eosinophils and lymphocytes. Methotrexate at a dose of $1\,mg\,kg^{-1}$ reduced the increase in lymphocytes but not in eosinophils (Figure 7).

Discussion

Methotrexate has immunosuppressive effects and is used for the treatment of rheumatoid arthritis clinically, but it often causes side effects, such as gastrointestinal toxicity, bone-marrow suppression and fever, as well as hepatic and pulmonary fibrosis. These adverse effects are considered to be based on the toxicity caused by the high concentration of methotrexate intracellular storage. Against this background, MX-68 was developed as a derivative of methotrexate with the aim of avoiding severe side effects. In this study, although other side effects were not confirmed, a decrease in bodyweight was not observed after administration of MX-68. This result demonstrates one of the aspects of safety of MX-68, which is clearly different from prednisolone.

In this study, we examined the effect of MX-68 on airway inflammation and hyperresponsiveness using the antigen-induced airway hyperresponsiveness model in mice and guinea-pigs. In these models, repeated antigen inhalation causes airway hyperresponsiveness, accumulation of inflammatory cells such as eosinophils in BALF, production of Th2 cytokines and elevation of serum immunoglobulin levels. These features reflect the actual clinical condition of bronchial asthma.

As shown by the results, MX-68 at a dose of 0.1 or $0.3\,mg\,kg^{-1}$ clearly inhibited airway inflammation, airway hyperresponsiveness and production of Th2 cytokines in mice. In guinea-pigs, the effects of MX-68 at a dose of $0.5\,mg\,kg^{-1}$ on airway inflammation and airway

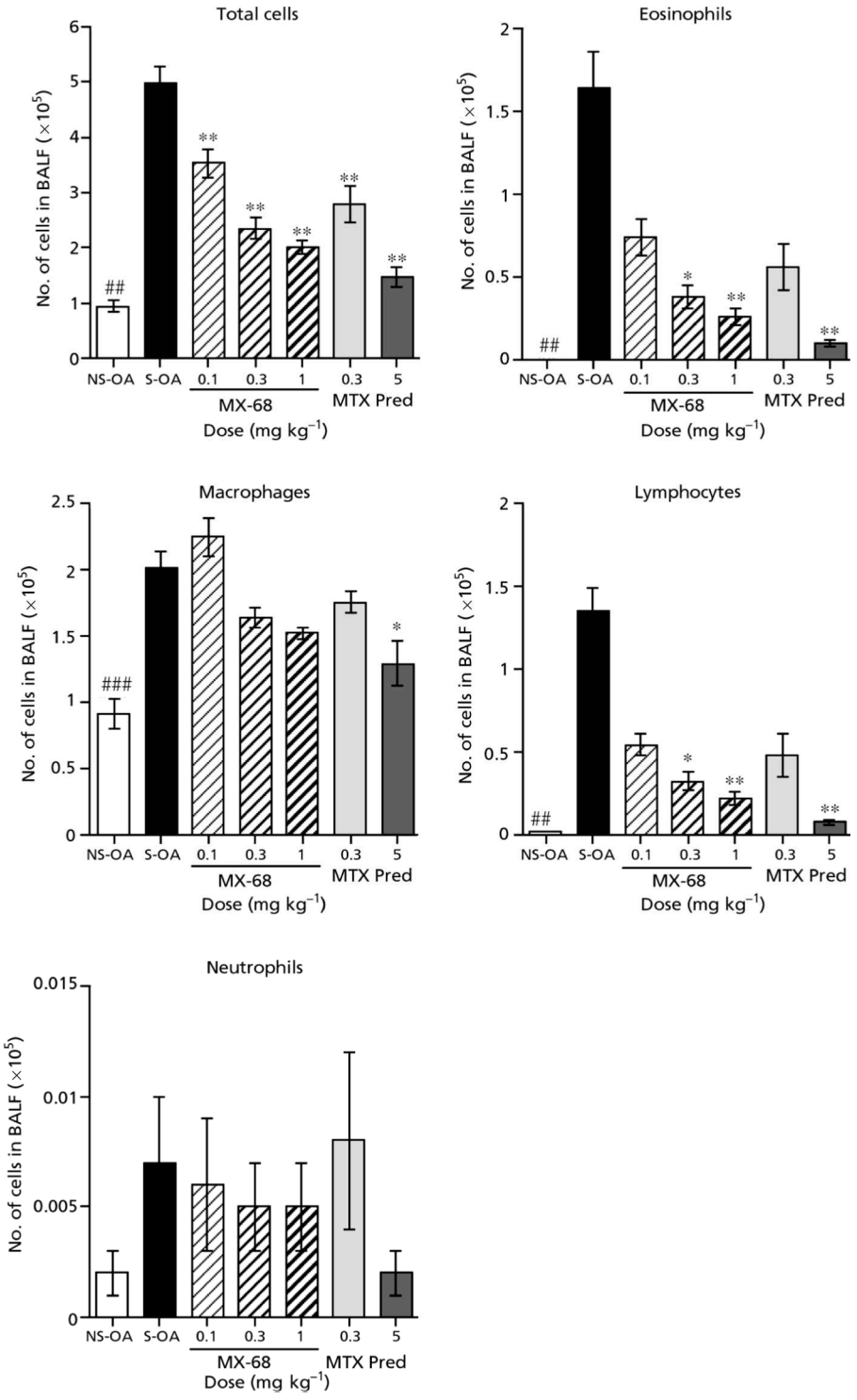

Figure 3 Effect of MX-68 on antigen-induced airway inflammation after repeated antigen challenge in BALB/c mice. Results are represented as the means ± s.e.m. of 6 or 7 mice. NS, non-sensitized; S, sensitized; OA, ovalbumin inhalation; MTX, methotrexate; Pred, prednisolone. ##$P < 0.01$, ###$P < 0.001$ vs S-OA (Student's *t*-test or Mann-Whitney test); *$P < 0.05$, **$P < 0.01$ vs S-OA (Dunnett's multiple comparison test).

hyperresponsiveness were not significant. The differences of administration route, method of sensitization and challenge may affect these results.

In addition, MX-68 at a dose of 1 mg kg^{-1} inhibited the production of immunoglobulin. These data indicate that MX-68 has an anti-inflammatory effect due to both

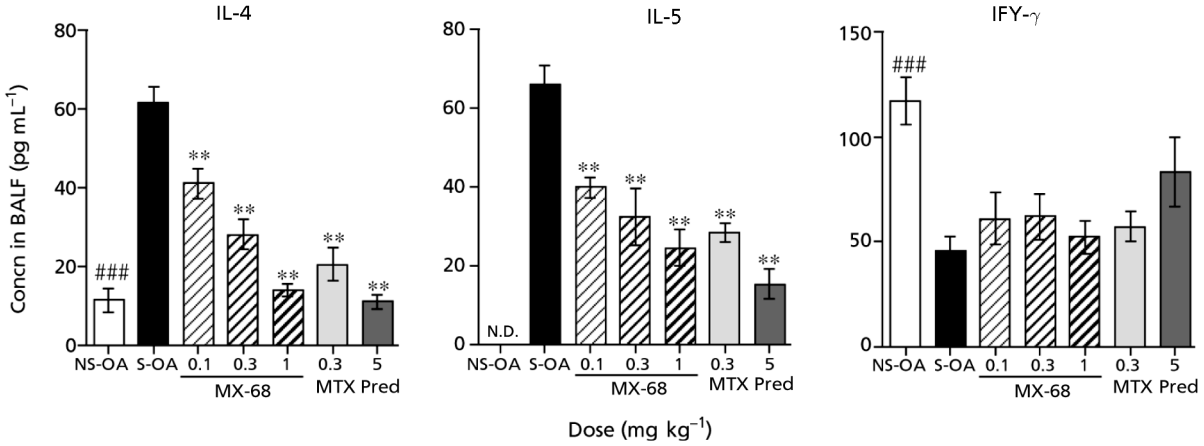

Figure 4 Effect of MX-68 on cytokine production in bronchoalveolar lavage fluid (BALF) after repeated antigen challenge in BALB/c mice. Results are represented as means ± s.e.m. of 6 or 7 mice. N.D., Not detected; NS, non-sensitized; S, sensitized; OA, ovalbumin inhalation; MTX, methotrexate; Pred, prednisolone. ###$P < 0.001$ vs S-OA (Student's t-test); **$P < 0.01$ vs S-OA (Dunnett's multiple comparison test).

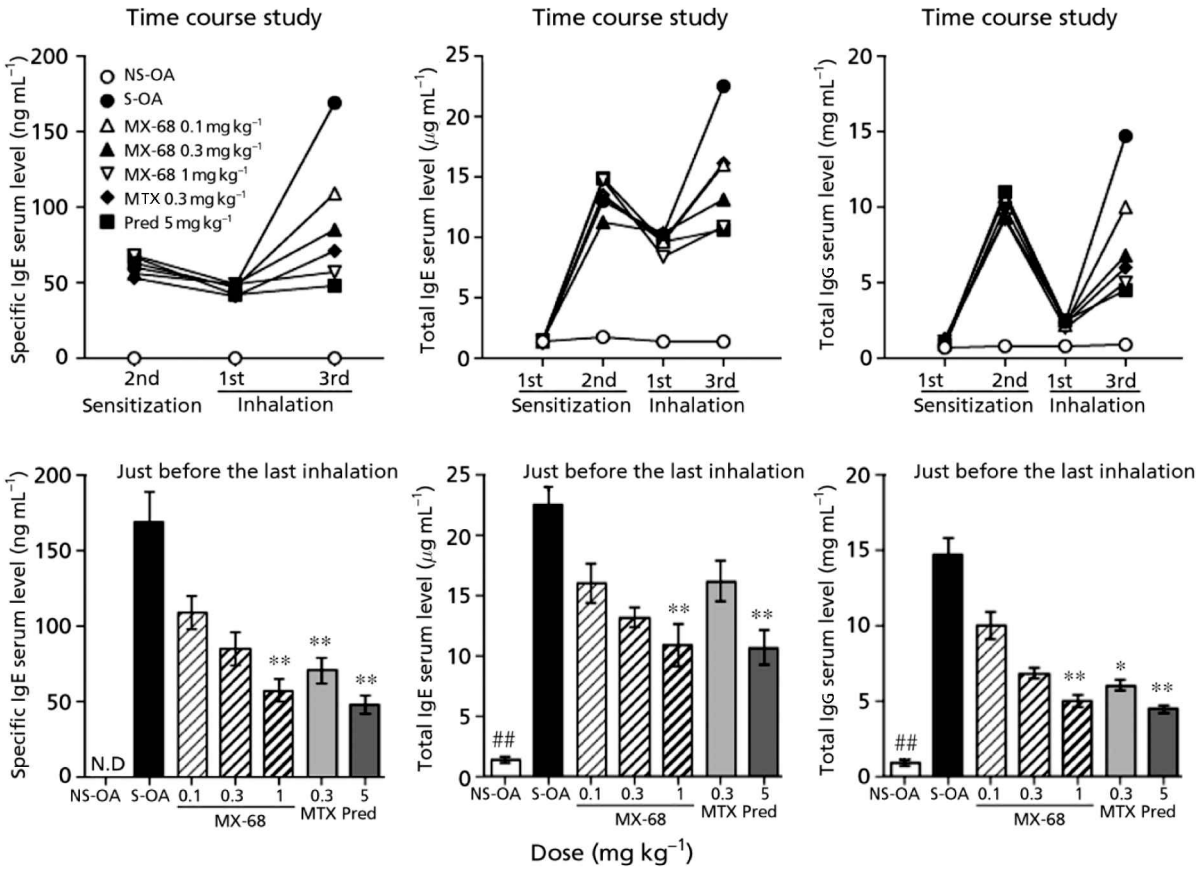

Figure 5 Effect of MX-68 on increases in serum antigen-specific IgE, total IgE and total IgG after repeated antigen challenge in BALB/c mice. Results are represented as the means ± s.e.m. of 6 or 7 mice. N.D., Not detected; NS, non-sensitized; S, sensitized; OA, ovalbumin inhalation; MTX, methotrexate; Pred, prednisolone. ##$P < 0.01$ vs S-OA (Mann-Whitney test); *$P < 0.05$, **$P < 0.01$ vs S-OA (Dunnett's multiple comparison test).

immunosuppressive and non-immunosuppressive actions. As far as the immunosuppressive effects are concerned, there are some reports showing a close relationship between AHR and serum IgE level (Sears et al 1991). Therefore, one of the anti-asthmatic mechanisms of methotrexate may be closely related to immunosuppression, as shown in the study of rheumatoid arthritis (suppression of rheumatoid factors and immunoglobulin levels) (Spadaro

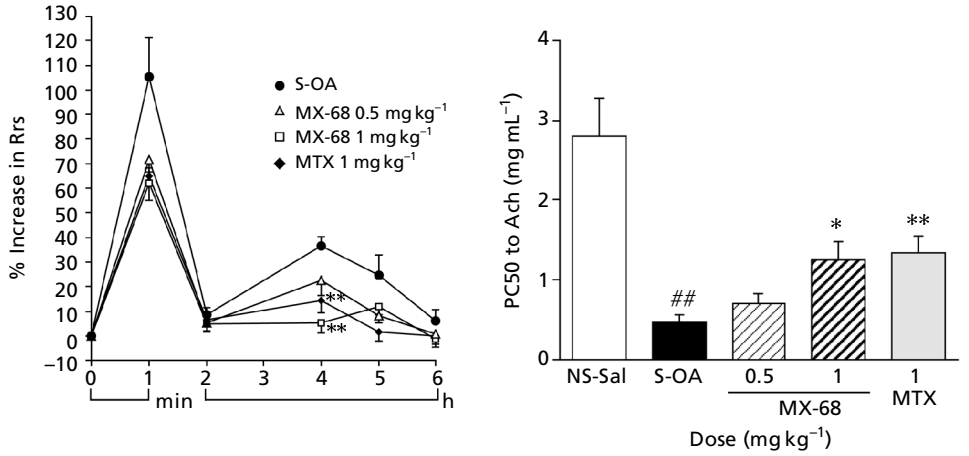

Figure 6 Effect of MX-68 on antigen-induced increase in respiratory resistance (Rrs) and airway hyperresponsiveness to acetylcholine (Ach) in conscious guinea-pigs. Results are shown as the mean ± s.e.m. of 6 guinea-pigs. NS, non-sensitized; S, sensitized; Sal, Saline inhalation; OA, ovalbumin inhalation; MTX, methotrexate. ##$P < 0.01$ vs NS-Sal (Mann-Whitney test); *$P < 0.05$, **$P < 0.01$ vs S-OA (Dunnett's multiple comparison test).

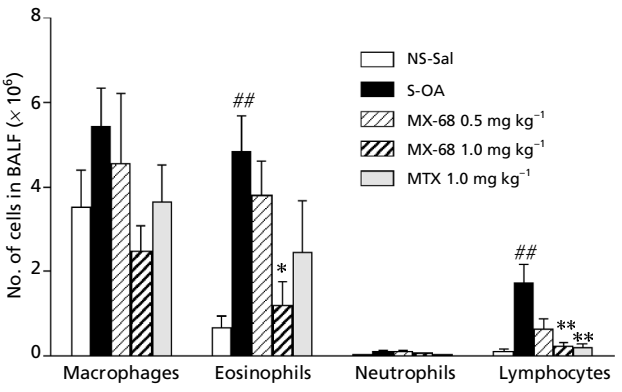

Figure 7 Effect of MX-68 on antigen-induced airway inflammation in guinea-pigs. Results are shown as the mean ± s.e.m. of 6 animals. NS, non-sensitized; S, sensitized; Sal, Saline inhalation; OA, ovalbumin inhalation; MTX, methotrexate. ##$P < 0.01$ vs NS-Sal (Mann-Whitney test); *$P < 0.05$, **$P < 0.01$ vs S-OA (Dunnett's multiple comparison test).

et al 1993; Rackham et al 2002). O'Meara et al (1985) and Rosenthal et al (1987) reported that methotrexate inhibited the production of immunoglobulin from human monocytes and mouse splenocytes. Our study confirmed that methotrexate, as well as MX-68, inhibited the increase in immunoglobulin levels in serum. The effects of both drugs are similar because MX-68 was chemically designed to avoid toxicity while retaining the activity of methotrexate.

We previously examined the effect of rapamycin on the mouse model of asthma with other immunosuppressors. Rapamycin is a macrolide antibiotic immunosuppressor, which inhibits the immunological response (Martel et al 1977). However, in our previous study, instead of inhibiting immunoglobulin production, rapamycin did not inhibit the AHR to acetylcholine (Nagai et al 1997). These results mean that the modulation of immunoglobulin production does not always correlate with the AHR. Complex mechanisms may contribute to the inhibition of the AHR by MX-68.

Another reason may be related to the unique anti-inflammatory actions of MX-68. Methotrexate has certain anti-inflammatory effects other than the inhibition of immunoglobulin production. For example, it inhibits neutrophil and macrophage functions (Hu et al 1988; Crostein et al 1991; Sperling et al 1992). Therefore, MX-68 also may have additive effects.

It is considered that in allergic diseases including asthma, the balance between Th1 and Th2 shifts to the Th2 side and an imbalance results. For example, in this study, Th2 cytokines (IL-4 and IL-5) increased in BALF and the Th1 cytokine (IFN-γ) decreased after antigen inhalation. In many cases, anti-asthmatic drugs modify the Th1 and Th2 balance (Bentley et al 1996; Lin et al 2002). But MX-68 only significantly inhibited the increase in Th2 cytokines, and had no effect on the decrease of Th1 cytokine. This is the difference between MX-68 and other drugs reported to be useful for the treatment of asthma. MX-68 may affect only the abnormal increase in the Th2 response.

MX-68 inhibited antigen-induced airway inflammation and Th2 cytokine production at a dose of 0.3 mg kg^{-1} and had an anti-inflammatory effect at this low dose. However, it had no effects on the immunoglobulin level in serum at this low dose. Therefore, the anti-asthmatic effects of MX-68 may relate to its anti-inflammatory activity based on both immunosuppressive and non-immunosuppressive effects. These effects are different from anti-rheumatoid arthritis effects. Although the mechanism is still unclear in its details, this study revealed the efficacy of MX-68 as an immunosuppressor for the treatment of asthma.

Furthermore, inhaled corticosteroids play an important role in the therapy of asthma and a drug that is used with steroids is wanted clinically (Pedersen & Byrne 1997). Many studies have been carried out with combinations of steroids and long-acting β_2-agonists or cysteinyl leukotriene antagonists wherein the efficacy was proved (Woolcock et al 1996; Nayak et al 1998). Because methotrexate, a preform of MX-68, also has a steroid-sparing effect (Dyer et al 1991; Stewart et al 1994; Marin 1997), and MX-68 possesses similar activity to methotrexate, the safety is greatly expected to be useful clinically.

Conclusion

MX-68, a newly synthesized antifolate, is a derivative of methotrexate, which is used as an immunosuppressor for the treatment of rheumatoid arthritis. Because it is not polyglutamated, MX-68 was developed to avoid severe side effects. Therefore, we think that it is a very promising compound.

In this study, for further research on immunosuppressive drugs, we examined the effect of MX-68 on the AHR to acetylcholine and airway inflammation in mice and guinea-pigs. As a result, MX-68 showed inhibitory effects on airway inflammation, hyperresponsiveness, the increase in the levels of Th2 cytokines (IL-4 and IL-5) and production of immunoglobulin. It did not, however, affect the decrease in the level of Th1 cytokine (IFN-γ). MX-68 may affect only the abnormal increase of the Th2 response. Furthermore, the anti-asthmatic effects of MX-68 may relate to its anti-inflammatory effects based on both immunosuppressive and non-immunosuppressive effects.

Taking these results into consideration, we think that MX-68 may be useful as an immunosuppressor for the treatment of bronchial asthma.

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