**Prevention of Immunological Disorders in MRL/l Mice
by a New Synthetic Analogue of Vitamin D₃:
22-Oxa-1 α ,25-Dihydroxyvitamin D₃**

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Summary We examined the immunoregulating effect of 22-oxa-1 α ,25-dihydroxyvitamin D₃ (22-oxa-1 α ,25(OH)₂D₃), a synthetic analogue of vitamin D₃ with an oxygen atom at C₂₂ in the side chain skeleton, on spontaneously developing autoimmune disorders in MRL/Mp-lpr/lpr (MRL/l) mice. The oral administration of the compound significantly prolonged the average life span of the mice and showed a significant reduction in proteinuria. Histopathological investigations also revealed that pathological conditions such as renal arteritis, granuloma or arthritis of the knee joints were much lighter in the treated group than in the untreated group. Furthermore, the lymphocyte phenotypes in thymus, lymphnode, and spleen were partially normalized and became similar to those found in young control animals by the treatment with 22-oxa-1 α ,25(OH)₂D₃. These results suggest that this compound inhibits the development of lupus nephritis in MRL/l mice and may be therapeutically effective on the mice.

Key Words 22-oxa-1 α ,25(OH)₂D₃, 1 α ,25(OH)₂D₃, autoimmune disorders, MRL/Mp-lpr/lpr mice, immunoregulating activity

1 α ,25-Dihydroxyvitamin D₃ (1 α ,25(OH)₂D₃) is a hormonal form of vitamin D₃ active in the regulation of calcium homeostasis. Since the first report in 1981 (1) that 1 α ,25(OH)₂D₃ inhibited the proliferation and induced the differentiation of murine myeloid leukemic M1 cells into monocyte-macrophages, many studies have demonstrated that 1 α ,25(OH)₂D₃ is a potent modulator not only in suppressing proliferation and inducing differentiation of numerous normal and tumor cells, but

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also in regulating the functions of immunologically competent cells such as T and B lymphocytes (2,3). These findings are consistent with the fact that receptors for $1\alpha,25(\text{OH})_2\text{D}_3$ are present in many kinds of cells (4,5). Nevertheless, the major function of active vitamin D is to elevate the plasma calcium level as $1\alpha,25(\text{OH})_2\text{D}_3$ induces hypercalcemia in addition to cellular differentiation and immunomodulation. These results prompted us to synthesize vitamin D_3 analogues which had a potent differentiation-inducing activity without inducing hypercalcemia.

We have synthesized numerous analogues of vitamin D_3 compounds with an oxygen atom in the side chain (6,7). Among them, only 22-oxa- $1\alpha,25(\text{OH})_2\text{D}_3$ had a more potent differentiation-inducing effect (8) and a more strongly augmented primary immune response (9) than $1\alpha,25(\text{OH})_2\text{D}_3$ without inducing hypercalcemia. These results indicated that the immunoregulating activity of vitamin compounds could be separated structurally from their calcium-regulating activity.

MRL/Mp-lpr/lpr (MRL/l) mice spontaneously develop various pathological changes which resemble human autoimmune disease such as systemic erythematosus (SLE) or rheumatoid arthritis (RA) (10–13). These changes include lupus nephritis and vasculitis. These age-dependent progressive features are characterized by the expansion and accumulation of an abnormal T cell subset in lymphoid and other organs, which express the phenotypes, Thy1^+ , dull Ly-1 , Lyt-2^- , L3T4^- (14). Thus it is established that the fundamental abnormality of MRL/l mice is the immunological dysfunction.

This paper describes the effects of 22-oxa- $1\alpha,25(\text{OH})_2\text{D}_3$ on MRL/l mice as an autoimmune model, suggesting that it may be a candidate for a therapeutic agent against autoimmune disease.

MATERIALS AND METHODS

MICE. MRL/Mp-lpr/lpr (MRL/l) mice were provided by the Jackson Laboratory, Bar Harbor, ME., and have been bred at the Laboratory Animal Center in our Research Laboratories. Six-week-old specific-pathogen-free males were kept in cages in a room maintained at $24 \pm 2^\circ\text{C}$ and 50–60% relative humidity.

22-OXA- $1\alpha,25(\text{OH})_2\text{D}_3$. The new derivative of vitamin D_3 , 22-oxa- $1\alpha,25(\text{OH})_2\text{D}_3$, was synthesized in our laboratory. The detailed synthetic procedure was described previously (6,7). Various amounts of 22-oxa- $1\alpha,25(\text{OH})_2\text{D}_3$ dissolved in 1% ethanol in water were administered to the mice orally.

For the mortality study, two groups of mice were given orally either 2 or 100 ng/kg of 22-oxa- $1\alpha,25(\text{OH})_2\text{D}_3$ 5 days a week from 6 weeks of age until death. A control group was similarly given the same volume of vehicle. Each group started with more than ten mice. In the subsequent experiment, six mice were sacrificed at 24 weeks of age to examine the histopathological changes and the phenotypes of lymphocytes.

Appearance of proteinuria. Proteinuria was examined at 6, 11, 16, 21, 26, and 30 weeks of age by means of Combistix paper (Sankyo, Tokyo). Five stages of

proteinuria were determined semiquantitatively, 0–30, 30–100, 100–300, 300–1,000, and >1,000 mg/dl, respectively. The last two stages, 300–100 and >1,000 mg/dl, were considered positive.

Histological examination. The kidneys and knee joints were removed, fixed in 10% buffered formalin, embedded in paraffin, and sectioned at 2 μ m thickness. After staining with hematoxylin-eosin (HE), Azan and periodic acid-Schiff (PAS), the specimens were observed by light microscopy.

Cell staining and flow cytometry. Fluorescein isothiocyanate (FITC)-conjugated anti-B220 and phycoerythrin (PE)-conjugated anti-Thy1.2 monoclonal antibodies were kindly provided by Dr. K. Okumura (Department of Immunology, School of Medicine, Juntendo University, Tokyo). FITC-conjugated anti-Lyt-2 and PE-conjugated anti-L3T4 monoclonal antibodies were purchased from Becton Dickinson, Mountain View, CA. The thymus, lymphnode, and spleen were removed at 24 weeks of age and a single cell suspension of each was prepared by teasing the tissues, washing with phosphate-buffered saline (PBS) containing 1% fetal calf serum (FCS), and passing through nylon mesh. The spleen cells were used after depletion of erythrocytes.

Aliquots of 1×10^6 cells were directly stained with FITC-conjugated anti-B220 or FITC-conjugated anti-Lyt-2 monoclonal antibody at 4°C for 30 min, and sequentially followed by PE-conjugated anti-Thy1.2 or PE-conjugated anti-L3T4 monoclonal antibody. After washing with PBS containing 1% FCS, the stained cells were applied to a FACS star (Becton Dickinson). The two-color analysis was performed for 10,000 viable cells gated by light scatter.

Statistical analysis. Results were expressed as the mean $\pm$ SEM. Values of the proteinuria and survival time were analyzed by Wilcoxon's T-test and the others were assessed by Student's *t*-test.

RESULTS

Effect of 22-oxa-1 α ,25(OH) $_2$ D $_3$ on survival of MRL/l mice

MRL/l mice were treated with 22-oxa-1 α ,25(OH) $_2$ D $_3$ at 2 or 100 ng/kg from 6 weeks old until their death. As shown in Fig. 1, while the half survival time for both the control group and the group given the high dose of 100 ng/kg was the same, 39 weeks, that for the group given 2 ng/kg was 51 weeks, which was significantly longer ($p < 0.05$ against the control group).

Effect of 22-oxa-1 α ,25(OH) $_2$ D $_3$ on proteinuria in MRL/l mice

The animals used in the above mortality study were monitored periodically at 6, 11, 16, 21, 26, and 30 weeks of age for the appearance of proteinuria as an indicator of renal dysfunctions (Fig. 2). The mice in the untreated control group clearly showed increased protein excretion in urine. Approximately 50% of them showed positive in proteinuria at 21 weeks of age. Compared with this control group, the group treated with 2 ng/kg of the drug showed a significant inhibition of

Fig. 1. Effect of 22-oxa-1 α ,25(OH) $_2$ D $_3$ on survival of MRL/l mice. More than ten mice were treated with 22-oxa-1 α ,25(OH) $_2$ D $_3$ at 2 ng/kg (■), 100 ng/kg (-----), or none (—) as the control. The mice treated with 2 ng/kg of this compound had significantly prolonged survival compared to the control mice ($p < 0.05$, Wilcoxon's T-test).

Fig. 2. Effect of 22-oxa-1 α ,25(OH) $_2$ D $_3$ on appearance of proteinuria at 6, 11, 16, 21, 26, and 30 weeks of age. The level of proteinuria was represented as the cumulative percentage of positive mice treated with 0 (control) (▲), 2 ng/kg (●), and 100 ng/kg (■) of 22-oxa-1 α ,25(OH) $_2$ D $_3$. Significantly different from the control group, ** $p < 0.01$.

proteinuria at 21, 26, and 30 weeks of age. The 100 ng/kg-treated group also showed a considerable inhibition of proteinuria at 26 and 30 weeks of age, although the effect was weaker than that induced by 2 ng/kg. In the control and 100 ng/kg-treated groups, all the mice that died showed high levels of proteinuria prior to death. In the 2 ng/kg-treated group, all mice lived until at least 30 weeks of age.

Histological analysis

The kidneys and knee joints of the MRL/l mice were histologically examined. All kidneys in the control mice at 24 weeks of age were infiltrated with lymphoid cells around the medium-sized arteries and granulomatous lesions developed (Fig.

Fig. 3. Histopathological changes in the kidney of MRL/l mice at 24 weeks of age. Compared with the control group (A), there was a marked infiltration of lymphoid cells. Lesions were reduced in the 22-oxa-1 α ,25(OH) $_2$ D $_3$ -treated group (2 ng/kg, B, HE 20 $\times$). (Inset) Higher magnification of the same field (HE 100 $\times$).

3A). The oral administration of 22-oxa-1 α ,25(OH) $_2$ D $_3$ at the dose of 2 ng/kg prevented the development of granuloma for all the mice and improved the arteritis for 70% of the mice (Fig. 3B). In addition, calcium was not deposited in the renal tubuli. In the group treated with 100 ng/kg of 22-oxa-1 α ,25(OH) $_2$ D $_3$, the effects of this compound on the formation of granuloma and arteritis were observed on half of the mice.

In the 24-week-old mice, the synovial lining was replaced by vasculitis and infiltration of lymphoid cells (Fig. 4A). On the other hand, the lymphoid cell infiltration and the pannus formation in the mice given 22-oxa-1 α ,25(OH) $_2$ D $_3$ at 2 ng/kg were milder than those in the control mice (Fig. 4B). The improvements in the knee joints by the treatment with 22-oxa-1 α ,25(OH) $_2$ D $_3$ at the dose of 2 ng/kg were observed commonly in 80% of the mice.

Fig. 4. Histopathological changes in the knee joint of MRL/l mice at 24 weeks of age. Compared with the control group (A), the hypertrophy of the synovial lining and infiltration of lymphoid cells were reduced in the 22-oxa-1 α ,25(OH) $_2$ D $_3$ -treated group (2 ng/kg, B, HE 20 $\times$). (Inset) Higher magnification of the same field (HE 100 $\times$).

Effect of 22-oxa-1 α ,25(OH) $_2$ D $_3$ on the abnormal expression of surface antigens

The composition of the subsets of thymus, lymphnode, and spleen in MRL/l mice was analyzed by flow cytometry. Table 1 summarizes the ratio of the cells which showed various cellular phenotypes.

Figure 5 shows a typical pattern by two-color analysis of B220 and Thy1.2 antigens of thymus, lymphnode, and spleen. In normal thymus and lymphnode, almost all lymphocytes are Thy1.2 positive, B220 negative. As shown in the control group, Thy1.2 positive normal T lymphocytes were greatly reduced with age, but Thy1.2 and B220 double positive abnormal T lymphocytes were increased. In the thymus, 22-oxa-1 α ,25(OH) $_2$ D $_3$ significantly changed the expression of Thy1.2 and B220 on the cells, but there was only a slight change in the lymphnode. Also Thy1.2 and B220 double positive cells appeared in the spleen with age. 22-Oxa-1 α ,

Table 1. Effect of 22-oxa-1 α ,25(OH) $_2$ D $_3$ on the occurrence of cell surface antigens in thymus, lymphnode, and spleen of MRL/l mice.

Tissue	Cellular phenotype	Untreated	22-oxa-1 α ,25(OH) $_2$ D $_3$		Young
			2 ng/kg	100 ng/kg	
Thymus	Thy1.2 $^+$, B220 $^-$	45 $\pm$ 14 ^a	81 $\pm$ 5*	70 $\pm$ 21	99 $\pm$ 0**
	Thy1.2 $^+$, B220 $^+$	50 $\pm$ 12	18 $\pm$ 5*	28 $\pm$ 21	1 $\pm$ 0**
	L3T4 $^+$, Lyt-2 $^+$	24 $\pm$ 9	64 $\pm$ 8*	52 $\pm$ 21	89 $\pm$ 0**
	L3T4 $^-$, Lyt-2 $^-$	47 $\pm$ 14	13 $\pm$ 4*	25 $\pm$ 19	2 $\pm$ 0**
Lymphnode	Thy1.2 $^+$, B220 $^-$	17 $\pm$ 2	24 $\pm$ 6	18 $\pm$ 1	81 $\pm$ 3**
	Thy1.2 $^+$, B220 $^+$	78 $\pm$ 2	70 $\pm$ 5	77 $\pm$ 0	15 $\pm$ 2**
	L3T4 $^+$, Lyt-2 $^-$	23 $\pm$ 1	33 $\pm$ 12	20 $\pm$ 1	47 $\pm$ 2**
	L3T4 $^-$, Lyt-2 $^+$	5 $\pm$ 2	8 $\pm$ 1	8 $\pm$ 1	38 $\pm$ 0**
Spleen	L3T4 $^-$, Lyt-2 $^-$	70 $\pm$ 0	58 $\pm$ 11	70 $\pm$ 0	10 $\pm$ 2**
	Thy1.2 $^+$, B220 $^-$	24 $\pm$ 4	35 $\pm$ 6	32 $\pm$ 2	12 $\pm$ 0
	Thy1.2 $^+$, B220 $^+$	50 $\pm$ 6	29 $\pm$ 5*	29 $\pm$ 5*	4 $\pm$ 1*
	Thy1.2 $^-$, B220 $^+$	14 $\pm$ 4	26 $\pm$ 1*	25 $\pm$ 2*	20 $\pm$ 2
	L3T4 $^+$, Lyt-2 $^-$	26 $\pm$ 4	41 $\pm$ 2*	35 $\pm$ 2	11 $\pm$ 1*
	L3T4 $^-$, Lyt-2 $^+$	11 $\pm$ 3	9 $\pm$ 1	8 $\pm$ 3	7 $\pm$ 0

^a Values (mean $\pm$ SEM) are percentage of positive cells. Tissues of three mice from each group at 24 weeks of age were individually teased and analyzed by two-color flow cytometry after staining with two kinds of combinations of anti Thy1.2 and B220 antibodies or anti L3T4 and Lyt-2 antibodies. Significantly different from the control group. * p < 0.05, ** p < 0.01.

Fig. 5. Two-color analysis by flow cytometry of cell surface antigens of Thy1.2 and B220 in the thymus (A), lymphnode (B), and spleen (C) of MRL/l mice treated with 0 (control) (a) and 2 ng/kg (b) of 22-oxa-1 α ,25(OH) $_2$ D $_3$. Young control (c).

Fig. 6. Two-color analysis by flow cytometry of cell surface antigens of L3T4 and Lyt-2 in the thymus (A), lymphnode (B), and spleen (C) of MRL/l mice treated with 0 (control) (a) and 2 ng/kg (b) of 22-oxa-1 α ,25(OH) $_2$ D $_3$. Young control (c).

25(OH) $_2$ D $_3$ suppressed significantly the occurrence of these abnormal cells and increased the proportion of either Thy1.2 or B220 single positive cells as seen in young controls.

Figure 6 shows the changes in the L3T4 and Lyt-2 antigens in the thymus, lymphnode, and spleen. In lymphnodes of the untreated control group, approximately 70% of the cells were double negative. In contrast, L3T4 or Lyt-2 single positive mature cells appeared in the young control group. The 22-oxa-1 α ,25(OH) $_2$ D $_3$ -treatment had increased the single positive cells and decreased the double negative cells. More than 95% of thymocytes in young controls were L3T4 and Lyt-2 double positive, while less than 25% of those in the untreated old controls were double positive. The treated group showed a marked increase in L3T4 and Lyt-2 double positive thymocytes.

DISCUSSION

This is the first report to describe the beneficial effects of 22-oxa-1 α ,25(OH) $_2$ D $_3$ on autoimmune disorders developing with age in MRL/l mice.

Our previous study (9) revealed that the dose-response curve of 22-oxa-1 α ,25(OH) $_2$ D $_3$ on the *in vivo* immunoregulating activity made a bell-shape form, being most effective at 2 ng/kg and less effective at dosages higher than 2 ng/kg. The 1 α ,25(OH) $_2$ D $_3$ also showed the similar patterns, a bell-shape form, on the immune response, even though the most effective dosage was 100 ng/kg. These patterns are often seen, especially in immune responses.

We examined in advance the dose response of this compound on the changes of the appearance of proteinuria in MRL/l mice. At 24 weeks of age, 80% of control mice showed the severe elevation in proteinuria. 0.1, 0.5 ng/kg of 22-oxa-1 α ,25(OH) $_2$ D $_3$ induced 80, 40% of the mice positive in proteinuria, respectively. The

mice treated at 2 ng/kg showed all negative in proteinuria. However, both 10 and 100 ng/kg of this compound induced again the elevation in proteinuria for 50% of the mice. Since it was estimated that 22-oxa-1 α ,25(OH) $_2$ D $_3$ exhibited the most potent effect in MRL/l mice at 2 ng/kg and rather no effect at 100 ng/kg, the two dosages of this compound were chosen for the mortality study.

MRL/l mice treated with 2 ng/kg of the compound survived significantly one and a half times longer than the untreated controls in average. Nor did the treated group of mice develop proteinuria significantly. This shows that the prolongation of the life span of these mice may be due to the prevention of renal disease by this compound. The pathological changes in kidneys were also corrected in the 2 ng/kg-treated group, suggesting that 22-oxa-1 α ,25(OH) $_2$ D $_3$ should prolong the life span by preventing the renal tissue damage, which would otherwise result in their death. Moreover 22-oxa-1 α ,25(OH) $_2$ D $_3$ at a dose of 2 ng/kg significantly failed to increase the plasma blood urea nitrogen (BUN) levels at 28 weeks of age (control, 42.3 $\pm$ 4.6; 2 ng/kg, 31.5 $\pm$ 1.5, p < 0.05 against the control group; 100 ng/kg, 36.1 $\pm$ 5.8 mg/dl). These results supported that the effectiveness of this compound was due to reducing the renal pathological and functional changes.

The present studies confirm that there was an abnormal intrathymic T cell development in MRL/l mice. Thymocytes in the mice older than 18 weeks of age were highly enriched in B220 $^+$, L3T4 $^-$, Lyt-2 $^-$ cells, suggesting an abnormal intrathymic T cell maturation. The neonatal thymectomy of MRL/l mice prevented the autoimmune disorders. Therefore, the abnormal peripheral lymphocytes in MRL/l mice were considered to be under thymic influence (15,16). The treatment with 22-oxa-1 α ,25(OH) $_2$ D $_3$ induced a drastic change in lymphocyte populations, increasing the L3T4, Lyt-2 double positive cells and Thy1.2 positive cells while reducing Thy1.2, B220 double positive cells. These changes in lymphocyte subpopulations in MRL/l mice effected by 22-oxa-1 α ,25(OH) $_2$ D $_3$ occurred much more in the thymus than in the lymphnode. Lymphadenopathy, one of the characteristic abnormalities in MRL/l mice, was closely associated with the abnormal T cells, whose phenotypes were L3T4 $^-$ and Lyt-2 $^-$. The 22-oxa-1 α ,25(OH) $_2$ D $_3$ showed a distinct tendency of reducing the L3T4 $^-$ and Lyt-2 $^-$ cells. In the spleen, 22-oxa-1 α ,25(OH) $_2$ D $_3$ also suppressed significantly the increment of Thy1.2, B220 double positive cells. The splenic IgG and IgM secreting cells at 24 weeks of age were suppressed in the mice treated with this compound at 2 ng/kg. In addition, we examined the IL-2 production by splenic T cells at 24 weeks of age. These mice produced IL-2 very poorly, and the IL-2 productivity in splenic T cells recovered when the mice were treated with 22-oxa-1 α ,25(OH) $_2$ D $_3$ (data not shown). These abnormal T cells were known to activate relatively premature B cells to differentiate into antibody-producing cells, and to induce polyclonal B cell activation (17). In this study, levels of serum anti-ssDNA antibody, rheumatoid factor, and immune complex were all elevated at 24 weeks of age, but the treatment with 22-oxa-1 α ,25(OH) $_2$ D $_3$ did not show a significant reduction of the levels of these serum factors.

The administration of $1\alpha,25(\text{OH})_2\text{D}_3$ over a long period of time sometimes induces severe hypercalcemia. But the oral administration of 22-oxa- $1\alpha,25(\text{OH})_2\text{D}_3$ for more than 4 months did not change calcium levels in these mice significantly. Moreover, there was no calcium deposition in renal tubuli, which was often associated with hypercalcemia.

These results clearly indicate that 22-oxa- $1\alpha,25(\text{OH})_2\text{D}_3$, a new synthesized analogue of vitamin D_3 , at such a low dose has now been found to be therapeutically effective for autoimmune disorders in MRL/l mice without inducing hypercalcemia, which has been an inherent problem in almost all vitamin D_3 analogues. The mechanism by which 22-oxa- $1\alpha,25(\text{OH})_2\text{D}_3$ works on the T cell maturation/differentiation in MRL/l mice is unknown, although it may be through a pathway similar to that in its well-known effects on monocyte-macrophage maturation.

In view of recent reports on T cell dysfunction in patients with rheumatoid arthritis, 22-oxa- $1\alpha,25(\text{OH})_2\text{D}_3$ may become a valuable therapeutic agent for rheumatoid arthritis. Although many feasible studies are needed, we believe that there is a possibility that this newly synthesized drug may become a useful drug for various autoimmune diseases. Viewing this goal, we are now examining the *in vivo* metabolism and tissue distribution of 22-oxa- $1\alpha,25(\text{OH})_2\text{D}_3$ using its labeled form. The mechanism of the action of this drug is also under study.

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