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Lobenzarit Disodium (CCA) Inhibits *in Vitro* Immunoglobulin Production *via* Direct Interaction with B Lymphocytes

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The regulatory effects of lobenzarit disodium (CCA), a therapeutic agent for treating rheumatoid arthritis (RA), on polyclonal immunoglobulin production by human lymphocytes were investigated *in vitro*. CCA inhibited the production of immunoglobulin in all the classes examined at a clinically relevant concentration. Moreover, it inhibited the immunoglobulin production as well as lymphocyte proliferation even when purified B lymphocytes preactivated by *Staphylococcus aureus* Cowan I were cultured with recombinant lymphokines such as IL2 and IL6. These results suggest that CCA acts directly on B lymphocytes.

The analysis at each of two different stages of B lymphocyte activation lineage, *i.e.*, the primary activation stage and a stage of proliferation and differentiation to antibody secreting cells, has indicated that CCA inhibits the proliferation-differentiation stage of B lymphocytes. CCA does not inhibit B lymphocytes at the primary activation stage; actually, it augments them, resulting in the subsequent enhancement of immunoglobulin production.

Keywords lobenzarit disodium (CCA); antirheumatic drug; human B lymphocyte; immunoglobulin production

Introduction

Lobenzarit disodium (CCA; disodium 4-chloro-2,2'-iminodibenzoate) is an effective therapeutic agent for the treatment of rheumatoid arthritis (RA).[1] Despite its structural similarity to mefenamic acid, a potent nonsteroidal anti-inflammatory agent, CCA has no anti-inflammatory effect, as shown in several experimental animal models of acute inflammation.[2] Also, it does not inhibit cyclo-oxygenase enzyme activity, whereas most non-steroidal anti-inflammatory agents do.[1]

Though the precise mechanism of action of CCA still remains unknown, it appears to exert its therapeutic effects by modulating the hosts' immunologic functions. The immunomodulating activities of CCA have been demonstrated in some animal models. It prevents the spontaneous development of autoimmune diseases in NZB/WF$_1$ mice[3] as well as MRL/*l* mice,[4,5] and these CCA-treated mice have lowered levels of serum autoantibodies, such as naturally occurring anti-deoxyribonucleic acid (DNA) antibodies.[6]

In recent clinical studies it has been demonstrated that the treatment of RA patients with CCA lowered their elevated serum immunoglobulin levels, including auto-antibodies, while decreasing their increased number of peripheral B lymphocytes, without any significant alteration of T cell subsets.[7] These results suggest that CCA may affect B lymphocytes directly, although no direct evidence of this has been reported yet.

Therefore, we investigated the direct effect of CCA on human B lymphocytes in an *in vitro* culture system, assessing its effect on B lymphocyte proliferation and antibody production. The results indicate that (1) CCA has direct inhibitory effects on B lymphocytes at the stage of their proliferation and differentiation to antibody secreting cells, while (2) it does not inhibit B lymphocytes at the primary activation stage, or it may augment them somewhat resulting in the subsequent enhancement of the immunoglobulin production, and (3) B lymphocyte functions are inhibited, as a whole, when CCA is present during the culture.

Materials and Methods

Cell Preparation Mononuclear cells (MNCs) were isolated from the heparinized venous blood of healthy adult volunteers, or from tonsils obtained from children with chronic tonsillitis, by centrifugation over Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradients.

B lymphocytes were purified from tonsillar MNCs as follows. Tonsillar MNCs were separated into sheep red blood cell (SRBC) binding (E+) and nonbinding (E−) subsets using 2-aminoethylisothiouronium bromide hydrobromide (Sigma, St. Louis, MO, U.S.A.) to treat SRBCs according to the method of Madsen and Johnsen.[8] High-density resting E− cells and low-density E− blast cells were separated by 45%(v/v) Percoll (Pharmacia) fractionation, and the high-density E− cells were further purified by treatment with OKT3, OKM1 (Ortho, Raritan, NJ, U.S.A.) and baby rabbit complement (Cedarlene, Hornby, Ontario, Canada). The purified high-density B lymphocytes contained >99% Leu12(CD19)-positive cells and <1% Leu4(CD3)-positive cells.

Culture Medium RPMI 1640 culture medium (Gibco, Grand Island, NY, U.S.A.) supplemented with 5% fetal calf serum (Bocknek, Toronto, Canada), streptomycin (100 μg/ml), penicillin G (100 U/ml), and 2 mM glutamine was used. All cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

Cell Cultures for Immunoglobulin Production Almost all immunoglobulin production was from a single culture because of the limited number of human cells obtained. So all experiments were repeated more than three times to confirm the reproducibility of the results.

(1) MNCs Cultures: Peripheral blood MNCs were cultured at 1×10^6 cells/ml in polypropylene tubes (Falcon #2053; Becton Dickinson, Lincoln Park, NJ, U.S.A.) with 0.3 μg/ml of pokeweed mitogen (PWM; Honen Corp., Tokyo, Japan) for 7 d or indicated periods in the presence or absence of CCA (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan). The culture supernatants were obtained, and an equal volume of phosphate-buffered saline (PBS) (pH 7.4, 10 mM) containing 0.05% sodium azide was added. The preparation was stored at 4 °C until determination of immunoglobulin concentrations. In some experiments, incorporation of ^{3}H-TdR ([methyl-^{3}H]thymidine, 1 μCi/well; Amersham, Buckingham, UK; code TRK. 120) was determined for the last 4 h of the culture.

(2) Two-Step Cultures of Purified B Lymphocytes: Purified tonsillar B lymphocytes were cultured at 1×10^6 cells/ml in polypropylene tubes for 48 h in the presence of 0.005% (v/v) *Staphylococcus aureus* Cowan I (SAC; Pansorbin; Calbiochem, La Jolla, CA, U.S.A.). The lymphocytes were washed three times, resuspended in fresh medium, and cultures for an additional 5 d in microplates with flat-bottom wells (Nunc, Roskilde, Denmark) at 5×10^4 cells/0.2 ml/well in the presence of recombinant IL2 (rIL2; Behringer Mannheim, Mannheim, W. Germany) or recombinant IL6 (rIL6; Genzyme, Boston, MA, U.S.A.). CCA was present during the

first and/or the second incubation periods. The subsequent procedures were as described above.

Cell Cultures for Proliferation Studies Purified tonsillar B lymphocytes were cultured with SAC(0.005%) or anti-μ beads (5 μg/mg; Immunobead rabbit anti-human immunoglobulin M (IgM); Bio-Rad, Richmond, CA, U.S.A.) in microplates with flat-bottom wells at 5×10^4 cells/0.2 ml/well in the presence or absence of CCA, and ^{3}H-uridine ([5-^{3}H]uridine, 1 μCi/well; Amersham; code TRK. 178) incorporation was determined for the last 4 h of the 24-h culture. Radioactivity incorporated in the cells was measured with a scintillation counter (Betaplate liquid scintillation counter; LKB, Turku, Finland).

For two-step incubation, B lymphocytes were activated by preincubation for 2 d with SAC (0.005%) or anti-μ beads (5 μg/ml) at 1×10^6 cells/ml in a polypropylene tube. Washed cells were recultured in microplates for 3 d at 5×10^4 cells/0.2 ml/well with rIL2 (5 U/ml) or 1×10^5 cells/0.2 ml/well with 100 U/ml of recombinant IL4 (rIL4; Genzyme, Boston, MA, U.S.A.). ^{3}H-TdR (1 μCi/well) incorporation was determined for the last 4 h.

Measurement of Immunoglobulin The immunoglobulin concentration was determined by enzyme-linked immunosorbent assay (ELISA). Rabbit antibodies to human immunoglobulins were purchased from Dako (Glostrup, Denmark). Nunc-Immunoplate I was coated with 100 μl/well of either anti-IgG, anti-IgM, or anti-IgA diluted 1:5000 with 50 mM carbonate–bicarbonate buffer (pH 9.6) at 37 °C for 1 h, and blocking was carried out at 37 °C for 2 h with 200 μl/well of 3% bovine serum albumin (BSA) (Cohn fraction V; Armour, Kankakee, IL, U.S.A.) in 10 mM PBS. After washing three times with PBS containing 0.05% Tween-20 (PBS-Tween), a 100-μl sample of diluted culture supernatant was added and incubated at 37 °C for 1 h. Then the plate was washed with PBS-Tween, and horse radish peroxidase-labeled antibody to either IgG (1:5000), IgM (1:4000), or IgA (1:5000) diluted with 1% BSA in PBS, was added and incubated for 30 min. After a thorough washing with PBS-Tween, 0.4 mg/ml o-phenylenediamine (Tokyo Kasei, Tokyo, Japan) solution (citrate buffer: 50 mM, pH 5.0, and the reaction was stopped by adding 50 μl/well of 2.5 M sulfuric acid. The absorbance at 492 nm was measured using a microplate reader (Flow Lab., Helsinki, Finland) and the immunoglobulin concentration was assessed on the basis of a standard curve of IgG (purified polyclonal human IgG; Serotec, Blackthorn, Bicester, England), IgM (purified human myeloma IgM; Jackson Immunoresearch, West Grove, PA, U.S.A.), or IgA (purified polyclonal human IgA; Tago, Burlinger, CA, U.S.A.).

Results

CCA Inhibits PWM-Induced Immunoglobulin Production by MNCs CCA inhibited polyclonal immunoglobulin secretion by MNCs stimulated with PWM in a dose-dependent manner, and there were no significant differences in the intensity of CCA-dependent inhibition of immunoglobulin production among immunoglobulin classes, IgG, IgM, and IgA. Figure 1 shows representative data
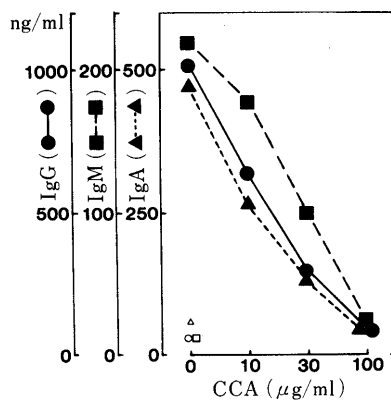

Fig. 1. CCA Inhibition of PWM-Induced Immunoglobulin Production

Peripheral blood MNCs were cultured at 1×10^6 cells/ml in polypropylene tubes with (closed symbols) or without (open symbols) 0.3 μg/ml of PWM for 7 d in the presence or absence of CCA. Concentrations of IgG ($\bullet$, $\bigcirc$), IgM ($\blacksquare$, $\square$) and IgA ($\blacktriangle$, $\triangle$) represent the mean of duplicate cultures. The graph represents five experiments.

from five experiments. CCA also inhibited the spontaneous secretion of immunoglobulins from tonsillar low-density E− blast cells which had already been activated in ·the human body chronic tonsillitis (Fig. 2).

CCA showed no crucial cytotoxicity according to the trypan blue dye exclusion test in any of the experiments.

Kinetic Studies of the Inhibitory Effect at CCA on PWM-Induced Responses Adding PWM to MNC cultures induced immunoglobulin secretion from the third day of the culture, and immunoglobulin production was inhibited dose-dependently by adding CCA (Fig. 3a, b). CCA inhibited ^{3}H-TdR incorporation as well (Fig. 3c), but much less than it did immunoglobulin production.

CCA Inhibits Immunoglobulin Production as a Result of Direct Interaction with B Lymphocytes in the Proliferation-
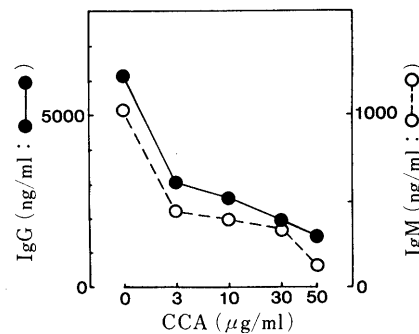

Fig. 2. CCA Inhibition of Spontaneous Immunoglobulin Production in Tonsillar Blast Cells

Tonsillar blast cells fractionated on 40% (v/v) Percoll were cultured at 1×10^6 cells/ml in polypropylene tubes for 7 d in the presence or absence of CCA. Concentrations of IgG ($\bullet$) and IgM ($\bigcirc$) in the culture supernatants were assessed by ELISA. The graph is representative of three experiments.
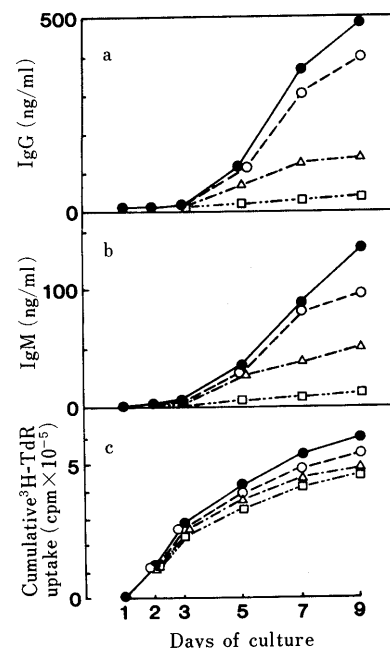

Fig. 3. Time Course of the Effect of CCA on PWM-Induced Responses

Peripheral blood MNCs were cultured at 1×10^6 cells/ml in polypropylene tubes with 0.3 μg/ml of PWM in the absence ($\bullet$) or presence ($\bigcirc$: 10 μg/ml, $\triangle$: 30 μg/ml, $\square$: 100 μg/ml) of CCA. On each day (absissa), culture supernatants were obtained to determine immunoglobulin concentrations, and the ^{3}H-TdR incorporation was assessed. Concentrations of IgG (a) and IgM (b) were determined by ELISA, and the cumulative ^{3}H-TdR uptake (c) is indicated. Data is representative of three similar experiments.

Differentiation Stage We used purified B lymphocytes to determine whether or not CCA affects B lymphocytes directly. It is well-known that resting B lymphocytes can be activated to be responsive to IL2 or IL6 when cultured with SAC or anti-μ beads. Indeed, SAC-activated B lymphocytes cultured with rIL2 or rIL6 produced considerable amounts of immunoglobulin, and the simultaneous addition of CCA to the culture dose-dependently inhibited immunoglobulin production (Fig. 4a, b). In the same experiment, ^{3}H-TdR incorporation was examined on day 3 of the rIL2-supplemented culture. The addition of CCA at more than 10 μg/ml inhibited the incorporation of ^{3}H-TdR induced by rIL2 (Fig. 4c), though the inhibition was weaker than that of immunoglobulin production, as shown in Fig. 4a.

It has been demonstrated that the proliferation of anti-μ beads-activated B lymphocytes is induced by adding IL4.[9] A representative of three similar experiments is shown in

Fig. 5. CCA inhibited ^{3}H-TdR incorporation in the anti-μ bead-activated B lymphocytes cultured with rIL4, though the inhibition was weaker than that of the immunoglobulin production. As purified B lymphocytes were used in these experiments, the data indicates that CCA affects B lymphocytes directly.

Interaction of CCA with B Lymphocytes at the Primary Activation Stage Dose not Inhibit but May Actually Augment Immunoglobulin Production In the preliminary experiments with PWM-induced immunoglobulin production by unfractionated peripheral blood MNCs, CCA more strongly inhibited when it was absent during the initial 3 d of the 7 d-culture (CCA exposure period = days 3 to 7) than when it was present from the initiation of the culture (CCA = day 0 to 7). And the presence of CCA during the initial 3 d of the culture (CCA = day 0 to 3) actually enhanced immunoglobulin production (data not shown).
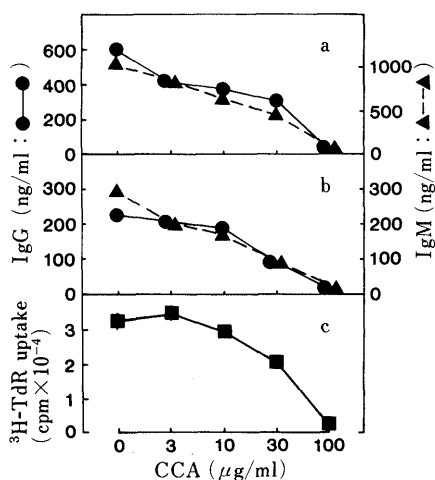

Fig. 4. CCA Inhibition of the IL2- and IL6-Responsiveness of SAC-Activated B Lymphocytes

SAC-activated B lymphocytes, cultured for 48 h at 1 × 10⁶ cells/ml with 0.005% SAC, were washed three times and recultured in microplates at 5 × 10⁴ cells/well with rIL2 (5 U/ml) or rIL6 (10 U/ml) in the absence or presence of CCA. The concentrations of secreted IgG (●) and IgM (▲) in the cultures with rIL2 (a) or rIL6 (b) were determined on day 5. Concentrations of spontaneously secreted immunoglobulins without the addition of any lymphokines were 126.5 ng/ml for IgG and 142.3 ng/ml for IgM. The values of ^{3}H-TdR uptake in the presence of rIL2 (c; ■), assessed on day 3, represent the mean ± S.D. of triplicate cultures. Spontaneous ^{3}H-TdR incorporation was 2773 ± 481 cpm. The graphs represent three similar experiments.

TABLE I. Effect of CCA on B Lymphocyte Activation and on the Subsequent IL2-Responsiveness of Activated B Lymphocytes

1st incubation of of B cells added with:		CCA (μg/ml)	2nd incubation added with:			
			rIL2 (U/ml)			
			0		10	
			IgG	IgM	IgG	IgM
			ng/ml			
Exp. 1	SAC	0	14.4	21.5	855.6	199.5
	SAC	30	—	—	236.6	94.3
	SAC+CCA	0	9.1	14.7	1035.2	274.6
	SAC+CCA	30	—	—	333.7	131.0
Exp. 2	SAC	0	21.5	41.1	345.3	374.3
	SAC+CCA	0	18.3	59.8	493.8	442.2
Exp. 3	SAC	0	17.7	28.4	970.4	179.9
	SAC	30	—	—	357.4	80.4
	SAC+CCA	0	16.8	31.3	1521.3	286.4
	SAC+CCA	30	—	—	435.7	125.8

Purified tonsillar B lymphocytes were cultured with 0.005% SAC for 2 d at 1 × 10⁶ cells/ml in the absence or presence of 30 μg/ml of CCA (1st incubation). After being washed three times, activated B lymphocytes were recultured at 5 × 10⁴ cells/well in microplates in the presence or absence of rIL2 (10 U/ml) for 5 d with or without CCA (30 μg/ml; 2nd incubation). The immunoglobulin concentrations in culture supernatant were determined by ELISA. —: not tested.
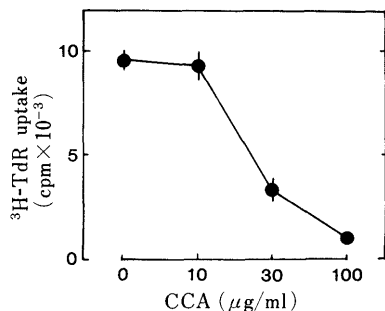

Fig. 5. CCA Inhibition of the IL4-Induced Proliferation of B Lymphocytes Activated by Anti-μ Beads

Purified tonsillar B lymphocytes were cultured for 2 d with 5 μg/ml of anti-μ beads, washed three times, and recultured with rIL4 (100 U/ml; ●) at 1 × 10⁵ cells/well in microplates for 3 more days in the absence or presence of CCA. The ^{3}H-TdR uptake for the last 4 h was determined. Data is expressed as the mean ± S.D. of triplicate cultures and are representative of three similar experiments. The incorporation in control culture, without the lymphokine, was 399 ± 160 cpm.
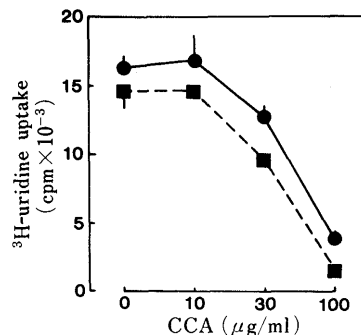

Fig. 6. Effect of CCA on RNA Synthesis of B Lymphocytes Stimulated in Vitro

Purified tonsillar B lymphocytes were cultured with SAC (0.005%; ●) or anti-μ beads (5 μg/ml; ■) at 5 × 10⁴ cells/well in microplates in the absence or presence of CCA, and ^{3}H-uridine incorporation was determined for the last 4 h of the 24-h culture. Data is expressed as the mean ± S.D. of triplicate cultures and is representative of two experiments. The ^{3}H-uridine incorporation of nonactivated B lymphocytes was 2169 ± 109 cpm.

For further analysis, a two-step culture was performed using purified B lymphocytes. The presence of CCA (30 μg/ml) during the second incubation of SAC-activated B lymphocytes (proliferation-differentiation stage of B lymphocytes) with 10 U/ml of rIL2 clearly suppressed the immunoglobulin production, similar to the results in Fig. 4a. But the presence of CCA during the first incubation with SAC (primary activation stage of B lymphocytes) did not inhibit, but rather augmented, subsequent immunoglobulin production by the B lymphocytes (for example, as shown in exp. 1, Table I; IgG: 1035.2 vs. 855.6, and 333.7 vs. 236.6 ng/ml, IgM: 274.6 vs. 199.5, and 131.0 vs. 94.3 ng/ml). The increase in immunoglobulin production is marginal. However, three repeated experiments confirmed the reproducibility of the augmentative effect of CCA (exp. 2 and 3 in Table I).

Effect of CCA on RNA Synthesis in B Lymphocytes
The effect of CCA on ribonucleic acid (RNA) synthesis in purified B lymphocytes activated with SAC or anti-μ beads was examined using ^{3}H-uridine incorporation for the last 4 h of a 24 h-culture. CCA clearly inhibited RNA synthesis (Fig. 6), though the inhibition was weaker than the inhibition of immunoglobulin production shown in Fig. 4.

Discussion
The present results have demonstrated that CCA inhibits in vitro immunoglobulin production by human peripheral blood and tonsillar lymphocytes. CCA also inhibited immunoglobulin production when MNCs from patients with RA were stimulated with PWM or SAC (data not shown). Furthermore, CCA inhibited the spontaneous immunoglobulin production by tonsillar E− blast lymphocytes (Fig. 2). It should be noted that CCA inhibits the immunoglobulin production not only by the lymphocytes activated in vitro by the artificial stimulators such as PWM and SAC, but also by the tonsillar blast lymphocytes obtained from patients with chronic tonsillitis, which have been activated in vivo by natural activation mechanisms.

Since such inhibition occurred in a culture of purified B lymphocytes without any critical decrease of cell viability (Fig. 4 and Table I), CCA apparently acts directly on B lymphocytes. The same results were obtained when monocytes were depleted by treatment with L-leucine methyl ester, as described by Thiele et al.,[10] instead of OKM1 plus complement after the deletion of T lymphocytes by the treatment with OKT3 plus complement. The direct effect of CCA on B lymphocytes is supported by the result that CCA inhibited the IL6-dependent immunoglobulin production[11] by Epstein–Barr virus (EBV)-transformed human B lymphocyte cell lines SKW6-CL4 and CESS (data not shown). Also, CCA inhibited B lymphocytes directly when purified mouse B lymphocytes were stimulated with a combination of lipopolysaccharide and B151-TRF2 (T cell hybridoma B151 derived B cell differentiation factor[12]) to produce immunoglobulins polyclonally.[13] Although it has been reported that CCA inhibits the functions of monocytes/macrophages and T lymphocytes,[14-16] no evidence of the direct effect on B cell activation has been documented yet.

Immunoglobulin production and B lymphocyte proliferation were inhibited in vitro by adding CCA at more than 10 μg/ml. As the blood concentration of CCA is maintained at 10 to 40 μg/ml in RA patients,[17] the immunoglobulin production and B lymphocyte proliferation are supposed to be inhibited in CCA-treated RA. And in fact, a remarkable decrease in B lymphocyte numbers followed by a decrease in immunoglobulin concentrations, including autoantibodies, occured in the peripheral blood of RA patients treated with CCA.[7] Moreover, it is likely that CCA inhibits the proliferation of B lymphocytes in preference to T lymphocytes, because it inhibited TdR incorporation more profoundly in purified B lymphocyte cultures (Figs. 4 and 5) than it did in PWM-stimulated cultures of unfractionated peripheral MNC containing both T and B lymphocytes (Fig. 3).

The presence of CCA during the proliferation–differentiation stage inhibited immunoglobulin production. But in contrast, the presence of CCA during the activation stage of B lymphocytes in our experiments did not inhibit, but slightly augmented the subsequent production of immunoglobulins. Although the increase in immunoglobulin production was marginal, this biphasic effect of CCA was reproducible in repeated experiments. This slight but reproducibly augmentative effect of CCA occurred at concentrations of 10—30 μg/ml in the culture medium, while at a higher dose of CCA (100 μg/ml) the inhibitory effect was obvious, probably because it was impossible to completely wash out the CCA. Moreover, immunoglobulin production was also augmented when SAC-activated B lymphocytes were cultured with other lymphokines such as rIL6 and the culture supernatant of E+ cells stimulated with PHA (data not shown). The same results were obtained when monocytes were depleted by treatment with L-leucine methyl ester instead of OKM1 plus complement after the deletion of T lymphocytes by the treatment with OKT3 plus complement. This is significant because, under some experimental conditions in vivo, CCA exhibits an immunopotentiating profile.[2,18,19] This is consistent with our finding that the immunopotentiating effect of CCA occurs in mice only when it is given at the early stage of the immune response.[2]

Since the RNA synthesis of B lymphocytes, which occurs in the late G_1 (G_{1B}) phase of the cell cycle,[20] was also inhibited by adding CCA, the interaction of CCA with B lymphocytes was considered to occur in the G_{1B} phase or earlier. However, ^{3}H-uridine uptake and ^{3}H-TdR uptake were scarcely inhibited at 10 μg/ml of CCA in contrast with the significant inhibition of immunoglobulin production which occurred at the same concentration of CCA. The difference in the intensity of these inhibitions may be attributed to the inhibition by CCA of the differentiation stage of B lymphocytes, as was the IL6-induced production of the immunoglobulins inhibited by CCA.

In spite of our studies of cell surface expressions of HLA-DR and IL2-receptor on SAC-activated and anti-μ-activated B lymphocytes, we could not detect any notable effects of CCA, and we have no information yet to explain how CCA promotes immunoglobulin production when it is present in the primary activation stage. Moreover, the inhibition of ^{3}H-uridine uptake by CCA (Fig. 6) is in conflict with the results showing that CCA augments immunoglobulin production. At present, there is no appropriate explanation for this discrepancy. Although the precise

mechanisms of CCA are still unclear, some cyclic adenosine monophosphate (cAMP)-modulating agents were reported to have a biphasic effect on immunoglobulin prodution.[21-23] So, we have started to study the effects of CCA on some intracellular events.

Anyway, CCA may inhibit immunoglobulin production, including autoantibodies, and the B lymphocyte proliferation accompanied by autoimmune diseases such as RA, when the blood concentration of CCA is maintained at its effective level. Recent clinical studies by Miyasaka and Nishioka[7] demonstrated the B lymphocyte-oriented immunoinhibitory effect of CCA in RA in accordance with the findings reported in this peper. So it appears that B lymphocyte is, at least, one of the targets of the CCA used to treat rheumatoid arthritis.

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