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SUPPRESSION OF CYTOTOXIC T LYMPHOCYTE ACTIVATION BY L-ORNITHINE

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The effect of L-ornithine on several types of immune reactions was analyzed. L-ornithine was found to suppress the activation of cytotoxic T lymphocytes (CTL) in vivo and in vitro. This suppressive effect was not observed with the structural analogues D-ornithine, L-lysine, or putrescine or with the amino acids L-histidine or L-alanine. The concentration of 9×10^{-3} M L-ornithine was found to mediate a practically complete suppression of the cytotoxic response in vitro if applied on day 0 or day 1 of the culture, but a comparably weak suppression if applied on day 3. The same concentration of L-ornithine had no effect on the production of the lymphokines interleukin 2 (IL 2) and γ-interferon (IFN-γ). This concentration of ornithine had also no substantial effect on several types of proliferative responses, including the allogeneic mixed lymphocyte reaction, the concanavalin A-activated IL 2-dependent proliferation of thymocytes, and IL 2-dependent proliferation of the T cell clone W-2.

These observations suggest that L-ornithine inhibits selectively the differentiation of CTL effector cells. By the criteria tested, the immunosuppressive effect of L-ornithine is more selective than that of cyclosporine A, which was previously found to suppress not only the activation of cytotoxic activity but also proliferative responses and the production of the lymphokines IL 2 and IFN-γ.

Activated macrophages were shown to contain increased arginase-activity (1, 2) and to release arginase into the extracellular space (3–5). Arginase converts L-arginine into urea and the amino acid L-ornithine, which serves as a key precursor for the polyamine biosynthesis. Increased cellular levels of the polyamines spermidine and spermine are reportedly required for optimal DNA-synthesis in concanavalin A-activated lymphocytes (6) (as well as in other types of proliferating cells and tissue (reviewed in References 7, 8)). Ornithine decarboxylase activity, the key enzyme in polyamine biosynthesis, increases dramatically within a few hours after mitogenic or antigenic stimulation of lymphocytes (9–11).

Taken together, these observations suggested the possibility that the available concentration of ornithine may have a strong influence on the activation process and that the release of arginase by activated macrophages and the resulting increase of the L-ornithine concentration in the extracellular space may serve as a physiologic regulatory signal from activated macrophages to responding lymphocytes.

We have therefore analyzed the effect of L-ornithine on several T cell functions. Our experiments demonstrate a selective immunosuppressive effect of L-ornithine on the early induction of cytotoxic T lymphocyte (CTL)[1] responses. At comparable concentrations, ornithine was found to have no effect on several types of proliferative T cell responses and on the production of the lymphokines interleukin 2 (IL 2) and γ-interferon (IFN-γ). The immune suppressive effect of L-ornithine is in this respect more selective than that of cyclosporine A, which is widely used for immunosuppressive therapy in organ transplantation. Cyclosporine A inhibits not only the activation of CTL but also inhibits the production of the lymphokines IFN-γ (12) and IL 2 (13–17). As a consequence, cyclosporine A inhibits not only the development of the cytotoxic effector function but also the proliferative response (18–20) of the T cell system. Recently, cyclosporine A was also found to inhibit the induction of ornithine decarboxylase activity (11).

MATERIALS AND METHODS

Animals. Mice were purchased from Bomholtgard, Ry, Denmark. Eight- to 12-wk-old mice were used in most of the experiments.

Activation of cytotoxic responses in vivo. Mice were treated with 3 mg cyclophosphamide (Endoxan, Asta, Brackwede, West Germany) in 0.15 ml saline i.p. 2 days before immunization (21–23). The immunization against trinitrophenylated syngeneic cells was performed by painting the mice with 6 × 0.01 ml 20% trinitrochlorobenzene (TNCB) in acetone on the four footpads and two abdominal sites (22, 23). Immunization against allogeneic cells was performed by distributing 5×10^7 irradiated (1500 rad) allogeneic spleen cells equally into the four footpads in a total volume of 0.2 ml. The indicated amounts of L-ornithine were also injected into the four footpads at the time of immunization. Five days after immunization, the mice were sacrificed and the pooled inguinal and axillary lymph nodes were directly tested in a 4-hr ^{51}Cr-release assay for cytotoxic activity on concanavalin A-activated lymphoblasts as target cells.

Activation of cytotoxic responses in macrocultures. Twenty million responder cells (usually C3H spleen cells) were incubated with 1×10^6 irradiated (1500 rad) allogeneic or trinitrophenylated syngeneic stimulator cells in a total volume of 4.5 ml culture medium (RPMI 1640, GIBCO medium supplemented with 10 mM L-glutamine [GIBCO], streptomycin/penicillin [GIBCO, 100 U/ml], 0.5% HEPES [GIBCO], 10% fetal calf serum [GIBCO], and 3×10^{-5} M 2-mercaptoethanol) for 5 days at 37°C in 5% CO_2. The responder and stimulator cells were spleen cells. The cultures were tested after 5 days for cytotoxic activity against concanavalin A-activated allogeneic or trinitrophenylated syngeneic spleen cell blasts in a 4-hr ^{51}Cr-release assay as described (24, 25).

Activation of cytotoxic responses in the presence of IL 2-containing cell supernatants in microcultures. C3H spleen cells (5 ×

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[1] Abbreviations used in this paper: CTL, cytotoxic T lymphocyte; IFN, interferon; IL 2, interleukin 2; PMA, phorbol myristic acetate; TNCB, trinitrochlorobenzene.

10^4) were incubated as responder cells with 3×10^5 trinitrophenylated and irradiated (1500 rad) C3H spleen cells and the indicated amounts of IL 2-containing EL-4 supernatant in 0.2-ml microcultures. The cytotoxic activity against 2×10^4 trinitrophenylated concanavalin A-activated C3H spleen cells blasts was tested after 5 days in a 4-hr ^{51}Cr-release assay as described (24).

Assay of DNA synthesis. The DNA synthesis in microcultures was assayed by adding 1 μCi of [^{3}H]thymidine per 0.2 ml culture. The microcultures were usually incubated for another 8 hr, then were harvested with a Scatron-cell-harvester (Flow Laboratories), and the filters were counted in a liquid scintillation counter. Each point was assayed in quadruplicate if not indicated otherwise.

Assay for IL 2 activity. The IL 2 titers were determined with the IL 2-dependent W-2 T cell line as described (26).

Assay for IFN activity. IFN titrations were performed by using a microtiter assay with L929 cells (0.2 ml of medium [RPMI 1640 + 5% FCS] per well) and vesicular stomatitis virus (Indiana strain) as challenge virus. One unit corresponds to the minimal amount of IFN capable of conferring protection to 50% of the cells. One microtiter unit/0.2 ml corresponds to 2 NIH reference units/ml. All titers are expressed in laboratory units.

The preparation of IL 2-containing supernatants. Supernatants were obtained from an IL 2-producing EL-4 thymoma subline after induction with phorbol myristic acetate (PMA) as described (27). Briefly, 10^6 EL-4 cells/ml were incubated together with 10 ng PMA/ml (SIGMA) for 48 hr. The superantant was collected and stored frozen at −20°C.

RESULTS

The suppressive effects of L-ornithine on cytotoxic responses. Ornithine was found to exert a strong suppressive effect on cytotoxic responses in vivo and in vitro against haptenated syngeneic or allogeneic target cells (Fig. 1). The cytotoxic response of cyclophosphamide-treated C3H mice against allogeneic C57BL/6 cells in vivo was always a priori relatively weak but was also suppressed by the injection of L-ornithine (data not shown). The dose-response curves for the effects of ornithine in vivo and in vitro are shown in Figure 2 and Figure 3, respectively. Figure 3 shows also that the suppression is more pronounced when ornithine is added in the early phase, i.e., on day 0 or day 1, rather than on day 3 of the cytotoxic response. The suppressive effect is not observed with the structural analogues D-ornithine, L-lysine, or putrescine or with the amino acids L-histidine or L-alanine (Table I).

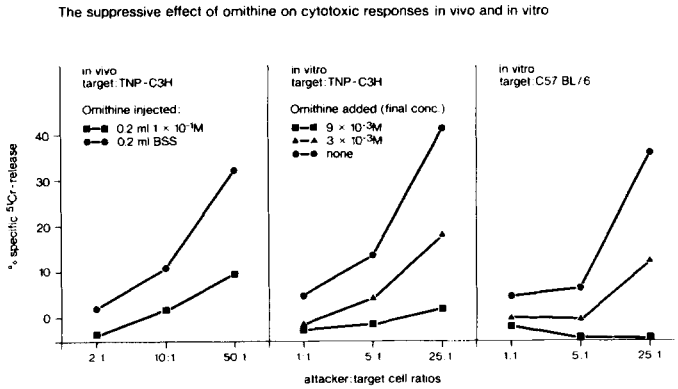

Figure 1. The suppressive effect of ornithine on cytotoxic responses in vivo and in vitro. Effect in vivo (*left panel*): C3H mice received 3 mg cyclophosphamide in 0.15 ml BSS i.p. and were immunized 2 days later with 6 × 0.01 ml of a 20% solution of TNCB in acetone painted on the skin at two abdominal sites and on the four footpads. Immediately before immunization, 4 × 0.05 ml BSS containing the indicated concentrations of ornithine were injected into the four footpads. The mice were sacrificed 5 days later, and the cytotoxic activity of the pooled inguinal and axillary lymph node cells against ^{51}Cr-labeled trinitrophenylated C3H target cells was tested at the indicated attacker to target cell ratios. Effect in vitro (*middle and right panels*): C3H spleen cells (2×10^7) were incubated with 1×10^6 irradiated and trinitrophenylated C3H spleen cells (*middle panel*) or 1×10^6 irradiated C57BL/6 spleen cells (*right panel*) and with the indicated final concentrations of ornithine in 4.5 ml cultures for 5 days. The cytotoxic activity against the corresponding target cells was tested after 5 days.

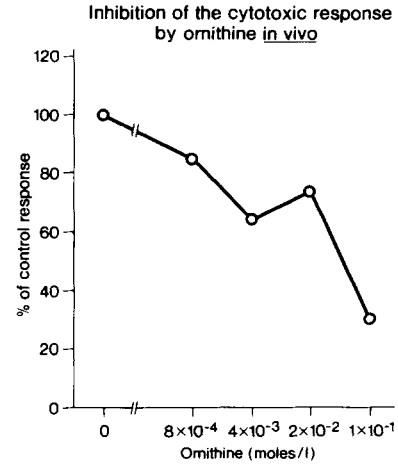

Figure 2. Effect of graded doses of L-ornithine on the cytotoxic response in vivo. C3H mice were treated with cyclophosphamide and immunized with TNCB as described in the legend to Figure 1. Immediately before immunization, 4 × 0.05 ml BSS with graded concentrations of ornithine were injected into the four footpads. After 5 days, the cytotoxic activity of the pooled and inguinal axillary lymph node cells against trinitrophenylated C3H target cells was tested at attacker to target cell ratios of 50:1, 10:1, and 2:1. The figure shows the relative cytotoxic activity at attacker to target cell ratio 50:1. (The control response without ornithine showed 32% specific ^{51}Cr release.)

In order to distinguish whether ornithine inhibits the activation of CTL precursor cells directly or only indirectly through the inhibition of helper T cells, experiments were performed in IL 2-containing microcultures. The results of these experiments (Fig. 4) revealed that ornithine also exerts a strong suppressive effect on cytotoxic responses in IL 2-containing cultures. The suppressive effect again was more pronounced if ornithine was added on day 0 or day 1 rather than on day 3 of the culture.

The effect of L-ornithine on lymphokine production. The concentration of 9×10^{-3} ornithine, which has been found to suppress cytotoxic responses completely, had no substantial effect on the production of IL 2 or IFN-γ (Fig. 5). Graded concentrations of ornithine were added to cultures of concanavalin A-activated C3H spleen cells (10^7 cells + 5 μg concanavalin A/ml). The resulting supernatants were harvested 24 hr later and were tested for IL 2 and IFN activity. The results (Fig. 5) show that the production of both lymphokine activities was not markedly affected by the addition of ornithine. Similar results were obtained with respect to the IL 2 and IFN titers after 48 hr of culture and also with respect to the IL 2 titers in ornithine containing cultures of PMA activated EL-4 thymoma cells (data not shown). C3H spleen cell cultures with 1 μg/ml concanavalin A and allogeneic mixed lymphocyte cultures containing 10^7 nonirradiated C3H spleen cells plus 10^7 nonirradiated C57BL/6 spleen cells were also set up with graded concentrations of ornithine. However, their supernatants showed no IL 2 or IFN activity irrespective of the amount of ornithine added (data not shown). Control solutions containing graded amounts of ornithine in culture medium also showed no lymphokine activities in the two assay systems (data not shown). These control experiments showed that ornithine by itself has no IL 2 or IFN activity and does not induce lymphokine production in lymphocyte cultures. Also, ornithine had no enhancing or inhibitory effect on

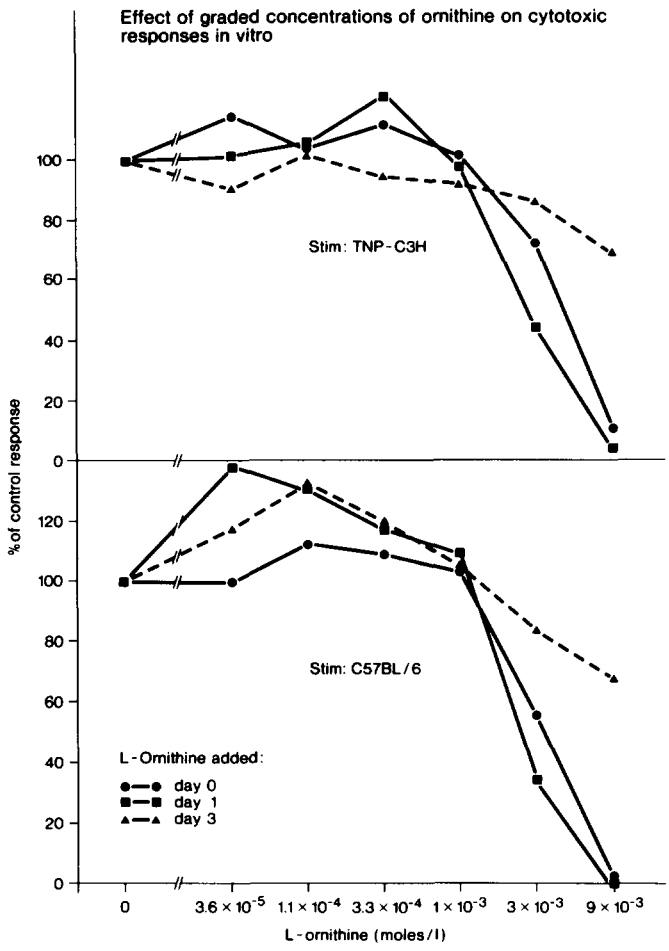

Figure 3. Effect of graded doses of ornithine on cytotoxic response in vitro. C3H spleen cells (2×10^7) were incubated with 1×10^6 irradiated and trinitrophenylated C3H spleen cells (*upper panel*) or 1×10^6 irradiated C57BL/6 spleen cells (*lower panel*) in 4.5 ml cultures for 5 days. At day 0, day 1, or day 3, the cultures were supplemented with 0.2 ml culture medium containing graded concentrations of L-ornithine. The abscissa indicates the final concentration of ornithine in the culture. The cytotoxic activity against the corresponding target cells was tested after 5 days at attacker to target cell ratios of 25:1, 5:1 and 1:1. The data indicate the relative cytotoxic activity at the attacker to target cell ratio 25:1. (The control responses without ornithine showed 54% [*upper panel*] and 50% ^{51}Cr-release [*lower panel*].)

TABLE I

Effect of different amino acids and putrescine on the activation of CTL[a]

Expt.	Added Component	Stimulator and Target					
		TNP-C3H			C57BL/6		
I	none	54.9	32.0	16.3	33.5	17.7	4.8
	L-ornithine	7.9	3.7	3.2	1.8	−1.7	−0.2
	L-lysine	60.7	39.9	21.5	41.7	19.1	9.0
	L-histidine	48.5	27.8	11.1	24.5	11.4	5.1
	L-alanine	40.5	25.3	9.8	19.2	5.3	6.7
	putrescine	44.2	24.8	9.6	30.2	11.9	7.2
II	none	63.9	34.1	13.8	67.8	40.6	12.3
	L-ornithine	17.6	2.9	−1.8	17.5	2.5	1.5
	D-ornithine	64.3	38.4	13.7	64.2	43.6	15.0

[a] C3H spleen cells (2×10^7) were incubated with 5×10^6 irradiated trinitrophenylated C3H spleen cells or C57BL/6 spleen cells in 5-ml cultures containing, in addition to the standard medium, components of the indicated amino acids or putrescine at a concentration of 1×10^{-2} mol/liter. The data indicate the cytotoxic activity (percent of ^{51}Cr release) against homologous target cells after 5 days at attacker to target cell ratios of 25:1, 5:1, and 1:1.

the two assay systems.

The effect of ornithine on proliferative responses. A final set of experiments was designed to test the effects

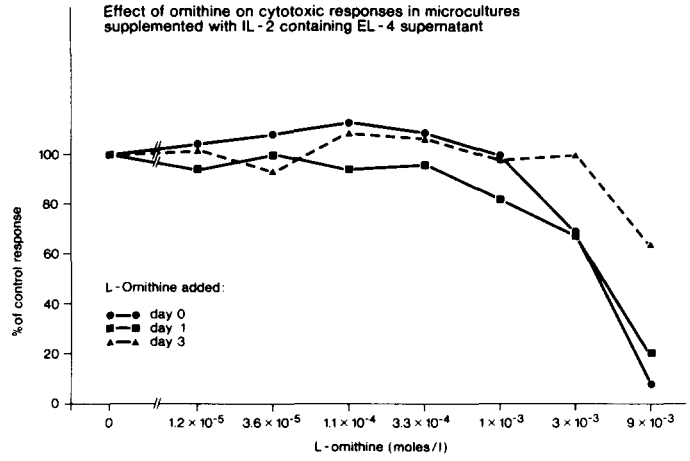

Figure 4. Effect of ornithine on cytotoxic responses on microcultures supplemented with IL 2-containing EL-4 supernatant. C3H spleen cells (5×10^4) were incubated with 3×10^5 irradiated (1500 rad) trinitrophenylated C3H spleen cells and 0.015 ml EL-4 supernatant (2 U of IL 2). The cultures were supplemented on day 0, day 1, or day 3 with 0.02 ml culture medium containing graded concentrations of L-ornithine. The abscissa indicates the final concentration of ornithine in the cultures. The cytotoxic activity against TNP-haptenated syngeneic target cells (2×10^4/well) was tested at day 5. (The control response showed 54% ^{51}Cr-release.)

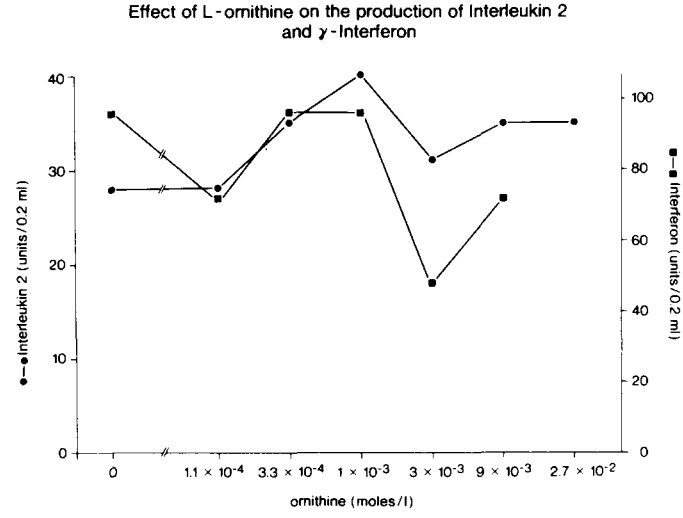

Figure 5. Effect of L-ornithine on the production of IL 2 and IFN-γ. C3H spleen cells (1×10^7/ml) were incubated with concanavalin A (5 μg/ml) and the indicated final concentrations of L-ornithine for 24 hr in 4-ml macrocultures. The resulting supernatants were then isolated by centrifugation and tested for IL 2 and IFN activity as described in *Materials and Methods.* IL 2 production by PMA-activated EL-4 thymoma cells was also tested and was also not suppressed by these concentrations of L-ornithine (data not shown). The slight suppression of the IFN titer at 3×10^{-3} M L-ornithine is not a reproducible and meaningful effect as judged from three independent experiments.

of ornithine on three types of proliferative T cell responses. First, mixed lymphocyte cultures containing 3×10^5 C3H spleen cells and 3×10^5 irradiated (1500 rad) C57BL/6 spleen cells as stimulator cells with or without 0.15 ml EL-4 supernatant (2 U IL 2/culture) were incubated with graded doses of L-ornithine. The results (Fig. 6) revealed that ornithine had no effect on the DNA-synthesis response ([^{3}H]thymidine incorporation) at any of the concentrations tested (Fig. 6). Second, C3H thymocytes (1×10^5) were incubated with IL 2 (2 U/culture) and with graded concentrations of concanavalin A and L-ornithine and in 0.2-ml microcultures. The results (Fig. 7) showed again that ornithine had no effect on the DNA synthesis of the responding lymphocytes. Third, 10^4 cells of the W-2 T cell clone were incubated with IL 2 (0.5 U/

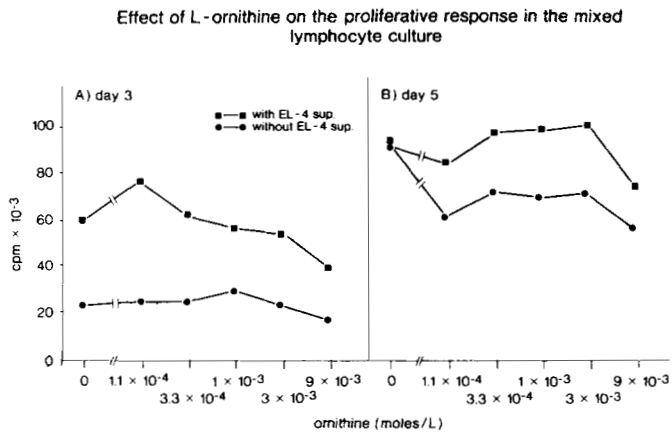

Figure 6. Effect of L-ornithine on the proliferative response in the allogeneic mixed lymphocyte culture. C3H spleen cells (3×10^5) were incubated with 3×10^5 irradiated (1500 rad) C57BL/6 spleen cells with or without 0.015 ml EL-4 supernatant in 0.2-μl microcultures. The DNA-synthesis was determined 3 days and 5 days later as described in *Materials and Methods*.

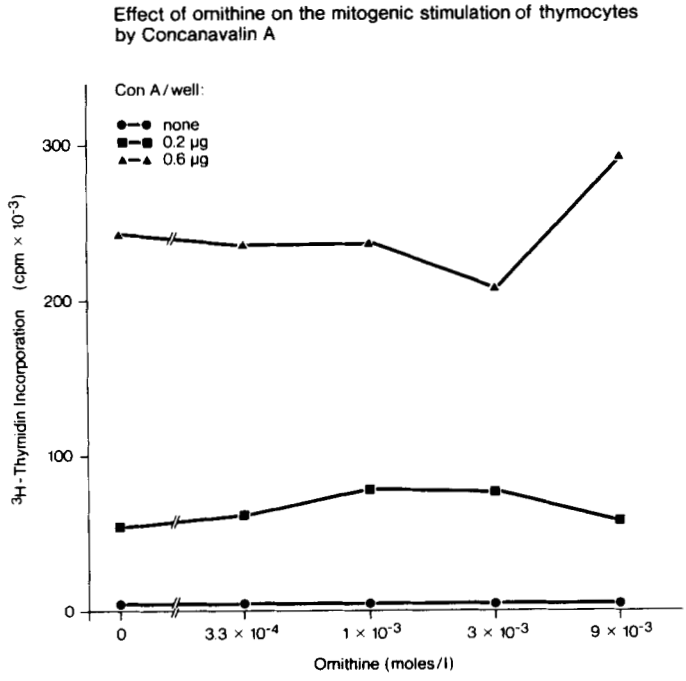

Figure 7. Effect of ornithine on the mitogenic stimulation of thymocytes by concanavalin A. C3H thymus cells (1×10^5) were incubated with the indicated amounts of concanavalin A and with 2 U of IL 2 and the indicated concentrations of L-ornithine in 0.2-ml microcultures. The DNA synthesis was tested 3 days later as described in *Materials and Methods*.

culture) and graded doses of L-ornithine in 0.2-ml microcultures for 20 hr at 37°C. The W-2 cells were then assayed for DNA synthesis as described for the IL 2 assay (*Materials and Methods*). Again, ornithine had no effect on the DNA synthesis of this T cell clone (Fig. 8).

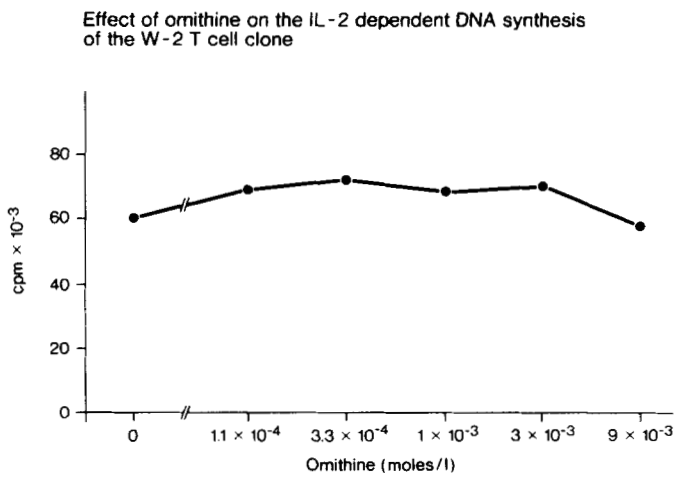

Figure 8. Effect of L-ornithine on the IL 2-dependent DNA synthesis of the W-2 T cell clone. Cells of the W-2 T cell clone (10^4) were incubated with IL 2 (0.5 U) and graded concentrations of L-ornithine in 0.2-ml microcultures for 20 hr. The cultures were then assayed for DNA synthesis as described for the IL 2 assay (see *Materials and Methods*).

DISCUSSION

Our experiments demonstrated a strong immunosuppressive effect of L-ornithine on the activation of CTL in vivo and in vitro. On the other hand, ornithine in similar concentrations had no effect on the production of IL 2 or IFN-γ by concanavalin A-activated C3H spleen cells or PMA-activated EL-4 thymoma cells. It had also no effect on several types of proliferative responses, including the allogeneic mixed lymphocyte reaction, the IL 2-dependent proliferative response of concanavalin A-activated thymocytes, and the IL 2-dependent proliferation of the W-2 T cell clone. These observations indicated that L-ornithine at the concentrations tested had no general toxic effect on the responding cell populations but exerted a selective immunosuppressive effect on the activation of CTL precursor cells. This conclusion was also supported by the fact that the cytotoxic responses were strongly suppressed when ornithine was added during the first 2 days of the response, but they were much less affected if ornithine was added on day 3. Whether ornithine at these concentrations selectively suppressed the maturation of CTL activity or whether it suppressed the proliferation of CTL precursor cells without inhibiting the proliferation of other T cell subsets cannot be distinguished with the available experimental information. It should be emphasized that the responder cells from in vivo and the macroculture experiments (Figs. 2 and 3) were washed before they were mixed with the target cells. This point, and the fact that cytotoxic responses were strongly inhibited when L-ornithine was added in the early and not in the late phase of the response, argued against the possibility that L-ornithine exerted its effect on the target cell rather than on the CTL precursor.

The mechanism of this suppressive effect is unknown. Its structural specificity, however, is remarkable: a similar effect is not observed with the structural analogues D-ornithine, L-lysine, or putrescine or with some other amino acids. It is well established that antigenically or mitogenically activated lymphocytes express increased intracellular levels of ornithine decarboxylase (9–11), a key enzyme for the biosynthesis of polyamines, which are required for cell growth and differentiation. It therefore seems possible that the external addition of L-ornithine may lead to an excess of polyamines or may induce a feedback suppression of the ornithine generating enzymes (i.e., arginase), and thereby generate an ornithine deficiency in the later phase of the response.

Activated macrophages express high arginase activity (3–5) and are thereby expected to increase the concentration of L-ornithine in the extracellular space. Cultured macrophages accumulate L-ornithine in the supernatant

(H. Kriegbaum and W. Dröge, unpublished observation). It is conceivable that a concentration of 10^{-2} M L-ornithine may also occur in vivo in the *immediate vicinity* of the ornithine-producing cells. The selective inhibition of the development of CTL activity by L-ornithine may therefore be part of a physiologic regulatory signal. Unfortunately, it is difficult to test the L-ornithine concentration in the immediate vicinity of individual cells in vivo. The fact that the late phase of the cytotoxic responses is less sensitive against L-ornithine than the early induction may also provide an explanation for the reported observation (28–30) that activated lymphocyte populations can prevent nonactivated CTL precursor cells from being recruited into the activated pool without inhibiting the previously induced CTL precursor cells to mature into functional CTL. This selective inhibition of the nonactivated precursor cells may well be mediated by L-ornithine-producing cells in the activated cell population. On the other hand, in view of the relatively high effective concentration of L-ornithine, one should also consider the possibility that L-ornithine may only mimic a natural analogue with a similar function.

Irrespective of whether a local concentration of 10^{-2} M L-ornithine occurs in physiologic situations, L-ornithine may turn out to be a useful immune response modifier because of its relatively selective regulatory effects. A comparison of L-ornithine with the immune suppressive agent cyclosporine A revealed remarkable differences. In contrast to L-ornithine, cyclosporine A was shown to inhibit not only the induction of cytotoxic responses but also proliferative responses against mitogens and alloantigens (18–20) and the production of the lymphokines IL 2 (13–17) and IFN-γ (12). In view of its more selective immunosuppressive effect, it seems possible that ornithine will find a field of application complementary to cyclosporine A as an immunosuppressive agent in human therapy.

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