Cyclosporin A prevents induction of the interleukin 2 receptor gene in cultured murine thymocytes

(control of cell proliferation/immunosuppression/blast formation/mitotic activation/chemotherapy of mammalian gene expression)

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Communicated by V. Prelog, April 16, 1986

ABSTRACT Blast formation and mitotic activation of G0-arrested mouse thymocytes were triggered by the addition of concanavalin A plus interleukin 2 (IL-2) to the culture medium. When added alone, Con A induces within 6 hr a complex reprogramming ("priming") that comprises the activation of the IL-2 receptor gene. The primed thymocytes are competent to interact with IL-2 and to respond to its growth-promoting effect, which corresponds to blast formation and mitotic activation. Cyclosporin A, an immunosuppressive cyclic peptide of fungal origin, prevents in T lymphocytes the activation of a set(s) of genes encoding lymphokines and the IL-2 receptor but does not affect their expression once they have been activated. The biomedical implications of these observations are discussed.

Cyclosporin A (CsA), a cyclic undecapeptide (M, 1203) of fungal origin, is a powerful immunosuppressive drug of considerable clinical importance. CsA prevents blast formation and mitotic activation of T lymphocytes derived from different mammalian species (1–3). It inhibits the production of interleukin 2 (IL-2) and of other lymphokines (4–8) and prevents the activation by phorbol 12-myristate 13-acetate of the IL-2 gene in a human T-cell line (Jurkat; ref. 9) and a murine T-cell line (EL-4; ref. 10). It was suggested that CsA exerts its immunosuppressive action by interfering with or by preventing the appearance of receptors for lymphokines or growth factors (11–13); however, when tested in the Jurkat T-cell line, CsA had no inhibitory effect on the expression of the IL-2 receptors, as judged by indirect immunofluorescence (9). It was reported (14) that CsA directly suppresses the response of T-lymphocyte-independent B lymphocytes to anti-immunoglobulin antibodies. Although several hypotheses have been proposed, no coherent picture of the mechanism of action of CsA has as yet emerged (15, 16).

Lectin-induced proliferation of peripheral T lymphocytes requires the production and release by the stimulated cells of IL-2 and its interaction with the IL-2 receptors (17) on the cell surface. To simplify the experimental approach, we used in the present work G0-arrested mouse thymocytes, which upon stimulation with lectin produce little if any IL-2; thymocytes are therefore unable to undergo blast formation and mitotic activation unless exogenous IL-2 is added to the culture medium (18). We report here the results from a study on the sequence of molecular events during mitogen-induced thymocyte activation and on the inhibitory effect of CsA.

MATERIALS AND METHODS

Thymocytes isolated from five 1-month-old female BALB/c mice (obtained from IFFA-Credo, France; ref. 19) were mixed and distributed in aliquots of 2 or 25 ml (about 2 × 10^6 cells per ml) in modified RPMI medium (20) supplemented with 10% fetal bovine serum (GIBCO). After preincubation for 20 hr at 37°C, more than 80% of the cells were viable, as determined by the use of fluorescein diacetate. IL-2 was obtained from the supernatants of rat spleen cells (a gift from S. Cammisuli, Sandoz Pharmaceutical, Basel; ref. 21) or a mouse cell line (EL-4; ref. 22) by gel filtration of the concentrated supernatants (21, 22). The preparations were used at a concentration that led to maximal mitotic activation. To stimulate the thymocyte cultures, either Con A alone (2 μg/ml; Pharmacia) or Con A plus IL-2 was added to the medium at "time zero" (i.e., 20 hr after explantation). CsA (Sandoz Pharmaceutical) was solubilized (3) and added at time zero to the cultures at a final concentration of 0.3 μg/ml, unless indicated otherwise. Duplicate 2-ml cultures were labeled by incubation with [3H]thymidine (5 μCi, 25 Ci/mmol; 1 Ci = 37 GBq) or [35S]methionine (10 μCi, 1000 Ci/mmol) for 1 hr or with [3H]thymidine (5 μCi, 45 Ci/mmol, Amersham) for 4 hr (19); acid-precipitable radioactivity was determined and aliquots of [3H]thymidine-labeled cells were processed for autoradiography (19, 23). For colorimetric determinations of DNA, RNA, and protein content (24), about 10^6 cells were used per experimental point. IL-2 receptors were visualized on the cell surface by indirect immunofluorescence (all steps performed on ice): 2 × 10^6 cells were washed with culture medium supplemented with 10 mM methyl α-D-mannoside and were incubated for 1 hr with a rat monoclonal antibody (diluted 1:10) directed against the mouse IL-2 receptor (PC61; ref. 25) and then for 1 hr with fluorescein-conjugated goat anti-rat serum (Nordic, Lausanne, Switzerland). For each experimental point, two aliquots (4000 cells) were examined by phase-contrast and ultraviolet microscopy (Leitz Prahx 22 EB).

To measure synthesis of the IL-2 receptor protein, thymocyte cultures (10^6 cells) were metabolically labeled with [35S]methionine (200 μCi/ml) for 15 min at 15 hr. Membrane proteins were extracted (26), and the IL-2 receptor was isolated by immunoaffinity chromatography using PC61 antibody (25) and subjected to NaDodSO4/PAGE and revealed by autoradiography (7 days exposure).

To measure steady-state levels of IL-2 mRNA, total RNA was extracted from 10^6 thymocytes with phenol at 65°C (27) and containing DNA was removed by treatment with pancreatic DNase I (Boehringer Mannheim). RNA (5 μg) was subjected to electrophoresis in 1% agarose gels containing 6% formaldehyde (28), electrophoretically transferred to GeneScreen nylon membranes (New England Nuclear-DuPont), and fixed by UV irradiation (29). The membranes were stained with methylene blue to localize and quantitate the 28S and 18S rRNA and then hybridized with a 32P-labeled nick-translated probe (pmIL-2R-1; ref. 30) specific for the IL-2 receptor mRNA(s); radioactivity was revealed by ex-

Abbreviations: CsA, cyclosporin A; IL-2, interleukin 2; hnRNA, heterogeneous nuclear RNA; kb, kilobase(s).
posure of the membranes to Kodak XAR-5 films for 2–15 hr at −70°C in the presence of an intensifying screen.

RESULTS

DNA Synthesis and Thymoblast Formation. Unstimulated thymocyte cultures consist mainly of thymocytes arrested in phase G0 of the mitotic cycle (A. Grieder, personal communication); only 0.8–1.5% of the cells synthesize DNA, as judged by autoradiography (Fig. 1A). Throughout the experiments the cell number remained virtually stable. To induce blast formation and mitotic activation the cultures were stimulated with Con A plus IL-2, added at time zero; the percentage of DNA-synthesizing cells began to increase by 20–25 hr (3–5%) and reached a maximum by 30–60 hr, when 35 ± 5% of the cells were engaged in DNA synthesis. A slight increase in the cytoplasmic rim could be observed by phase-contrast microscopy in 5–10% of the cells around 15 hr, whereas by 40 hr and later 30–40% of the cells exhibited the morphology of thymoblasts. Con A alone (i.e., without added IL-2) induced DNA synthesis in only 2–5% of the cells, whereas IL-2 alone was, as expected (18), without effect.

In unstimulated cultures, CsA (0.3 μg/ml) had no effect on residual DNA synthesis, even after exposure for up to 100 hr. In Con A-plus-IL-2-stimulated cultures, CsA, added at time zero, reduced the maximum number of DNA-synthesizing cells to 2–3% (Fig. 1A); these corresponded to thymocytes which escaped the inhibitory effect of CsA and underwent blast formation and mitotic activation (see below). When CsA was added at 8 hr or later (after Con A plus IL-2) it no longer inhibited blast formation and DNA synthesis induced by Con A plus IL-2 (data not shown). In cultures stimulated with Con A alone, CsA reduced the number of DNA-synthesizing cells to that observed in unstimulated cultures (data not shown).

RNA Synthesis. Determined colorimetrically, the RNA content of unstimulated thymocyte cultures was very low (0.5–1 μg of RNA per 10⁶ cells; Fig. 1B), being less than 10% of the RNA content of G0-arrested mouse kidney tissue culture cells (24); in contrast, the DNA content (6.5 μg per 10⁶ thymocytes) corresponded to that expected for quiescent, diploid mouse cells (24). In unstimulated thymocyte cultures, the rate of incorporation of [3H]uridine into RNA was low, as determined by 1-hr labeling (Fig. 2 Inset); it mainly reflected residual heterogeneous nuclear RNA (hnRNA) synthesis (Fig. 2), whereas synthesis of 5S RNA, tRNA, 4S prerRNA, 18S rRNA, and 28S rRNA was barely detectable, even after labeling for 3 hr (data not shown). Actinomycin D (0.05 μg/ml) had no detectable effect on residual hnRNA synthesis (19).

In cultures stimulated with Con A plus IL-2, RNA content began to increase around 12 hr, reaching a maximum plateau by 60–80 hr, when the cultures contained 4–5 μg of RNA per 10⁶ cells (Fig. 1). Rate of [3H]uridine incorporation increased around 3 hr and reached a maximum plateau by 50–60 hr (Fig. 2 Inset and ref. 19). Increased incorporation of [3H]uridine was resistant to actinomycin D (0.05 μg/ml) until about 9 hr and reflected “early” stimulation of hnRNA synthesis (Fig. 2 and ref. 19). A rather sudden and massive increase in nucleolar and nucleoplasmic transcription (45S pre-rRNA, 32S rRNA, 28S rRNA, 18S rRNA, hnRNA, 5S RNA, and tRNA) began by 10–11 hr (19). Judged from the high sensitivity of transcription toward actinomycin D (0.05 μg/ml) and from the migration pattern of phenol-extracted RNA during gel electrophoresis (A₂₆₀ and radioactivity), the bulk of RNA synthesized by 10–11 hr (and later) was rRNA (19). Direct evidence for the activation of the ribosomal genes was obtained by measuring steady-state levels of 45S pre-rRNA (ref. 19 and unpublished results). Con A alone led to early stimulation of hnRNA synthesis indistinguishable from that induced by Con A plus IL-2 (Fig. 2), whereas the subsequent stimulation of overall RNA synthesis did not occur (data not shown).

In unstimulated cultures, CsA (tested up to 80 hr) remained without effect on RNA content and decreased incorporation of [3H]uridine by only about 5% (data not shown). In cultures stimulated with Con A plus IL-2 or with Con A alone, CsA had no detectable effect on the early stimulation of hnRNA synthesis, until 6–7 hr (Fig. 2); CsA prevented, however, the subsequent stimulation of overall RNA synthesis and the increase in total RNA (Figs. 1B and 2 Inset). The small increase in RNA content (3–5%; Fig. 1) in the presence of CsA reflects residual blast formation. When CsA was added to Con A-plus-IL-2-stimulated cultures by 8 hr or later, it exerted no detectable effect on synthesis and accumulation of RNA (data not shown).
Protein Synthesis. Unstimulated cultures contained about 15 μg of protein per 10^7 thymocytes (Fig. 3), corresponding to less than 10% of the protein content of G4-32 mouse kidney cells (24). After addition of Con A plus IL-2, protein content increased by 20–25 hr and reached a maximum around 80 hr (Fig. 3).

Unstimulated cultures and cultures stimulated with Con A plus IL-2 and incubated in the absence or presence of CsA were labeled for 1 hr with [35S]methionine at different times between 3 and 70 hr, and acid-precipitable radioactivity was determined (Fig. 3A and ref. 19); aliquots of all preparations were analyzed by electrophoresis in one (NaDodSO4/PAGE) or two dimensions (32), and the proteins were revealed by staining the gels with Coomassie blue and by autoradiography or fluorography. From the results (19), we concluded that in unstimulated cultures, residual incorporation of [35S]methionine reflected overall protein synthesis, similar to that observed in G4-32 mouse kidney cells (24), though at a much lower rate (19). In cultures stimulated with Con A plus IL-2, the apparent rate of protein synthesis increased around 6 hr—i.e., about 3 hr after the onset of stimulated hnRNA synthesis—reaching a maximum by 60–80 hr (Fig. 3A). The stimulation of [35S]methionine incorporation that was induced by Con A and IL-2 reflected, at all times tested, increased overall protein synthesis (19).

In unstimulated cultures, CsA had no detectable effect on protein content, on the incorporation of [35S]methionine, or on the protein patterns (ref. 19 and unpublished data). In cultures stimulated with Con A and IL-2, CsA virtually prevented the increase in protein content (Fig. 3B). However, stimulation of [35S]methionine incorporation became noticeable around 6 hr, reached a maximum around 15 hr (Fig. 3A), and then decreased to the low level of unstimulated cultures; when tested between 10 and 12 hr, incorporation of [35S]methionine was 30–50% lower than in parallel cultures stimulated in the absence of CsA. The protein patterns of cultures stimulated with Con A plus IL-2 in the presence of CsA were indistinguishable from those observed in parallel cultures incubated without CsA, with the exception that radioactivity was markedly lower (unpublished results).

Expression of the IL-2 Receptor Gene. The IL-2 receptors on the cell surface were visualized by indirect immunofluorescence using a monoclonal antibody (PC61; ref. 25) directed against the mouse IL-2 receptor. In unstimulated cultures and also in Con A-plus-IL-2-stimulated cultures, tested at 0.5 and 3 hr, less than 0.3% of the thymocytes exhibited an immunofluorescence reaction, whereas by 6 hr after stimulation with Con A plus IL-2, 15–20% of the cells showed on their surface numerous, rather regularly distributed fluorescent dots (Table 1); the relative number of immunofluorescent cells then increased, reaching a maximum plateau of 35 ± 5% by 20–25 hr (tested until 60 hr). A very strong increase in the intensity of the immunofluorescence reaction, which then covered the entire cell surface, was noted on thymoblasts around 20 hr and later. Actinomycin D (1 μg/ml) or cycloheximide (25 μg/ml) added at 3 hr or 4 hr, respectively, prevented the appearance of the IL-2 receptors, determined at 7 hr; in contrast, exposure of Con A plus IL-2-stimulated cultures to cycloheximide from 36–40 hr had no detectable effect on the immunofluorescence reaction. After addition of Con A alone, the time course of the appearance of fluorescent dots was indistinguishable until about 20 hr, when the percentage of fluorescent cells rapidly decreased, corresponding by 40–60 hr to only 2–3% strongly positive thymoblasts. In cultures stimulated with Con A plus IL-2, CsA reduced the percentage of immunofluorescent cells to 3–5% when tested at different times between 6 and 20 hr, and by 40–60 hr only 1–3% of the cells (thymoblasts) exhibited a strong immunofluorescence reaction (Table 1). In contrast, when CsA was added at 8 hr or later, the time course of cell surface IL-2 receptor expression, as judged from the immunofluorescence reaction (Table 1 and unpublished data), was indistinguishable from that observed in the absence of CsA. In cultures incubated with Con A alone, CsA reduced the maximum percentage of positive cells to about 3%, and by 40 hr and later, less than 1% immunofluorescent cells could be detected.

Table 1. Cell surface expression of IL-2 receptor

<table>
<thead>
<tr>
<th>Cultures</th>
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<th>40 hr</th>
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<td>4</td>
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<td>2*</td>
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<tr>
<td>Plus CsA (8 hr)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>28*</td>
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</tbody>
</table>

Stimulated cultures were exposed to Con A plus IL-2 from time zero. ND, not determined.

*Strong immunofluorescence reaction.
Fig. 5. Sequence of the major molecular events during mitogen-induced blast formation and mitotic activation of G0-arrested mouse thymocytes.

To study biosynthesis of the IL-2 receptor, cultures were incubated with \(^{35} \text{S}\)methionine for 15 min at 15 hr, and the receptor was isolated by immunochromatography and subjected to NaDodSO\(_4\)/PAGE. Autoradiography of the gels (Fig. 4A) revealed that isolates from Con A-plus-IL-2-stimulated cultures contained radioactive polypeptides of apparent molecular mass ranging from 30 to 65 kDa; the broad upper band (50–65 kDa) corresponds to the surface-labeled IL-2 receptor (25), whereas the group of faster-moving bands most likely corresponds to the unglycosylated (30) and underglycosylated forms of the IL-2 receptor. In contrast, no radioactive bands could be detected in extracts from unstimulated cultures or from cultures stimulated with Con A plus IL-2 but incubated in the presence of CsA.

Steady-state levels of IL-2 receptor mRNA(s) were determined in total RNA extracted with phenol 8, 15, and 30 hr after stimulation by Con A plus IL-2 in the absence or presence of CsA and from unstimulated parallel cultures. Aliquots of RNA were subjected to electrophoresis, transferred to GeneScreen membranes, fixed by UV irradiation (29), and then hybridized with \(^{35} \text{P}\)-labeled nick-translation pmlIL-2R-1, a probe specific for the mouse IL-2 receptor mRNA(s) (30). Autoradiography revealed, in samples taken 8 hr after the addition of Con A plus IL-2, radioactive bands at the positions expected for mouse IL-2 receptor mRNA(s) (3.5, 2.2, and 1.5 kilobases (kb) and trace amounts at 4.5 kb (Fig. 4B); the mRNA(s) markedly increased around 15–20 hr, coinciding in time with the strong increase in the immunofluorescence reaction for the IL-2 receptor (see above). In unstimulated cultures and also in cultures incubated with Con A plus IL-2 in the presence of CsA, only trace amounts of 3.5-kb IL-2 mRNA could be detected (Fig. 4), possibly reflecting the small percentage of immunofluorescent thymocytes observed in unstimulated cultures. When CsA was added at 8 hr or later, steady-state levels of IL-2 mRNA(s) were indistinguishable from those found in parallel cultures stimulated with Con A and IL-2 but incubated without CsA (data not shown).

**DISCUSSION**

In unstimulated, G0-arrested mouse thymocytes, transcription is very low and virtually restricted to hnRNA, whereas residual translation reflects overall protein synthesis. Addition of Con A plus IL-2 leads in 35 ± 5% of the thymocytes to blast formation and mitotic activation. The sequence of the major molecular events reported here is shown in Fig. 5. "Priming" (phase 1) is induced by Con A alone; "early," low-level stimulation of hnRNA and protein synthesis begins around 3 and 6 hr, respectively, and is paralleled by the expression of the IL-2 receptor gene; phase 1 apparently reflects a complex, Con A-induced reprogramming that renders the thymocytes competent to interact with IL-2 and to respond to its growth-promoting effect, referred to as phase 2. This phase begins around 10 hr; it comprises blast formation, mitotic activation, and, by 15–20 hr, a marked increase in the expression of the IL-2 receptor gene. The sequence of the events in phase 2 is similar to that observed during mitotic activation triggered in quiescent mammalian cells by oncogenic DNA viruses and serum growth factors (23, 24, 33).

In unstimulated thymocytes, CsA (0.3 \(\mu\)g/ml) has virtually no effect on residual DNA, RNA, and protein synthesis. When added at time zero (i.e., together with Con A plus IL-2), CsA prevents the expression of the IL-2 receptor gene and phase 2, whereas the early stimulation (until 6–7 hr) of hnRNA and protein synthesis is not detectably inhibited. If CsA is added at 8 hr or later, after priming, it no longer exerts any inhibitory effects; this is in accordance with the observation that CsA acts early in T-lymphocyte activation (3).

Prevention of IL-2 receptor gene activation seems to be a major, though most likely not the only, effect by which CsA blocks mitogen-induced blast formation and mitotic activation. In quiescent mouse 3T3 fibroblast and mouse kidney cell cultures, CsA (1 \(\mu\)g/ml) inhibits neither cellular RNA synthesis nor the virus- or serum-induced mitotic reaction (19); this tends to exclude the possibility that CsA exerts its immunosuppressive effect by inhibiting mammalian RNA polymerase II (34) or by blocking the translation of mammalian cells from a quiescent to a proliferative state.

The experimental observations now available indicate that CsA prevents T lymphocytes the activation of a set(s) of genes encoding lymphokines and the IL-2 receptor but that it does not affect the expression of these genes once they have been activated; they suggest that prevention of the activation of specific genes in T lymphocytes plays a pivotal role in the immunosuppressive action of CsA. We may expect that the understanding of the molecular mode of action of CsA will provide a step toward the chemotheraphy of mammalian gene expression, leading to the development of drugs that modulate the activity of specific genes.

We are grateful to Dr. S. Cammisuli (Basel) for the EL-4 cell line; Drs. H. R. MacDonald and M. Nabholz (Lausanne, Switzerland) for the monoclonal antibody PC61; Dr. M. Nabholz for performing the radioimmunoassay shown in Fig. 4; Prof. T. Honjo (Kyoto, Japan) for the pmlIL-2R-1 plasmid; and Drs. S. Cammisuli, B. Ryffel, and H. Stähelin (Sandoz A.G., Basel, Switzerland) for useful discussions and suggestions. We gratefully acknowledge the critical reading of the manuscript by Drs. R. Lamers and T. Stuehelin (Max Planck Institut für Immunobiologie, Freiburg, F.R.G.) and Prof. J.-C. Jaton (University of Geneva). We thank Mrs. Y. Eprecht and Mr. O. Jenni for preparing the figures. This work was supported by Grant 3.072.81 from the Swiss National Science Foundation and by grants from the Sandoz Research Foundation and the Swiss Cancer Society.