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# CYCLOSPORIN A AND DEXAMETHASONE SUPPRESS T CELL RESPONSES BY SELECTIVELY ACTING AT DISTINCT SITES OF THE TRIGGERING PROCESS[1]

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**The mechanism by which mitogenic lectins induce proliferation in murine T cells has recently been dissected into two major events: a rapid, accessory cell-independent acquisition of responsiveness to growth factors, and an accessory cell-dependent elaboration of T cell growth factors (TCGF) occurring later in time.**

**Within this background, the mode of action by which two different immunosuppressive drugs, namely, cyclosporin A and dexamethasone, inhibit T cell responses was studied. It was shown that cyclosporin A inhibits the lectin-dependent acquisition of responsiveness to growth factors in resting T cells at concentrations that have no effect on the production of TCGF. Furthermore, cyclosporin A does not interfere with the proliferative responses of preformed blasts to TCGF at these concentrations, demonstrating that lack of responsiveness to TCGF is not due to nonspecific toxicity of the drug. In contrast, dexamethasone at pharmacologic concentrations ($10^{-6}$ M) did not inhibit the acquisition of responsiveness to growth factors, whereas it drastically reduced TCGF production. Furthermore, dexamethasone also failed to interfere with the mitogenic activity of preformed TCGF on T cell blasts, indicating that T lymphocytes involved in mitogenic responses are insensitive to nontoxic concentrations of dexamethasone, both at the initiation of the responses and at later phases of proliferate activity.**

**These results demonstrate the distinct sites of action for two drugs, providing further insight into the mechanisms of T lymphocyte physiology and developing present possibilities of controlled application of such drugs in clinical practice.**

Recent experiments have provided further understanding on the mechanism by which T cells are activated. It is now well established that T cell proliferation is dependent on T cell growth factors (TCGF)[2] (1, 2). Such factors, however, do not induce growth in resting small T cells (3), which are rendered sensitive to TCGF by a short pulse with specific ligands (4, 5). On the basis of absorption experiments (3, 4), we have postulated before that this lectin-dependent acquisition of responsiveness to growth factors by resting cells corresponds to the expression of growth receptors for TCGF and demonstrated the specificity of the induction (5) and its dependence on protein synthesis.[3] This paper will refer to the expression of growth receptors, although they are putative, or to acquisition of TCGF responsiveness indiscriminately. Once the cells express growth receptors for TCGF, the inducing ligand is no longer needed, and the availability of TCGF becomes the only limiting factor for growth. This initial event in T cell triggering does not result in growth by itself, implying that an *in situ* production of TCGF must occur (4).

In light of the above observations, it appeared interesting to study the mode of action by which immunosuppressive drugs inhibit T cell proliferation in order to increase both the effectiveness of their clinical use and our understanding of T cell responses. The fungus metabolite, cyclosporin A (CyA), has been demonstrated to have a selective immunosuppressive effect on T cells, since it inhibits cell-mediated cytolysis, graft-*vs*-host and delayed-type hypersensitivity (6–8) reactions, and T cell but not B cell colony formation (9).

A large number of reports have described various effects of glucocorticoids on lymphocytes and on immune responses. In particular, it has been found that glucocorticoids inhibit mitogen- or antigen-induced T cell proliferation (10, 11). Some studies demonstrate that this effect is due to an inhibitory effect by the drug at an early state in the activation process (12), and that activated lymphocytes are insensitive to the suppressive effect of glucocorticoids (12, 13). In contrast, Smith and coworkers (14) first reported that both unstimulated and stimulated lymphocytes are equally sensitive to glucocorticoids, although more recently these authors ascribe to TCGF production the major inhibitory action of glucocorticoids (15).

In this communication, results on the mode of action of these two immunosuppressive agents are presented, demonstrating that they are sharply different in their target mechanisms in the process of T cell proliferation. Thus, CyA acts by inhibiting the presentation of growth receptors by resting T lymphocytes, but it does not interfere with TCGF production. Dexamethasone (DM), on the other hand, does not inhibit the presentation of growth receptors but inhibits TCGF production. Although both of these drugs display the same effect, i.e., inhibition of T cell growth, the causes for such inhibition are completely disparate.

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[1] This work was supported in part by the Swedish Medical Research Council.
[2] Abbreviations used in this paper: TCGF, T cell growth factor; CyA, cyclosporin A; DM, dexamethasone; α-MM, α-methyl-D-mannoside; CM, conditioned medium; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.
[3] E.-L. Larsson, T cell activation: An analysis of the triggering process (doctoral thesis, Umeå, 1980).

## MATERIALS AND METHODS

*Mice.* All experiments reported here were performed with C3H/HeJ mice obtained from the Institut für Biologische-Medizinische Forschung AB, Füllingsdorf, Switzerland. Mice from both sexes between 2 to 3 months of age, were used.

*Chemicals.* Concanavalin A (Con A, Pharmacia Fine Chemicals, Uppsala, Sweden) and α-methyl-D-mannoside (α-MM, Sigma Chemical Co., St. Louis, Mo.) were dissolved in medium, CyA (Sandoz, Basel, Switzerland) was dissolved in methylsulphoxide, and DM (Ciba Geigy, Basel, Switzerland) was dissolved in ethanol.

*Cell cultures.* Spleen cells were prepared as described earlier (16) and cultured in RPMI 1640 medium supplemented with 10 mM HEPES (see Abbreviations), 50 μg/ml neomycin, $5 \times 10^{-5}$ M 2-mercaptoethanol and 10% fetal calf serum (FCS, Gibco-Biocult, Glasgow, Scotland, batch U 18710.11). T cell blasts were prepared as described earlier (17) and maintained in 50% standard conditioned medium (CM) (see below) for more than 3 weeks before being tested.

*Preincubation and cultures of cells.* Spleen cells ($3 \times 10^6$/ml) were incubated for 4 hr at 37°C in medium with or without Con A (5 μg/ml) in the presence or absence of various concentrations of CyA or DM as indicated in the text. As controls, parallel cultures were set up with the corresponding concentrations of the solvents, namely, dimethyl sulphoxide and alcohol. The cells were then washed four times in medium containing 20 mg/ml of α-MM and were recultured ($2 \times 10^5$/ml) in microtiter plates in 0.2 ml of either medium, TCGF-containing medum (50% v/v of conditioned to fresh medium), or medium containing Con A (5 μg/ml).

*Preparation of TCGF.* Standard CM containing TCGF were obtained by incubating mouse spleen cells ($5 \times 10^6$/ml) for 24 hr in the presence of Con A (5 μ/ml). TCGF production was also attempted as above but in the presence of various concentrations of CyA and DM as indicated in the *Results* section. The supernatants were thereafter collected, 20 mg/ml of α-MM were added, and the mixture was filtered through 0.45 μ Nalgene filters and stored at −20°C until used. This mixture was used at a final concentration of 50% v/v in all experiments presented.

*Assay.* The proliferative responses in triplicate cultures were assayed at various days of culture as indicated after a 3-hr pulse of $^3$H-thymidine (Radiochemical Center, Amersham, GB, TRK 310; specific activity 2 Ci mmole; 5 μCi/ml). Cultures were harvested in a microharvesting apparatus, and incorporated thymidine was determined by scintillation counting, expressed as cpm.

## RESULTS

As already described by others, both CyA (18) and glucocorticoids (10, 11) inhibit the proliferative responses of lymphocytes.

Table I demonstrates such an experiment where Con A-induced proliferation is reduced in the presence of these drugs. The Con A response of normal spleen cells is reduced approximately 60% and 50% in the presence of 5 μg/ml of CyA or $10^{-6}$ M of DM, respectively. We have recently found that the mitogen-induced T cell activation can be dissected into two major events; 1) a rapid accessory cell-independent acquisition of responsiveness to growth factors by resting T cells, and 2) an accessory cell-dependent elaboration of TCGF. These findings provide a useful system for studying where in the activation-process different drugs exert their action, in addition to investigating their influence in proliferating T cell blasts.

The following experiments were therefore designed to investigate whether the inhibitory effects of CyA and DM were due to blocking the presentation of growth receptors for TCGF by resting cells, or to inhibition of TCGF production. Additional experiments for studying how these drugs interfered with the TCGF-dependent T cell blast proliferation were also performed. Each experiment was performed repeatedly with reproducible results.

*CyA inhibits the Con A-dependent induction of responsiveness to TCGF in resting T lymphocytes.* Murine spleen cells were incubated for 4 hr at 37°C with or without Con A present. To these cultures various concentrations of CyA or, as a control, the solvent used (ethanol) were added. The cultures were thereafter washed four times in medium containing α-MM and were tested for their responsiveness to preformed TCGF and to Con A. As shown in Figure 1 (upper panel), normal spleen cells

TABLE I

*Inhibition of Con A-induced responses by CyA and DM*

| Additions to Cultures[a] | Spleen Cell Responses (cpm/cult × $10^{-3}$)[b] after: | |
|---|---|---|
| | 3 days | 5 days |
| None | 0.8 | 0.6 |
| Con A | 15.0 | 40.7 |
| CyA | 0.8 | 0.7 |
| Con A + CyA | 6.3 | 14.0 |
| None | 0.5 | 0.5 |
| Con A | 33.4 | 64.6 |
| DM | 0.9 | 0.6 |
| Con A + DM | 17.4 | 37.1 |

[a] Normal C3H/HeJ spleen cells ($2 \times 10^5$/ml) were incubated in either Con A (5 μg/ml), CyA (5 μg/ml), and DM ($10^{-6}$ M) or in mixtures of Con A + CyA and Con A + DM, respectively.

[b] $^3$H-thymidine uptake was measured at days 3 and 5 of culture. Mean values of triplicate cultures are expressed as cpm.

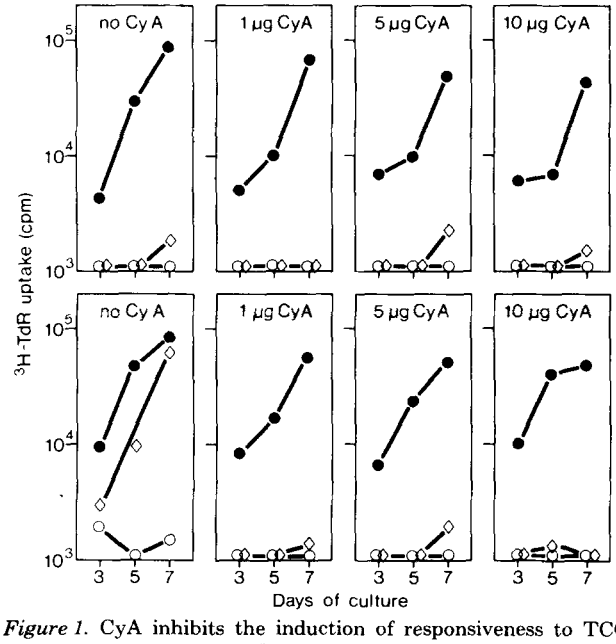


*Figure 1.* CyA inhibits the induction of responsiveness to TCGF. C3H/HeJ spleen cells ($5 \times 10^6$ ml) were incubated for 4 hr with various concentrations of CyA (1 μg, 5 μg, and 10 μg/ml) as indicated, and in the presence (*lower panel*) or absence (*upper panel*) of Con A (5 μg/ml). The cells were thereafter washed four times in α-MM-containing medium, and recultured ($2 \times 10^5$/ml) in medium (○), in TCGF (◇), or in Con A (●). Incorporation of $^3$H-thymidine was measured at the indicated days of culture. In this, and in all the following figures, the bars indicating the standard deviation of the means of triplicate samples were smaller than the symbols, and are therefore not shown.

preincubated in medium do not respond to TCGF but respond to Con A. The presence of CyA during the preincubation did not inhibit the further responses to Con A. Spleen cells that were preincubated for 4 hr in the presence of Con A acquired responsiveness to TCGF, as shown in Figure 1 (lower panel). If this preincubation, however, is preformed in the presence of as little as 1 μg/ml of CyA, this induction of responsiveness is abolished. These cells do, however, respond well to Con A when recultured, indicating that the inhibitory effect is not due to toxic effects, and it pertains to the induction of growth receptors on resting T cells only.

*Production of TCGF occurs in the presence of CyA.* It has earlier been demonstrated that the presentation of growth receptors and the production of TCGF are separable by time and cellular requirements (4). It was of interest, therefore, to investigate whether CyA would interfere with the elaboration of TCGF. For standard TCGF production, spleen cells were incubated at $5 \times 10^6$/ml for 24 hr at 37°C in the presence of 5 μg/ml of Con A. Parallel cultures received, in addition, various concentrations of CyA. The different supernatants from these cultures were then assayed for TCGF activity on full T cell blasts that had been kept in a standard CM for more than 3 weeks. The results from such an experiment are presented in Figure 2. T blast proliferation was measured on days 2, 3, 4, and 5. As shown, TCGF is elaborated at control levels in the presence of 1 and 5 μg/ml of CyA, but its production appears inhibited in the presence of 10 μg/ml of CyA.

*Interaction of CyA with T cell blast responses to preformed TCGF.* The fact that at 1 μg/ml CyA inhibits the presentation of growth receptors on resting T cells but has no influence in TCGF production indicated that CyA acts at an early stage in the activation process. In addition, CyA could have a similar effect on full T blasts. This was investigated by adding various concentrations of CyA directly to cultures containing T cell blasts and preformed TCGF. Figure 3 demonstrates the results from two different experiments by using different TCGF-containing CM. T cell blasts are fully responsive to TCGF in the presence of concentrations up to 10 μg/ml of CyA. The response is abolished at 50 μg/ml of CyA, probably due to nonspecific toxicity of the drug.

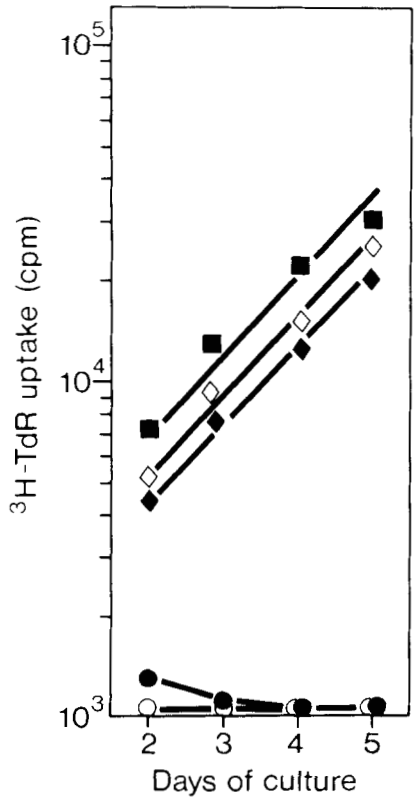


*Figure 2.* Production of TCGF in the presence of CyA. Spleen cells ($5 \times 10^6$/ml) were incubated with Con A (5 μg/ml) in the presence of various concentrations of CyA, for 24 hr. The supernatants were collected and tested for TCGF-activity on T cell blasts ($5 \times 10^4$/ml). Supernatants induced without CyA (■), 1 μg/ml CyA (◇), 5 μg/ml CyA (◆), 10 μg/ml CyA (●). The control cultures of T cell blasts in fresh medium are also shown (○). The proliferative responses were measured at the indicated days of culture.

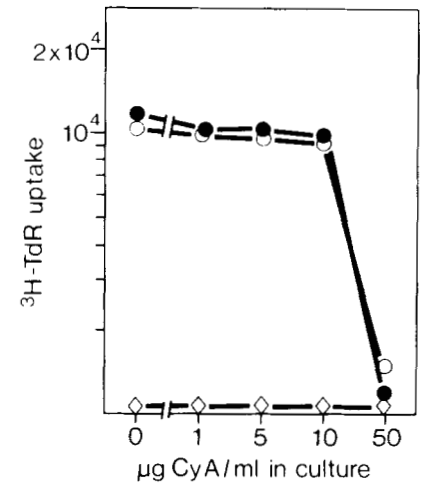


*Figure 3.* CyA does not interfere with TCGF-dependent T cell blast growth. Various concentrations of CyA were added to cultures containing T cell blasts ($5 \times 10^4$/ml) and preformed TCGF. Two different batches of TCGF containing CM are presented (○) and (●). Control cultures in fresh medium (◇) are also shown. The proliferative responses were measured on day 3 of culture.

*DM does not inhibit induction of responsiveness to TCGF.* The same protocol used above for the stepwise analysis of T cell activation was followed to investigate the effects of DM. Normal spleen cells were preincubated in the presence or absence of Con A (5 μg/ml) and exposed to various concentrations of DM. Four hours later, and after washing, the cells were recultured and assayed for responsiveness to TCGF. Results from such an experiment are presented in Figure 4, where proliferation was determined on days 2, 4, and 6 of culture. Preincubation with Con A in the presence of $10^{-8}$ to $10^{-6}$ M DM did not alter the presentation f growth receptors, since such cells showed full sensitivity to the TCGF mitogenic activity in secondary cultures. TCGF responsiveness of cells preincubated in $10^{-5}$ M DM, however, was reduced and reached only 20% of control cultures. Figure 4 also depicts the unresponsiveness to TCGF of spleen cells preincubated in medium, with or without DM, demonstrating the lectin dependence of the acquisition of TCGF responsiveness.

*DM inhibits the production of TCGF.* Since DM appeared not to affect the induction of responsiveness to TCGF (at $10^{-8}$ to $10^{-6}$ M), the inhibition of T cell proliferation by glucocorticoids was likely to be due to lack of availability of TCGF. As demonstrated in Figure 5*a*, this was found to be the case, in that DM inhibited Con A-induced TCGF production. When $10^{-8}$ M of DM was present during elaboration of TCGF, a 75% reduction of TCGF-induced T blast growth was observed. A linear decrease of TCGF activity was found with increasing amount of DM added to the cultures for TCGF production,

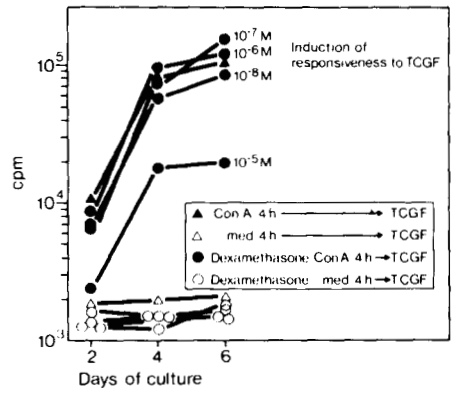


*Figure 4.* Induction of responsiveness to TCGF in the presence of DM. Normal spleen cells were preincubated as in Figure 1, with or without Con A, but in the presence of various concentrations of DM as indicated. The cells were recultured in preformed TCGF and the proliferative responses assayed on the indicated days of culture.

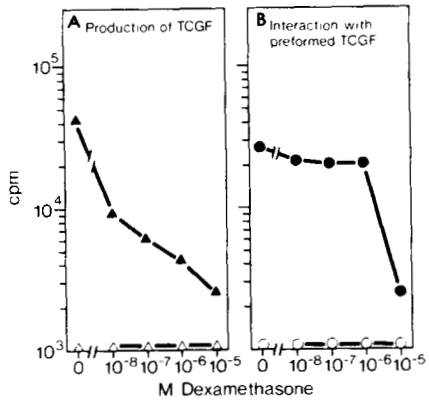


*Figure 5.* DM inhibits TCGF production but not TCGF-dependent T cell blast growth. *A, left panel:* spleen cells ($5 \times 10^6$/ml) were incubated with Con A (5 μg/ml) in the presence of the indicated concentrations of DM. The 24-hr supernatants were tested for TCGF activity on T cell blasts ($5 \times 10^4$/ml) and the results from day 3 of culture are presented. Supernatants induced with (▲) and without (△) 5 μg/ml of Con A. *B, right panel:* various concentrations of DM were added to cultures of T cell blasts ($5 \times 10^4$/ml) in fresh medium (○) or in TCGF-containing CM (●). The proliferative responses measured on day 3 of culture are presented.

resulting in an almost complete loss of TCGF activity at $10^{-5}$ M of DM.

*Interaction of DM with T cell blast responses to preformed TCGF.* The results shown in Figures 4 and 5*a*, i.e., the lack of effect of DM ($10^{-8}$ to $10^{-6}$ M) on the expression of growth receptors by resting T cells, and the drastic inhibition of TCGF production in the presence of $10^{-8}$ to $10^{-5}$ M DM, respectively, would suggest that glucocorticoids exert their effect at a late stage in the activation process, namely, at the stage of TCGF production. The lack of TCGF activity in these supernatants could, however, be due to an inhibitory effect of DM on the proliferative T cell blasts themselves and not to a real lack of TCGF. To test for this possibility, T cell blasts were cultured with TCGF, and various concentrations of DM were titrated into such cultures. It is shown in Figure 5*b* that $10^{-8}$ to $10^{-6}$ M DM does not inhibit T blasts to respond to TCGF, and only at $10^{-5}$ M of DM is T blast growth inhibited. These findings indicate, therefore, that the major mechanism by which DM suppresses T cell responses is by inhibiting TCGF production.

## DISCUSSION

Recent progress on the understanding of the proliferative responses of T cells to antigens and mitogens have established that continuous proliferation of T cells is maintained by growth factors released by activated cells and not by the antigens or mitogens themselves (2, 17). TCGF only induce growth in activated T cells, but not in resting T cells (3, 4, 19), which obtain sensitivity to TCGF by the interaction of specific ligands with the cell membrane (5). The acquisition of responsiveness occurs within 4 hr and is independent of accessory cells (4). This first event in T cell triggering does not by itself result in growth, because proliferation necessarily requires the ultimate mitogen—TCGF. TCGF is elaboratted later in time and requires in addition to T cells the presence of I.A.-positive, θ-negative accessory cells (17, 20). The latter appear to function by secreting a soluble product, which in conjunction with a mitogenic lectin induces T cells to secrete a second product characterized on chemical and functional basis as TCGF (20). Once TCGF has been elaborated (and the resting T cells rendered responsive), there is no further requirement for the lectin, the concentration of TCGF being the sole variable determining the extent of proliferation of the TCGF-reactive T cells (17). Since these are not capable of producing TCGF themselves (17, 20), T cell proliferative responses appear as a cooperative process dependent upon macrophage-T cell and T-T cell interactions.

It should be pointed out that although only crude conditioned media were used in the experiments as a source of growth factors, we have previously shown that Con A-derived blasts and normal cells pulsed with Con A for 4 hr, which were the responder cell types used here, are only induced to proliferate by a single protein peak obtained after three purification steps of crude supernatants (20). We and others have defined this chemically homogenous activity as TCGF, and therefore, we are certain of dealing with such growth factors even when using crude supernatants.

The results presented herein demonstrate the distinct selective effects of two different immunosuppressive drugs. The theoretical and technical background presented above provides a useful way of dissecting the mechanisms by which immunosuppressive drugs exert their effects.

The cyclic-peptide, CyA, is a fungus metabolite that has been isolated and reported to have high affinity for membrane lipids (21, 22). CyA has also been shown to inhibit cell-mediated cytolysis, graft-*vs*-host, and delayed-type hypersensitivity reactions (6–8). Furthermore, a direct inhibitory effect of CyA in T cell proliferation has been reported as the suppression of T cell colony formation (9), and of thymidine uptake in mass cultures (18). The present results demonstrate that the suppressive effect of CyA was primarily the result of inhibition of the lectin-dependent acquisition of responsiveness to TCGF by resting T cells. Concentrations of CyA that have no effect on other triggering-related events (such as production of TCGF) profoundly inhibit that first step in activation (1 to 5 μg/ml). At higher concentrations (10 μg/ml), however, CyA also inhibits the production of TCGF, indicating that at these concentrations the metabolite has composite suppressive effects in the proliferative responses. These inhibitory effects are not due to nonspecific toxicity of the drug, since TCGF-dependent T cell blast proliferation occurs at control levels in the presence of concentrations of CyA below 5 μg/ml. At these high doses, also, this phase of the response is inhibited, suggesting either a nonspecific toxic effect or yet a third mechanism of suppression mediated by CyA on T cell proliferation.

In contrast with CyA, DM was found to exert its effect on a different stage of the activation process, also resulting in inhibition of T cell growth. The well-established inhibition of mitogenic responses by glucocorticoids (10, 11) has been claimed to be related to the state of differentiation of the responding lymphocytes. Thus, it has been found that the major effect of glucocorticoids is at the initiation of a response (12). Smith and co-workers (14) have reported that lymphocytes were equally sensitive to glucocorticoid-induced inhibition, regardless of their state of activation. Recently, however, the same authors reported that the major effect of glucocorticoids was the inhibition of TCGF production (15). Although the authors maintain their earlier interpretation, their recent experiments show that proliferating T cells are, in fact, not inhibited by glucocorticoids (23).

DM was tested in our stepwise screening to investigate its capacity to inhibit the presentation of growth receptors or to inhibit the elaboration of TCGF, and finally, to determine its effect on full T cell blasts in their response to preformed TCGF. The results showed that DM did not interfere with the initiating step or with T cell blast to preformed TCGF at concentrations up to $10^{-6}$ M. In contrast, TCGF elaboration is drastically reduced in the presence of $10^{-8}$ M DM, and it is further suppressed with increasing concentrations of DM present in the cultures. Interestingly, even the highest concentrations of DM failed to inhibit completely TCGF production. It is possible that DM interferes at a specific point along this process and that some of the producing cells have already passed that point along the pathway at the time they are set in culture. At concentrations higher than $10^{-6}$ M, the drug inhibited both the presentation of growth receptors in resting T cells and the proliferative phase of the responses as well. It is not clear whether this is due to a nonspecific toxicity of DM or to some selective effects on T lymphocytes at these high concentrations.

It should be noted that although 5 μg/ml of CyA inhibits completely the acquisition of responsiveness to TCGF, and $10^{-6}$ M of DM inhibits 90% of TCGF production, the Con A-induced proliferation is only reduced by 50 to 60% when the same drugs are added at the start of cultures continuously stimulated by Con A. This is most likely due to a gradual inactivation of the drugs *in vitro*, thus resulting in recovery of the proliferative responses. This is to be expected, since, as demonstrated, the inhibitory effects of both drugs are reversible upon their removal. In addition, it is likely that our assays for each of the two steps are more sensitive to inhibition than *in situ* responses to Con A.

The practical importance of dissecting the "target mechanism" for various immunosuppressive drugs lies mainly in the evaluation of their suitability in various clinical protocols and on the indications for a particular schedule of administration. CyA appears to be advantageous to DM, in the sense that it exhibits its effect directly on the responding T cell, undergoing contact with the stimulus. It would appear that in the presence of CyA, antigen-sensitive cells would not proliferate in response to the antigen, whereas the cells that are already in cycle could proceed untouched, since TCGF is available.

DM, on the other hand, does not inhibit the triggering of antigen-sensitive cells and can only have general suppressive effects on the proliferation of all T lymphocyte clones via the inhibition of TCGF production. Current pulse-chase experiments with these suppressive drugs will answer the question concerning the appropriate times for drug administration.

From a more basic point of view, these results demonstrate that production of TCGF and acquisition of responsiveness to it, can occur independently, whereas previous experiments had lead to the restriction that TCGF might be produced only by cell populations that had undergone the "first step" in triggering (5). This independence may be due to the cellular basis of those two events. It has been shown that Lyt-1-positive T cells (T helper cells) are involved in the production of TCGF (24) and that the proliferation of cytotoxic T cells is strictly dependent on TCGF activity (2, 25, 26). If in these experiments TCGF was elaborated by T helper cells and the cells that acquire functional receptors for TCGF were T killer cells, it could be speculated that CyA exerts its effect on T killer cells, since the presentation of growth receptors was inhibited, and not on T helper cells, since TCGF was produced.

Along the same line of speculation, DM would not exert its effect on T killer cells, but instead directly or indirectly on T helper cells, to inhibit TCGF production. DM could, in that case, inhibit TCGF production, either by interacting with elaboration of the macrophage product, which is necessary for induction of TCGF by the T cells (20), or by directly inhibiting the T cells responsible for TCGF production. Preliminary results indicate that the former assumption is correct, in that the reduced Con A response of normal spleen cells in the presence of DM can be partially overcome upon addition of a soluble macrophage product from the WEHI-3 macrophage cell line. These studies are under further investigation.

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