Growth, Interleukin-2 Production, and Responsiveness to IL-2 in T4-Positive T Lymphocyte Populations From Malignant Cutaneous T Cell Lymphoma (Sézary’s Syndrome): The Effect of Cyclosporine A

By Werner Solbach, Claus-Ekkehard Lange, Martin Röllinghoff, and Hermann Wagner

The immunosuppressive activity of cyclosporine A (CsA) includes suppression of graft rejection in several types of allografts in various species or suppression of graft-versus-host disease after bone marrow transplantation in man. In vitro, CsA seems to exert its immunosuppressive effects primarily by acting on T lymphocytes, although some effect on certain subsets of B lymphocytes has been reported in the mouse.

It has been shown in this laboratory, as well as by others, that CsA can prevent the generation of cytotoxic T lymphocytes in primary mixed lymphocyte cultures (MLCs). It has been proposed that the observed suppression is due to CsA-induced blockade of production and/or release of soluble T lymphocyte factors, such as interleukin-2 (IL-2). IL-2 promotes the proliferation of T cells by binding to specific antigen/lectin-induced membrane receptors. In man, the majority of IL-2 producer cells has been confined to the T4-positive T helper lymphocyte subset, whereas IL-2 receptor-positive cells among other T cells include activated T8-positive cytotoxic T cell precursors.

Recent analysis of the surface phenotype of peripheral blood cells (PBMs) from patients with Sézary’s syndrome, a cutaneous T cell lymphoma, has revealed that the malignant T cells from these patients express surface antigens identical to those expressed by T helper cells in normal individuals, ie, OKT3+, T4+, T6-, T8+. Moreover, it has been shown that Sézary PBMs can be stimulated to produce IL-2.

The aim of the experiments reported here was to determine, first, whether or not the immunosuppressive effect of CsA as reported for normal T cells in vitro can be observed in peripheral blood cells from Sézary patients as well, and second, whether or not exogenous IL-2 can restore CsA-mediated suppression.

Materials and Methods

Medium

The medium used was RPMI 1640 (Seromed, Munich) supplemented with penicillin G (100 U/ml), streptomycin (100 μg/mL), L-glutamine (1mmol/L; Seromed), HEPES buffer (10 mmol/L; GIBCO, Glasgow, Scotland), sodium bicarbonate (0.15%; GIBCO), and fetal calf serum (FCS) (5% vol/vol; Seromed).

CsA

CsA was a kind gift of Dr J.F. Borel, Sandoz Ltd, Basel, Switzerland. One milligram was dissolved in 0.1 mL ethanol 96%, 0.02 mL Tween-80 (Serva, Heidelberg, FRG), and culture medium without FCS under continuous stirring to a total volume of 1 mL. This stock solution (1 μg/mL) was further diluted as indicated. Ethanol concentration in the culture medium did not exceed 0.2%. As a control, the solvents without CsA were added to the cultures.

Cell Preparations

Peripheral blood mononuclear cells (PBMs) were obtained from heparinized blood samples of five patients with the confirmed clinical and microscopical diagnosis of Sézary’s syndrome or from consenting healthy volunteers by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden).
sala, Sweden) density gradient centrifugation (specific density 1.078 g/mL). The cells were used either freshly drawn or after thawing from liquid nitrogen storage. The patients with Sézary's syndrome did not receive specific treatment at the time of blood collection. Cell viability was assessed microscopically by the eosin exclusion test.

**Immunofluorescence**

For determination of cell surface antigens, $1 \times 10^7$ PBMs were incubated in 100 mL of culture medium (5% FCS) for 45 minutes at 4°C in the presence of saturating amounts of the following mouse anti-human monoclonal antibodies: OKT3, T4, T6, T8, T11; T4, T6, T8, and T11 antibodies were a kind gift from Dr Ellis Reinherz, Sidney Farber Cancer Institute, Boston; OKT3 was the commercially available antibody (Ortho Pharmaceuticals, Raritan, NJ). After incubation, the cells were washed twice and incubated for 45 minutes with saturating amounts of affinity-purified, fluorescein-isothiocyanate (FITC) conjugated goat anti-mouse Ig F(ab)2 fragments (Tago, Burlingame, Calif). After washing twice, 200 cells were examined on a Zeiss fluorescence microscope for the presence of membrane fluorescence. Background fluorescence was below 5%.

For determination of surface membrane immunoglobulins (SIg), a one-step staining procedure was employed using FITC-labeled goat anti-human Ig F(ab)2 fragments (Tago).

**Cell Cultures**

**Cell proliferation.** For determination of proliferative responsiveness, $2.5 \times 10^7$/mL PBMs were cultured in round-bottom microtiter plates (Greiner, Nürtingen, FRG) in a total volume of 200 mL culture medium (5% FCS) for 48 to 84 hours. The following stimulators were used: (1) purified phytohemagglutinin (PHA) (0.5 μg/mL; Wellcome Research Laboratories, Beckenham, England); (2) phorbol myristate acetate (PMA) (10 ng/mL; Sigma Chemie, Munich); or (3) a combination of PHA and PMA. In prior experiments, the doses applied had been determined as being appropriate to give maximum responses. Four hours prior to termination, each culture was pulsed with $^3$H-thymidine (2.5 μCi/mL) for 4 hours. The incorporated radioactivity (cpm) of each sample was compared by probit analysis to that of the standard IL-2 preparation. The dilution of the IL-2 standard, which resulted in 50% of the maximum $^3$H-thymidine incorporation, was arbitrarily defined as having an IL-2 concentration of 100 U/mL. This was usually the case at a dilution between 1:64 and 1:128. Maximum radioactivity incorporation resulted in 30,000 to 40,000 cpm.

**Interleukin-2 (IL-2) production.** IL-2-containing supernatants were generated by incubating $2 \times 10^7$/mL pooled tonsillar lymphocytes, from consenting patients undergoing tonsillectomy, in the presence of PHA (0.5 μg/mL) and PMA (10 ng/mL) at 37°C, 5% CO₂ atmosphere, in 800-mL plastic flasks. After six hours, the cells were washed twice, thus removing as much of the free stimulating agents as possible, and further incubated for additional 20 hours in fresh, prewarmed culture medium (2% FCS). The supernatants were then harvested and subjected to the following purification procedures: ammonium sulfate precipitation (50% to 80%), dialysis (buffer: PBS plus 0.1% polyethylene glycol 6,000), and ion-exchange chromatography (DEAE-Sepharose). Active fractions after the chromatography step were pooled and used as semipurified IL-2.

**IL-2 assay.** IL-2 activity was determined by measuring proliferation of the cloned, IL-2-dependent murine cell line, CTLL (a kind gift of Dr Max Schreier, Basel Institute for Immunology, Switzerland). CTLL (2 x $10^6$) were incubated in the presence of serial twofold dilutions of the supernatant to be tested (including an IL-2 standard preparation, generated as described above) for IL-2 activity in a total volume of 200 μL. After 15 hours incubation time, the cultures were pulsed with $^3$H-thymidine (2.5 μCi/mL) for four hours. The incorporated radioactivity (cpm) of each sample was compared by probit analysis to that of the standard IL-2 preparation. The dilution of the IL-2 standard, which resulted in 50% of the maximum $^3$H-thymidine incorporation, was arbitrarily defined as having an IL-2 concentration of 100 U/mL. This was usually the case at a dilution between 1:64 and 1:128. Maximum radioactivity incorporation resulted in 30,000 to 40,000 cpm.

**Statistical Analysis**

The statistical significance of differences between groups was determined by the use of Student’s t test for paired and unpaired data, with $P < .05$ as the minimum level of significance.

**RESULTS**

**Surface Phenotype of Sézary PBMs**

The surface phenotype of circulating PBMs from five patients with Sézary’s syndrome is shown in Table 1. The majority of PBMs in all patients were T3⁺, T11⁺ T cells; the relative percentage of T3⁺, T11⁺ T cells varied from 65% in patient 5, to nearly 100% in patient 1. Using the anti-T4 antibody, a remarkable homogeneity was found: in all patients tested, the T4:T11 ratio exceeded the value of 0.8, whereas in normal individuals, the ratio is approximately 0.65 ($P < .05$). On the other hand, the percentage of T8⁺ T cells was low, ranging from 13% in patient 3 to levels below 1% in patient 1. All cells in each patient were negative for the thymus antigen, T6. The percentage of SIg-positive cells varied from normal levels (15%) to nearly 100% in patient 2 to 1% in patient 1. Thus, the results presented in Table 1 confirm previous work, reporting Sézary T cells as being phenotypically identical to a subset of normal T4⁺ helper cells.

**Table 1. Phenotype of Peripheral Blood Cells From Patients With Sézary’s Syndrome as Defined by Monoclonal Antibodies**

<table>
<thead>
<tr>
<th>Antibody Reactivity</th>
<th>Patient 1 (Male)</th>
<th>Patient 2 (Female)</th>
<th>Patient 3 (Female)</th>
<th>Patient 4 (Male)</th>
<th>Patient 5 (Male)</th>
<th>Normal*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-T3, mature T cells</td>
<td>98 (95-99)*</td>
<td>75 (71-79)</td>
<td>79 (74-81)</td>
<td>88 (86-95)</td>
<td>65 (63-67)</td>
<td>67 ± 4</td>
</tr>
<tr>
<td>Anti-T4, helper/inducer T cells</td>
<td>98 (93-99)</td>
<td>71 (68-75)</td>
<td>67 (63-71)</td>
<td>84 (81-90)</td>
<td>60 (58-62)</td>
<td>42 ± 5</td>
</tr>
<tr>
<td>Anti-T6, mostly thymocytes</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>Anti-T8, suppr./cytot. T cells</td>
<td>0 (0-3)</td>
<td>2 (0-5)</td>
<td>10 (8-13)</td>
<td>3 (1-7)</td>
<td>7 (5-9)</td>
<td>28 ± 5</td>
</tr>
<tr>
<td>Anti-T11, SRBC T cells</td>
<td>98 (93-99)</td>
<td>73 (70-80)</td>
<td>79 (75-85)</td>
<td>92 (88-96)</td>
<td>73 (70-76)</td>
<td>72 ± 5</td>
</tr>
<tr>
<td>Anti-Slg, B cells</td>
<td>1 (0-5)</td>
<td>13 (5-15)</td>
<td>12 (3-10)</td>
<td>7 (3-10)</td>
<td>9 (8-10)</td>
<td>12 ± 7</td>
</tr>
</tbody>
</table>

10⁶ PBMs from Sézary patients were labeled with the antibodies as indicated. The reactivity of the antibodies reacting with T cells was visualized with FITC-labeled goat anti-mouse Ig F(ab)2 fragments. B cells were detected using FITC-labeled goat anti-human Ig F(ab)2 fragments. Numbers indicate percent positive cells (range in parentheses) (200 cells analyzed).

*Mean of two to four determinations on different blood samples; range in parentheses.

†Based on data obtained from 57 apparently healthy individuals of both sexes, aged 19 to 45 years (mean ± SD).
**Proliferative Responses of Sézary PBMs**

Figure 1 shows the representative result of one of four experiments analyzing the proliferative response of PBMs from Sézary patients. A quantity of 5 x 10^5 PBMs per well from the patients was stimulated with PHA and/or PMA in the presence or absence of 1 μg/mL CsA (Fig 1). [H]-thymidine incorporation was measured after 72 hours in culture.

As can be seen (Fig 1), both PHA or PMA alone in all patients promoted a low, but significant, T cell response (P < .005) (between 2,500 and 10,600 cpm). In contrast, at least five times more proliferative response was induced by PHA in normal PBM populations.

In all but one patient, the combination of PHA and PMA induced a synergistic 2.5 to tenfold increment in T cell proliferation when compared to either stimulus alone.

The introduction of CsA inhibited cell proliferation to varying degrees, depending on the mitogenic stimulus used (black columns, Fig 1A). Thus, PHA-mediated T cell proliferation was significantly more inhibited by CsA (between 63.2% and 80.0%) than was the cell growth initiated by PMA alone or by the combination of PHA and PMA (between 39.1% and 53.2% inhibition) (P < .05). Interestingly, PHA-stimulated normal cells were significantly more sensitive to CsA inhibition than were Sézary PBMs (95.4% inhibition in the control v 80% inhibition in patient 3) (P < .05).

**Effect of CsA on IL-2 Production**

Aliquots of the same cell populations that were used for the analysis of PHA/PMA-induced cell proliferation (Fig 1A) were analyzed for their capacity to release IL-2 in the supernatant. Thus, 2 x 10^6/mL PBMs were incubated with PHA and/or PMA in the presence or absence of CsA for 24 hours. The culture supernatants were processed for purification and tested for IL-2 activity as described in Materials and Methods. The data in Fig 1B show that neither the patients nor the normal control produced IL-2 spontaneously (far right column). As expected, PHA stimulation of normal PBMs yielded high IL-2 production (92 U/mL). Sézary PBMs from all patients, however, produced only low amounts of IL-2 (between 6 and 35 U/mL). A different picture emerged with PMA stimulation. Here, normal PBMs were almost nonresponsive. In contrast, PBMs from all Sézary patients produced PMA-induced IL-2, which in all but one patient (No. 3), was significantly higher than the PHA-induced IL-2 secretion (P < .05). The combined action of PHA and PMA resulted in IL-2 activity between 91 U/mL and 119 U/mL; there was no difference between normal and Sézary PBMs.

CsA significantly suppressed IL-2 production in all patients. Apparently, PMA- or PHA-induced IL-2 production was more sensitive to CsA suppression than the IL-2 production induced by PMA plus PHA. The mean suppression was 74.1% in the PHA cultures, 76.3% in PMA cultures, and 54.5% in the PMA plus PHA cultures (P < .05).
The magnitude of PMA/PHA-induced IL-2 production was inhibited by CsA in a clear dose–response relationship (Fig 2). The slopes of the curves obtained from all patients essentially are equal and do not differ from the normal control. The maximum inhibition was achieved in all but one patient (No. 4) with a CsA concentration of 1,000 ng/mL.

**Effect of Exogenous IL-2 on CsA-Mediated Growth Inhibition**

From the previous experiments it can be concluded that the observed CsA-mediated suppression of cellular proliferation (Fig 1A) may be due to inappropriate IL-2 availability (Fig 1B) to those cells that require IL-2 for growth. Alternatively, it may be that the cells do not express the receptor for IL-2, thus being insensitive to its action. The following experiments addressed this latter question. PBMs from the Sézary patients were stimulated with PMA plus PHA in the presence of 1 μg/mL CsA. From the beginning of culture, increasing amounts of exogenous IL-2 were added to each culture, and proliferation was determined after 72 hours. Figure 3 shows a representative result from four independent experiments with PBMs from patients 1, 2, and 4.

In all patients tested, the proliferative response was clearly determined by the amount of added IL-2. The Sézary PBMs in all experiments incorporated two to fourfold more ³H-thymidine at 200 U/mL IL-2 than did normal PBMs.

Taken together, these experiments suggest that the main action of CsA on Sézary PBMs is the suppression of endogenous IL-2 production, thus inhibiting the growth of those cells requiring IL-2 for proliferation. Once appropriate amounts of IL-2 are available, activated Sézary PBMs start to proliferate. This indicates that Sézary PBMs do have functioning IL-2 receptors, the expression of which can be induced by PMA plus PHA. (Either compound alone is sufficient for induction of IL-2 receptor expression [data not given]). Nonstimulated Sézary PBMs are IL-2 receptor negative, since IL-2 alone (without PMA/PHA) does not enhance cell proliferation (Fig 3).

**DISCUSSION**

The most prominent feature of the lymphocytic cell population in the peripheral blood of patients with Sézary’s syndrome (Sézary PBMs) is the predominance of the T4⁺ subset with a concomitant low relative number of T8⁺ cells¹³ (Table 1). This finding, together with functional studies,¹⁴,¹⁵ suggests that the malignant cells in Sézary’s syndrome are similar or identical to normal T4⁺ lymphocytes, the members of which predominantly serve helper functions for the generation of effective T or B cell-mediated immune responses.¹²,¹⁶ Therefore, we studied PBMs from five Sézary patients for their capacity to produce IL-2, which can be generated by normal T4⁺ T cells,¹⁰ and to determine the effect of the T cell immunosuppressant CsA. Essentially, the PBMs from all five patients tested showed similar patterns of reactivity. When placed in vitro cultures, Sézary PBMs neither produced detectable amounts of IL-2 nor proliferated spontaneously (Fig 1A and B). Upon PHA stimulation, they produced significantly lower amounts of IL-2 than did normal PBMs (P < .05). In contrast, stimulation with the phorbol ester PMA resulted in low, but significantly higher, IL-2 yields than in normal controls (P < .05). Simultaneous stimulation with...
PMA and PHA lead to an IL-2 production of the same magnitude (90 to 120 U/mL) in both Sézary PBMs and normal PBMs.

The comparison of IL-2 production and cell proliferation showed a high correlation between both parameters in corresponding groups in the absence and presence of CsA (Fig 1). Thus, Sézary PBMs behave like normal cells, as the amount of PHA- or PMA-mediated cell proliferation is a function of the amount of IL-2 available.

In contrast to normal PBMs, Sézary PBMs only poorly produced IL-2 and proliferated in response to PHA. Possibly, Sézary PBM populations do not contain sufficient accessory cells (in number or function) for lectin-induced T cell growth. The recent finding that PMA can replace, in part, the accessory cell-dependent functions required for T-cell activation is supported by our finding that simultaneous stimulation of Sézary PBMs with PMA and PHA resulted in substantial amounts of IL-2 production and cell proliferation. PMA, however, certainly cannot fulfill complete accessory cell replacement, as Sézary PBM populations, which contain much more T4 lymphocytes than their normal counterparts (Table 1), were expected to produce more IL-2 than normal cell populations. This, however, could not be achieved. Mixing accessory cells with purified Sézary T lymphocyte populations will answer the question to what extent PMA can replace accessory cells.

In the presence of CsA, IL-2 production and cellular proliferation by PHA- and/or PMA-activated Sézary PBMs was inhibited in a dose-dependent manner in all patients tested and in normal PBMs (Fig 1, A and B, closed columns; Fig 2). PHA plus PMA-stimulated cells were less sensitive to CsA inhibition of proliferation and IL-2 production than those stimulated with PHA alone. This may reflect a PMA-mediated pathway of T cell activation that is different from the PHA-initiated activation and not susceptible to CsA suppression.

CsA inhibits the cellular parameters, as examined here, substantially, but not completely, even at high doses (up to 10 µg/mL; data not shown). Possibly, CsA suppresses IL-2-producing cells to a certain extent, leaving some residual IL-2 activity that is not inhibitable. Alternatively, the T4+ T lymphocyte population consists of two or more functionally heterogeneous subsets, some of which are CsA sensitive, while others are resistant. A similar finding has recently been reported from the T8+ T lymphocyte population, suggesting that the induction of cytotoxic T lymphocytes is CsA-sensitive, while the generation of suppressor cells is unaffected by CsA.

It cannot be determined from the experiments reported here whether or not the malignant T4+ Sézary T cells can be discriminated from the normal T4+ T lymphocytes (which are likely to represent a minor population within the patients' PBMs) on the basis of their susceptibility to CsA. Clarifying experiments using cloned malignant Sézary T4+ cells will help to resolve this and other questions. Despite serious efforts from different workers, all of the properties of Sézary T cells published so far were inferred from cell population studies. To our knowledge, none of the observed effects could be attributed to the malignant cell per se unequivocally.

To determine whether or not CsA inhibited the action of IL-2 on responsive cells, we added increasing amounts of exogenous IL-2 to PMA plus PHA-stimulated Sézary PBMs in the presence of inhibitory doses of CsA. The results (as shown in Fig 3) suggest that CsA does not interfere with cell growth, provided sufficient IL-2 is available. In contrast to IL-2 production by Sézary PBMs, which never exceeded that of normal PBMs, IL-2-driven proliferative responses of the patients’ cell populations was two to fourfold higher than the normal controls, thus reflecting either a higher content of IL-2 receptor-positive T cells in the patients' PBM populations or a higher IL-2 receptor density per cell, leading to more rapid proliferation. The cells did not respond to IL-2 in the absence of PHA/PMA, thus supporting recent reports in which no IL-2 receptors on resting Sézary T cells could be identified. In contrast to these and our findings, other reports state that Sézary T cells express IL-2 receptors immediately after isolation from peripheral blood. The cells analyzed in these reports, however, are most likely different from Sézary cells, as they express retroviral antigens, and some of the patients, who are from Japan, the Caribbean, the United States, or Israel, have antiviral serum antibodies. To our knowledge, this has not been described in any Sézary patient of mid-European origin.

In conclusion, PBMs from the Sézary patients studied resemble normal PBMs with respect to their capacity to produce IL-2 and to proliferate in response to PHA plus PMA. Both properties are susceptible to CsA-mediated inhibition substantially, but not completely. This may be of potential benefit for the treatment of patients with Sézary T leukemia.

CsA suppression of cell growth can be overcome in both populations by appropriate amounts of IL-2. Sézary PBMs differ from normal PBMs as only the former produce IL-2 and proliferate in response to PHA, while only the latter do so in response to PHA. Cell culture cloning techniques, which clearly distinguish malignant Sézary T4+ T lymphocytes from "normal" T4+ T lymphocytes, will help to further determine the intrinsic abnormalities in this T lymphocyte malignancy.
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Growth, interleukin-2 production, and responsiveness to IL-2 in T4-positive T lymphocyte populations from malignant cutaneous T cell lymphoma (Sezary's syndrome): the effect of cyclosporin A

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