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THE EFFECT OF CYCLOSPORIN A ON THE GROWTH AND PROLACTIN BINDING TO Nb-2 RAT LYMPHOMA CELLS

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Cyclosporin A, an immunosuppressive agent, inhibited the prolactin stimulated growth of rat lymphoma Nb-2 cells. In the presence of 1 ng/ml of prolactin, 50% inhibition of growth was at 5 x 10^{-6} M and the inhibition was reversible. The K_d of cyclosporin A binding to the Nb-2 cells was 10^{-7} M and was independent of prolactin. The K_d of prolactin binding to the Nb-2 cells was 2 x 10^{-10} M. Cyclosporin A did not influence the binding of prolactin to the cells and vice-versa. The inhibitory effect of cyclosporin A on the growth of Nb-2 cells is due to some step other than the binding of prolactin to the cells.

Cyclosporin A is a potent immunosuppressive agent preferentially active against proliferating T cells (1). It is postulated that immunosuppression is the result of reversibly blocking early mitogenic events in T cell activation (2) though the exact mechanism of this action is not clearly understood. Some evidence suggests that cyclosporin acts on activated T lymphocytes by blocking the transcription of the interleukin-2 gene (3-4) and other studies suggest that cyclosporin A might act by inhibiting calmodulin dependent cellular events (5).

Russell and coworkers (6,7) have demonstrated prolactin receptors on human peripheral blood lymphocytes and shown that cyclosporin inhibits the prolactin stimulated induction of ornithine decarboxylase in a variety of rat tissues, including the thymus and the spleen (8). These studies suggested that cyclosporin might be regulating the prolactin stimulated events by competing with prolactin for binding to its receptors (6,7). Similar observations in rat lymphocytes (9) suggested that the immunosuppressive effects of cyclosporin may be mediated by the displacement of prolactin from its binding site on rat lymphocytes.

In contrast, cyclosporin A did not affect the binding of ovine prolactin to isolated rabbit mammary gland membranes which have well characterized prolactin receptors (10). The Nb-2 rat lymphoma cell line requires lactogenic hormones for

Abbreviations: CsA, cyclosporin A; oPRL, ovine prolactin

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growth (11-13) and has well characterized prolactin receptors (14). This cell line then provides an excellent system for studying prolactin binding and biochemical events resulting from the interaction of prolactin with its receptor (15-19).

In this study, the effect of cyclosporin A on the prolactin induced mitogenic stimulation of the Nb-2 cells was examined as well as the effect of cyclosporin A on the binding of prolactin to the Nb-2 cells. The results showed that cyclosporin A inhibited reversibly the prolactin growth stimulation of the Nb-2 cells and that the binding of prolactin and cyclosporin A was independent of each other.

MATERIALS AND METHODS

Measurement of Cell Growth

The Nb-2 rat lymphoma cells were a gift from Dr. C. T. Beer (Vancouver, Canada). CsA was a gift from Sandoz Pharmaceutical Ltd. and [^{3}H]-CsA (0.05 μCi/μg) was purchased from the same manufacturer. Ovine prolactin was provided by the National Institute of Arthritis, Metabolism and Digestive Diseases (Bethesda, MD). [^{125}I] Ovine PRL was prepared as previously described (20). Fetal calf serum and horse serum were purchased from Dutchland Laboratories, (Denver, PA).

Nb-2 lymphoma cells were maintained as suspension culture in Fisher's medium (K.C. Biologicals, Lenexa, KS) supplemented with 10% fetal calf serum (FS), 10% horse serum (HS), 10^{-4} M 2-mercaptoethanol, 50 u/ml penicillin and 50 μg/ml streptomycin (21). Incubation was at 37°C in an atmosphere of 5% CO_2-95% air. Under these conditions, the cell numbers doubled every 20 hours reaching a maximum at 72 hours. Nb-2 cells were made quiescent by incubation at 37°C for 24 hours in Fisher's medium containing 10% horse serum and 1% fetal calf serum (slow down media). The cells were then transferred at a concentration of 10^5 cells/ml into Fisher's medium containing 10% horse serum (stationary media). In stationary media the cell population did not increase substantially, but following the addition of 1 ng/ml of ovine PRL, it doubled every 20 hours.

In the experiments measuring the inhibitory effect of CsA on the oPRL stimulated proliferation of the Nb-2 cells, CsA was added at the onset of culture along with oPRL and incubation was continued for 72 hours unless otherwise indicated. At the end of this time period, 0.4 ml of the cell suspension were removed and diluted to 10 ml with Hematol (Fisher Scientific Company). The number of cells was determined in the Sysmex cell counter.

Measurement of Binding [^{125}I] oPRL and [^{3}H] CsA to Nb-2 Lymphoma Cells

The effect of CsA on the binding of [^{125}I] oPRL to the Nb-2 cells (and vice versa) was examined using similar protocols. Actively growing cells (in maintenance media) were slowed down for 24 hours. At the end of the 24 hour incubation period, the cells were removed by centrifugation and resuspended in stationary media at 2 x 10^6 cells/ml. Assays were carried out in duplicate in 1.5 ml Eppendorf tubes. For each data point, two tubes were used. Both tubes contained the same amount of radiolabeled ligand (100,000 cpm of [^{125}I] oPRL or 80,000 cpm of [^{3}H] CsA) but only one tube received the cold ligand. For each determination, 2 x 10^6 cells/ml were used in a total volume of 1.1 ml. Incubations with labeled ligand were at 37°C for 1 hour. At the end of this time period, the cells were pelleted and washed three times with phosphate buffered saline solution. The radioactivity in the cell pellet was measured using a gamma counter for [^{125}I] oPRL binding , and the liquid scintillation counter for [^{3}H] CsA binding.

Specific binding was the difference between the binding of the labeled ligand (total) and that in the presence of cold ligand (non specific binding). Under these conditions, the nonspecific binding was 12-20% of the total binding for [^{125}I] oPRL in presence of 10 μg/ml cold PRL where as it was 45% for [^{3}H] CsA in the presence of 10^{-5} M CsA.

RESULTS

Prolactin is a potent mitogen for the Nb-2 cells (11-13) with maximum growth occurring at 1 ng/ml of prolactin. When cyclosporin A was added at the onset of culture along with 1 ng/ml oPRL, a marked decrease in the prolactin stimulated proliferation of the cells occurred over the total growth period. The inhibition of growth by CsA was dose-dependent Fig. 1, and the concentration of CsA required for 50% inhibition of cell growth (the IC_{50}) was 5 x 10^{-6} M while the EC_{50} or the concentration of prolactin required for a 50% increase in cell growth was 0.05 ng/ml (2.3 x 10^{-12} M). CsA at 10^{-5} M completely inhibited growth.

Cyclosporin A by itself had no effect on cell numbers since they were the same as those obtained in control samples maintained in stationary media. The viability of the cells, incubated with cyclosporin A, as determined by trypan blue staining experiments showed that CsA was non-toxic to the cells.

The inhibition of cell growth by cyclosporin A was completely reversible under the conditions used (data not shown). Cells which were held in the presence of 10^{-5} M CsA for 3 days did not grow but after washing with media to remove the CsA and then placed in media containing 1 ng/ml oPRL exhibited a normal growth curve.

Binding of [^{125}I] oPRL to the cells was saturable and specific. About 80-88% of the total binding was specific while only 12-20% of [^{125}I] oPRL bound non-specifically. Displacement of [^{125}I] oPRL from its receptor by varying concentrations of ovine prolactin (1 ng/ml to 10 μg/ml) showed that the apparent Kd (defined as the concentration of the unlabeled hormone required to displace 50% of the specifically bound radiolabeled prolactin) was 2 x 10^{-10} M [Fig. 2]. This was in good agreement with the Kd (1.4 x 10^{-10}) obtained by Scatchard analysis

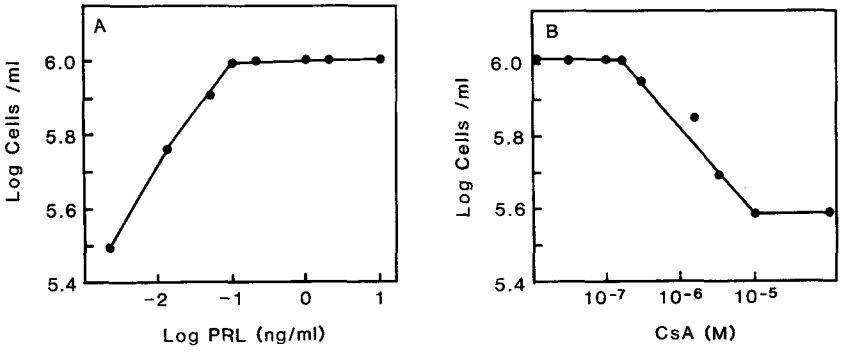
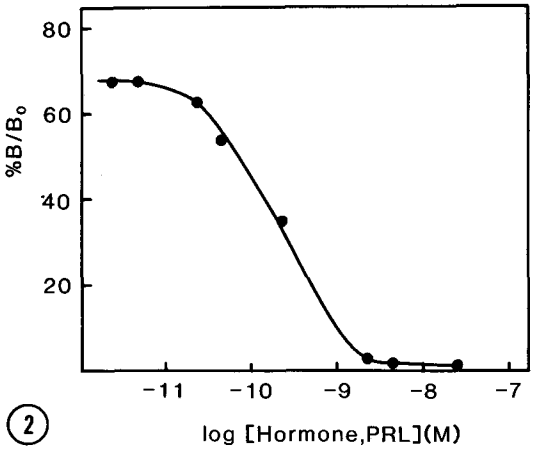


Fig. 1 Dose dependent inhibition of oPRL stimulated proliferation of Nb-2 cells by CsA

(A) Standard curve for the growth of Nb-2 cells at different concentrations of oPRL (from 0.01 ng/ml to 1 ng/ml). The EC_{50} for 50% stimulation of cell growth is 0.05 ng/ml oPRL.

(B) Inhibition of Nb-2 cells stimulated by 1 ng/ml oPRL by 5 x 10^{-6} M.



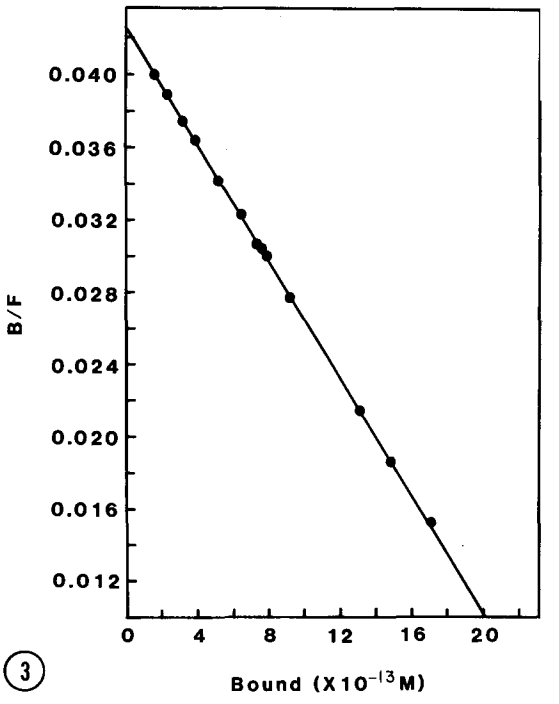


Fig. 2 Displacement curve for the binding of ^{125}I-oPRL to Nb-2 cells. Binding of [^{125}I]-oPRL to Nb-2 cells was carried out at varying concentrations of oPRL (from 0.05 ng/ml to 10 μg/ml). B represents specific binding of [^{125}I]-oPRL in the presence of 10 μg/ml of oPRL. B_0 is the maximum binding of [^{125}I]-oPRL. The data represent an average of two experiments.

Fig. 3 Scatchard analysis of [^{125}I]-oPRL binding to the Nb-2 cells. Data represents an average of two experiments

[Fig. 3]. Cyclosporin A over the concentration range 10^{-10} M to 10^{-5} M did not have any effect on the binding of [^{125}I] oPRL to the prolactin receptors on the Nb-2 cells [Fig. 4B]. Tritiated cyclosporin, however, bound to the cells with a Kd of 10^{-7} M. Competition with cold prolactin (up to 10 μg/ml had no effect on the binding of [^{3}H] CsA [Fig. 4A]. These results show that CsA and PRL do not compete for a common binding site on the Nb-2 cells.

Changes in the number and/or affinity of receptors can modulate the responsiveness of the cell to extracellular mitogenic signals. Also, the binding of structurally unrelated ligands to the cell can control the cellular affinity for the hormone. The possibility that cyclosporin A, by binding at a distinct site of its own on the Nb-2 cells, might somehow destabilize the binding of [^{125}I] oPRL to its receptor on the Nb-2 cells was explored. This was done by examining the Kd of [^{125}I] oPRL binding to the cells in the presence and absence of CsA by Scatchard analysis. No change was noted in the Kd of oPRL binding which was 1.4 to 2 x 10^{-10} in the absence of cyclosporin A and 2.7 x 10^{-10} in the presence of CsA at 10^{-5} M.

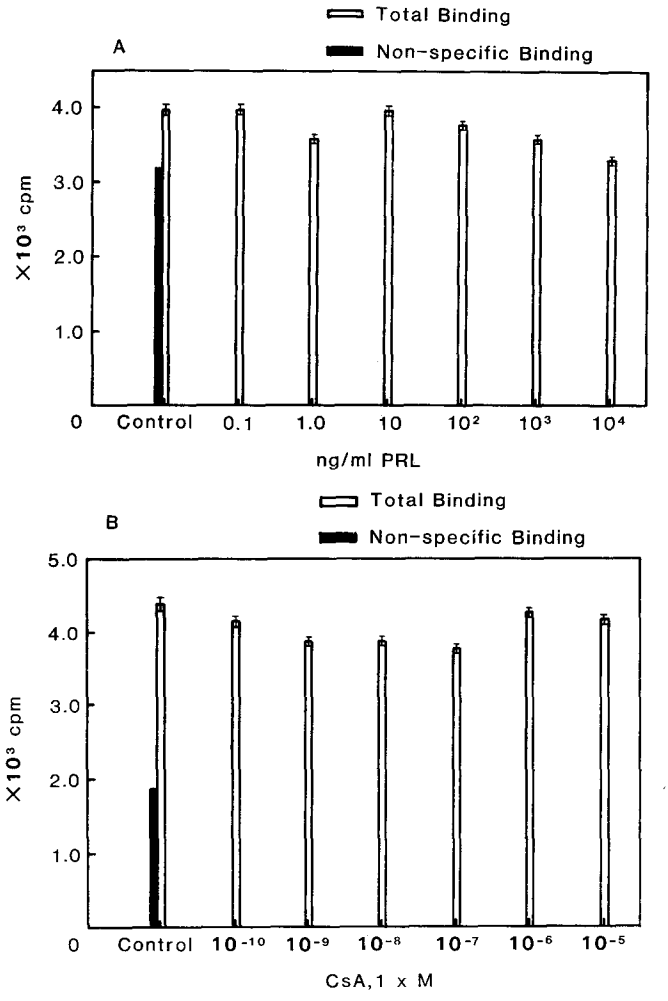


Fig. 4 Effect of ovine prolactin on the binding of [^{3}H]CsA (Fig. 5A) and of cyclosporin A on the binding of [^{125}I]-oPRL [Fig. 5B] to the Nb-2 rat lymphoma cells. Vertical bars represent an average of two experiments.

DISCUSSION

Previous studies (6,7) relating to the inhibitory action of cyclosporin A on human lymphocytes have implicated the prolactin receptor by competing for prolactin receptors. Such studies were difficult since these cells have low numbers of prolactin receptors which is in contrast to the Nb-2 rat lymphoma cells which have well characterized prolactin receptors and require prolactin for growth. Accordingly, the effects of cyclosporin A on the prolactin dependent growth and binding of prolactin to the Nb-2 rat lymphoma cells was examined in this study.

The results showed that cyclosporin A reversibly inhibited the prolactin stimulated growth of the Nb-2 cells with 50% growth inhibition at 5 x 10^{-6} M CsA. The K_d of binding of tritiated cyclosporin A to the Nb-2 cells was 1 x 10^{-7} M which is comparable to that found in other systems (22,23). In contrast, the K_d of

prolactin binding to the cells was 1.4 x 10^{-10} M and the concentration of prolactin to effect a 50% increase in growth was 2.3 x 10^{-12} M. These results are similar to binding constants observed for the binding of CsA (K_d = 2.2 x 10^{-6} M) and prolactin (K_d = 2 x 10^{-10} M) to isolated rabbit mammary gland membranes (10). Neither prolactin nor cyclosporin A competed with each other for binding suggesting that the sites were distinct. It is possible the CsA could modulate the binding of prolactin to the cells which occurred with the EGF receptor where PDGF which bound at a separate and distinct site deceased the affinity of EGF for its receptor (24-27). However, the presence of CsA did not change the K_d of prolactin binding to the Nb-2 cells indicating no evidence for transmodulation.

The results of this study show that in the Nb-2 rat lymphoma cell, cyclosporin A does not exert its action by interfering with the binding of prolactin to its receptor but acts at some point distal in the intracellular signal transduction pathway.

This concept is supported by the recent work of Bijsterbosch and coworkers (28) who have shown that in thymocytes or B cells, CsA does not interfere with the second messenger production initiated by PIP_2 breakdown, which is a receptor mediated event responsible for cell growth in many systems (29) including the Nb-2 cells (30). Cyclosporin A may inhibit an early step in lymphocyte activation by mitogens that induce PIP_2 degradation and Ca^{+2} mobilization (31-34).

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