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Immunology

Prolactin as a modulator of lymphocyte responsiveness provides a possible mechanism of action for cyclosporine

(immunosuppression)

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ABSTRACT **Lymphocyte responsiveness in rats was found to depend on serum prolactin levels. Blocking pituitary prolactin release with bromocriptine severely reduces lymphocyte reactivity *in vitro* (mixed lymphocyte reaction) as well as *in vivo* (graft-versus-host reaction). In addition, evidence for a prolactin/growth hormone-related mRNA species produced in mitogen- and antigen-stimulated lymphocytes has been obtained. Prolactin was shown to compete in a dose-dependent fashion with the immunosuppressant cyclosporine (cyclosporin A) for a common binding site on the surface of T lymphocytes. Further, stimulation of prolactin secretion reversed the immunosuppression induced by cyclosporine. We conclude that prolactin is involved in the maintenance of T-cell immunocompetence and that the immunosuppressive effects of cyclosporine may be mediated by the displacement of prolactin from binding sites on lymphocytes.**

Prolactin is a polypeptide hormone of pituitary origin with close structural relationships to growth hormone (GH). It produces pronounced physiological effects on growth, reproduction, and osmoregulation in a wide range of species (1, 2); it causes a marked proliferative effect on the crop sac of pigeons (3) and in mammals is involved in the development of various glandular tissues (1, 2). A variety of mammalian cells have receptors for prolactin, including those of the mammary gland, liver, kidney, brain, prostate, testis, and ovary (4). Although prolactin is a pituitary hormone, proteins that react with anti-prolactin antisera appear to be synthesized in nonpituitary tissues as well (5, 6).

Hypophysectomized rats fail to mount an immune response (7, 8). Treatment of those animals with prolactin or GH, but not with corticotropin or other pituitary hormones, restored their immunological status (9). Moreover, specific binding sites for prolactin have been demonstrated on human lymphocytes. The immunosuppressive peptide cyclosporine (cyclosporin A) displaces prolactin from these sites (10–13), whereas a biologically inactive derivative, cyclosporin H, does not (12).

The purpose of this report is to present further data demonstrating the involvement of prolactin in modulating the responsiveness of T lymphocytes to antigenic stimulation and to describe the interactions between prolactin and cyclosporine in this process.

MATERIALS AND METHODS

Mixed Lymphocyte Reaction. Equal numbers (2.5×10^5) of murine stimulator lymphocytes (female CBA mice, Iffa Credo, Lyon, France; lymphocytes irradiated *in vitro* with 2000 rads; 1 rad = 0.01 J/kg) and effector lymphocytes (female BALB/c mice, Iffa Credo) from control animals or from animals treated for 7 days with bromocriptine (Parlodel; 5 mg/kg of body weight, given s.c. daily), an inhibitor of prolactin secretion, were incubated together in a final volume of 0.2 ml for 5 days. The incubation medium consisted of RPMI 1640 medium supplemented with antibiotics, 2 mM L-glutamine, and 10% fetal bovine serum. 2-Mercaptoethanol was omitted in all studies. Immunocompetence of the effector cells, as shown by cell proliferation in response to the allogenic stimulus, was measured by incorporation of [*methyl*-^{3}H]thymidine (The Radiochemical Centre, Amersham Ltd., England; 5 Ci/mmol, 1 μCi per culture for 16 hr; 1 Ci = 37 GBq) into cellular DNA (14).

Localized Graft-Versus-Host Reaction. Spleen cells from Wistar Furth rats (female, 6 weeks old) were injected into one hind footpad of female (Wistar Furth × Fischer 344)F_1 hybrid rats (age 6 weeks). In this assay, recipient animals do not recognize the injected cells as foreign, whereas the injected cells mount a localized immune response towards the recipient animal, causing a swelling of the popliteal lymph node (15). Recipient animals were treated with bromocriptine (5 mg/kg per day, s.c.) for 7 days before and 4 days after the injection of the stimulator cells. The difference in weight of the left and right popliteal lymph node in untreated rats was taken as a reference for calculating inhibition occurring in animals receiving bromocriptine or cyclosporine treatment. In another experiment, bromocriptine treatment was initiated after spleen-cell injection and continued for 4 days. Studies were also done in rats receiving the immunosuppressant cyclosporine and/or a prolactin-releasing agent (compound 25-240, 1α,10β-dimethyl-9,10-dihydroergocristine; Sandoz Ltd., Basel, Switzerland).

Plaque-Forming-Cell Assay. Mice (OF1, female, Iffa Credo) were sensitized by i.v. injection of 10^8 sheep erythrocytes (SRBC). Treatment of the mice with potential immune-response-modulating agents started 3 hr after sensitization and was continued for the next 3 days. Two days after the last treatment, the spleens were removed and splenic lymphocytes were plated on soft agar in the presence of fresh antigen (SRBC) and complement. Sensitized lymphocytes release specific antibodies against SRBC, which in combination with complement causes local lysis of the surrounding SRBCs—i.e., gives rise to "plaque"-formation. Each plaque represents a single antibody-secreting B lymphocyte, which forms the basis of this assay, as previously described (16).

Binding and Displacement Studies Using Cytofluorometry. Human peripheral T cells were obtained from freshly collected blood and were enriched by erythrocyte-rosetting and by gradient centrifugation. Cells were labeled with dansylated [Lys^8]cyclosporine either in the presence or in the absence of human prolactin (for competitive binding or displacement studies, respectively). Dansylated cyclosporine and cytofluorometry were used in place of the usual labeling by [^{3}H]cyclosporine to confirm the reported findings

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Abbreviations: GH, growth hormone; SRBC, sheep erythrocytes.

(12) by an alternative procedure and to assess the number of labeled cells. Binding was achieved by incubating 2.5×10^6 cells with 0.3 μg of the dansylated cyclosporine in 1 ml (final volume) of RPMI 1640 medium with 2.5% fetal bovine serum for 60 min at 37°C, followed by a wash at 4°C. Displacement studies were done at 37°C for 60 min in the presence of the competing substance. Fluorescence was determined by flow cytofluorometry, counting 50,000 cells per sample by means of a Cytofluorograph 50HH (Ortho Diagnostic Systems, Westwood, MA) as published (17).

Dot Blots of Lymphocyte Cytoplasmic RNA. Cytoplasmic extracts containing RNA were prepared and bound to nitrocellulose sheets (GeneScreen, New England Nuclear, Zurich, Switzerland) according to published procedures (18). Hybridization of suitable DNA probes to the immobilized RNAs was done as described (18), using a rat prolactin-specific probe [plasmid pPrl-1 (19); gift of R. Maurer, University of Iowa] or a rat GH-specific probe [plasmid prGH-1 (20); gift of J. Baxter, University of California, San Francisco)]. Probes were uniformly labeled with a deoxynucleoside [α-^{32}P]triphosphate by nick-translation. A labeled plasmid preparation without specific inserts was used as a control to detect nonspecific hybridization signals. Rat pituitary cytoplasmic poly(A)$^+$ RNA was used to standardize the hybridization procedure. Hybridization with the denatured DNA probe was done for 24 hr at 42°C in 50% (vol/vol) formamide, as described by the manufacturers of the nitrocellulose sheets. Quantitation of the hybrids formed was by laser densitometry of the corresponding autoradiographs or by direct liquid scintillation counting of the hybrid dots.

Statistics. The *in vivo* data were analyzed by using either the unpaired *t*-test or Dunnett's multirange test. Before application of the latter test, the data were analyzed for normality (Wilk–Shapiro) and subjected to a two-way analysis of variance.

RESULTS

Mixed Lymphocyte Reaction Using Lymphocytes Obtained from Prolactin-Depleted Mice and Prolactin-Supplemented Lymphocytes. It is clear from Fig. 1 that pretreatment of BALB/c mice with the prolactin-secretion inhibitor bromocriptine markedly reduced lymphocyte responsiveness as assessed in this *ex vivo* assay; 40% residual lymphocyte reactivity was found under conditions where bromocriptine treatment lowered the concentration circulating prolactin below the detection level of radioimmunoassay (<1 ng/ml). Addition of exogenous rat prolactin (Table 1) yielded a slight stimulation of lymphocyte activity, whereas addition of porcine or human prolactin resulted in a strong inhibition of the mixed lymphocyte reaction. This inhibition was perhaps due to a competitive antagonism of the added prolactin preparation with the endogenous murine prolactin bound to lymphocyte surface receptors. Canine prolactin caused inhibition at unphysiologically high concentrations only. Ovine prolactin was without effect. Because of the unavailability of purified mouse prolactin, it was not possible to perform the homologous experiment. In addition, we cannot rule out the possibility that some sort of contamination in the rat prolactin preparation, or for that matter in any of the prolactin preparations used, caused these *in vitro* effects. However, the *ex vivo* experiment suggests that serum prolactin may be one of the factors determining immunological responsiveness of lymphocytes.

Graft-Versus-Host Reaction. Pretreatment of recipient animals with bromocriptine to lower the concentration of circulating prolactin before the injection of the donor lymphocytes resulted in a dose-dependent decrease in lymph-node weights (relative to control animals; see Table 2). It is also clear that administration of bromocriptine after injection

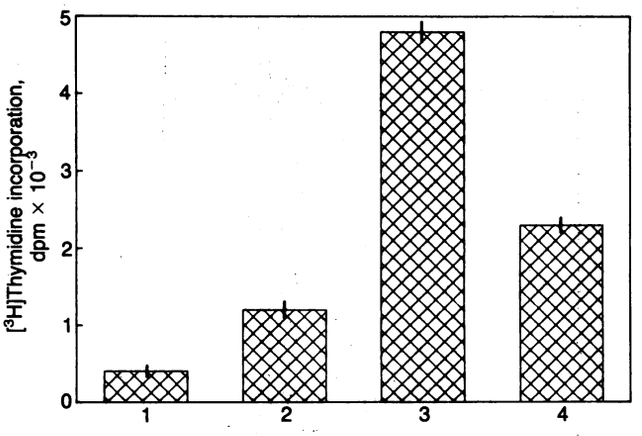

FIG. 1. Mixed lymphocyte reaction with lymphocytes from prolactin-depleted mice. Equal numbers of effector lymphocytes [from BALB/c mice, either untreated (controls) or treated with bromocriptine (5 mg/kg per day s.c. for 7 days)] and stimulator lymphocytes (from CBA mice; lymphocytes irradiated *in vitro* with 2000 rads) were coincubated in triplicate for 5 days. Lymphocyte response to allogenic stimulation was measured by following the incorporation of [^{3}H]thymidine. Bars 1 and 2: background incorporation in the absence of stimulator cells, with (bar 2) or without (bar 1) bromocriptine pretreatment of the BALB/c mice. Bar 3: response obtained with lymphocytes from untreated BALB/c mice. Bar 4: decreased response found after treating the effector-lymphocyte donor with bromocriptine. Values are given as means ± SD.

of the donor lymphocytes had no effect in this assay. In this context, we note that bromocriptine suppressed the graft-vs.-host response by not more than 75%, a fact that will be discussed below. Immunosuppression of female rats with cyclosporine at 36 mg/kg *per os* (initiated after donor-cell injection) resulted in a 45–60% reduction of lymph-gland weight, compared to matched controls. In contrast, male rats required only about 19 mg/kg (*per os*) for a similar suppression. Concomitant treatment of recipient animals with the immunosuppressant cyclosporine and with the prolactin-releasing agent 25-240 completely restored the reaction of these animals (Table 2).

Cyclosporine–Prolactin Interactions in T-cell-Mediated Humoral Immune Response. Treatment of mice with bromocriptine during or after sensitization with the T-cell antigen SRBC did not give rise to a lower lymphocyte responsiveness than corresponding controls (results not shown), an indication that circulating prolactin is not a limiting factor for T-cell function in a humoral immune response. However, when animals were treated with cyclosporine at an established immunosuppressive dosage in this system (60 mg/kg orally), it was found that T-cell function (as indicated by the number of plaque-forming cells) was reduced to 35% of control values (Table 3). As in the graft-vs.-host reaction, increase of the circulating prolactin levels by compound 25-240 overcame the inhibitory action of cyclosporine (Table 3).

Cyclosporine and Prolactin Compete for the Same Binding Site on Lymphocytes. [Dansyl-Lys^8]Cyclosporine binds to human peripheral T lymphocytes (Fig. 2). This binding seems to be specific, since only the biologically active molecule (cyclosporin A) but not the biologically inactive analogue (cyclosporin H) was able to compete with the dansyl-labeled cyclosporine (data not shown). Labeling of T cells with cyclosporine in the presence of prolactin or prior binding of cyclosporine to T cells, followed by an incubation with prolactin, resulted in a concentration-dependent reduction in the number of [dansyl-Lys^8]cyclosporine-labeled cells (Fig. 2). To remain within the linear portion of the cyclosporine binding curve, a concentration was chosen that caused up to 50% of the cells to be labeled and that corresponded to the

Table 1. Effect of prolactin from various species on the murine mixed lymphocyte reaction (MLR)

Prolactin*		[^{3}H]Thymidine incorporation,		
Species	Conc., ng/ml	dpm (mean ± SD)	% change†	IC_{50}, ng/ml
		One-way MLR‡		
—	—	4,767 ± 277	—	
Ovine	1000	5,391 ± 511	+15	
Canine	1000	2,207 ± 122	−61	600
	200	3,787 ± 287	−23	
Porcine	200	1,559 ± 30	−76	40
	40	2,648 ± 264	−50	
	8	4,189 ± 353	−14	
Human	1.6	1,065 ± 133	−88	<0.32
	0.32	1,365 ± 143	−81	
Human§	8	1,118 ± 136	−87	0.9
	1.6	2,626 ± 320	−51	
	0.32	2,732 ± 214	−48	
	0.064	4,177 ± 981	−14	
		Two-way MLR¶		
—	—	30,335 ± 2370	—	
Rat	1000	73,778 ± 1542	+162	
	200	48,213 ± 2607	+67	
	40	35,449 ± 1102	+19	

*Unless indicated otherwise, prolactin preparations used were reference preparations for RIA obtained from the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases (NIADDK, Bethesda, MD); the rat prolactin preparation was NIADDK RP1.
†Relative to minus-prolactin control. Incorporation in the absence of stimulator cells (556 ± 168 dpm for one-way MLR; 3455 ± 382 dpm for two-way MLR) was subtracted from both the control and the experimental value before computation of % change.
‡BALB/c responder lymphocytes and irradiated CBA stimulator lymphocytes.
§From Calbiochem (Lucerne, Switzerland).
¶BALB/c responder cells and nonirradiated CBA stimulator cells.

IC_{50} value (concentration causing 50% inhibition) of this compound in a mixed lymphocyte reaction.

Identification of mRNA Hybridizing with Prolactin- and GH-Specific Probes in Rat Lymphocytes After Mitogen Stimulation. In dot blot hybridization experiments, cytoplasmic RNA from lymphocytes stimulated *in vitro* with concanavalin A hybridized with both the prolactin- and the GH-specific probe (Fig. 3). The hybridization signal increased 3- to 4-fold on stimulation of the cells with Con A and leveled off after 24 hr of stimulation. Characterization of the prolactin/GH-specific RNAs by blot-hybridization analysis of electrophoretically fractionated poly(A)$^+$ RNA showed that the hybridizing RNA species were larger than the corresponding precursor and mature RNA species from pituitary (Fig. 4). Thus, it is clear that the pituitary and the lymphocyte cytoplasmic RNAs that hybridize with the prolactin-specific probe pPrl-1 are not the same species. Dot blot analysis of rat liver RNA showed no hybridization signal with either the prolactin- or the GH-specific probe (Fig. 3).

DISCUSSION

This study provides evidence for the involvement of prolactin in the maintenance of T-cell immune competence. Our data show that a reduction of serum prolactin levels by bromocriptine leads to a decrease of lymphocyte responsiveness toward antigenic stimulation. This phenomenon was observed both *in vitro* (mixed lymphocyte reaction) and, more important, *in vivo* (graft-vs.-host reaction). A further indication that prolactin is involved in regulating immune responses is the presence of prolactin-binding sites on lymphocyte cell surfaces (10–13). Displacement of bound prolactin from these sites by a biologically active cyclosporin (cyclosporine, or cyclosporin A) has been shown (12). On the other hand, another cyclosporine, cyclosporin H, which lacks immunosuppressive activity (unpublished observations) was found to be completely ineffective in competing with prolactin for this binding site (12). The present study also

Table 2. Graft-versus-host reaction

Treatment			Lymph node weight, mg (mean ± SD, n = 8)	% change*
Compound(s)	Daily dose, mg/kg	Route		
		Administered days 0–3		
Solvent	—	s.c.	16.8 ± 6.9	—
Bromocriptine	5	s.c.	15.1 ± 6.5	−10
		Administered days −7 to 3		
Solvent	—	s.c.	16.4 ± 8.9	—
Bromocriptine	1	s.c.	9.6 ± 8.9	−42†
Bromocriptine	5	s.c.	8.0 ± 4.5	−51†
		Administered days 0–3		
Solvent	—	s.c.	30.3 ± 8.9	—
Cyclosporine	36	Oral	13.1 ± 5.1	−60‡
25-240	0.25	s.c.	40.4 ± 8.8	+33†
Solvents	—	Oral + s.c.	23.4 ± 8.6	—
Cyclosporine	36	Oral	12.8 ± 5.4	−45†
Cyclosporine + 25-240	36 0.25	Oral s.c.	13.7 ± 4.6	−41‡
Cyclosporine + 25-240	36 2.5	Oral s.c.	22.4 ± 3.5	−4§

Solvents used were ethanol/olive oil, 1:20 (for oral administration), and ethanol/0.9% NaCl, 1:20, containing an equimolar amount (relative to drug) of D-tartaric acid (for subcutaneous injection).
*Relative to corresponding solvent control.
†$P < 0.05$ (Dunnett's test, t-test) compared to solvent control.
‡No significant difference when compared to animals treated with cyclosporine alone. $P < 0.01$ (Dunnett's test, t-test) when compared to solvent control.
§$P = 0.0004$ (t-test) or $P < 0.01$ (Dunnett's) when compared to animals treated with cyclosporine alone. No significant difference when compared to solvent-control animals.

Table 3. Stimulation of prolactin secretion can counteract inhibitory effects of cyclosporine treatment on antibody-producing cells

Treatment			pfc* per spleen (mean ± SD, n = 5)	% change
Compound(s)	Dose (mg/kg) on days 0–3	Route		
Solvent	—	Oral	85,132 ± 24,083	—
Solvent	—	s.c.	99,349 ± 26,329	—
Cyclosporine	60	Oral	30,092 ± 15,433	−65†
25-240	0.25	s.c.	112,979 ± 37,487	+14‡
25-240	2.5	s.c.	81,111 ± 21,503	−18‡
Cyclosporine + 25-240	60 0.25	Oral s.c.	64,238 ± 54,676	−30‡
Cyclosporine + 25-240	60 2.5	Oral s.c.	110,330 ± 45,282	+20§

*Plaque-forming cells (i.e., IgM-secreting cells).
†$P = 0.0001$ (Student's t-test) compared to corresponding solvent control.
‡Not significant.
§$P = 0.0028$ (Student's t-test) when compared to cyclosporine treatment alone. Value not significantly different from the mean of the two solvent controls. There was no significant difference between the two solvent controls.

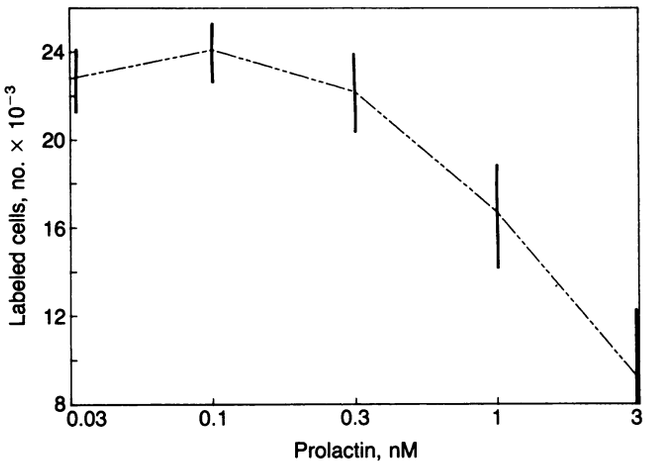

FIG. 2. Cyclosporine competes with prolactin for binding sites on human lymphocytes. [Dansyl-Lys[8]]Cyclosporine was bound to isolated peripheral human T lymphocytes in the presence of various concentrations of human prolactin (Calbiochem); each experimental point was determined in triplicate (error bars indicate mean ± SD). Fluorescence was determined by flow cytometry as described (17); values represent the number of T lymphocytes labeled with [dansyl-Lys[8]]cyclosporine. The value for the solvent control is 21,798, and for the background, 2997.

provides new data suggesting the interaction of prolactin and cyclosporine on immune competence. First, we have demonstrated that human prolactin displaces cyclosporine from the surface of human lymphocytes in a concentration-dependent fashion. Second, administration of a prolactin-releasing agent (25-240 at a dose of 2.5 mg/kg s.c.) causes an increase in the concentration of circulating prolactin (P. Marbach, personal communication). This may lead to a competitive displacement of cyclosporine on lymphocytic prolactin receptors, thus restoring lymphocyte responsiveness to antigen (Table 3) in cyclosporine-treated animals. T-cell interactions, as in cellular immune responses and as seen in the graft-vs.-host reaction, were partially inhibited by lowering circulating prolactin levels. Again, immune responsiveness in cyclosporine-treated (immunosuppressed) animals was restored by raising circulating prolactin levels. That reduction of circulating prolactin by bromocriptine did not reduce the number of antibody-producing cells in response to the injection of SRBC might be explained if the number of lymphocyte prolactin receptors is directly related to the concentration of circulating prolactin (23).

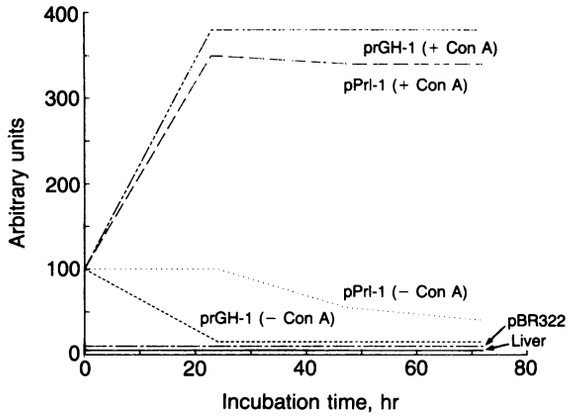

FIG. 3. Prolactin/GH-specific RNA in rat lymphocytes. Rat splenic lymphocytes were isolated and cultured in triplicate at a density of 5×10^6 cells per ml in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics, in the presence or the absence of the T-cell mitogen Con A (2 μg/ml). At 0, 24, 36, and 72 hr, extracts containing total cytoplasmic RNA from 10^6 cells were prepared as described (18), and dot blot hybridization with plasmids containing inserts specific for rat prolactin or rat GH mRNA (plasmids pPrl-1 and prGH-1, respectively) was carried out in duplicate. Rat liver cell extracts were dot blotted as negative controls and labeled plasmid pBR322 (without specific inserts) was used to check for nonspecific hybridization signals. Relative hybridization values were obtained by densitometry of the dot-blot autoradiograms and by relating each hybridization signal to the 0-hr (control) value.

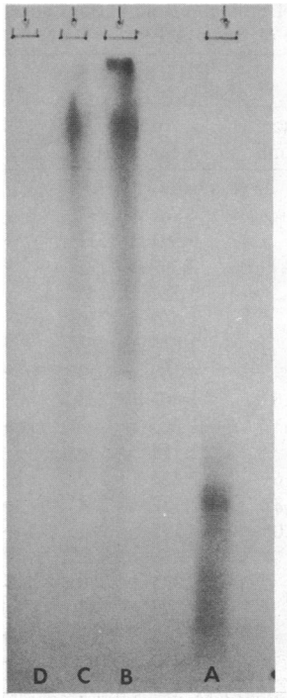

FIG. 4. Size of prolactin-related RNA. Poly(A)$^+$ RNA, isolated from splenic lymphocytes of graft-vs.-host-stimulated rats according to published procedures (21), was electrophoresed in 1.5% agarose under denaturing conditions as described (22). Nitrocellulose blots were incubated with ^{32}P-labeled nick-translated plasmid pPrl-1 (19) as described (21) and then were autoradiographed. Lane A: 1 μg of pituitary poly(A)$^+$ RNA. Lane B: 50 μg of lymphocyte poly(A)$^+$ RNA. Lane C: 25 μg of lymphocyte poly(A)$^+$ RNA. Lane D: plasmid control.

Since we were not able to completely abolish the T-cell-mediated immune response, even after reducing circulating prolactin to virtually undetectable levels with bromocriptine, we investigated whether lymphocytes themselves could produce prolactin (or a functionally related product) on antigenic stimulation. Dot blot hybridization showed that cytoplasmic RNA from lymphocytes hybridized with both prolactin and GH cDNA probes. Lymphocytes from either rats (Fig. 3) or mice (data not shown) reacted with an increase in these RNAs after mitogenic stimulation. We therefore suggest that the ability of T lymphocytes to respond fully to antigenic stimulation may depend on the presence of prolactin, of pituitary origin, bound to receptors on their outer membrane. On presentation of antigen these lymphocytes then produce a secondary signal in a form of a prolactin/GH-related polypeptide that can amplify either the response of the same cell to produce lymphokines (autocrine action), or the mitogenic response of neighboring lymphocytes (exocrine action), or both.

Our results also suggest that cyclosporine may exert its immunosuppressant action by displacing pituitary prolactin, and perhaps also the prolactin/GH-related polypeptide, from

binding sites on the T-lymphocyte surface, thereby making the cell unresponsive to antigenic stimulation.

Note. Linzer and Nathans (24) have described the presence of a prolactin/GH-related polypeptide in serum-stimulated 3T3 fibroblasts.

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