Evidence for the Release of a Prolactin-like Substance by Mouse Lymphocytes and Macrophages (43671)

RICHARD R. GALA[1,2] AND ETHAN M. SHEVACH

Department of Physiology, Wayne State University School of Medicine, Detroit, Michigan 48201; and Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892

Abstract. **The influence of co-cultures of irradiated mouse thymocytes, splenocytes, and mesenteric lymph node cells on the proliferation of Nb2 cells in synthetic medium were studied. Irradiated thymocytes with or without the addition of ovine prolactin (oPRL) decreased the incorporation of ^{3}H-thymidine into Nb2 cells. Irradiated splenocytes and lymph node cells when co-cultured with Nb2 cells and stimulated with concanavalin A (Con-A) induced Nb2 cell proliferation. Lipopolysaccharide stimulation of irradiated splenocytes and lymph node cells, however, did not alter Nb2 cell proliferation. Isolated thioglycolate-induced peritoneal macrophages stimulated Nb2 cells to incorporate ^{3}H-thymidine while IFN-γ-stimulated macrophages decreased PRL-induced Nb2 cell proliferation when isolated 48 but not 24 hr before co-culture. The addition of a mouse PRL antiserum to macrophage/Nb2 cell co-cultures did not alter the proliferative activity induced by macrophages. The preliminary data presented indicate that Con-A-stimulated, irradiated splenocytes and lymph node cells, and isolated peritoneal macrophages secrete a PRL-like activity. The PRL-like activity of macrophages, however, does not appear to be of mouse origin, and it is suggested that macrophages, which have surface PRL receptors, sequestered PRL from the fetal calf serum in the medium used to isolate them and release this PRL when co-cultured.** [P.S.E.B.M. 1994, Vol 205]

Numerous studies in the past few years have implicated prolactin (PRL) in the regulation of the immune system (1). Hypophysectomized rats are immunocompromised for both humoral and cellular immunity and either PRL or growth hormone can correct them (2). Dwarf mice, which lack growth hormone and prolactin, have been reported to have immunologic defects (3–5) and anterior pituitary kidney capsule transplants, known to secrete large amounts of PRL, or human growth hormone, which has prolactin activity, can provide relief from some of the defects (5, 6). Bromocriptine, a drug which specifically suppress PRL secretion, has similar effects to hypophysectomy on compromising immune function (7–9) and prolactin administration significantly improves the immunoreactions (8, 10). Some investigators have been unable to show PRL actions on lymphocytes in vitro (7, 11) while others have been able to stimulate proliferation and cytokine production only in cells from ovariectomized animals (12, 13). A consistent finding by a number of investigators has been the ability of PRL antisera to block mitogen-stimulated lymphocyte proliferation in vitro in serum-free medium (11, 14).

The first indication that lymphocytes may produce a PRL-like factor was the observation of Hiestand *et al.* (7) that splenocytes from graft vs host-stimulated rats had a mRNA similar to that of PRL. Montgomery *et al.* (15) subsequently reported that concanavalin A (Con-A)-stimulated murine splenocytes released into serum-free medium a substance similar to PRL since

[1] To whom requests for reprints should be addressed at Department of Physiology, Wayne State University, School of Medicine, 540 East Canfield, Detroit, MI 48201.

[2] Supported in part by NIH IPA Grant #CAN 2-8325806 while on sabbatical at NIH.

Received March 29, 1993. [P.S.E.B.M. 1994, Vol 205]
Accepted July 29, 1993.

0037-9727/94/2051-0012$10.50/0

cultured supernatants stimulated Nb2 cell proliferation and an anti rat-PRL serum blocked this response. They subsequently identified in the medium of Con-A-stimulated rat thymocyte and splenocyte cultures a 22 kDa protein that was similar to PRL and was immunoprecipitated with a specific rat PRL antisera (16). Using immunocytochemistry, others reported that Con-A-stimulated murine T and B splenocytes contained a PRL-like protein (17). O'Neal *et al.* (18) using pediatric thymocytes stimulated by Con-A demonstrated by cDNA cloning the expression of PRL mRNA, which was larger than that of pituitary PRL mRNA. This observation was rapidly confirmed and extended to include the secretion of bioactive PRL-like proteins from human thymocytes and peripheral blood mononuclear cells (19). In addition, other recent reports indicated that human peripheral blood mononuclear cells contained a high molecular weight (60 kDa) PRL-like material which was secreted when stimulated by mitogens (20) while another group separated human peripheral mononuclear cells into T-cells, B-cells and monocytes and found the genetic expression of PRL to be mainly associated with the T-cell fraction (21).

The evidence available indicates that immunocompetent cells appear to be capable of expressing a PRL-like molecule that may be secreted when stimulated by mitogens. The object of this study was to determine whether irradiated lymphocytes from the murine thymus, spleen, and mesenteric lymph nodes when co-cultured with Nb2 cells and stimulated by mitogens would release a PRL-like substance as evidenced by Nb2 cell proliferation. In addition, we also examined isolated peritoneal macrophages in co-culture to drive Nb2 cell proliferation. The advantage of this approach is that it is more sensitive and that it would detect labile material that might be lost if one collected the supernatant from the lymphoid cells and then mixed the supernatant with Nb2 cells in a two-step assay.

Material and Methods

Nb2 Cell Line. The Nb2 cell line, which was cloned from a rat lymphoma and whose proliferation is specific for lactogenic hormones (22), was originally obtained from Dr. P. W. Gout (Vancouver, BC, Canada). The line was propagated in our laboratory (23) using RPMI 1640 medium supplemented with 10% fetal calf serum (Biofluids), 10% horse serum (Sigma), 3.0×10^{-5} *M* 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.0 m*M* L-glutamine, and 1.0 m*M* HEPES at final concentration. When used to bioassay for PRL activity, the cells were washed twice with the synthetic medium AIM-V (Gibco cat. #320-20055 PK) supplemented with 0.1 m*M* MEM nonessential amino acids, 1 m*M* sodium pyruvate, 3.0×10^{-5} *M* 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.0 m*M* L-glutamine and 1.0 m*M* HEPES at final concentration. The cells were then adjusted to 50,000 cells/ml with the AIM-V medium and 100 μl added to each well of a 96-well microtiter plate. Nb2 cells were cultured with 100 μl of either lymphocytes or macrophages for 72 hr at 37°C in a humidified 7% CO_2 gas environment. One μCi of ^{3}H-thymidine (NEN cat no. #NET 027)/20 μl AIM-V was added 16 hr prior to harvesting. The cells were harvested using Tomtec Mach II 96-well harvester and counted with a Wallac Betaplate liquid scintillation counter. The addition of oPRL (NIDDK oPRL #19) to the Nb2 cells alone indicated a sensitivity of 5 pg/ml and a dose-response curve between 5 and 5000 pg/ml.

The specificity of Nb2-cell proliferation for PRL was determined using the following cytokines: r human IL-1 (Genzyme), r human IL-2 (Genzyme), r mouse IL-3 (Genzyme), r mouse IL-4 (Genzyme), r mouse IL-5 (Genzyme), r mouse IL-6 (Genzyme), r mouse IL-7 (Genzyme), r mouse IL-8 (Genzyme), r mouse IL-10 (Bachem Bioscience), r mouse IFNγ (Biosource International), r mouse IFNα (Biosource International), r mouse GM-CSF (Genzyme), r mouse TNFα (Genzyme). Each cytokine was serially diluted in triplicate wells either alone or with either 500 or 1000 pg/ml of oPRL to determine whether they inhibited PRL-induced proliferation. All preparations were shown to have bioactivity in the appropriate cell systems.

Thymus, Spleen, and Lymph Node Cells. The thymus, spleen and mesenteric lymph nodes were removed from five 6–10-week-old female BALB/c mice, and pooled. The lymphocytes were obtained by crushing the tissue on fine-meshed stainless steel screens and splenic red blood cells were lysed using ACK lysing buffer. All cells were washed twice with Hanks balanced salt solution and counted with a hemocytometer after the addition of 0.1% trypan blue. Cell viability was greater than 95%. The lymphocytes were washed once with AIM-V medium supplemented as above and diluted to give 16×10^6 thymocytes/ml, 4.0×10^6 splenocytes/ml and 8.0×10^6 lymph node cells/ml. The cells were then divided into two aliquots, and one aliquot was exposed to 3000 rads using a 137 Cs source while kept on ice. Both irradiated and nonirradiated cells were added to triplicate wells of a 96-well microtiter plate at a final concentration of 4.0×10^6 thymocytes/ml, 1.0×10^6 splenocytes/ml and 2.0×10^6 lymph node cells/ml. Concanavalin-A (Con-A; Pharmacia #17–0450–01) was added to triplicate wells at a final concentration of 0.5, 0.75, and 1.0 μg/ml. Lipopolysaccharide (LPS; Calbiochem, *E. coli* 0111:B4, cat no. 437627) was added to triplicate wells of splenocyte and lymph node cells at a final concentration of 1, 5, and 10 μg/ml. The cells were cultured in AIM-V

medium for 72 hr in a humidified 7% CO_2 gas environment with and without the addition of Nb2 cells. ^{3}H-thymidine (1.0 μCi/20 μl) was added 16 hr prior to harvesting the cells. The data presented represents the results of a single experiment.

Isolation of Peritoneal Macrophages. Mouse peritoneal macrophages were obtained from Dr. Linna Ding (NIH) (24) in two separate experiments and prepared as follows. Ten female BALB/c mice 6–12 weeks of age were injected ip with 3 ml of a 3 g/ml solution of thyoglycolate and three days later the peritoneal cavity was rinsed three times with 10 ml of DMEM-5% FCS. The T and B cells were lysed by adding 1 ml each of a 1:10 supernatant dilution of anti-Thy 1.2 (clone H 13.4), a 1:5 supernatant dilution of anti-B220 (clone 6B2), a 1:5 supernatant dilution of anti-Ia (clone M5/114), and a 1:10 dilution of rabbit complement per 1.0×10^8 cells. The mixture was incubated at 37°C for 45 min to lyse T and B cells. The cells were then washed twice with DMEM-5% FCS and 1 ml of a 1:10 supernatant dilution of mouse anti-rat (clone MAR 18.5) and 1 ml of 1:10 dilution of rabbit complement were added per 1.0×10^8 cells and incubated at 37°C for 45 min. The level of purity was determined using anti-Thy 1.2 PE (clone 30-H12; Pharmingen), anti B220 PE (Pharmingen), and anti Mac-1 FITC (clone M1/70; Caltag Labs) by flow cytometry. The isolated macrophages were greater than 98% pure. Aliquots of the cells were either kept on ice for 24 hr in one experiment or cultured immediately for 24 hr in DMEM-5% FCS at 37°C in a humidified 7% CO_2 environment with 100 U/ml of r mouse IFNγ in the other experiment. Nonstimulated cells were kept on ice for either 24 or 48 hr. The cells were then washed twice with AIM-V medium and checked for viability with 0.1% trypan blue. The viability at the end of 24 hr was greater than 85% and at the end of 48 hr was greater than 70%. The cells were then adjusted to 1.0×10^6 viable cells/ml with AIM-V medium and an aliquot was irradiated with 3000 rads using a 137 Cs source. Irradiated macrophages were added in triplicate to 96-well microtiter plates, serially diluted and then 100 μl of Nb2 cells at a concentration of 50,000 cells/ml were added. The co-cultures were cultured for 72 hr at 37°C in a humidified 7% CO_2 environment. Sixteen hours prior to harvesting, 1 μCi ^{3}H-thymidine/20 μl was added to each well to assess the proliferative activity of Nb2 cells.

Mouse PRL Antiserum and Ovine PRL. A polyclonal anti-mouse PRL serum (25) was obtained as a gift from Dr. Y. N. Sinha (Whittier Institute, CA) and added to some cultures to neutralize Nb2 proliferative activity. The ability of the antiserum to neutralize Nb2-cell proliferation induced by a saline extract of the mouse anterior pituitary was determined in a separate experiment. This antiserum was specific for mouse PRL and did not alter the Nb2 proliferative activity of either purified ovine PRL or bovine PRL (data not shown). Ovine PRL (NIDDK oPRL #19) was obtained as a gift from the NIDDK National Hormone and Pituitary Program.

Analytical Flow Cytometry. Flow cytometric analyses were performed using the Becton-Dickinson FACScan. Dead cells were eliminated from the analyses by adding 10 μl of a 50 μg/ml of propidium iodide and gating these cells out. A total of 10,000 cells were counted for each sample.

Results

The levels of Con-A and LPS selected resulted in a substantial proliferation of nonirradiated thymocytes, splenocytes, and lymph node cells when cultured alone in the synthetic medium AIM-V (Fig. 1, upper panel). However, when the same cells were irradiated, Con-A and LPS were unable to induce proliferation as evidenced by the lack of ^{3}H-thymidine incorporation (Fig. 1, lower panel). These results indicate that any increase ^{3}H-thymidine incorporation of co-cultures of irradiated lymphocytes with Nb2 cells must be due to Nb2 cell proliferation.

Irradiated thymocytes co-cultured with Nb2 cells decreased ^{3}H-thymidine incorporation, and Con-A stimulation did not alter the response. Con-A stimulation of irradiated splenocytes and lymph node cells, however, induced a 2.8- and 2.3-fold increase, respectively, of the incorporation of ^{3}H-thymidine into Nb2 cells. Stimulating splenocyte and mesenteric lymph nodes with LPS did not alter the proliferation of Nb2 cells from that of control cultures (Fig. 2, upper panel). Two hundred fifty picogram per milliliter of oPRL was added to each well to determine whether factors secreted by irradiated lymphocytes would suppress Nb2-cell proliferation. Thymocytes, whether stimulated by Con-A or not, decreased the PRL-induced proliferation of the Nb2 cell. Splenocytes and mesenteric lymph node cells whether stimulated by Con-A or LPS did not alter ^{3}H-thymidine incorporation (Fig. 2, lower panel). Thus, thymocytes appear to suppress Nb2-cell proliferation, while splenocytes and mesenteric lymph node cells do not.

The co-culturing of irradiated macrophages 48 hr after isolation with Nb2 cells resulted in a significant proliferation of Nb2 cells (Table I). The degree of Nb2-cell proliferation was proportional to the number of macrophages in the co-culture. The culturing of irradiated or nonirradiated macrophages alone resulted in little ^{3}H-thymidine incorporation above that noted for background (data not shown). Irradiated macrophage isolated 48 hr earlier and stimulated with IFNγ for the last 24 hr had considerably less proliferative activity on Nb2 cells. However, when a known amount of oPRL was added to the co-culture, IFNγ-stimulated

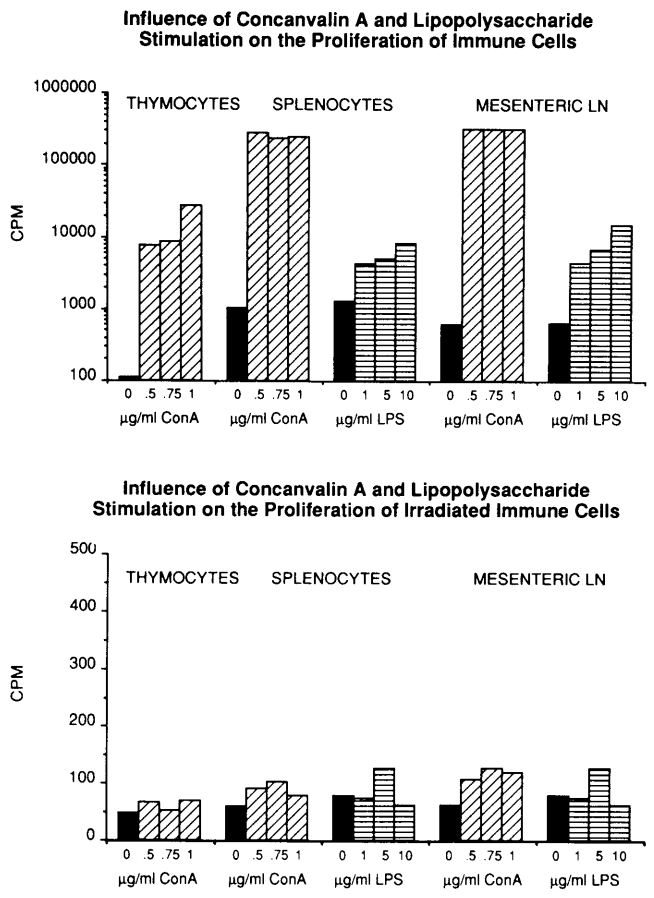

Figure 1. The influence of concanavalin A (Con-A) and lysopolysaccharide (LPS) stimulation on the proliferation of thymocytes, splenocytes, and mesenteric lymph nodes. Upper panel: nonirradiated cells; Lower panel: irradiated cells. Cells irradiated with 3000 rads using ^{137}Cs. Thymocytes were added to triplicate wells at a final concentration of 4.0×10^6 cells/ml. Splenocytes were added at a final concentration of 1.0×10^6/ml. Mesenteric lymph node cells were added at final concentration of 2.0×10^6/ml. Culture medium was the synthetic medium, AIM-V and total volume per well was 200 µl. Each group represents a pool of cells from five 6–10-week-old BALB/c mice.

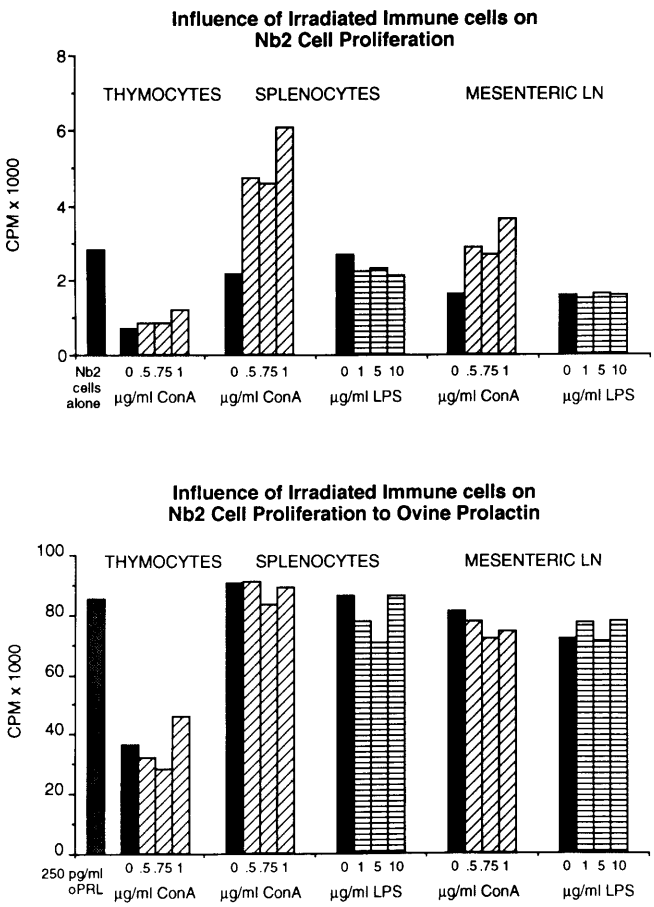

Figure 2. The influence of concanavalin A (Con-A) and lypopolysaccharide (LPS) stimulation on co-cultures of irradiated thymocytes, splenocytes, and mesenteric lymph node cells with Nb2 cells. Upper panel: co-cultures with no added oPRL; Lower panel: co-cultures with the addition of 250 pg/ml of oPRL. See legend of Figure 1 for additional details.

macrophages markedly inhibited the proliferative activity of Nb2 cells (Table I). Unstimulated macrophages also were able to partly block PRL activity on Nb2 cell proliferation, but this was only observed at the highest concentration of macrophages; and as the number of macrophages was decreased, there was an increase in Nb2 cell proliferation over that seen for PRL alone (Table I).

Macrophages co-cultured 24 hr after isolation also induced Nb2-cell proliferation and potentiated the action of added oPRL (Table II). A 24-hr-stimulation of macrophages with IFNγ immediately after isolation induced a lower proliferation of Nb2 cells when compared with unstimulated macrophages, and when oPRL was added, there was only a slight inhibition of Nb2-cell proliferation. A specific antiserum to mouse PRL did not alter the proliferation of Nb2 cells when co-cultured with macrophages (Table II). The inability of the mouse PRL antiserum to block the proliferative activity of co-cultures of macrophages was not due to the inability of the antiserum to block the proliferative activity of mouse PRL. A saline extract of the mouse anterior pituitary when added to Nb2 cells induced a marked proliferation and the antiserum blocked this activity (Table III). The possibility that one of the known cytokines produced by lymphocytes and macrophages may have altered the proliferation of Nb2 cells was eliminated when a large panel of cytokines were cultured with Nb2 cells, with and without the addition of PRL, and no effect was observed (Table IV). The Nb2 cells used here do not respond to IL-2.

Discussion

The results indicated that Con-A stimulated murine splenocytes secrete a PRL-like substance and confirm the work of Montgomery *et al.* (15). Irradiated lymphocytes are capable of secreting cytokines when mitogen-stimulated, however, they do not proliferate (26). This fact allows us to ascribe the incorporation

Table I. Influence of Irradiated Macrophages 48 Hr After Isolation on Nb2-Cell Proliferation

Number of macrophages per 5000 Nb2 cells per well	CPM			
	Unstimulated	IFN γ stim*	Unstimulated plus 1 ng/ml oPRL	IFN γ stim* plus 1 ng/ml oPRL
0	695	673	168,681	178,058
50,000	114,863	3,386	149,684	28,656
25,000	46,878	1,598	184,386	59,765
12,500	33,526	718	189,702	93,352
6,250	19,495	467	201,118	138,873
3,125	6,743	612	160,543	171,486
1,560	2,673	632	199,432	197,176
780	1,133	605	186,120	206,673
390	872	541	189,960	206,803

* Stimulated with 100 U mouse rIFN γ/ml last 24 hr before irradiation and co-culture.

Table II. Influence of Irradiated Macrophages 24 Hr After Isolation on Nb2-Cell Proliferation

Number of macrophages per 5000 Nb2 cells per well	CPM					
	Unstimulated	IFN γ stim*	Unstimulated +250 pg/ml oPRL	INF γ stim* +250pg/ml oPRL	Unstimulated +α mPRL**	INF γ stim* +α mPRL**
0	2,020	2,444	37,638	55,111	2,132	2,160
50,000	14,142	—	93,428	105,994	14,365	11,508
25,000	12,560	5,022	57,930	44,808	11,011	4,840
12,500	7,080	3,415	50,239	36,838	6,664	3,409
6,250	5,427	2,456	46,610	44,175	5,284	2,299
3,125	3,535	2,422	44,683	47,396	3,861	2,887
1,562	3,245	2,305	42,745	45,181	3,468	2,616
780	2,540	2,385	36,228	37,769	2,890	2,383
390	2,080	2,538	38,340	47,009	2,270	1,996

* Macrophages stimulated with 100 U mouse rIFN γ/ml for 24 hr before irradiation and co-culture.
** α mouse PRL at 1:10,000 dilution.

Table III. Influence of a Mouse Prolactin (PRL) Antiserum on the Proliferation of Nb2 Cells Stimulated by a Saline Extract of the Mouse Anterior Pituitary (AP)[a]

Dilution of AP extract	CPM				
	Control	Anti-mouse PRL serum			
		1:640	1:2,560	1:5,120	1:10,240
No extract	3,542	3,902	3,379	3,498	3,537
1:4,000	69,817	4,134	5,948	30,809	47,988
1:8,000	50,123	4,065	4,794	15,471	44,641
1:16,000	42,748	4,206	4,160	6,432	28,537
1:32,000	40,645	4,097	4,275	5,616	18,669

[a] Responsiveness of Nb2 cells to ovine PRL: 10 pg/ml = 8,407 CPM; 78 pg/ml = 33,530 CPM; 625 pg/ml = 87,936 CPM.

^{3}H-thymidine to the Nb2 cells and any induced proliferation of the Nb2 cells must be originating from the cells in co-culture. The addition of Con-A to Nb2 cell cultures alone did not alter ^{3}H-thymidine incorporation from that noted for controls (data not shown). Although the proliferation induced by Con-A stimulated splenocytes and lymph node cells was not extensive, two factors must be considered. First, the splenocyte and lymph node cells producing a PRL-like substance may be a minor subset of the cells derived from these tissues and the isolation and stimulation of this subset would result in a greater proliferation of Nb2 cells. Second, irradiation prevented the expansion of the cells by Con-A-stimulation, thus one would not expect as great a stimulation of Nb2 cells as has been previously reported. Others have stimulated splenocytes with Con-A for varying periods of time and then assayed the culture medium for PRL-like activity (15, 16). Under their conditions splenocyte proliferation would greatly increase the cells producing a PRL-like material. This could explain the apparent higher PRL-like activity produced by their Con-A-stimulated cultures when compared to ours. The identification of the Nb2-cell proliferative activity from Con-A-stimulated co-cultured irradiated splenocytes and lymph node cells as a PRL-like substance is tentative pending experiments demonstrating that a specific antiserum to mouse PRL will block the activity. Future experiments will be directed to providing this evidence.

Co-culturing of thymocytes with Nb2 cells resulted in a suppression of Nb2-cell proliferation, with and without the addition of oPRL. This was an unexpected finding since others have found a PRL-like mol-

Table IV. Influence of Interleukins or Cytokines on Nb2-Cell Proliferation

Exper	Interleukins or cytokines	Dose range	CPM	
			IL or cytokines alone	1 or 0.5 ng/ml oPRL + IL or cytokines
1	Nb2 cells alone	—	340	13,877
	r human IL-2	0.39–50 U/ml	325–464	12,648–14,172
	r mouse IL-4	1.50–200 U/ml	295–545	13,172–13,695
	r mouse IL-10	15.00–2000 pg/ml	207–417	13,247–14,521
2	Nb2 cells alone	–	1,508	141,872
	r human IL-1	0.75–100 U/ml	1,237–1,686	155,240–167,002
	r mouse IL-3	76.00–10,000 pg/ml	1,211–1,374	137,082–158,356
	r mouse IL-5	7.50–1,000 U/ml	1,134–1,333	135,931–150,834
	r mouse IL-6	7.50–1,000 U/ml	1,034–1,152	124,470–138,964
	r mouse IL-7	7.50–1,000 U/ml	1,221–1,636	144,340–154,376
3	Nb2 cells alone	–	3,775	135,483
	r human IL-8	0.39–50 ng/ml	3,856–4,162	121,846–133,670
	r mouse IFNγ	2.00–250 U/ml	2,755–3,194	134,393–141,725
	r mouse IFNα	7.50–1,000 U/ml	2,916–3,835	136,845–142,196
	r mouse GMCSF	0.75–100 ng/ml	3,705–4,155	124,241–129,807
	r mouse TNFα	0.75–100 ng/ml	4,034–4,811	138,064–142,720

r = recombinant.
IL = interleukin.

ecule in Con-A stimulated murine (16) and human thymocyte lysates (18, 19, 21). One of these reports (19) indicated that human thymocytes secreted into the medium a PRL-like molecule capable of stimulating Nb2-cell proliferation. The difference may be due to our use of murine thymocytes while the other group (19) studied human cells.

The differential effect of Con-A and LPS in inducing the proliferation of Nb2 cells in co-culture suggested that the spleen and lymph node cells producing the PRL-like activity may be of T-cell origin. It is generally accepted that Con-A stimulates cytokine production and proliferation of T-cells while LPS stimulates B-cells (27, 28). In addition, the lack of an effect by LPS indicates that the Con-A response is not a general phenomenon of cross-linking cell surface receptors. LPS and Con-A when added to Nb2 cells alone does not alter the proliferation from that of control cultures (data not shown).

Co-cultured irradiated macrophages induced Nb2-cell proliferation. The proliferation was more marked when the co-cultures were initiated 48 hr after macrophage isolation than when isolated for 24 hr. The reason for this is not readily apparent. Stimulation of macrophages with IFNγ suppressed the PRL-induced proliferation only when macrophages were stimulated 24 hr after isolation and not when stimulated immediately. It appears that aging of the isolated macrophages alters the response. In fact, IFNγ stimulation of macrophages immediately after isolation removed the inhibitory activity of these cells on PRL-induced Nb2-cell proliferation, and a stimulatory activity was now evident but less than that observed for unstimulated macrophages (Table II). The identification of the macrophage inhibitory substance is unknown since a number of cytokines produced by macrophages did not alter the proliferative response of Nb2 cells (Table IV).

One possible factor responsible for the suppression of Nb2-cell proliferation when co-cultured with IFNγ-stimulated macrophages may be PGE_2. It has been observed that PGE_2 is released by IFNγ-stimulated macrophages (29) and that PGE_2 suppress lymphocyte proliferation (30). Another possible factor responsible for the suppression may be TGFβ, which is known to be secreted by macrophages (31). Both PGE_2 and TGFβ have been reported to suppress the proliferative activity of Nb2 cells when stimulated by human growth hormone (32).

The proliferative action of macrophages on Nb2 cells was not altered when a polyclonal anti-mouse PRL serum, shown to block pituitary PRL activity (Table III), was added to the co-cultures. This was surprising since previous reports indicated that PRL antisera from a number of species would block the proliferation of Con-A-stimulated lymphocytes (11) and IL-2-stimulated L-2 cells, a murine T-helper cell line (14). The latter investigators further noted that although the L-2 cell required PRL for proliferation and PRL was secreted from the cells, no mRNA for PRL was detected (14). These authors suggested that the PRL observed was sequestered by the cells (since they have PRL receptors) from the maintenance medium and was released when stimulated by IL-2. In a subsequent publication, these investigators reported that intracellular PRL was present within L-2 cells and that this PRL was of bovine origin (33). This observation may explain why the specific antiserum to mouse

PRL we used did not block the Nb2-cell proliferative activity induced by macrophage co-culture. This mouse PRL antiserum does not alter the Nb2-cell proliferative activity of purified bovine PRL (data not shown). We have recently observed that similarly isolated macrophage have a high surface expression of PRL-receptors using FITC-labeled PRL-R monoclonal antibodies and flow cytometry (34). Thus, the possibility exists that the bovine PRL in the fetal calf serum (35, and our unpublished observations) added to DMEM used to isolate the macrophages was sequestered by them and released when co-cultured with the Nb2 cells inducing them to proliferate. This may explain why macrophages maintained *in vitro* for 48 hr in cell culture were more potent stimulators of Nb2 cell proliferation than macrophages maintained *in vitro* for 24 hr. Subsequent experiments will be initiated with this hypothesis in mind.

The authors express their appreciation to Dr. Linna Ding from the Laboratory of Immunology, NIAID, NIH for providing the macrophages. The authors acknowledge the National Hormone and Pituitary Program of NIDDK for providing as a gift the ovine prolactin (NIDDK oPRL #19).

1. Gala RR. Prolactin and growth hormone in the regulation of the immune system. Proc Soc Exp Biol Med **198**:513–527, 1991.
2. Berczi I, Ed. Pituitary Function and Immunity. Boca Raton: CRC Press, 1986.
3. Duquesnoy RJ, Kalpaktsoglou PK, Good RA. Immunological studies on the Snell-Bagg pituitary dwarf mouse. Proc Soc Exp Biol Med **133**:201–206, 1970.
4. Fabris N, Pierpaoli W, Sorkin E. Hormones and the immunological capacity. III: The immunoefficiency disease of the hypopituitary Snell-Bagg dwarf mouse. Clin Exp Immunol **9**:209–225, 1971.
5. Murphy WJ, Durum SK, Anver MR, Longo DL. Immunologic and hematologic effects of neuroendocrine hormones. Studies on DW/J dwarf mice. J Immunol **148**:3799–3805, 1992.
6. Esquifino AI, Villanua MA, Szary A, Yau J, Bartke A. Ectopic pituitary transplants restore immunocompetence in Ames dwarf mice. Acta Endocrinol **125**:67–72, 1991.
7. Hiestand PC, Mekler P, Nordmann R, Grieder A, Permmongkol C. Prolactin as a modulator of lymphocyte responsiveness provides a possible mechanism for cyclosporine. Proc Natl Acad Sci USA **83**:2599–2603, 1986.
8. Bernton EW, Meltzer MS, Holaday JW. Suppression of macrophage activation and T-lymphocyte function in hypoprolactinemic mice. Science **239**:401–404, 1988.
9. Neidhart M. Bromocriptine microcapsules inhibit ornithine decarboxylase activity induced by Freund's complete adjuvant in lymphoid tissues of male rats. Endocrinology **125**:2846–2852, 1989.
10. Berczi I, Nagy E. Prolactin and other lactogenic hormones. In: Berczi I, Ed. Pituitary Function and Immunology. Boca Raton: CRC Press, pp161–183, 1986.
11. Hartman DP, Holaday JW, Bernton EW. Inhibition of lymphocyte proliferation by antibodies to prolactin. FASEB J **3**:2194–2203, 1989.
12. Mukherjee P, Mastro AM, Hymer WC. Prolactin induction of interleukin-2 receptors on rat splenic lymphocytes. Endocrinology **126**:88–94, 1990.
13. Viselli SM, Stanek EM, Mukherjee P, Hymer WC, Mastro AM. Prolactin-induced mitogenesis of lymphocytes from ovariectomized rats. Endocrinology **129**:983–990, 1991.
14. Clevenger CV, Russell DH, Appasomy PM, Prystowsky MB. Regulation of interleukin 2–driven T-lymphocyte proliferation by prolactin. Proc Natl Acad Sci USA **87**:6460–6464, 1990.
15. Montgomery DM, Zukoski CF, Shah NG, Buckley AR, Pacholczyk T, Russell DH. Concanavalin A-stimulated murine splenocytes produced a factor with prolactin-like bioactivity and immunoreactivity. Biochem Biophys Res Commun **145**:692–698, 1987.
16. Montgomery DW, LeFevre JA, Ulrich ED, Adamson CR, Zukoski CF. Identification of prolactin-like proteins synthesized by normal lymphocytes. Endocrinology **127**:2601–2603, 1990.
17. Kenner JR, Holaday JW, Berton EW, Smith PF. Prolactin-like protein in murine splenocytes: Morphological and biochemical evidence. Prog Neuroendoimmuno **3**:188–195, 1990.
18. O'Neal KD, Montgomery DW, Truong TM, Yu-Lee LY. Prolactin gene expression in human thymocytes. Mol Cell Endocrinol **87**:R19–R23, 1992.
19. Montgomery DW, Shen GK, Ulrich ED, Steiner LL, Parrish PR, Zukoski CS. Human thymocytes express a prolactin-like messenger ribonucleic acid and synthesize bioactive prolactin-like proteins. Endocrinology **131**:3019–3026, 1992.
20. Sabharwal P, Glaser R, Lafuse W, Varma S, Liu Q, Arkins S, Kooijmans R, Kutz L, Kelley KW, Malarkey WB. Prolactin synthesized and secreted by human peripheral blood mononuclear cells: An autocrine growth factor for lymphoproliferation. Proc Natl Acad Sci USA **89**:7713–7716, 1992.
21. Pellegrini I, Lebrun JJ, Ali S, Kelly PA. Expression of prolactin and its receptor in human lymphoid cells. Mol Endocrinol **6**:1023–1031, 1992.
22. Gout PW, Beer CT, Noble RL. Prolactin-stimulated growth of cell cultures established from malignant Nb rat lymphoma. Cancer Res **40**:2433–2436, 1980.
23. Lawson DM, Sensui N, Haisenleder DJ, Gala RR. Rat lymphoma cell bioassay for prolactin: Observations on its use and comparisons with radioimmunoassay. Life Sci **31**:3063–3070, 1982.
24. Ding L, Shevach EM. IL-10 inhibits mitogen-induced T-cell proliferation by selectively inhibiting macrophage costimulatory function. J Immuno **148**:3133–3139, 1992.
25. Sinha YN, Lewis UJ, Vanderlaan WP. Effects of administering antisera to mouse growth hormone and prolactin on gain in litter weight and on mammary nucleic acid content of lactating C3H mice. J Endocrinol **55**:31–40, 1972.
26. Ben-Sasson SZ, Le Gros G, Conrad DH, Finkelman FD, Paul WE. IL-4 production by T cells from naive donors. IL-2 is required for IL-4 production. J Immunol **145**:1127–1136, 1990.
27. Sprent J. T lymphocytes and the thymus. In: Paul WE, Ed. Fundamental Immunology (2nd ed). New York: Raven Press, pp69–93, 1989.
28. Kincade PW, Gimble JM. B lymphocytes. In: Paul WE, Ed. Fundamental Immunology (2nd ed). New York: Raven Press, pp41–68, 1989.
29. Mauel J, Pham TV, Kreis B, Corradin-Betz S, Bauer J. Evaluation of assay procedures measuring macrophage stimulation by immunomodulators *in vitro*. Develop Biol Stand **77**:71–77, 1992.
30. Baker PE, Fahey JV, Munek A. Prostaglandin inhibition of T-cell proliferation is mediated at two levels. Cell Immun **61**:51–61, 1981.

31. Assoian RK, Fleurdelys BE, Stevenson HC, Miller PJ, Madtes DK, Raines EW, Ross R, Sporn MB. Expression and secretion of type β transforming growth factor by activated human macrophages. Proc Natl Acad Sci USA **84**:6020–6024, 1987.
32. Rayhel EJ, Prentice DA, Tabor PS, Flurkey WH, Geib RW, Laherty RF, Schnitzer SB, Chen R, Hughes JP. Inhibition of Nb_2 T-lymphoma cell growth by transforming growth factor–β. Biochem J **253**:295–298, 1988.
33. Clevenger CV, Stillman AL, Prystowsky MB. Interleukin-2 driven nuclear translocation of prolactin in cloned T-lymphocytes. Endocrinology **127**:3151–3159, 1992.
34. Gala RR, Shevach EM. Identification by analytical flow cytometry of prolactin receptors on immunocompetent cell populations in the mouse. Endocrinology **133**:1617–1623, 1993.
35. Honn KV, Singley JA, Chavin W. Fetal bovine serum: A multivariate standard. Proc Soc Exp Biol Med **149**:344–347, 1973.