Enhanced Yields of Gamma Interferon in Prolactin Treated Human Peripheral Blood Mononuclear Cells (43683)

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Abstract. **Prolactin is a peptide hormone with effects on a number of target organs including the immune system. It has been shown that animals rendered hypoprolactinemic have impaired delayed hypersensitivity, impaired macrophage activation and altered secretion of gamma interferon (IFN). Using peripheral blood mononuclear cells (PBMC) and inducing the cells to produce gamma IFN with a range of inducers, we have studied the effects of a number of hormones on IFN production. Using cells from normal donors, we have found that prolactin in concentrations of 10^{-8} *M* or greater, can significantly enhance the production of gamma IFN. The effect was dose related and was observed when lectins (PHA and Con A), but not anti CD_3 antibodies, ionophones, or IL-2 were used to induce the cells. The presence of prolactin in concentrations above that encountered in the fetal bovine serum used to incubate the cells resulted in a doubling or more of the IFN produced. The tests were performed on 30 occasions with cells drawn from 21 individuals. On all but three occasions, yield enhancement was observed in the presence of prolactin. The mechanism of the effect was investigated, and genistein, a tyrosine kinase inhibitor, was found to abort the influence of prolactin on gamma IFN production. These studies indicate prolactin in physiological concentrations can enhance the production of gamma IFN from cells from normal donors.** [P.S.E.B.M. 1994, Vol 205]

Prolactin is a pituitary hormone with pleiotropic effects (1). It is known that prolactin induces the synthesis of milk proteins (2), helps to sustain testosterone levels by increasing the sensitivity of the testis to LH stimulation (3), and has a role in osmoregulation at least in certain species (1). Of special interest however is the role of prolactin in immune regulation. It has been shown that T and B lymphocytes bear prolactin receptors (4) and that hypophysectomized rats fail to develop contact sensitization to dinitrochlorobenzene and do not form antibodies appropriately to sheep red blood cells (5). Treatment with prolactin as well as growth hormone restores this competence (5). Furthermore, bromocriptine treatment, which blocks pituitary prolactin release, has been shown to result in reduced lymphocyte proliferation in the mixed lymphocyte reaction and altered graft versus host reactivity in rodents (6). Additionally, Bernton *et al.* (7) have reported that bromocriptine-treated mice have defective macrophage activation, diminished gamma interferon production, and altered lymphocyte proliferation to mitogens. These defects were corrected by administration of prolactin. Finally, it has been demonstrated that prolactin competes with cyclosporin for binding sites on the surface of T cells (5).

In this report, we have examined the effects of prolactin on the production of interferons (IFN) by human peripheral blood mononuclear cells from normal donors. We have found that exogenously added prolactin can significantly enhance yields of gamma IFN in lymphocytes from normal donors.

Methods

Reagents. Phytohemagglutinin (PHA) was purchased from Difco, Inc. (Detroit, MI). Human inter-

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leukin-2 (IL-2) was obtained from Amgen, Inc. (Thousand Oaks, CA). Ionomycin and A23187 were purchased from Calbiochem (San Diego, CA), and phorbol myristic acetate (PMA) was purchased from Sigma, Inc. (St. Louis, MO). Anti CD_3 antibody was obtained from Coulter Immunology (Hialeah, FL). Prolactin and growth hormone were purchased from Sigma, Inc. (St. Louis, MO). Hydrocortisone was the gift of the Upjohn Co. (Kalamazoo, MI). All hormones were of human origin. H7(1-5-isoquinolinyl sulfonyl)-2-methyl piperazine and calmidazolium (R24571) were obtained from Sigma (St. Louis, MO). Cholera toxin was purchased from List Biochemical (Campbell, CA). Fetal bovine serum was purchased from Gibco (Santa Clara, CA), and RPMI 1640 medium was obtained from Irvine Scientific (Irvine, CA). Recombinant human interferons alpha and gamma were the gift of Hoffman LaRoche (Nutley, NJ). Antiserum to human interferon gamma was obtained from Interferon Sciences (New Brunswick, NJ), and antiserum to human IFN alpha from the National Institutes of Health (Bethesda, MD).

Peripheral Blood Mononuclear Cells (PBMC). Human PBMC were obtained as the buffy coat fraction from normal human donors. PBMC were separated on Ficoll Hypaque gradients (8), washed, and adjusted to a final concentration of 5×10^6 cells/ml in RPMI 1,640 supplemented with 10% fetal bovine serum (FBS), 250 units/ml of penicillin and 150 ugm/ml of streptomycin.

Mitogenic Stimulation of PBMC. PBMC, separated as above, were stimulated with either 25 ugm/ml (unless otherwise indicated) of PHA, 25 ugm/ml of concanavalin A, 150 units of IL-2, 1 μM ionomycin, (with 50 ngm PMA) or a 1:400 dilution of anti CD_3 antibody. Preliminary experiments determined these to be the optimal concentrations of mitogens used for the induction of interferon. Induction using PHA was routinely accomplished in the presence of 10% FBS as indicated above, although in certain experiments bovine serum albumin (1 mg/ml) was substituted for FBS. Induction with anti CD_3 or IL-2 was performed using bovine serum albumin but induction in the presence of ionomycin was done in 10% FBS.

Incubation of the PBMC was carried out in 15 ml plastic tubes in a 5% CO_2 incubator at 37°C for three days. At the end of that time, the cells were removed by centrifugation, tested for viability using trypan blue, and exposed to ^{3}H-thymidine to assess the proliferative response as described below. PBMC were used for assay only if the viability of the cells was 95% or greater. All supernatants were harvested from the cells, dialyzed overnight, and frozen for subsequent assay. Dialysis membranes selected excluded molecules greater than 6,000 Daltons. In those experiments in which the effects of various reagents were being studied, the reagent underwent final dilution just prior to addition to the PBMC and was added simultaneously with the mitogen, unless otherwise indicated. Appropriate controls were performed for each test sample. Controls were simultaneously prepared and treated identically in every way to the test samples, save for the absence of the test reagent or reagents.

Proliferative Responses. Proliferative responses were determined by pulsing stimulated and unstimulated PBMC at a concentration of 1×10^6 cells/ml for 4 hr in a CO_2 incubator with ^{3}H-thymidine (0.5 uCi/well) having a specific activity of 6.7 Ci/mM (ICN, Irvine, CA). After pulsing, the cells were harvested using a multisample automatic cell harvester. The ^{3}H-thymidine incorporation was measured using a liquid scintillation counter. PBMC from different individuals were tested on different days, each with their own controls and comparisons made between yields in the control and test samples.

Induction of Human Interferon Alpha. Human PBMC, separated as described above, were adjusted to a concentration of 5×10^6 cells/ml in media supplemented as described above and containing 1 mg/ml of bovine serum albumin. These cells were added to 15 ml plastic centrifuge tubes and poly rI:rC was added along with DEAE-D as described in detail in the past (9). After a 30-min incubation carried out in a CO_2 incubator overnight, the inducing solution was washed from the cells and replaced with media as described above. The following morning, the supernatants were harvested after centrifugation, dialyzed as described above, and frozen for subsequent assay. In the experiments where the effects of individual hormones on the production of human interferon alpha were being investigated, control and test aliquotes of PBMC were processed simultaneously and under identical circumstances, except for the presence or absence of the test reagent. Individual hormones were added to both the inducing solution (poly rI:rC with DEAE-D) and subsequent incubation media (supplemented RPMI) in the indicated concentrations.

Interferon Assays. Interferon (IFN) was assayed as previously reported (10). Briefly WISH cells were grown to confluence at the bottom of individual wells of 96-well microtiter plates (Costar, Boston, MA). Serial dilutions of samples were added, allowed to incubate with the cells overnight and then removed. After the cells were washed, they were challenged with encephalomyocarditis virus and reincubated for an additional 24 hr. At that time, the cells were examined by light microscopy for cytopathic effect. The reciprocal of the last dilution of the sample where cytopathic effect was inhibited by 50% was termed the titer of the sample. All assays were carried out in duplicate. A standard alpha IFN preparation titered against NIH alpha standard G023–901–530 and a gamma standard

titered against NIH gamma standard Gg23–901–530 were included in all assays. In this system, the NIH alpha standard titers 7,200 units and the gamma standard titers 16,000 units. All results are expressed in international reference units.

Typing of Interferons. For purposes of identifying the specific IFN species present after the induction with a given inducing agent, aliquots of IFN containing supernatants were independently mixed with specific antisera to each IFN species. Quantities of antisera were selected to neutralize 100 units of IFN bioactivity and supernatants were adjusted to contain approximately 100 international IFN units. Incubation was carried out for 1 hr, at 36°C, with the specific antisera or with normal control serum. The IFN was then titered as described above and the amount of IFN bioactivity compared between samples incubated in normal control serum or specific antiserum.

Effects of Hormones on IFN Bioactivity. Media containing appropriate concentrations of the individual hormones were prepared and added to appropriate rows of microtiter plates. Samples of recombinant human IFN's alpha and gamma were then titered on media with the various hormones present or absent, and the titers were compared. Interferons were titered by this method on at least two occasions.

Statistical Analysis. Statistical analysis was performed using Student's Paired *t*-test.

Results

Initial studies using specific antibody demonstrated that PHA and Con A induce gamma IFN in human PBMC. Additionally, prolactin alone, without lectin, did not induce detectable IFN levels (data not shown).

Figure 1 shows the effects of prolactin in concentrations ranging from 10^{-7} *M* to 10^{-11} *M* on the production of gamma IFN by PBMC. Concentrations of prolactin as low as 10^{-8} *M* significantly enhanced yields of gamma IFN. In three separate experiments (i.e., PBMC from three different individuals), Con A at a concentration of 25 μg/ml was used to induce the cells instead of PHA. In these experiments using Con A, the yield of gamma IFN in the presence of 10^{-8} *m* prolactin was again increased 2-fold over yields induced by Con A in the absence of prolactin (data not shown). In three additional experiments (PBMC from three individuals), albumin was substituted for FBS in order to eliminate the influence of bovine prolactin present in the media. Use of albumin did not alter results, and, just as in FBS, levels of gamma IFN doubled with the prolactin concentration (10^{-7} *M*) employed under these conditions. Figure 2 shows the effect of the 10^{-8} *M* prolactin concentration on enhancement of gamma IFN yields in the presence of a range of PHA concentrations. Yield enhancement approximately doubled over the range of lectin concentrations employed. Of interest is the fact that the presence of prolactin permitted the induction of detectable IFN levels at PHA concentrations too low to induce IFN when the lectin was used alone. In contrast, substitution of anti CD_3 antibody, IL-2, or ionomycin for PHA or Con A was not accompanied by yield enhancement in the presence of prolactin.

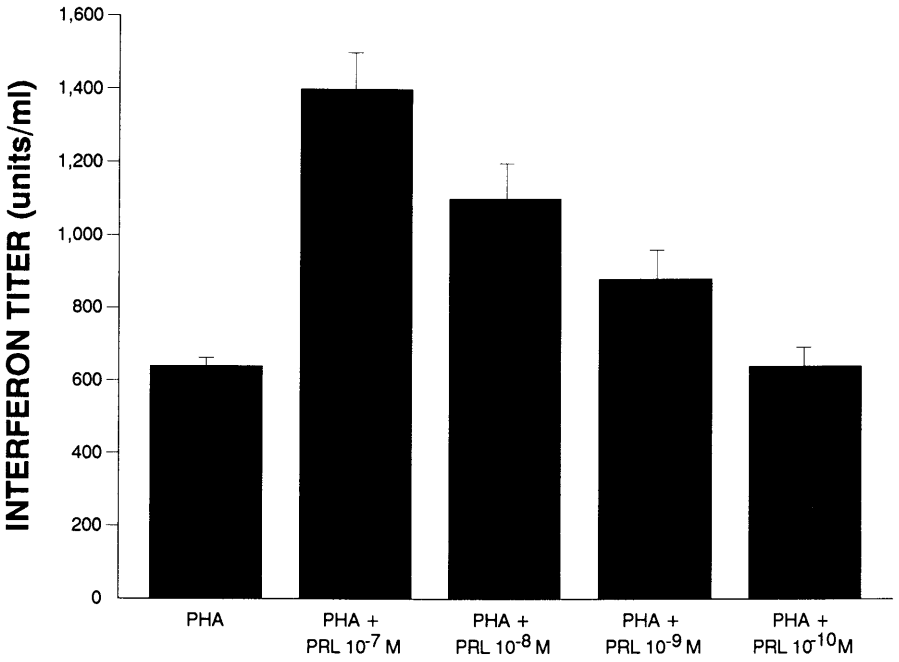

Figure 1. The effect of prolactin on the yield of PHA induced interferon in human peripheral blood mononuclear cells. The concentration of PHA utilized was 25 μg/ml. The difference in yield between the samples with PHA alone and PHA with prolactin (PRL) at 10^{-7} and 10^{-8} *M* was significant ($P < 0.05$). Each bar is based on a mean or eight or more determinations (PBMC from four individuals).

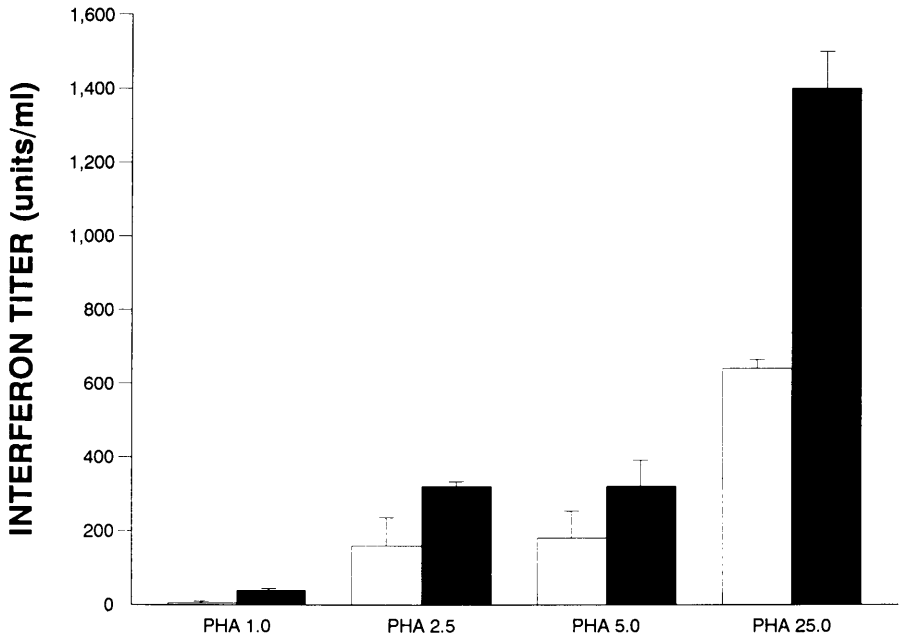

Figure 2. The effect of PHA concentration on prolactin enhancement of gamma IFN yields in peripheral blood mononuclear cells. The numeral beside each PHA designation is the concentration of PHA in μg/ml. The difference between the IFN yield with PHA alone and PHA + Prolactin is statistically significant with PHA at the 2.5, 5.0, and 25 μg/ml concentrations. Each bar is based on a mean of eight or more determinations (PBMC from four individuals). □, PHA alone. ■, PHA + PRL.

The influence of the time of addition of the prolactin was also studied. The addition of prolactin 24 hr prior to PHA resulted in the best enhancement in gamma IFN yields. IFN yields were enhanced by 400% or greater under these circumstances (data not shown).

Overall, 30 different experiments (i.e., tests performed on 30 occasions) were conducted with prolactin and plant lectins. These experiments employed PBMC from a total of 21 different individuals. On 27 occasions, using blood from 18 different individuals, significant enhancement of gamma IFN production occurred in the presence of prolactin as compared to controls without prolactin. Additionally, specific antiserum was used to verify that IFN induced by PHA in the presence of prolactin was indeed gamma IFN. Anti gamma IFN, but not anti alpha IFN, neutralized the antiviral activity induced in the presence of prolactin.

Since prolactin had an enhancing effect, another hormone of similar size was also tested to determine if the results with prolactin were specific. Yields of gamma IFN did not increase in the presence of growth hormone concentrations ranging from 10^{-7} *M* to 10^{-9} *M*. Indeed yields of gamma IFN in the presence of 10^{-7} *M* growth hormone were 10% lower than controls.

In Table I, we have examined the effects of various pharmacologic agents on the enhancing effect of prolactin on gamma interferon yields. We tested the effects of calmidazolium and H7 to determine if alterations in either calmodulin or protein kinase C dependent systems would eliminate the enhancing influence of prolactin on gamma IFN yields. Neither agent abolished this effect. Genistein, however, an inhibitor of tyrosine kinases was also tested. This agent reduced the titer of total IFN produced and also eliminated the enhancing influence of prolactin on interferon yields.

Table I. The Effect of Various Treatments on Enhancement of Interferon Yields by Prolactin

Treatment	IFN yield (U/ml)	*P* value[5]
Cells alone	0	—
PHA (5.0 ug/ml)	640 ± 160	—
PHA + PRL[1]	1280 ± 320	<0.05
PHA + H7[2]	700 ± 120	—
PHA + H7 + PRL	1280 ± 200	<0.5
PHA + CALMID[3]	640 ± 160	—
PHA + CALMID + PRL	1280 ± 300	<0.5
PHA + GENIS[4]	320 ± 200	—
PHA + GENIS + PRL	300 ± 80	NS

[1] PRL = Prolactin and used at a concentration of 10^{-8} *M*.
[2] H7 = 1-(5-isoquinolinyl sulfonyl)-2-methyl piperazine and used at a concentration of 10^{-7} *m*.
[3] CALMID = Calmidazolium and used at a concentration of 10^{-6} *M*.
[4] GENIS = Genistein and used at a concentration of 10.0 μg/ml.
[5] *P* value = *P* value for difference between this value and the result with PHA and the indicated compound without PRL.
[6] IFN yield = the titer is derived from a mean of eight determinations using PBMC from four individuals.

Table II. The Effect of Prolactin on the Proliferative Response

Treatment	Proliferative response[1] CPM × 10^3
Cells alone	1.5 ± 0.2
PHA (25 μg/ml)	27.1 ± 3.0
PHA (25) + PRL 10^{-9} *M*	25.8 ± 4.0
PHA (25) + PRL 10^{-8} *M*	25.8 ± 3.0
PHA (25) + PRL 10^{-7} *M*	29.0 ± 4.0
PHA (2.5 g/ml)	18.5 ± 5.0
PHA (2.5) + PRL 10^{-9}	18.9 ± 4.3
PHA (2.5) + PRL 10^{-8}	17.1 ± 4.3
PHA (2.5) + PRL 10^{-7}	20.5 ± 4.0

[1] Proliferative response represents the mean of 24 determinations using PBMC from 12 individuals.

Not shown in Table I is the effect of cholera toxin, a stimulator of adenyl cyclase. This agent was without effect on the prolactin mediated enhancement of IFN yields.

Table II shows the effect of prolactin on the proliferation of PBMC. No significant increase in proliferation was seen.

Figure 3 shows the effect of prolactin on the yield of human gamma IFN in the presence of hydrocortisone. It can be seen that even when using the lowest dose of hydrocortisone necessary to significantly decrease IFN yields, we could not demonstrate any ability of prolactin to reverse the adverse influences of hydrocortisone. Similarly, prolactin did not reverse the effect of hydrocortisone on proliferation (data not shown).

Finally, we determined if prolactin would also boost the level of alpha IFN. Using poly rI:rC as an inducer of alpha IFN, we were unable to demonstrate any influence of prolactin on alpha IFN production. Such tests were performed on three occasions using PBMC from three different individuals.

Discussion

In this communication we have established statistically that prolactin can significantly enhance interferon yields from PBMC when induced by either PHA or Con A. That prolactin can influence immune function is not new. Previous communications have established that lymphocytes bear prolactin receptors (4), that the binding of prolactin is blocked by cyclosporin

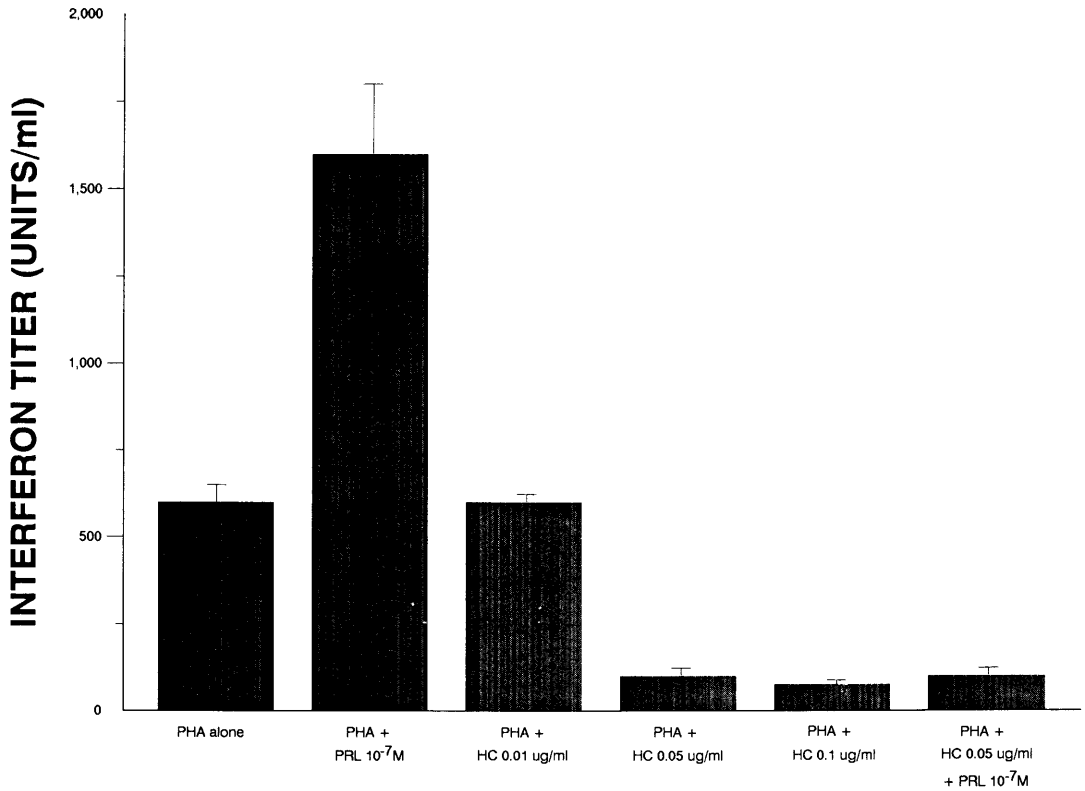

Figure 3. The effect of prolactin on gamma interferon production in the presence of hydrocortisone (HC). PHA was used at a concentration of 10 μg/ml. Each bar is based on a mean from five separate experiments done on different days using the blood of different individuals. The difference between the yield of gamma interferon in the presence of PHA with prolactin is significantly greater than that in the presence of PHA alone ($P < 0.05$). The difference between the yield of gamma interferon in the presence of PHA alone and PHA in the presence of each concentration of hydrocortisone is also significant ($P < 0.05$) save for the lowest concentration of hydrocortisone used (i.e., 0.01 μg/ml).

(4), and that inhibition of prolactin secretion in animals depresses lymphocyte proliferation (6, 7) and the ability to produce gamma IFN (7). Recently, nuclear prolactin has been shown essential for proliferation of T lymphocytes (11) in the presence of IL-2. What has not been heretofore appreciated is the fact that the presence of prolactin can result in significantly enhanced yields of gamma interferon by human PBMC taken from normal individuals not rendered hypoprolactinemic. Thus, we have shown that PBMC taken from donors denying significant underlying disease can produce increased amounts of gamma IFN in the presence of prolactin concentrations known to exist in the body (1). That this is the case is illustrated by the facts that the concentration of the IFN produced doubled in the presence of prolactin at 10^{-7} *M*, that there was a progressive increase in the titer of the IFN produced when the concentration of prolactin was increased, and that in 27 of the 30 experiments performed the titer of the IFN produced in the presence of prolactin was greater than the titer produced in its absence. In the other three cases, the titers in test and control samples were identical.

While prolactin enhanced production of gamma IFN, it did not increase the ability of lymphocytes to proliferate. Thus, in the presence of prolactin the proliferative response did not statistically significantly differ from that observed in the absence of prolactin. Similarly prolactin failed to reverse the adverse effect of hydrocortisone on gamma IFN production.

It is of interest that the use of genistein, a known antagonist of tyrosine kinases, was able both to reduce total IFN yields and to eliminate the prolactin mediated increase in IFN production. These results would suggest tyrosine kinases are involved in the production of gamma IFN and the enhancement of this process by prolactin. Although viability studies did not demonstrate that this concentration of genistein had an effect on cell viability we can not exclude the possibility that the effect of the genistein was merely a toxic one. Current studies are examining the effects of prolactin on the transcription of gamma IFN messenger RNA.

In contrast, prolactin in the same concentrations failed to influence production of human alpha IFN. This disparate result is similar to those we have observed in the past. Thus, while hydrocortisone (12) and somatostatin (13) have both been shown to decrease yields of human gamma IFN, neither affected yields of human alpha IFN.

These studies expand the known role of prolactin in the immune response and suggest that this hormone may not only be required for normal production but when present in high concentrations can serve to allow progressively enhanced production of gamma IFN. Increased production of gamma IFN was seen at a prolactin concentration of 10^{-8} *M* which is the concentration normally found in serum (1). The increased production of IFN was seen with PHA and Con A but not with the other inducers tested. That these results with increasing prolactin concentrations are specific is shown by the fact that growth hormone and other hormones tested previously (14) do not affect production of human gamma IFN under these circumstances.

Finally, we should ask what is the relevance of these findings? For these findings to have a physiological importance it will be necessary to find a natural equivalent of the lectins we have used or to find the enhancement in IFN yields associated with prolactin also occurs with natural antigens. It should be emphasized that the concentrations of prolactin employed here are within those concentrations encountered in the body (1). Of particular relevance is the high level of chorionic prolactin seen in amniotic fluid. Here prolactin may play an important role in regulating gamma IFN concentrations locally during pregnancy to enhance the expression of cell surface antigens and permit immune recognition of fetal antigens by maternal cells. It is also possible that these levels of prolactin are reached in certain tissues to allow for paracrine effects to occur on IFN production.

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