Original Article

Histochemical and Immunocytochemical Localization of Prolactin Receptors on Nb2 Lymphoma Cells: Applications of Confocal Microscopy¹

EDUARD MICHEL and JONATHAN A. PARSONS²

Department of Cell Biology and Neuroanatomy, University of Minnesota Medical School, Minneapolis, Minnesota.

Received for publication May 30, 1989 and in revised form January 31, 1990; accepted February 20, 1990 (9A1706).

We studied prolactin (PRL) binding sites on Nb2 lymphoma cells using two different light microscopic methods. First, histochemical detection was accomplished by using an aminomethyl coumarin–acetic acid-conjugated ovine prolactin molecule (AMCA-oPRL) on both glutaraldehyde-fixed and unfixed Nb2 lymphoma cells. Binding of AMCA-oPRL was studied after UV illumination and appeared as punctate fluorescence associated with many but not all cells. Binding was abolished when tissue sections were treated with excess unlabeled lactogenic hormones and was unchanged when a non-lactogenic hormone was used for displacement. Counting revealed significant differences between the number of labeled cells in populations known to exhibit up- or down-regulated PRL receptors. Second, indirect immunocytochemistry of Nb2 PRL receptors was accomplished by immunological detection of exogenously added ovine PRL using two antisera directed against ovine PRL. Visualization of the ligand–antibody complexes was accomplished by confocal laser scanning microscopy. Staining was restricted to a subpopulation of cells. The morphological results presented here add to the previous physiological and biochemical data on the presence of lactogenic hormone receptors on Nb2 lymphoma cells. (J Histochem Cytochem 38:965–973, 1990)

KEY WORDS: Nb2 lymphoma cells; Prolactin receptors; Histochemistry; Immunocytochemistry; Confocal microscopy.

Introduction

Nb2 lymphoma cells are dependent on lactogenic hormones for proliferation (Gout et al., 1980; Noble et al., 1980). Since being adapted to long-term tissue culture (Gout et al., 1980), these cells have been a widely used model for studying the interaction between lactogenic hormones and their receptors and the intracellular events that follow this interaction. This system has been used to address questions of structure–function relationship of lactogenic hormones (Gertler et al., 1986), receptor modulation (Larsen and Dufau, 1988; Ashkenazi et al., 1987a,b,c) and the effects of lactogens on gene expression and other events leading to cell proliferation (Murphy et al., 1988; Rayhel et al., 1988; Elsholtz et al., 1986; Fleming et al., 1985; Gertler et al., 1985).

High-affinity binding sites specific for lactogenic hormones were first demonstrated on Nb2 lymphoma cells by competitive binding assays by Shiu et al. (1983). Subsequent work confirmed the initial results on binding of ovine prolactin (oPRL) and human growth hormone (hGH) to these cells and generated additional data on the properties of these binding sites. The affinity of the Nb2 PRL binding sites was reported as being up to twentyfold higher than that of lactogenic receptors in other tissues, having Kd values between 3.7 x 10⁻⁹ M and 6.5 x 10⁻¹¹ M (Michel and Parsons, 1988; Ashkenazi et al., 1987a; Shiu et al., 1983). Several species of the Nb2 lactogenic receptor binding units have been reported. Shenk et al. (1987) reported two units with molecular weights of 65 and 42 KD identified by radioactive hormone crosslinking; Ashkenazi et al. (1987a) found two binding subunits with molecular weights of 29 and 75 KD lacking intermolecular disulfide bonds; and a 72–88 KD binding unit polymerized via disulfide bonds to the rest of the lactogenic receptor was reported by Webb and Wallis (1988). The density of lactogenic receptors on Nb2 cells also varied among studies, with reported values of 2900 (Ashkenazi et al., 1987a), 5000 (Michel and Parsons, 1988), and 12,000 (Shiu et al., 1983) receptors per cell. Despite being the object of such extensive investigations, the lactogenic receptors on Nb2 lymphoma cells have not been detected histochemically.

Fluorochrome-labeled ligands have been proven useful to study receptor–hormone interactions. The advantages of this method are the short time course of experiments and the elimination of intermediate steps present in indirect immunocytochemical methods which may lead to increased nonspecific binding. Fluorochrome-

¹ Supported by funds from the Minnesota Medical Foundation. The funds for acquisition of the confocal microscope and supporting equipment were provided by the Minnesota Medical Foundation, the University of Minnesota Medical School, and the University of Minnesota Graduate School.

² Correspondence to: Jonathan A. Parsons, PhD, Department of Cell Biology and Neuroanatomy, University of Minnesota, 321 Church Street SE, Minneapolis, MN 55455.
labeled corticotropin-releasing factor (Schwartz et al., 1986), calmodulin (Welsh, 1983), α-bungarotoxin (Ravdin and Axelrod, 1977), and low-density lipoproteins (Anderson et al., 1980) have been used as receptor markers for both fixed and unfixed tissues and for study of initial steps of receptor–ligand interactions. Similarly, oPRL can be conjugated to the fluorochrome FITC without compromising its bioactivity (Burleigh and Erickson, 1981), but the lack of reports on histochemical applications of FITC–PRL suggests that this molecule may not be appropriate for use as a histochemical probe for PRL receptors. Biotin-conjugated PRL (Michel and Parsons, 1988) retains both receptor binding activity and biological activity; however, it has not proven useful for histochemical use in conjunction with an avidin probe.

The development and validation of a novel oPRL–fluorochrome probe that is useful for localization of PRL binding sites was described recently (Michel and Parsons, 1989). The protein labeling agent employed was 7-amino-4-methylcoumarin-3-acetic acid [AMCA (Khalfan et al., 1986)], a compound that emits in the blue spectrum (440–460 nm) on activation with UV light (355 nm). With the use of this probe, histochemical evidence is presented on the heterogeneous distribution of PRL receptors on Nb2 cells. In addition, the histochemical findings are supported with immunocytochemical evidence of the presence of PRL receptors on Nb2 lymphoma cells. The indirect detection of PRL receptors in these studies was facilitated by the use of confocal microscopy, a technique that optimizes epifluorescent images by eliminating out-of-focus fluorescence and allowing optical sectioning of a specimen by adjusting the plane of focus (White et al., 1987).

Materials and Methods

Hormones. Ovine PRL (lot AFP-8277E), bGH (lot AFPA-7938), and rat GH (rGH, preparation RPI) were received from the National Hormone and Pituitary Program of the NIDDK. University of Maryland School of Medicine (Bethesda, MD).

Conjugation of oPRL. The reactive N-hydroxysuccinimide ester derivative of 7-amino-4-methylcoumarin-3-acetic acid (NH3-AMCA; Bio Carb Chemicals, Lund, Sweden) was linked to oPRL according to the procedure of Khalfan et al. (1986) as described previously (Michel and Parsons, 1989). Briefly, NH3-AMCA in DMSO was added at a five times molar excess to a solution of oPRL in 0.1 M NaHCO3, pH 8.1. The mixture was allowed to react at room temperature for 1 hr. The reaction was stopped by the addition of 1 ml ice-cold NaHCO3, followed by gel filtration on a 10 × 0.5-cm Bio-Gel P-4 column (BioRad; Richmond, CA) eluted with the NaHCO3 buffer. The protein fractions containing fluorescence were identified by illumination with an UV lamp and pooled. The buffer salts contained in the pooled fractions were removed by three washes with distilled water using Centricon-10 microconcentrators (Amicon Corp; Danvers, MA). The final product was concentrated and stored in frozen aliquots at −20°C for up to 4 months. The protein and AMCA concentrations of the product were determined by the BioRad method and absorbance at 355 nm (Khalfan et al., 1986), respectively. The AMCA content of the probe was expressed as the fluorochrome:protein ratio (F/P). In all the studies reported here, a probe with a F/P of 5:1 was used.

Nb2 Lymphoma Cells. Nb2 lymphoma cells (gift of Dr. C. T. Beer, University of British Columbia, Vancouver, BC) were maintained in culture as described previously (Parsons et al., 1984). Briefly, cells were maintained as suspension cultures in Fisher’s medium for leukemic mouse cells (FM) supplemented with 7% fetal calf serum, 10% horse serum, 100 µM β-mercaptoethanol, and antibiotics (penicillin 100 U/ml and streptomycin 100 µg/ml) at 37°C in a humidified atmosphere of 95% air–5% CO2. All sera and media were obtained from Gibco (Grand Island, NY). Sixteen to eighteen hours before use the cells were synchronized in stage G1 as described by Gout et al. (1980) by transfer to the above medium from which fetal calf serum was omitted (conditioning medium; CM).

Histochemistry. Both the histochemical and the immunocytochemical studies described below studied the distribution of PRL binding sites on Nb2 cells before and after cell fixation. To avoid possible blocking of lactogen–receptor interaction by harsh fixation (Salih et al., 1979), cells were fixed with 0.01% glutaraldehyde in 0.05 M PBS, pH 8.5, for 30 min at room temperature.

On the day of the experiment the cells were suspended in FM at a concentration of 5 × 105 cells/ml and placed on poly-L-lysine-coated glass slides. Both synchronous and asynchronous cells were allowed to settle onto slides for 30 min, after which they were rinsed with FM and fixed as described above. Unreacted aldehyde groups were blocked by washing the cells for 30 min with 0.2% glycine in PBS, pH 7.6. After fixation, the cells were rinsed with PBS and then incubated with the appropriate reagents diluted in PBS containing 3% BSA (PBS-BSA). All incubations were done in a humidified chamber at room temperature.

For total binding conditions, cells were incubated with 10 nM AMCA-oPRL in PBS-BSA for 1 hr. Competition studies were done by incubating the cells for 1 hr with a mixture of 10 nM AMCA-oPRL and 5 µM unlabeled oPRL, hGH, or rGH in PBS-BSA. For nonspecific binding controls using oPRL or AMCA, the cells were incubated for 1 hr with either of the two reagents at the concentration they were present in the probe (i.e., 10 nM oPRL and 50 nM AMCA for oPRL and AMCA, respectively). The cells were then washed with PBS-BSA (three times for 10 min) and coverslipped using a glycerol-based medium containing the anti-fade agent p-phenylenediamine (Johnson et al., 1982). The slides were viewed with a Zeiss standard microscope equipped for transmitted darkfield illumination, using a LP410 barrier filter. Photographs were taken on Technical Pan film (Kodak).

Labeled cells were counted by four independent observers on coded photomicrographs (×252 magnification) of total binding and nonspecific binding conditions. Cells were considered positive if punctate staining was present in association with the cell profile. The results were expressed as percent of the cells showing punctate fluorescence to the total number of cells evaluated. The values obtained by observers for each experimental group were expressed as mean ± SEM. Significance of differences between groups was determined using Student’s t-test.

Histochemistry of PRL receptors on unfixed Nb2 cells in suspension was done on synchronized cells. Nb2 cells were re-suspended in CM and incubated with 10 nM AMCA-oPRL (total binding) or 10 nM AMCA-oPRL and 5 µM oPRL (nonspecific binding) for various periods of time (5 min to 4 hr) at 37°C in a humidified atmosphere of 95% air–5% CO2. After incubation, cells were collected by centrifugation (3 min at 300 × g), washed with PBS (three times for 10 min), and fixed with 2% paraformaldehyde in PBS for 30 min on ice. After rinsing with PBS (three times for 10 min), the cells were coverslipped and viewed with the same Zeiss microscope set-up.

Immunocytochemistry. The primary antisera used in these studies were raised in adult guinea pigs against oPRL NIH-PS12 (antiserum 1-C and 5-B) and rPRL NIH-B-1 (antiserum 14-A) as described previously (Schulte et al., 1980). In radioimmunossay, at the titer used for immunocytochemistry, antiserum 1-C and 5-B bound 82.8% and 79.6% of the [125I]-oPRL, respectively, and antiserum 14-A bound 85.7% of [125I]-rPRL (unpublished observations).

For immunocytochemical studies employing fixed cells, synchronized Nb2 lymphoma cells were allowed to settle on poly-L-lysine-coated slides, fixed, and rinsed with 0.2% glycine as described above for histochemical studies. Cells were then incubated with (total binding) or without (nonspecific binding) 5 µM oPRL for 1 hr in a humidified chamber at room
temperature. The cells were rinsed with PBS (three times for 10 min) and then fixed with 2% paraformaldehyde in PBS for 10 min on ice. After washing in PBS (three times for 10 min), the cells were incubated with one of the three anti-PRL antisera (diluted 1:100 in PBS buffer containing 0.1% Triton X-100 and 1% normal goat serum (PTGS)) for 1 hr at room temperature and then washed overnight in the same buffer. The following day the cells were incubated for 1 hr with fluorescein isothiocyanate (FITC)-conjugated goat anti-guinea pig IgG (Cappel Laboratories; West Chester, PA) diluted 1:50 with PTGS which had been pre-absorbed overnight with mouse liver powder (Cappel). Before coverslipping and viewing, cells were washed with the PTGS buffer (three times for 1 hr).

Unfixed NB2 lymphoma cells were incubated in suspension with or without 5 mM oPRL in CM for 1 hr at 37°C in a humidified atmosphere of 95% air-5% CO₂. The cells were collected by centrifugation (3 min at 300 × g), washed with FM (three times for 10 min), and then fixed with 2% paraformaldehyde in PBS for 30 min on ice. All washes and the incubations with the guinea pig anti-oPRL antisera and with FITC-conjugated secondary antibodies were done as described above. Before viewing with the confocal microscope, both sets of cells were coverslipped using the antifade agent p-phenylenediamine described above.

The system used in this study consists of a MRC-500 Confocal Imaging System (BioRad Microscience; Boston, MA) and an Olympus BH-2 microscope equipped for epifluorescence. The incident beam (the 488-nm wavelength of an argon ion laser) passes through a pinhole aperture and is scanned in a raster pattern across the specimen being viewed by a pair of computer-controlled galvanometer mirrors. The fluorescent light from the illuminated spot is returned to the same galvanometer mirrors and focused on a pinhole aperture in front of a low-noise photomultiplier tube. This imaging technique is called confocal because the image of the illumination pinhole and the detector pinhole have a common focus in the specimen (White et al., 1987; Wilson and Sheppard, 1984). The digitized photomultiplier output at each point in the raster scan is assembled into an image of up to 768 × 512 pixels by means of a frame buffer in the microcomputer. The frame buffer is also used to integrate a series of successive frames to improve the signal-to-noise ratio. In the studies reported here, each photomicrograph was the average of 100 successive frames.

Polyacrylamide Gel Electrophoresis and Protein Blotting. Polyacrylamide gel electrophoresis (PAGE) was carried out on 8% polyacrylamide gels under both non-reducing and reducing conditions. Analytical PAGE was accomplished at pH 8.9 in a buffer containing 25 mM Tris and 192 mM glycerine according to the procedure of Davis (1964) and Ornstein (1964). After staining with Coomassie, the gels were scanned using an Eikonix digitizing camera (Bedford, MA) and densitometer-like curves were generated. The relative amount present in each of the protein bands was calculated by integrating the area under the peaks corresponding to each of the bands, using software developed by International Imaging Systems (Milpitas, CA). PAGE under reducing conditions was accomplished on 8% polyacrylamide slab gels containing 0.1% SDS and 2% β-mercaptoethanol in a pH 8.4 discontinuous buffer system, as described by Laemmli (1970). After electrophoresis, the gels were transblotted to nitrocellulose membranes (Towbin et al., 1979). Detection of transferred proteins was accomplished by UV illumination (Michel and Parsons, 1989) and by immunostaining (Michel and Parsons, 1988), as described previously.

Results
The histochemical probe used in these studies was obtained by conjugating the chemically reactive A-hydroxyaceticimide ester derivative of the fluorochrome AMCA to oPRL. Using the conjugation protocol described above, we obtained a probe with a 5:1 F/P. As seen in Figure 1, AMCA was linked to several forms of immunoreactive oPRL (21, 23, and 45 KD PRL) with no apparent change in

![Figure 1](attachment:image1.png)  
**Figure 1.** Protein blots of SDS-PAGE under reducing conditions of oPRL (lanes 1 and 1′) and AMCA-oPRL with a F/P of 5:1 (Lanes 2 and 2′). (Lanes 1 and 2) Immunodetection with anti-oPRL antiserum. (Lanes 1′ and 2′) Detection by UV illumination. Approximately 10 μg protein was applied to each lane.

![Figure 2](attachment:image2.png)  
**Figure 2.** Analytical polyacrylamide gel of unreacted oPRL (Lane 1) and AMCA-oPRL (5:1 F/P) stained with Coomassie (Lane 2) and seen with UV illumination (Lane 3). The densitometer tracing shown in the top panel corresponds to the protein bands in lane 2. The area under the peaks corresponding to the protein bands was calculated and the unreacted oPRL was expressed as percent of total protein present. The arrow labeled alb indicates the migration position of bovine serum albumin.
the molecular weight of the complex. Some dissociation of AMCA from oPRL occurred, either during the preparative steps for PAGE or during the electrophoresis run itself, as illustrated by the fluorescent band migrating with the ion front ahead of the protein bands. A more evident change in the characteristics of the AMCA-PRL complex was evident on analytical PAGE. Under non-reducing conditions the AMCA-PRL complex migrated more rapidly than oPRL owing to loss of positive charges secondary to conjugation (Figure 2). Densitometric analysis of non-SDS, non-reducing gels revealed that approximately 14% of the oPRL present in the histochemical probe was not conjugated to AMCA.

The AMCA-conjugated oPRL molecule was found suitable for labeling of PRL binding sites on Nb2 lymphoma cells. As seen in Figure 3, binding of AMCA-oPRL to fixed Nb2 cells resulted
in a punctate fluorescence associated with some of the cells and in some cases appeared to surround the entire cell. Competition of AMCA-oPRL with 5 μM unlabeled oPRL or hGH resulted in marked decrease of bound fluorescence (Figures 3c and 3d, respectively), whereas competition with the same dose of rGH, a nonlactogenic hormone, resulted in minimal decrease of binding (Figure 3e). Furthermore, when asynchronous Nb2 cells were used for these studies, the binding pattern closely resembled that of synchronized cells. However, the number of fluorescent cells was significantly reduced (Figure 3f; Table 1). Incubation of cells with AMCA or oPRL administered individually resulted in no noticeable fluorescence (data not shown). When Nb2 cells were incubated with AMCA-oPRL in suspension before fixation, maximum binding was noted at 1 hr and appeared as cap-like patterns visible on most cells (Figure 4a). Binding was abolished when excess unlabeled oPRL was included in the incubation medium in addition to AMCA-oPRL (Figure 4b).

Indirect immunocytochemistry of PRL receptors employed incubation of synchronous Nb2 lymphoma cells with oPRL followed by fixation of ligand to binding sites, immunodetection, and confocal microscopy. Use of two different anti-oPRL antisera revealed a heterogeneous pattern of binding similar to the one observed by histochemistry with AMCA-oPRL (Figures 5a and 5c; Table 2). Immunoreactivity appeared to be distributed throughout the cell, which was confirmed with optical sectioning. Control cells that were not incubated with oPRL or were incubated with oPRL followed by anti-oPRL antiserum were not immunoreactive (Figures 5b and 5d–f; Table 2). In addition, no fluorescence was associated with cells that were incubated with normal guinea pig serum followed by FITC-labeled second antibody, or with secondary antiserum alone (data not shown). If the cells were placed on slides and fixed before exposure to oPRL, the staining was limited to the periphery of the cells, in a pattern similar to the binding of AMCA-oPRL to fixed cells (Figure 6a; Table 3). No immunoreactivity was present when either the primary ligand or the primary antiserum were omitted (Figures 6b and 6c; Table 3). Use of a confocal microscope for observation of the cells was essential for these immunocytochemical studies. The confocal microscope eliminated out-of-focus noise present in conventional epifluorescence microscopy which made it difficult to localize immunohistochemical staining. In addition, the signal-to-noise ratio for excitation of AMCA prevented us from analyzing the localization of AMCA-oPRL on unfixed Nb2 cells by the confocal microscopy technique. Therefore, even though AMCA-oPRL appeared not to be internalized by Nb2 cells, a definite conclusion as to whether AMCA-oPRL gained entry into the cells could not be drawn.

**Discussion**

For the present studies, an AMCA-conjugated oPRL molecule was used to visualize lactogenic receptors on Nb2 lymphoma cells. The morphological results presented in this report agree with previous biochemical and physiological data and provide histochemical and immunocytochemical evidence on the presence of PRL binding sites on Nb2 lymphoma cells. Binding of the probe was specific, as illustrated by the fact that competitive binding with lactogenic hormones resulted in diminished fluorescence. The AMCA-oPRL probe bound to select cells only, serving as a marker for cells expressing PRL receptors in heterogeneous populations. The fact that not all Nb2 lymphoma cells

---

**Table 1. Distribution of AMCA-oPRL-labeled cells as a function of culture condition**

<table>
<thead>
<tr>
<th>Cell population</th>
<th>Incubation conditions</th>
<th>% cells labeled*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synchronous</td>
<td>TB</td>
<td>30.1 ± 2.1 (1390)</td>
</tr>
<tr>
<td></td>
<td>NSB</td>
<td>26.7 ± 4.1 (1267)</td>
</tr>
<tr>
<td>Asynchronous</td>
<td>TB</td>
<td>28.5 ± 1.3 (1087)</td>
</tr>
<tr>
<td></td>
<td>NSB</td>
<td>12.8 ± 0.6 (1065)</td>
</tr>
</tbody>
</table>

* Synchronous and asynchronous cultures were obtained by culturing cells in the presence or absence of 7% fetal calf serum, respectively.

**Figure 4. Histochemical demonstration of PRL binding sites.** Unfixed Nb2 lymphoma cells were incubated with 10 nM AMCA-opPRL (a) or 10 nM AMCA-oPRL + 5 μM oPRL (b) for 1 hr at 37°C. Bar = 10 μm.
Figure 5. Indirect immunocytochemistry of PRL binding sites. Synchronized Nb2 lymphoma cells were immunostained for oPRL or rat PRL after incubation with 10 μM oPRL or vehicle for 1 hr at 37°C and then fixed with 2% paraformaldehyde for 1 hr on ice. Cells represented in the panels above were treated as indicated in Table 2. After incubation with FITC-conjugated goat anti-guinea pig IgG the cells were viewed with a confocal microscope. To improve signal-to-noise ratio, 100 successive frames were integrated for each optical section obtained. Bar = 10 μm.

Table 2. Treatment of Nb2 cells for indirect immunocytochemistry of PRL binding sites before fixation

<table>
<thead>
<tr>
<th>Panel</th>
<th>Ligand</th>
<th>1° Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>oPRL</td>
<td>GP-anti-oPRL #1-C</td>
</tr>
<tr>
<td>b</td>
<td>-</td>
<td>GP-anti-oPRL #1-C</td>
</tr>
<tr>
<td>c</td>
<td>oPRL</td>
<td>GP-anti-oPRL #3-B</td>
</tr>
<tr>
<td>d</td>
<td>-</td>
<td>GP-anti-oPRL #3-B</td>
</tr>
<tr>
<td>e</td>
<td>oPRL</td>
<td>GP-anti-rPRL #14-A</td>
</tr>
<tr>
<td>f</td>
<td>-</td>
<td>GP-anti-rPRL #14-A</td>
</tr>
</tbody>
</table>

expressed PRL binding sites is not surprising in view of a previous report describing a similar heterogeneous pattern of expression of lactogenic receptors on cultured mammary tumor cells (Paterson et al., 1982). Furthermore, the present study showed that significantly more synchronous Nb2 cells bound AMCA-oPRL than asynchronous cells. A study by Ashkenazi et al. (1987a) addressed the question of receptor up- and down-regulation as a function of the growth medium. Their results show that cells maintained in lactogen-supplemented medium have fewer receptors on their surface, whereas synchronous cells conditioned in lactogen-free medium
express higher numbers of lactogenic receptors. Our results provide morphological evidence that maintenance of Nb2 cells in lactogen-supplemented medium induces receptor down-regulation. As illustrated, fewer asynchronous cells bind AMCA-oPRL; however, because the sensitivity of this technique with regard to the number of receptors necessary for visualization of bound AMCA-oPRL is not known, a conclusion as to whether cells possess greater numbers of receptors as well cannot be drawn.

When Nb2 cells were incubated with AMCA-oPRL before fixation, no diffuse fluorescence over the entire surface of the cells, suggestive of internalization, was noted. Instead, most cells showed fluorescent "caps" even after 4 hr of incubation at 37°C, suggesting that the fluorochrome-conjugated lactogen could not be internalized by the Nb2 lymphocytes following binding to the cell surface. With respect to our previous observations on bioactivity of AMCA-oPRL probes (Michel and Parsons, 1989), these observations suggest two possibilities. First, the interaction between a bivalent ligand and lactogenic receptors is the only signal necessary for inducing proliferation of Nb2 lymphoma cells. A similar finding was reported by Shiu et al. (1983), who found that divalent (Fab2) but not monovalent (Fab) fragments of anti-PRL receptor antibodies could produce a PRL-like effect with respect to cell proliferation, presumably by cross-linking the binding sites. A second possibility suggested by our results is that AMCA-oPRL was completely devoid of biological activity and that the limited biological effects noted in bioassays were due to the unconjugated PRL present. Our previous studies have found a limited ability of AMCA-oPRL to induce proliferation of Nb2 lymphoma cells in vitro (Michel and Parsons, 1989). However, the present findings indicate that the histochemical probe used for histochemical studies and for bioassay determinations contains a minor amount of unconjugated PRL as a contaminant. This limited amount of oPRL could be sufficient to produce the biological effects noted in vitro.

Indirect immunocytochemistry of PRL binding sites employing incubation with oPRL followed by fixation, immunodetection, and confocal microscopy was performed to support the findings obtained by direct histochemistry using AMCA-oPRL. The similarity of the staining pattern obtained using two different specific antisera against oPRL supports our claims for staining specificity. Moreover, the immunocytochemical results were not related to the use of guinea pig serum per se, as neither normal pig serum nor guinea pig anti-rat PRL bound to cells that had been incubated with oPRL before fixation. When immunocytochemistry was attempted using the rat species of PRL as primary ligand followed by anti-rPRL antiserum, the results obtained were negative. We assume this was due to the tenfold decrease in the biopotency of the rat hormone when compared to oPRL (Tanaka et al., 1980).

Both histochemical and immunocytochemical methods for localization of PRL binding sites on glutaraldehyde-fixed Nb2 lymphoma cells produced similar results; in both cases the fluorescence was localized mainly at the cell periphery, suggesting binding of AMCA-oPRL and oPRL to cell surface-fixed binding sites. The detergent Triton X-100 was included in all buffers used for immuncytochemistry after incubation with PRL, therefore allowing access of primary and secondary antisera to the interior of the cell. The fact that no immunoreactivity was detected inside the cell led to the conclusion that the ligand was not internalized following cell fixation. In contrast, the cells incubated with oPRL before fixation showed immunoreactivity distributed throughout the cell, suggesting uptake of the lactogen. As discussed by Shiu et al. (1983), Nb2 cells incubated with radiolabeled lactogen at 37°C show maximum binding at 1 hr followed by ligand degradation, presumably after intracellular uptake. In addition, several morphological studies have detected internalization of the lactogenic receptors, both at the ultrastructural level (Josefsberg et al., 1979) and the light microscopic

Table 3. Treatment of Nb2 cells for indirect immunocytochemistry demonstration of PRL binding sites after fixation

<table>
<thead>
<tr>
<th>Panel</th>
<th>Ligand</th>
<th>Antiserum</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>oPRL</td>
<td>GP-anti-oPRL #1-C</td>
</tr>
<tr>
<td>b</td>
<td>oPRL</td>
<td>Normal guinea pig serum</td>
</tr>
<tr>
<td>c</td>
<td></td>
<td>GP-anti-oPRL #1-C</td>
</tr>
</tbody>
</table>

See Figure 6.
level (Meuris et al., 1983; Dunaif et al., 1982; Nolin, 1978; Witorsch and Smith, 1977; Nolin and Wittorsch, 1976). The results obtained by incubating Nb2 cells with oPRL before fixation followed by immunodetection confirm previous indirect observations that PRL is taken up into the cells.

As discussed by Hughes et al. (1987), studies on PRL receptor regulation in heterogeneous tissues will require a combination of quantitative biochemical studies to measure changes in receptor binding parameters and morphological studies to identify the cells and tissues responsible for the observed changes. The morphological tools suggested were fluorochrome-labeled lacticgenic hormones, as FITC-labeled hormones have been used previously to distinguish between receptor-positive and -negative cells in heterogeneous tissues (Dandliker et al., 1978). The present results suggest that the fluorescent molecule AMCA-oPRL may also be a useful tool for identification of cells and tissues responsible for PRL binding in lacticogenic organs.

Acknowledgments

We wish to thank Todd Breije for his assistance with the confocal microscopy and Dr. Martin Wiesenfeld for helpful discussions on fluorescence techniques.

Literature Cited


of corticotropin-releasing factor (CRF) target cells and effects of dexamethasone on binding in anterior pituitary using a fluorescent analog of CRF. Endocrinology 119:2376