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PROLACTIN-DEPENDENT Nb2 LYMPHOMA CELL LINE

Origin

The cultured, prolactin-dependent Nb2 cell line was established from a transplant of a lymphoma which originally developed in the thymus/lymph node of a male Noble (Nb) strain rat following prolonged treatment with estrogen.

Reference #1: Gout, P.W., C.T. Beer and R.L. Noble.
Cancer Research 40: 2433-2436, 1980.

General Properties

The Nb2 cells are of pre-T cell origin (1-3) (see also page 6). Their proliferation depends on prolactin (or other lactogen) acting as a growth factor (1). The mitogenic response of the cells is highly specific for mammalian lactogens (4) and is mediated by specific cell surface receptors (5). The cells have a modified prolactin receptor, i.e. the Nb2 form (6).

Nb2 cells can also be mitogenically stimulated by interleukin-2 (7).

When Nb2 cells are injected subcutaneously into Noble rats, they give rise to rapidly growing, malignant tumours. These tumours are highly sensitive to treatment with vinca alkaloids, such as vinblastine and vincristine, even when they are very far advanced (8).

Karyotypic analysis has shown that the original, prolactin-dependent Nb2 cell line has only five well-defined chromosomal abnormalities (9).

General applications

1. *in vitro* bioassay of lactogenic hormones of a variety of species, including prolactins, placental lactogens, and primate growth hormones (4). The assay is useful for measuring levels of lactogens in body fluids and for lactogen structure-function studies (10). See "Bioassay section" below.

2. studies into the molecular mechanisms mediating lactogen-induced mitogenesis (11,12,18). This usually involves the use of lactogen-starved, early G_1-arrested cells (please see below).

3. studies of the mechanism of action of vinca alkaloids (13).

4. studies of tumour progression, including development of growth factor (lactogen) independence and metastasis. This involves the use of a number of cloned sublines (14,9,11,16,17).

Recent review: Ref. 19, December 1995 Editorial of "Endocrinology."

Nb2 Cell Culture Propagation

The prolactin-dependent Nb2 cells are maintained at 37°C in suspension culture, in closed tissue culture flasks, in an atmosphere of 5% CO_2-95% air. Details of culture medium composition and cell handling are given below.

The cells should not run out of lactogen, in order to avoid the development of lactogen-independent cells. In view of this, the culture maintenance should contain high concentrations of lactogen. This is usually provided by having fetal bovine serum (containing placental lactogen and prolactin) in the medium at 10%. It may be noted that bovine serum, in contrast to fetal bovine serum, contains much lower lactogen levels.

Also, one should avoid overgrowth of the cell cultures. "Eye-balling" of the cultures to evaluate the need for subculturing is not recommended. A preferred method for subculturing is given below.

2-Mercaptoethanol (2-ME; 33-100 uM) is essential for a proper mitogenic response of the cells to lactogens; higher 2-ME concentrations are toxic. While 2-ME was previously used at 100 uM, 50 uM may be better. 2-ME mediates uptake of cystine, an essential amino acid for Nb2 cells, which is present in Fischer's medium at relatively low amounts.

CULTURES ARE STORED AS ALIQUOTS IN LIQUID NITROGEN (NOT AT -70C) AND RESURRECTED WHEN REQUIRED. AS A ROUTINE, CULTURES ARE NOT MAINTAINED AT 37C FOR MORE THAN TWO MONTHS.

SUB-CULTURING

A. General procedure

All handling of maintenance cultures is done under sterile conditions (e.g., flaming) in a laminar flow hood.

We routinely maintain the cell line as two parallel cultures in separate, closed 80 sq. cm. tissue culture flasks.

Before subculturing, the cultures are inspected under the microscope for contamination (e.g., bacterial), general health (as indicated by the refractility of the cells), morphology and cell size.

The flasks are then lightly shaken to ensure lifting of cells off the bottom and a homogeneous cell suspension. A 1.0 ml portion is then taken for cell counting (usually by electronic cell counter).

Following cell counting, appropriate amounts of fresh medium (37C) are put in new 80 sq.cm. flasks (minimum 15 ml, maximum 40 ml). Appropriate amounts of cell suspension (see below), calculated on the basis of the cell counts, are then added. The flasks are gassed with 5% CO_2-95% air (through a sterile, cotton-plugged pipet) to replace the air (e.g., for 20 sec), capped and put at 37C for incubation, in a horizontal position.

Notes:

1. Avoid using dilutions of cell suspensions with fresh medium at ratios greater than 1:10. Instead, the appropriate number of cells should be centrifuged (3-4 min at 350xg) and the cell pellet resuspended in fresh medium.

2. **PROTECT FISCHER'S MEDIUM (and cultures) FROM WHITE FLUORESCENT LIGHT SINCE THE LATTER GIVES RISE TO RIBOFLAVIN-MEDIATED PHOTOTOXICITY. USE OF YELLOW FLUORESCENT TUBES (OR WHITE FLUORESCENT TUBES IN UV-LIGHT ABSORBING SLEEVES, OR TUNGSTEN LIGHT) IS RECOMMENDED.** Ref. #20.

B. Starting cell concentrations

The cells have a culture doubling time of 12-13 hours.

As a routine we subculture the cells twice a week, using initial cell concentrations of 4,000 cells/ml and 12,000 cells/ml for growth periods of 96 and 72 hours, respectively. During maintenance we avoid growing the cells to concentrations higher than 900,000 cells/ml. We always make a note of the culture doubling time, to monitor growth.

C. Media composition - on basis of 100 ml

	Medium for active growth	Medium for stationary phase
Fetal bovine serum (containing lactogens needed for mitogenesis)	10 ml	--
Horse (gelding) serum#, lactogen-deficient	10 ml	10 ml
Fischer's medium*	77 ml	87 ml
Penicillin (5000 U/ml)/ streptomycin (5000 ug/ml)	1 ml	1 ml
Extra Na-bicarbonate (7.5%)	1 ml	0.7 ml
2-mercaptoethanol (5 mM, in saline, filter-sterilized, stored at -20C; added before use)	1 ml	1 ml

Incubation in 5% CO_2-95% air at 37C gives a pH of 7.5

#Lactogen deficiency is only important for bioassays, not for culture maintenance

*Containing bicarbonate as per manufacturer's instructions

Note:

For bioassays and mitogenic signaling pathway studies it is essential to use non-mitogenic horse (gelding) serum; as an alternative chemically defined medium can be used. Commercial horse serum is often obtained from a mixed herd and may then be mitogenic with regard to Nb2 cells. One may have to try several batches of horse serum. We have had good experiences with: ICN Flow Laboratories (CA). Other US suppliers include: Central Biomedia, (800) 448-0016
High Clone, Logan, Utah (800) 492-5663

To test the horse gelding serum, cells are washed free of fetal bovine serum (see below) and resuspended (e.g., at $3x10^5$ cells/ml) in medium, containing the test serum at 10% and 2-ME (50uM), for incubation at 37C. Culture growth should come to a stop within 24 hr and cells should decrease in size. Subsequently, major cell lysis should occur.

MODIFIED LACTOGEN Nb2 CELL BIOASSAY (August 1992)

Explanation of abbreviations:

FM = Fischer's medium containing penicillin (50 U/ml)/streptomycin (50 ug/ml)
10FBS = fetal bovine serum at 10%
1FBS = fetal bovine serum at 1%
10HS = lactogen-deficient horse (gelding) serum at 10%
2-ME = 2-mercaptoethanol, used at 50 uM final concentration
HEPES = HEPES buffer (1 M, pH 7.2) used at 10 or 15 mM

PROTOCOL

1. Grow cultures of PRL-dept Nb2 cells in 10FBS/10HS/FM/2-ME in 5% CO_2-air to (7-9)x10^5 cells/ml (log phase). Doubling time = 12-13 hr.

2. Centrifuge the cells 4 min at speed 3 [table top Clinical centrifuge (about 350xg)] and resuspend the cell pellet in lactogen content-reduced, 1FBS/10HS/FM/2-ME to a concentration of about 2.5x10^5 cells/ml.

3. Further incubate the cells for about 24 hours at 37C in 5% CO_2-air [expect to get (6-7)x10^5 cells/ml].

4. Then wash cells free of extracellular lactogen (present in FBS: i.e., bPL, bPRL) using HEPES/10HS/FM/2-ME, i.e. three times. Washing is done by centrifuging cells (see above) and resuspending the pellet in the medium (e.g., 50 ml). The third and final cell resuspension is done to a concentration of **2.2x10^5 cells/ml**. N.B. Cell losses up can go up to 20% during the washing procedure.

5. Distribute 1.80 ml aliquots in 12-well tissue culture plates.

6. Put plates in water-saturated 37C incubator in an atmosphere of 5%CO_2/95%air.

7. Add PRL standard solutions (200 ul; triplicates) in <u>a series of dilutions</u> to wells of the plates (to create a standard growth response curve). Then add samples at a series of dilutions (e.g., four different dilutions) to wells, one plate at a time. Also have standard PRL solutions in the last plate to monitor possible shifts in cell numbers during the assay. PRL standards are used at final conc's (ng/ml) of 0; 0.0039, 0.0078, 0.0156, 0.0312, 0.0625, 0.125, 0.25, 0.50, 1.0 (see prolactin solution preparation, below).

8. Incubate plates for about 48 hr at 37C.

9. Determine cell populations in plates, sampling homogeneous 1.0 ml portions from the plates in the same order as that used to add the standard PRL/samples to the wells.

10. Construct PRL standard-growth response curve for the determination of the "lactogen activity" in the undiluted samples.

Preparation of (standard) prolactin solutions

An amount of prolactin (0.5-1 mg) is carefully weighed using a microbalance. Following transfer to a glass tube, it is dissolved in 2.0 ml 0.01 N NaOH (4°C). The preparation usually dissolves readily with gentle vortexing. Then 500 ul of 0.05 M KH_2PO_4 is quickly added for neutralization (pH 7.4) and medium containing 10% non-mitogenic horse serum is added to get a solution of 100,000 nanog/ml. This solution is further diluted with the medium to 10,000 ng PRL/ml. Aliquots are snap-frozen and stored at -20C. It is not necessary to filter-sterilize PRL solutions for 2- or 3-day incubations; filter sterilization can lead to losses of the hormone.

Composition of 10HS/HEPES/FM/2-ME

100 ml basis:	10HS/FM	= 98.0 ml	
	HEPES (1M, pH 7.2)	= 1.0 ml	
	2-ME (5 mM)	= 1.0 ml	Final pH is about 7.4.

Notes

1. The HEPES buffer prevents major fluctuations in the pH, especially during during distribution of cell aliquots and addition of samples. However, one does not have to use HEPES. **In the absence of HEPES, medium has to be pre-gassed with 5% CO2 to get a pH of about 7.4.**

2. **Following incubation in 1FBS/10HS/FM/2-ME, the Nb2 cells are not stationary.** If one wants stationary, i.e. early G1-arrested Nb2 cells one should use the following procedure.

PROCEDURE FOR GETTING STATIONARY, EARLY G1-ARRESTED Nb2 CELLS

1. Grow cultures of PRL-dept Nb2 cells in 10FBS/10HS/FM/2-ME in 5% CO_2-air to (7-9)x10^5 cells/ml (log phase). Doubling time = 12-13 hr.

2. Centrifuge the cells 4 min at speed 3 [table top Clinical centrifuge (about 350xg)] and resuspend the cell pellet in lactogen-deficient HEPES/10HS/FM/2-ME (e.g., 50 ml). Repeat this once.

3. Centrifuge the cells for a third time and resuspend the cell pellet in the medium to a concentration of about 3x10^5 cells/ml.

4. Further incubate the cells for 18-24 hours at 37C in 5% CO_2-air. Expect to get an increase of about 60% in the cell numbers.

5. To get a mitogenic response, simply add PRL (lactogen) to the cultures. Resuspension of the cells in fresh medium is not needed and, in fact, not recommended since it may lead to a major disturbance of the cells, which are fragile, and e.g., an increase in ODC activity.

Notes

The average cell size in the culture so treated is substantially smaller than in log phase cultures (and some cells may escape being counted in the electronic cell counter); the cells are also more fragile. Flow cytometric analysis of DNA content has shown that 95% of the cells in the stationary culture are in the G1 phase of the cell cycle (14). Their biochemical response to addition of PRL indicates that they have accumulated in early G1 (15).

Serum-free medium:

The Nb2 cells can be grown in serum-free medium, for example, Fischer's medium (FM) containing:

HEPES (10 mM)

*iron-saturated human transferrin (8.25 ug/ml)
*bovine insulin (15 ug/ml)
*sodium pyruvate (165 ug/ml)
*sodium selenite (0.01005 ug/ml)
*ethanolamine (3 ug/ml)

penicillin (50 units/ml)/streptomycin (50 ug/ml)

2-mercaptoethanol (2-ME; 50 uM)

#(lactogen-free) fatty acid-free BSA (2 mg/ml; Boehringer Mannheim No. 1081489)

$prolactin

Note:*components come as one preparation from GIBCO (GMS-S, 100x), used at 1.5 ml per 100 ml medium. If GMS-S is not available, just using transferrin and insulin will be OK.

#BSA can be left out, but **be especially gentle when resuspending cells in protein-free medium**. Go for relatively high cell concentrations and then dilute to the desired final concentration.

$Use prolactin at a high concentration when BSA is left out, e.g, 100 ng/ml. Make the PRL up in protein-free medium. Do not filter-sterilize the solution since most of the hormone could be lost.

Pre-T cell origin of Nb2 lymphoma cells

I. As reported in Cancer Res. 42: 3138-3141, 1982 (ref. 2):
PRL-dependent Nb2 parent cells do not express surface immunoglobulin.
100% of cells bound monoclonal antibodies raised against:

a. rat thymocyte W3/25-HLK, a determinant on T-helper cells
b. OX8-HL, which identifies nonhelper T-cells.

II. As reported in Immunology 66: 83-89, 1989 (ref. 3):

Binding of PRL-dependent Nb2 cells by mAb, including:

OX18, NR3/31, NR5/10	95+ %	MHC class I
OX6	0 %	MHC class II
OX52	65+ %	Mature T cells and thymocytes
W3/13	90+ %	All T cells, myeloid & plasma cells
OX44	95+ %	All lymphoid cells, 12% thymocytes
OX12	0 %	B cells

See these references for more antibody information.

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