EFFECT OF ADDITION OF PROTON CARRIERS IN CULTURE MEDIUM ON GROWTH AND SECRETION OF HYBRIDOMA CELL LINE OKT3

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Abstract : Monoclonal antibodies (MAb) constitute the centre of all *in-vitro* diagnostic measures and almost all *in-vivo* therapeutic manoeuvres now. Production emphasis for these antibodies is having a current shift from animal-based large-scale culture to *in-vitro* bioreactor-based high-density culture. One of the major difficulties in high-density culture is end-metabolite accumulation in batch and fed-batch cultures in the forms of H^+, NH_4^+ etc.. thereby reducing cellular growth and secretions. In the present study, effects of added proton carries --- NAD and NADP --- over and above the metabolic pools of the molecules, were examined on the cellular growth and secretion kinetics. Although NADP fortification showed a remarkable improvement in cellular growth (time dependent 200–300% improvements compared to controls) and size, cumulative MAb titre was better with NAD fortification. Combined additional loads of the proton carriers would be interesting to study in high density culture conditions.

Key words : chemical additives   high-density culture
       hybridoma   cell growth

INTRODUCTION

Cationic oligomeric amino acids, poly-I-lysine and poly-l-arginine stimulate cellular productivity of immunoglobulins (1). Proteins rich in cationic amino acids such as histones and protamine also stimulate IgM production rate to varying degrees (1). Monomeric L-lysine has been observed to have stimulatory effect on cellular hybridoma growth *in vitro* (2). Innovation of strategies to achieve higher yields of monoclonal antibodies (MAb) of defined affinity and specificity through high-density culture for clinical applications is currently a thrust area of research. Various approaches have been made and these include varying media formulations (3), new additives-both of biochemical and chemical nature (4), induction of physical means of stimulations (5–8). Though the use of highly supplemented media yields a higher cell density and elevated MAb titre (9–10), the drawbacks are the increasingly high amounts of excreted metabolites such as lactate, ammonia, etc. On repeated supplementation, these metabolites lead to cell death and decrease in MAb titre.

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However, both NAD (Nicotinamide Adenine Dincleotide) and NADP (Nucleotide Adenine Dincleotide Phosphate) quench protons and thereby reduce the lactate load. They also act as coenzymes in the *de novo* synthesis and salvage pathways of nucleotide formation (14). Thus, it appears feasible that addition of these coenzymes promote cellular growth and secretion *in vitro*. To this effect, the objective of the present study was to supplement such coenzymes in the media in order to reduce the metabolic burden on the growing cells, thereby enabling augmented growth promotion and/or secretory functions of the cells (MAbs, in the present study).

METHODS

Cell culture

Mouse hybridoma cell OKT3 (National Centre for Cell Science, Pune, India) was cultured for 96 h in a 24 well plate (Flow Labs, USA). The cells were grown in Iscove's modification of Dulbecco's medium (IMDM) (Sigma Chemical Company, USA), supplemented with 10% FCS (Gibco BRL, U.K), 0.0359 M $NaHCO_3$ (Loba, Chemie, India), 40 unit/ml penicillin G, 400 µg/ml streptomycin sulphate, 40 mg/ml gentamycin sulphate, 400 µg/ml mycostatin (India Pharma, India). The culture medium was further supplemented with NAD or NADP (25 µg/ml) (Loba Chemie, India), respectively. The combinations of supplementation was as follows : Control wells [complete IMDM media], NAD wells [complete IMDM + NAD (25 µg/ml], NAD + L-lysine wells [complete IMDM + NAD (25 µg/ml) + L-lysine (7 µg/ml)]. For NADP, the following combinations were studied : Control wells [complete IMDM media], NADP wells [complete IMDM + NADP (25 µg/ml)], NADP + L-lysine

wells [complete IMDM + NADP (25 µg/ml) + L-lysine (7 µg/ml)]. The sodium salts of NAD and NADP were dissolved in phosphate buffered saline (PBS) solution, filter sterilised and then used for supplementing the cell culture media. The cells were incubated in 5% CO_2 atmosphere at 37°C in an incubator (Jouan, France), and viable cells counts were taken at 24 h intervals using trypan blue dye exclusion principle (13). The culture media were isolated, centrifuged at 100 rpm, and the supernatants stored at –20°C for further analyses.

Analysis of culture supernatant

Culture supernatants were analysed qualitatively and quantitatively for identification of changes in the metabolite patterns and secretion profile of the cell line using SDS-PAGE (sodium dodecyl sulphate-polycrylamide gel electrophoresis) (SRL, India), ELISA (enzyme liked immunosorbent assay), and FPLC (fast performance liquid chromatography). The percentage of gel used was 8%, the volume of sample loaded in each lane was in the range 10 µl to 15 µl. The current applied during the passage of sample through stacking gel, was 15 mA and that during the running gel was 20 mA. The gels were stained with Coomassie Brilliant Blue. Fast Performance Liquid Chromatography (FPLC) (Pharmacia, Sweden) parameters were as follows : 1MPa pressure, at a flow rate of 0.4 ml/min. Absorbance of the eluted volume was screened (280 nm) in UV spectrophotometer off-line. The column used was Superose - 12 (Pharmacia, Sweden) and the volume loaded was 100 µl. The antibody titre, measured by direct ELISA technique, was carried out according to the following protocol: 50 µl of samples from OKT-3 supernatants were incubated in separate wells of the microtiter

plate at 4°C overnight. Washings were done with phosphate buffer saline (PBS)-Tween-20 (3X) (Bangalore Genei, India) and then 0.2% Bovine Serum Albumin (w/v) (Sigma Chemical Company, USA) was added to the wells and incubated at 37°C for 2 hours. Washing was again done with PBS-Tween-20 (3X) to remove unbound components. Anti mouse – rabbit antibody conjugated with enzyme alkaline phosphatase was added to the wells and incubated at 37°C for one and half hours. Any non-specific binding was removed by washing with PBS-Tween-20 for 5 to 6 times and substrated solution (p-nitrophenol phosphate) was added to each well and incubation was done at 37°C for 10 minutes. Control samples of known IgG content were also assayed simultaneously. Absorbance was taken at 405 nm.

Determination of specific growth rate and stimulation index

The specific growth rate was calculated using the following equation (11).

$$\mu = \frac{\overline{X}_T}{\overline{X}_V \cdot \Delta t} \ln \frac{X_T(t)}{X_T(t-1)} + \mu_1^* \frac{\overline{X}_d}{\overline{X}_V}$$

$\overline{X}_V$ = average values of two consecutive viable cell concentrations (cells/ml), $\overline{X}_T$ = average values of two consecutive total cell concentrations (cells/ml), $\overline{X}_d$ = average values of two consecutive dead cell concentrations (cells/ml), t = time (hrs), for static culture system, without any agitation, specific lysis rate (μ_1) was taken as zero.

The stimulation index (S.I.) is defined as the ratio of population of the experimental cells to the population control cells at a particular given hour. As it is a ratio same physical parameters, it

does not have any units. It gives an insight to the effect (stimulatory or repressive) that a molecule has over the cells under experimentation versus the control population.

$$S.I = \frac{Experimental_{(T)}}{Control_{(T)}}$$

Number of times the experiments were repeated is 4.24 wells plate was used in each case having equivalent internal sets each. Each 24 well plate was marked in the following manner : the first three columns were designated for control, the next three for NAD and the last three were reserved for NAD + Lysine. Again the first row was meant for 24 hour reading, second row 489 hours, third and fourth rows for 72 and 96 hours respectively.

RESULTS AND DISCUSSION

L-lysine has very significant growth promoting effect(s) on hybridoma cell AE9D6 *in vitro* with spurt in cellular growth rate taking place within first 48 hrs of culture (2). Addition of excess in NAD/NADP was done as a measure to prevent the build up of lactate (in confined

TABLE I : Stimulation Index for OKT3 cells cultured in presence of NAD (25 µg/ml) and NADP (25 µg/ml), alone and in combination with L-lysine HCl (7 µg/ml) in IMDM/10% FCS/4% PSGM media.

Time (hrs)	NAD	NAD+L-lysine	NADP	NADP+L-lysine
0	0	0	0	0
24	2	1.57	2.14	1.86
48	1.38	1.77	1.31	1.54
72	1.31	1.38	1.46	1.46
96	1.7	1.5	2.6	3.1

TABLE II : Specific growth rate profile of OKT3 cells cultured in presence of NAD (25 μg/ml) and NADP (25 μg/ml), alone and in combination with L-lysine HCl (7 μg/ml) in IMDM/10% FCS/4% PSGM media.

Time (hrs)	Control (hr^{-1})	NAD (hr^{-1})	NAD+L-lysine (hr^{-1})	NADP (hr^{-1})	NADP+L-lysine (hr^{-1})
0	0	0	0	0	0
24	0.03	0.06	0.05	0.06	0.05
48	0.02	0	0.03	0	0.02
72	0	0	0	0	0
96	0	0	0	0.01	0.02
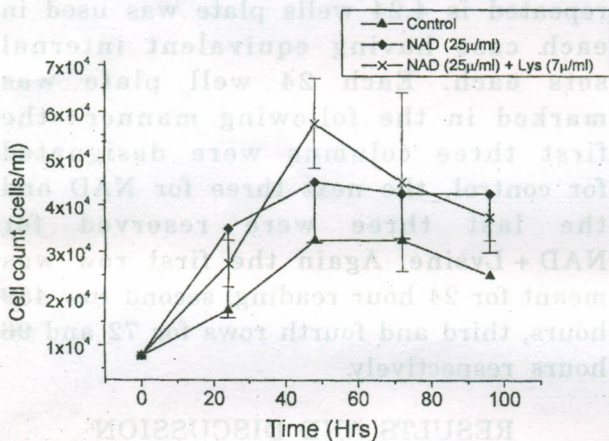
Fig. 1 (a):Growth profile of OKT 3 cells cultured in complete IMDM/10%FCS/4%PSGM supplemented with NAD (25 μg/ml), and NAD (25 μg/ml) + monomeric L-lysine (7 μg/ml).
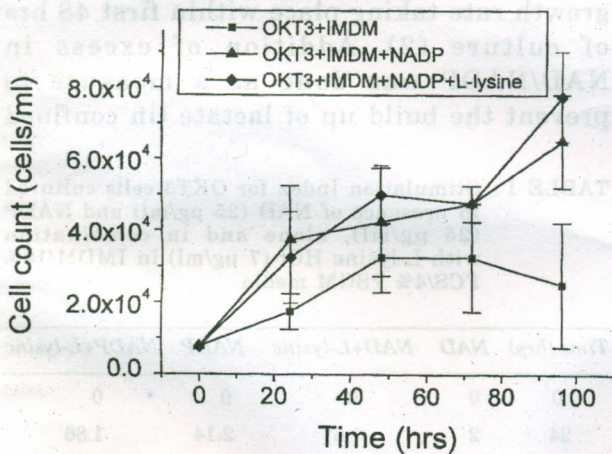

Fig. 1 (b):Growth profile of OKT 3 cells cultured in complete IMDM/10%FCS/4%PSGM supplemented with NADP (25 μg/ml), and NADP (25 μg/ml) + monomeric L-lysine (7 μg/ml).

culture in presence of relative lack of oxygen). In all the populations, initial spurt of cellular growth within 24 and 48 hrs, although on a much varied scale, were evident.

NADP, being the dedicated proton carrier (in contrast to NAD) in all
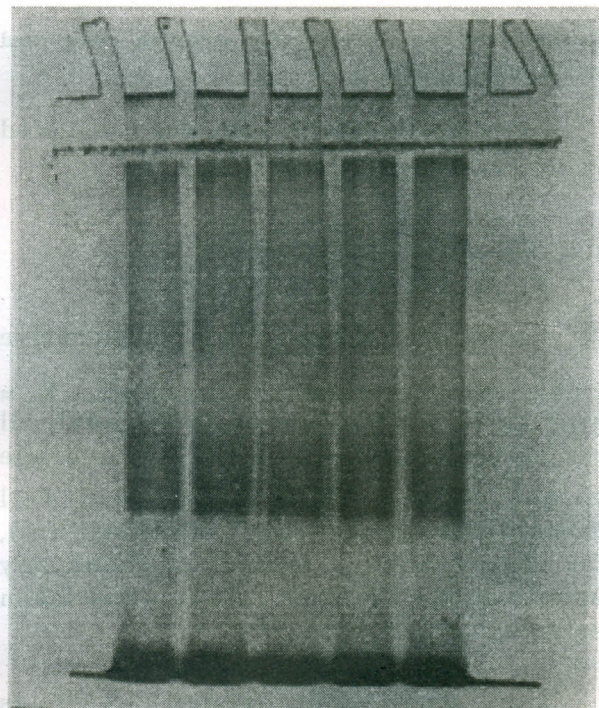

Fig. 2 : 8% SDS-PAGE of the spent media (supernatants) of OKT-3 cells grown in complete IMDM : Lane (1) Control; Lane (2)NAD (25 μg/ml); Lane (3) NAD (25 μg/ml) + L-Lysine (7 μg/ml); Lane (4) NADP (25 μg/ml); Lane (5) NADP (25 μg/ml) + L-lysine (7 μg/ml).

biosynthetic pathways *in vivo*, could possibly be a better supportive agent against the building lactate concentrations as well as in supporting synthetic activity in dividing cells in *in-vitro* culture where lactate has been shown to have a direct inhibitory role on cellular growth and secretion (12). Dual role of NADP – both in biosynthesis and as a proton carrier could be of profound significance in *in-vitro* continuous cultures with continuous harvest facility, especially in conditions of apparent anaerobisis e.g. in batch culture conditions.

Culturing of cells in presence of NAD + L-lysine had a lower cell count compared to

NADP or NADP + L-lysine at 48 hrs, with the S.I values for NAD (24 h) reaching at 2. Also we had a very interesting phenomenon where we observed that in presence of NADP or NADP + L-lysine, the cellular growth kinetics had an additional spurt at a very late phase (at 96 hrs.) with S.I. at 2.6 and 3.1, respectively. And this was also evident from the specific growth rate values (Fig. 1a,b,; Tables I, II). On the other hand, the cumulative 96 hrs MAb titre was higher for NAD and NAD + L-lysine group, with NAD, NAD + L-lysine, NADP, NADP + L-lysine supplemented cells having 11.6 mg/L, 13.6 mg/L, 6.5 mg/L, and 10.2 mg/L, respectively. Though the NADP
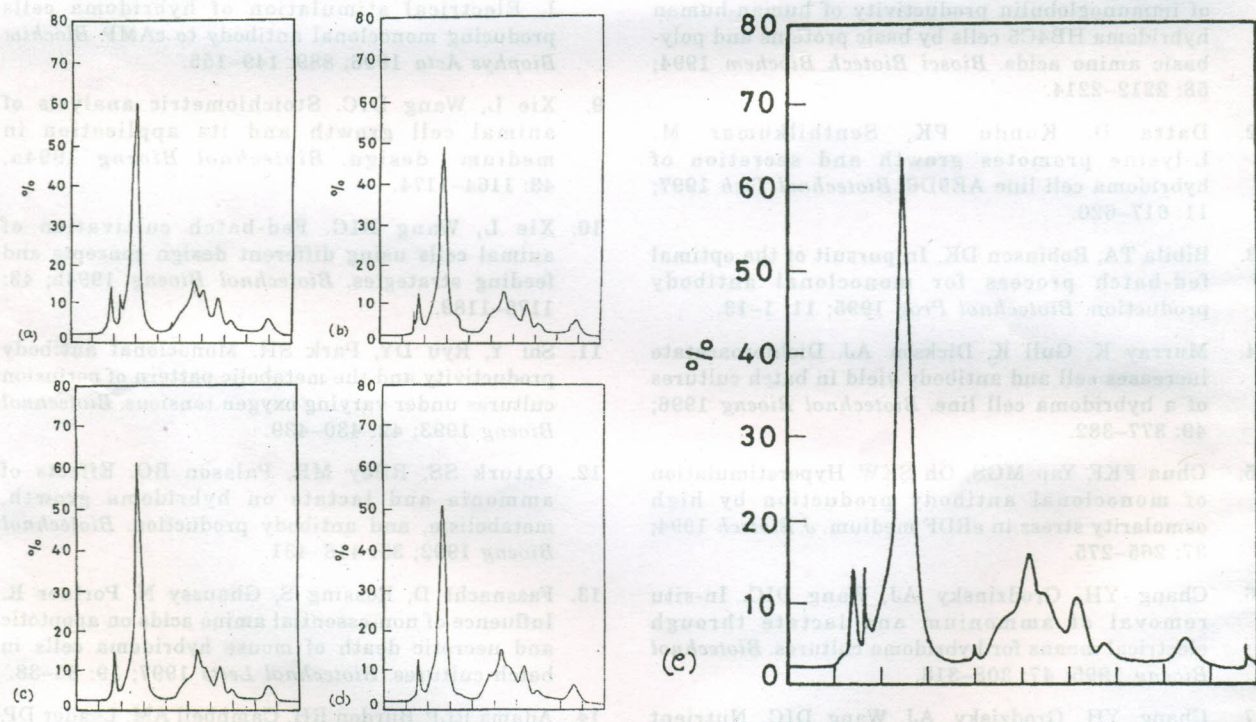

Fig. 3 : EPLC analyses of spent media of OKT-3 cells cultured in presence of NAD and NAD/L-lysine as well as NADP and NADP/L-lysine. 100 µl sample was loaded on Superose™ 12 prepacked 10/30 gel-filtration column and eluted with 0.1 M phosphate buffer, pH 7.4 at a flow rate of 0.4 ml/min and UV sensitivity of 0–0.1. (Each division corresponds to 4 ml). (a) Control, (b) OKT3 cells in IMDM and NAD, (c) OKT3 cells in IMDM + NAD + L-lysine, (d) OKT3 cells in IMDM and NADP and (e) OKT3 cells in IMDM + NADP + L-lysine.

or NADP + L-lysine populations had a higher final cell count, the MAb titre was not reflective. It showed that though the cells were proliferating and appeared much bigger in size, specific secretion rate had decreased, which corroborates with the fact that MAb production is a secondary stage, non-growth associated phenomenon.

SDS-PAGE analysis did not show any qualitative or quantitative difference between the groups, nor do we get any difference in the FPLC plot analyses

(Figs. 2, 3). Thus, it appears that NADP was a better supporter of growth, probably acting against the inhibitory effect of lactate build up in *in-vitro* culture (12), however higher MAb titre was yielded in presence of NAD + L-lysine.

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