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Hybridoma Ped-2E9 cells cultured under modified conditions can sensitively detect *Listeria monocytogenes* and *Bacillus cereus*

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Abstract Lymphocyte origin hybridoma Ped-2E9 cell-based cytotoxicity assay can detect virulent *Listeria* or *Bacillus* species, and its application in a cell-based biosensor for onsite use would be very attractive. However, maintaining enough viable cells on a sensor platform for a prolonged duration is a challenging task. In this study, key factors affecting the survival and growth of Ped-2E9 cells under modified conditions were investigated. When the Ped-2E9 cells were grown in media containing 5% fetal bovine serum in sealed tubes without any replenishment of nutrients or exogenous CO_2 supply, a large portion of the cells remained viable for 6 to 7 days and cells entered into G0/G1 resting phase. The media pH change was negligible and no cell death was observed in the first 4 days, then cells sequentially underwent apoptotic (fourth day onward) phase until day 7 after which a majority was dead. Subsequent cytotoxicity testing of 3- to 7-day stored Ped-2E9 cells sensitively detected virulent *Listeria* and *Bacillus* species. These data strongly suggest that Ped-2E9 cells can be maintained in viable state for 6 days in a sealed tube mimicking the environment in a potential sensor device for onsite use without the need for expensive cell culture facilities.

Keywords Ped-2E9 · Hybridoma · *Listeria monocytogenes* · *Bacillus* · Cytotoxicity · Cell-based sensor

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Introduction

The use of detection systems that incorporate whole cells or cellular components have a distinct advantage of responding in a manner that can offer insight into the physiological effect of an analyte (Pancrazio et al. 1999). Cell-based assays (CBAs) continue to serve as a reliable approach to probe the presence of pathogens in clinical, environmental, or food samples (Stenger et al. 2001; Ziegler 2000). The CBA systems can report perturbations in "normal" physiological activities of mammalian cells as a result of exposure to an "external" or environmental challenge (Stenger et al. 2001; Ziegler 2000). Some CBAs utilize the metabolic responses of cells (e.g., cyanobacteria) to detect biological products, such as oxygen and herbicides in water (Rawson et al. 1989). In other CBAs, mammalian cells or plasma membranes are used as electrical capacitors. The mechanical contact between cell–cell and cell–substrates is measured via conductivity or electrical impedance (Deng et al. 2003; Giaever and Keese 1993). In addition, mammalian cells can measure biochemical and metabolic endproducts (delivered from cultured cells to the medium) (Ziegler 2000). CBAs can also utilize the direct electrical response of electrogenic cells (neural cells, heart muscle cells, and pancreas beta cells) or a neural cell network

(Ziegler 2000). For example, neurons were used with great sensitivity to sense cell death dynamics, receptor–ligand interactions, alterations in metabolism, and generic membrane perforation processes.

Mammalian cell lines of different origins had previously been used to detect virulent *Listeria* species and some of the studies distinguished the differential cytotoxic response for virulent and avirulent *Listeria* species (Farber and Speirs 1987; Gracieux et al. 2003; Pine et al. 1991; Roche et al. 2001; Siddique 1969; Van Langendonck et al. 1998). *Listeria monocytogenes* can infect and produce detectable cytotoxicity in murine hybridoma B cells (Ped-2E9) (Bhunia et al. 1994, 1995). As a result, the hybridoma cells release alkaline phosphatase (ALP) that can be detected colorimetrically (Bhunia and Westbrook 1998). ALP release is directly related to the virulence potential of *Listeria*. *L. monocytogenes* kills hybridoma cells primarily by inducing apoptosis (Bhunia and Feng 1999; Menon et al. 2003). The ALP released from Ped-2E9 cells when challenged with *L. monocytogenes* was detected within 5–6 h using *p*-nitrophenyl phosphate (pNPP) as a substrate (Bhunia and Westbrook 1998). This assay is specific and sensitive in distinguishing virulent from avirulent species of *Listeria.* The detection time of this assay was further reduced to 2 h when reducing agent dithiothreitol (DTT) was added to the reaction mixture during incubation (Westbrook and Bhunia 2000). The assay time was again further reduced to 1 h when a fluorescence-based cytotoxicity assay was employed (Shroyer and Bhunia 2003). Ped-2E9-based assay was used to detect *L. monocytogenes* from food samples using a two-step method of immunobead capture and cytotoxicity analysis (Gray and Bhunia 2005). Recently, work from our laboratory showed that enterotoxigenic *Bacillus* species can be detected using the same Ped-2E9 cell-based cytotoxicity assay (Gray et al. 2005). The Ped-2E9 cell cytotoxicity assay is considerably faster than other conventional CBAs like MTT (3-[4,5-dimethyl thiazolyl-2]-2, 5-diphenyltetrazolium bromide) assay, showing a cytotoxic effect in just 1 h for *B. cereus* strains, which produce multiple toxins including hemolysins BL (with molecular weights 37.8, 38.5, and 43.5 kDa) (Lund and Granum 1997, 1999) and nonhemolytic enterotoxins (molecular weights 39, 45, and 105 kDa) (Pedersen et al. 2002). The sensitivity of Ped-2E9 hybridoma cells to pathogenic *Listeria* and *Bacillus* species makes this cell line a potential candidate to be employed in a cell-based biosensor device.

The challenge of using mammalian hybridoma cells in a cell-based sensor is to maintain the viability of the cells in the confinement of a sensor device, as the viability of the mammalian cells governs the "shelf-life" of the sensor. Various factors such as media formulations (Long et al. 1988), serum and other protein components (Franek and Dolnikova 1991), amino acids and fatty acid compositions (Butler and Huzel 1995; Franek and Sramkova 1996), oxygen and CO_2 concentrations, pH, and osmolality (deZengotita et al. 2002; Miller et al. 2000; Ozturk and Palsson 1991) affect the viability of hybridoma cells. Involvement of several stress factors may also cause cell death in a cell culture system. When stressors like nutrient and oxygen limitation occur, mammalian cell lines typically undergo programmed cell death or apoptosis (Simpson et al. 1999). Along with apoptotic cell death, cells can also undergo necrotic death.

The conventional determination of viability by trypan blue dye exclusion method may furnish overestimation of nonviable cells (Mascotti et al. 2000) or may not report precisely about the presence of membrane-damaged cells (necrotic or secondary necrotic cells) as opposed to membrane-intact cells (viable and apoptotic) (Fassnacht et al. 1997). Viability assays using the fluorescent dyes acridine orange (AO) and propidium iodide (PI) enable simultaneous visualization of both viable and nonviable cells (Mascotti et al. 2000). Also, the use of AO/PI was reported to furnish more accurate data about cellular physiology than trypan blue method (Fassnacht et al. 1997; Mascotti et al. 2000; Simpson et al. 1999). Many reports have suggested that hybridoma cells cease cell-cycle activity and rest in G0/G1 phase when nutrient (especially serum) is limited (Cooper 2003; Dedov et al. 2004; Pardee 1974). The cessation of proliferative activities may prolong the viability of the cells by resting in G0/G1 phase (Compton and Konigsberg 1988).

In this study, we optimized the growth conditions and media formulations to enable Ped-2E9 cells to grow and remain viable in a confined and closed environment outside the standard CO_2 incubator. The goal was to simulate the environment inside a simple sensor device where the cells will be grown and maintained until use. We further demonstrated that the sensitivity of Ped-2E9 cells to *L. monocytogenes* and enterotoxins extracted from *Bacillus-cereus* remained unaltered under the optimized cell culture conditions.

Materials and methods

Cell line, medium, and maintenance

For routine maintenance, lymphocyte origin murine Ped-2E9 cells (Bhunia et al. 1994) were cultured in Dulbecco's modified Eagle's medium with phenol red (DMEM, Cellgro, Mediatech, VA, USA) or without phenol red (DMEM-PRF, Invitrogen, Carlsbad, CA, USA) or phenol red-free Iscove's modified Dulbecco's media (IMDM-PRF, Invitrogen) supplemented with 10% fetal bovine serum

(FBS, Atlanta Biologicals, Norcross, GA, USA) at 37°C in 7% CO_2.

Adaptation of cells to reduced serum

Ped-2E9 cells were grown with different concentrations of FBS (10, 5, 2.5, and 1%) at 37°C in 7% CO_2. To grow the cells at reduced serum concentrations (5%, 2.5% and 1%) rather than the standard concentration (10%), a sequential serum adaptation (referred as "serum weaning") procedure was followed. Briefly, hybridoma cells from logarithmic phase of growth were inoculated at seeding density of 5×10^5 viable cells/ml in a 75:25 (v/v) mixture of serum-supplemented (DMEM, DMEM-PRF, or IMDM-PRF with 10% FBS) and serum-free medium (DMEM, DMEM-PRF, or IMDM-PRF without FBS). The culture was monitored until the density reached to 1×10^6 viable cells/ml. Then the cells from the above mixture were subcultured at the same seeding density into a 50:50 (v/v) mixture of serum-supplemented and serum-free medium and the cultures were monitored until the density reached to 1×10^6 viable cells/ml. The cells from the 50:50 mixtures were then subcultured similarly to a 25:75 (v/v) mixture of serum-supplemented and serum-free medium. Once the cell density reached approximately 1×10^6 viable cells/ml, the cells were subcultured in a 10:90 (v/v) mixture of serum-supplemented and serum-free medium. The Ped-2E9 cells from the above-mentioned 50:50, 25:75, or 10:90 mixtures were subcultured several times in either DMEM, DMEM-PRF, or IMDM-PRF with 5, 2.5, or 1% FBS to evaluate whether the cells were acclimated to the reduced serum conditions by measuring viability, doubling time, and cellular morphology. Cells originally from 50:50 (v/v) mixture of serum-supplemented and serum-free medium (and later grown in 5% FBS-supplemented medium) were designated as "Ped-2E9 cells weaned to 5% FBS"; similarly cells obtained from 25:75 and 10:90 (v/v) mixture were designated as "Ped-2E9 cells weaned to 2.5% and 1% FBS," respectively. These weaned cells were used in the current study and were also stored in liquid nitrogen for future use.

Determination of Ped-2E9 cell viability and growth kinetics

To determine the effect of FBS concentrations on Ped-2E9 cell growth and viability, DMEM, DMEM-PRF, and IMDM-PRF were supplemented with various concentrations of FBS (10, 5, 2.5, and 1%). Confluent Ped-2E9 cells were collected, counted, washed, and about 5×10^5 viable cells/ml were resuspended in DMEM supplemented with different concentrations of FBS in T-25 flasks (Nunc, Denmark) and maintained at 37°C under 7% CO_2 in a humidified incubator. Viability of the cells was enumerated and recorded at every 24 h for a period of 10 days by trypan blue dye (0.4%) exclusion test. The growth media containing 2.5% FBS (weaned) showed the best growth among the different FBS concentrations tested, and these cells were used for subsequent experiments.

To determine if the phenol red in the media could affect the growth or viability of the Ped-2E9 cells, a comparative study was done by growing the cells in medium with or without phenol red. Ped-2E9 cells (at a density of 5×10^5 viable cells/ml) were suspended in DMEM with phenol red or without phenol red (Invitrogen). Viable counts were recorded every day for a period of 10 days by trypan blue dye exclusion test.

Analytical methods: role of gaseous exchange and media pH on Ped-2E9 growth and viability

The goal of this experiment was to determine the growth pattern and viability of Ped-2E9 cells in a closed condition in tubes to minimize gaseous interchange in or out of the cell culture system. Aliquots of 4 ml of Ped-2E9 cells from logarithmic phase (initial cell concentration 1.6×10^6 viable cells/ml) in DMEM-PRF or IMDM-PRF (2.5% FBS) were dispensed in partially flat bottomed polystyrene cell culture tubes that were tightly closed with specially made rubber stoppers and wrapped with parafilm to minimize gaseous exchange. These polystyrene tubes have a negligible O_2 and CO_2 permeability values of 16.57 and 88.55 cm^3-mm/m^2 24 h bar, respectively, through the walls as indicated by the manufacturers (cat. no. 156758, Nunc). The tubes were maintained in a regular nonhumidified 37°C incubator without the external supply of CO_2. An identical set of tubes were kept in a humidified CO_2 incubator (7% CO_2), keeping their caps slightly loose to allow exchange of gases through the mouth. At 1- to 2-day intervals, two tubes were removed to determine CO_2/O_2 levels in each sealed tube by using a CO_2/O_2 dual headspace gas analyzer (PAC CHECK, Model 650, MOCON, Minneapolis, MN, USA). Viable and dead cell counts were determined by trypan blue dye exclusion test and culture media pH was recorded.

Bacterial cultures and toxins

Frozen stock of *Listeria* cultures listed in Table 1 were grown in brain heart infusion (BHI) broth at 37°C and subcultured twice. Fresh cultures (1 ml) were centrifuged (13,000×*g* for 10 min) at room temperature. The cell pellets were washed twice in filter-sterilized (filter pore size 0.45 μm) cell phosphate-buffered saline (C-PBS: 0.14 M NaCl, 5 mM KCl, 3 mM Na_2HPO_4, 4 mM NaH_2PO_4, and 10 mM glucose at pH 7.2) or in presterilized serum-free DMEM-PRF or IMDM and were resuspended in 1 ml of C-PBS or in DMEM-PRF or IMDM-PRF.

Table 1 Cytotoxicity assay with Ped-2E9 cells taken from air-tight sealed tubes grown in DMEM-PRF (2.5% FBS) and IMDM-PRF (2.5% FBS)

Treatments	Day 3		Day 4		Day 5		Day 6		Day 7		Day 8	
	VCC[a] (% death)	% Cytotoxicity[b]	VCC (% death)	% Cytotoxicity	VCC (% death)	% Cytotoxicity	VCC (% death)	% Cytotoxicity	VCC (% death)	% Cytotoxicity	VCC (% death)	% Cytotoxicity
(a) DMEM-PRF (2.5% FBS)												
Media only	15±0.16 (0)	–	15±0.04 (0)	–	15±0.09 (0)	–	15±0.06 (0)	–	15±0.01 (0)	–	15±0.19 (0)	–
Triton X- 100 (+control)	0 (100)	–	0 (100)	–	0 (100)	–	0 (100)	–	0 (100)	–	0 (100)	–
L.m Scott A	2.71±0.03 (81.9)	85.23	2.4±0.06 (84)	91.2	1.98±0.01 (86.8)	97.2	0.56±0.00 (96.2)	98.6	0 (100)	89.5	0 (100)	71.4
L.m V7	3.14±0.05 (79)	73.25	3.7±0.06 (75.33)	80.3	2.2±0.00 (85.33)	92.1	0.63±0.00 (95.8)	96.9	0.11±0.01 (99.2)	79.9	0 (100)	63.9
L. in F4247	15.6±0.83 (0)	0	15.12± 0.08 (0)	0	14.8±0.07 (0)	1.8	15±0.048 (0)	4.44	12.9±0.08 (14)	2.8	11.2±0.62 (25.33)	0
B.c MS1–9 toxin	0 (100)	97.2	0 (100)	109.6	0 (100)	99.7	0 (100)	101.3	0 (100)	96.6	0 (100)	76.9
B.s toxin	15.1±0.56 (0)	0	ND	ND	15.2±0.31 (0)	3.2	ND	ND	13.1±0.15 (13.3)	10.1	ND	ND
(b) IMDM-PRF (2.5% FBS)												
Media only	15±0.02 (0)	–	15±0.10 (0)	–	15±0.1 (0)	–	15±0.05 (0)	–	15±0.03 (0)	–	15±0.02 (0)	–
Triton X- 100 (+control)	0 (100)	–	0 (100)	–	0 (100)	–	0 (100)	–	0 (100)	–	0 (100)	–
L.m Scott A	3.8±0.01 (74.66)	94.3	3.13±0.06 (79.13)	92.9	2.36±0.03 (84.26)	95.8	1.3±0.01 (91.33)	98.5	0.69±0.01 (95.4)	81.3	0.17±0.00 (98.86)	69.9
L.m V7	4.23±0.08 (71.8)	64.0	3.9±0.08 (74)	78.9	2.1±0.03 (86.0)	82.5	1.66±0.07 (88.93)	96.6	0.89±0.03 (94.06)	73.9	0 (100)	60.6
L. in F4247	16.1±0.44 (0)	0	15.78± 0.56 (0)	0	15.2±0.31 (0)	2.0	15.27± 0.27 (0)	8.5	14.9±0.55 (0.6)	1.3	13.13±0.76 (12.46)	0
B.c MS1–9 toxin	0 (100)	95.1	0 (100)	102	0 (100)	99.4	0 (100)	100.4	0 (100)	97.3	0 (100)	99.1
B.s toxin	ND	ND	15.12±0.08 (0)	0	ND	ND	15.2±0.02 (0)	5.1	ND	ND	ND	ND

Values are the average of two separate experiments done in duplicate; values are presented as mean±SEM.

L.m. Scott A *L. monocytogenes* Scott A, L.m. V7 *L. monocytogenes* V7, L. in F4247 *L. innocua* F4247, B.c MS1–9, *B. cereus* MS1–9, B.s *B. subtilis*, ND *not done*

[a] VCC (viable cell counts) was determined by trypan blue dye exclusion test. Values in parenthesis are the percentages of cell death and were calculated using the formula: $1 - (\text{viable cell count at 2 h} \div \text{viable cell count at 0 h}) \times 100$

[b] Percent of cytotoxicity was calculated based on alkaline phosphatase release, which was calculated using the formula: $\left(A_{\text{experimental value}} - A_{\text{control(media)}}\right) \div \left(A_{\text{control(Triton-X)}} - A_{\text{control(media)}}\right) \times 100$

Crude toxins from *B. cereus* MS1-9 were prepared from bacterial cell-free culture supernatants as described previously (Gray et al. 2005). Briefly, *B. cereus* MS1-9 was grown in BHI for 18 h at 37°C in a shaker incubator (New Brunswick Scientific) at 140 rpm and centrifuged (10,000×*g* for 10 min at 4°C) and the supernatants were filter-sterilized (0.45 µm). The filtrate (crude toxin preparation) was immediately used for cytotoxicity assays or stored at 4°C for no more than 48 h. The protein concentrations of the toxin preparation were determined by using a BioRad protein assay kit following the manufacturer's protocol (BioRad, Hercules, CA, USA).

Cytotoxicity assay

Ped-2E9 cells were collected from tubes of different ages/incubation times (days 3 to 8), washed, counted, and resuspended in complete serum-free medium (Invitrogen). The cytotoxicity effects of *Listeria* spp. or crude toxin preparation from *B. cereus* on these cells were measured by trypan blue dye exclusion test and ALP release assay as described previously (Bhunia and Westbrook 1998). Briefly, the Ped-2E9 cells (1.5–2×10^6/ml) were inoculated with bacteria with multiplicity of infection of 100 bacteria to 1 hybridoma cell or 100-µl crude toxin preparations (~20 µg/ml added to 1.5–2×10^6 cells/ml) from *B. cereus*. DTT (0.5 mM) was added to the mixture (Westbrook and Bhunia 2000) and the hybridoma cells were incubated with bacteria for 2 h and with crude toxin preparations for 1 h. Viability of Ped-2E9 cells was determined by trypan blue dye exclusion test and the cell supernatants (0.1 ml each) obtained after centrifugation (1,800×*g* for 5 min) were assayed for ALP release by reacting with 0.1 ml of an ALP substrate solution containing ALP buffer (0.1 M Tris, 0.1 M NaCl, and 5 mM $MgCl_2$ at pH 9.5) and pNPP (1 mg/ml; Sigma Chemicals, St. Louis, MO, USA) in 96-well flat-bottomed plates. The plates were read after 3–5 min of incubation at room temperature at absorbance of 405/595 nm using a BioRad plate reader.

Determination of apoptosis and necrosis

Apoptosis and necrosis levels were determined by fluorescence microscopy by a method described previously (Simpson et al. 1999). Briefly, a solution containing 20 µg/ml of AO and 100 µg/ml PI (Sigma Chemicals) was prepared in C-PBS. Aliquots of 100 µl of cell suspension (1.5×10^6/ml) was mixed with 100 µl of staining solution and analyzed immediately with a fluorescence microscope (Leica, model DMLB, Wetzlar, Germany, with SPOT software, version 3.5.8, Diagnostic Instruments, Sterling Heights, MI, USA) using green (for AO) and red filters (for PI). All cells were permeable to AO and stained green (DNA-intercalated AO fluoresces green at 525 nm); only membrane-damaged cells took up PI and stained red (at 615 nm). Cells that excluded trypan blue [TB-negative (TB−)] were designated as membrane-intact viable cells. Cells that excluded trypan blue but showed green fluorescence (AO) showing nuclear condensation were designated as early apoptotic cells [AO-positive cells (AO+)]. Cells that took up trypan blue [TB-positive (TB+)] and PI [PI-positive (PI+)] were designated as late apoptotic and secondary necrotic.

Determination of DNA content and cell cycle

The DNA content of Ped-2E9 cells grown in DMEM with 2.5% FBS for different periods of time (0–7 days) in closed condition was enumerated by flow cytometric method according to a method described previously (Simpson et al. 1999) with some modifications. Briefly, dead cells were removed from the analysis by incubation of cells in a 0.5-mg/ml DNase solution (Sigma Chemicals) for 15 min at 37°C. The cells were then washed in C-PBS and the cell numbers were adjusted to 1×10^6/ml. Before analysis, cells were washed twice with C-PBS and resuspended in 500 µl of RNase solution (250 µg/ml, Sigma Chemicals) and incubated for 20 min at 37°C. Cells were again washed twice in C-PBS and resuspended in equal volume of modified Vindelov's PI (containing 50 µg/ml PI solution, 584 µg/ml NaCl, 1.21 mg/ml Tris base, 0.1 ml/100 ml NP-40, and 6.29 µl/100 ml RNase (Sigma Chemicals)). Cells were analyzed using a Beckman Coulter Cytomics FC 500 flow cytometer and cell cycle distributions were analyzed using the ModFitLT version 2 software (Verity Software House, Topsham, ME, USA).

Statistical analysis

Data were analyzed using the GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego CA, USA). Tukey's test was used to calculate mean±standard error of mean (SEM) at a significance level of $P<0.05$.

Results

Effect of serum concentration on viability of Ped-2E9 cells

Gradual adaptation or weaning of the Ped-2E9 cells to reduce serum seems to render a better viability when compared to higher serum concentration for a prolonged period. DMEM or IMDM supplemented with 2.5% serum (weaned) and 5% serum (weaned) resulted in significantly higher ($P<0.05$) Ped-2E9 cell growth between days 4 to 6 when compared with higher (10% FBS) or lower (1% FBS

weaned) serum concentrations at the same time points (Fig. 1a,b). Cell growth in different FBS concentration was found to be similar in the first 24 h, but from second day onward a progressively higher rate of net cellular growth was found in the media (IMDM and DMEM) supplemented with 5% FBS or 2.5% FBS (weaned). The initial higher rate of growth of the cells was observed in IMDM supplemented with 5 or 2.5% FBS. This trend of growth in both of the media tested continued until the fifth day, and then a progressive decline in cell number was observed. On day 5, a viable population of approximately a million (10^6) cells per milliliter was observed in the case of weaned cells (5 and 2.5%). Taken together, these results indicate that DMEM or IMDM supplemented with 2.5 or 5% FBS result in better growth and viability of Ped-2E9 cells compared to these media supplemented with 10 or 1% FBS for at least 5 days. Therefore, in subsequent experiments DMEM or IMDM supplemented with 5 or 2.5% FBS was used.

Viability of Ped-2E9 cells in air-tight tubes or in a humidified CO_2 incubator

Ped-2E9 cells displayed similar survival pattern when incubated in sealed tubes in a non-CO_2 incubator or in a CO_2 incubator (Fig. 2). The survival pattern of the cells cultured in IMDM or DMEM with either 5% FBS (Fig. 2a) or in 2.5% FBS (Fig. 2b) is similar and overall viable cell concentrations were found to be higher in IMDM than DMEM. Moreover, the viability of the cells tend to show a similar trend in sealed conditions as in a CO_2 environment indicating that an external CO_2 environment can be omitted for the growth and survival of Ped-2E9 cells in air-tight tubes with minimal gaseous exchanges.

The viable or live cell population was higher than dead or nonviable cells until the sixth day when the cells were incubated in closed conditions in DMEM with 5% FBS, and after day 6, the ratio was reversed (Fig. 3). By day 8, more than 65% of the cells were dead. This information is important as total number of viable cells contribute to the amount of ALP released (Bhunia and Westbrook 1998; Westbrook and Bhunia 2000). Along with these observations, it was also noticed that the cells did not grow when the seeding density was high in initial inoculations (~16×10^5 viable cells/ml, Fig. 2); in fact, the total viable cell numbers decreased but when the cells were seeded with low density (~8×10^5 viable cells/ml, Fig. 3), a typical growth curve was obtained where the total viable cell population increased till day 4, and after that the viable population started to decline (Fig. 3). With high inocula no further growth was achieved possibly because of nutrient limitation under those conditions. When lower inocula were used, the cells probably did not use up the nutrients as fast as in case of higher inocula, and therefore the viable cell density increased progressively until day 4 showing growth. After day 4 the viable cell density in tubes with lower inocula decreased probably because of the nutrient deficiency.

The growth pattern of Ped-2E9 cells when grown in DMEM supplemented with 5% FBS with or without phenol red were identical (data not shown), indicating that this pH

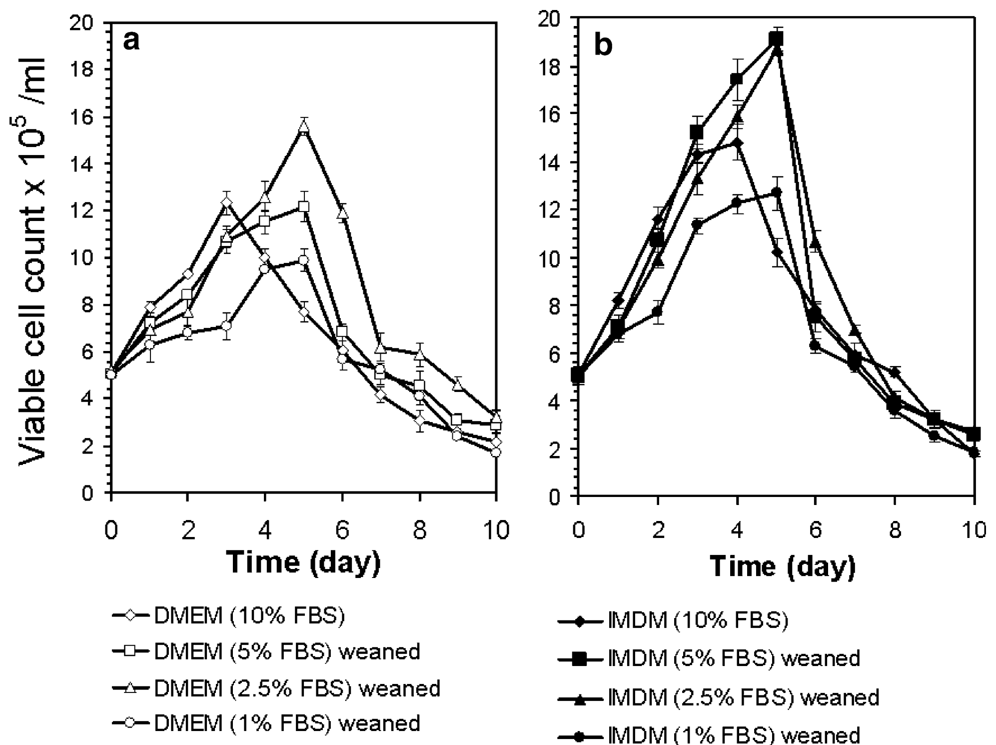

Fig. 1 Influence of FBS concentrations on Ped-2E9 cell growth. Ped-2E9 cells were cultured in different FBS concentrations in **a** DMEM-PRF or in **b** IMDM-PRF in 7% CO_2 supply at 37°C in a humidified incubator. Values are presented as mean±SEM of three experiments done in duplicate

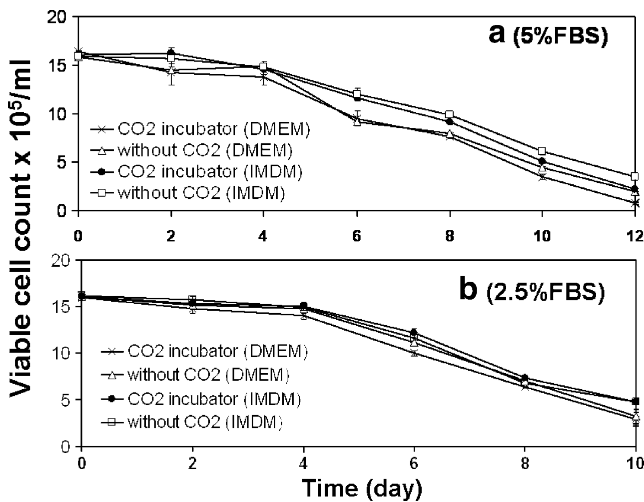

Fig. 2 Growth characteristics of Ped-2E9 cells in sealed tubes in a non-CO_2 37°C incubator and in a humidified CO_2 incubator at 37°C (7% CO_2). **a** DMEM and IMDM supplemented with 5% FBS. **b** DMEM and IMDM supplemented with 2.5% FBS (weaned cells). Values are presented as mean±SEM of three experiments done in duplicate

indicator dye (phenol red) in the medium does not impact Ped-2E9 cell growth or viability. Phenol red, therefore, can be eliminated from medium formulation of Ped-2E9 cell culture to improve the sensitivity of the colorimetry-based cytotoxicity assay.

Estimation of media pH and CO_2 and O_2 concentrations in sealed tubes

The pH of the culture medium of Ped-2E9 cells kept in closed tubes and in tissue culture flasks in a standard humidified CO_2 incubator (7% CO_2) showed no significant difference ($P<0.05$) at different time points (2, 4, 6, 8, and 10 days) (Fig. 4a). When high initial inoculum (1.6×10^6 viable cells/ml) was used, the population of live cells decreased with a decrease in pH (Fig. 4a). When low initial inoculum (5×10^5 viable cells/ml) was used, the number of live cells tended to increase even if there is a drop in pH (0–4 days, Fig. 4a). These observations indicate that the media pH profile, with a minimal pH drop, did not severely affect the viability (or growth) of the Ped-2E9 cells in closed tubes for the given window of time (10 days).

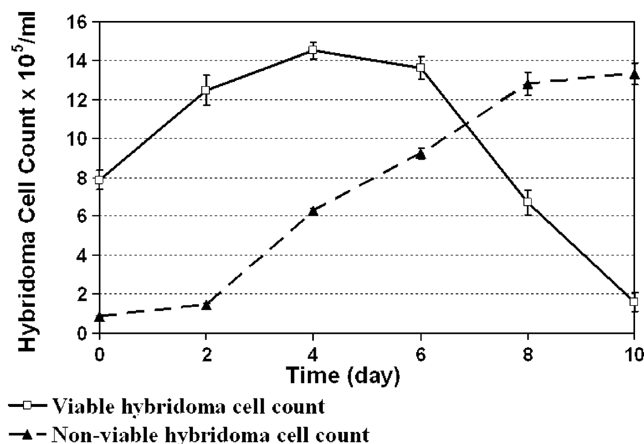

Fig. 3 Relative densities of viable and nonviable Ped-2E9 cells in sealed tubes in a non-CO_2 37°C incubator. Viable and nonviable cell counts were taken at different time points by trypan blue exclusion test. Values are presented as mean±SEM of two experiments done in duplicate

The CO_2 concentrations inside the sealed tubes containing Ped-2E9 (with initial viable cell density of 5×10^5/ml) increased progressively till the fourth day (0.6 to 10%) (Fig. 4b). From the fifth day onward the CO_2 concentrations dropped progressively. While the O_2 concentration decreased progressively until the fourth day, and thereafter showed an increasing trend. The viability of the Ped-2E9 cells showed a similar trend as in the previous studies described above and the cell viability showed strong correlation (R^2=0.8935) with the concentration of CO_2. When a higher initial inoculum (16×10^5 viable cells/ml) was used (Fig. 4c), correlation between cell viability and CO_2 was greatly reduced for the first 3 days ($R^2<0.2$). But a strong correlation (R^2=0.9155) was observed from day 4 through day 10. The oxygen concentrations in this period showed a similar trend as in case of the lower initial viable cell density. This datum indicates that the gaseous CO_2 concentration inside the sealed tube was probably dependent on two major factors, the cellular respiration and/or the conversion of bicarbonates to carbonic acid. The CO_2 concentration increased initially for the first 3–4 days and after that it decreased (irrespective of low or high initial cell densities; Fig. 4b,c). The minimal drop of pH (Fig. 4a) possibly due to buffering of medium by CO_2 produced by respiration and/or carbonate degradation may explain, at least in part, the correlation between cell viability and CO_2 concentration. But the actual biochemical interaction of cellular metabolites and byproducts (like lactate and ammonia) with gaseous or dissolved CO_2 in the sealed tubes remains to be elucidated.

Sensitivity of Ped-2E9 cells to *Listeria* cells or *Bacillus* toxin preparation

The hybridoma Ped-2E9 cells grown for 3 to 8 days were challenged with cells of *Listeria* spp. and toxin from *Bacillus* spp. The Ped-2E9 cells were highly sensitive to bacteria or toxins, as depicted by the percentage of cell death and the cytotoxicity until day 5 (Table 1). After day 6, the percent cytotoxicity receded and there was no direct relationship with percent cell death ($R^2<0.6$). This insensitivity after day 6 is probably due to the presence of increased nonviable cell populations. Ped-2E9 cell death and cytotoxicity apparently induced by avirulent *Listeria*

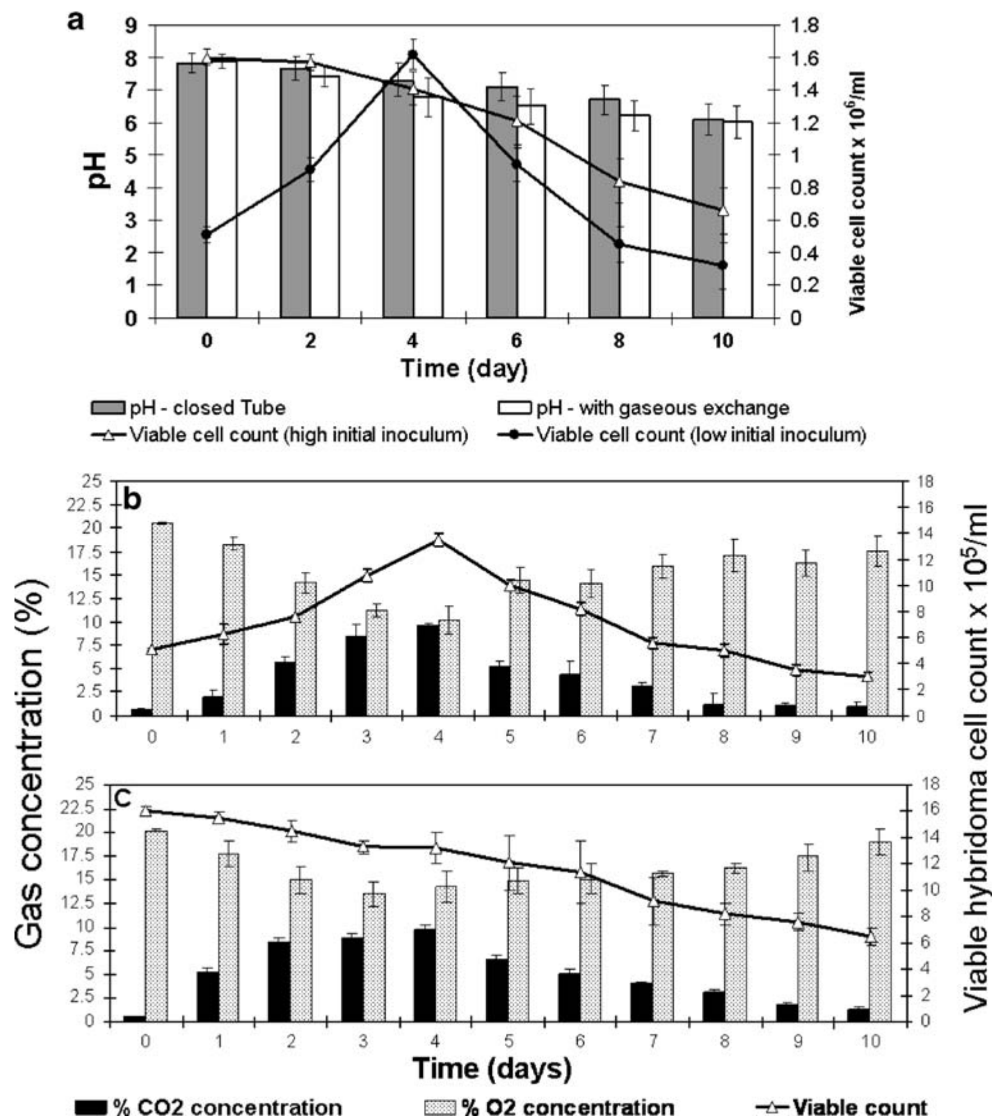

Fig. 4 Assessment of media pH, CO_2/O_2 concentrations, and the viability of Ped-2E9 cells. **a** Cell culture media pH and viability of Ped-2E9 cells grown in sealed tubes in a non-CO_2 incubator or in CO_2 incubator allowing gaseous exchange. *b*, **c** Relationship of CO_2 and O_2 concentration and Ped-2E9 cell viability in sealed tubes seeded with lower (**b**) or higher (**c**) inoculations of viable cells. Values are presented as mean±SEM of two experiments done in duplicate

innocua or *Bacillus subtilis* after day 6 may be attributed to the presence of large numbers of weakened or morbid cells in the tubes. These results demonstrate that Ped-2E9 cells maintained in air-tight tubes at different ages (for a period of 6 days) can effectively be used to assay *Listeria*- and *Bacillus*-mediated cytotoxicity by using ALP release assay or trypan blue dye exclusion test.

Analysis of Ped-2E9 cell death in closed tubes

Viable, apoptotic, or nonviable cells were visually determined by the intensity of nuclear fluorescence using a fluorescence microscope and SPOT software (as described in the "Materials and methods") (Fig. 5a). The fluorescence dye uptake profile by the Ped-2E9 cells at different days demonstrated that an apoptotic cell population (AO+ that also have signs of nuclear condensation but is PI-negative or TB−) could be visible from the fourth day onward (Fig. 5b). The membrane-damaged cells as designated by PI inclusion (PI+) have a similar trend with TB+ cells throughout the experiment. The population of apoptotic cells significantly increased ($P<0.05$) starting at day 6 (>8×10^5 cells/ml) while the membrane-intact viable cell population showed a steady decline from this point onward (~1×10^6 cells/ml). After day 7, the majority of the cells underwent either apoptotic or necrotic deaths.

DNA content and cell-cycle distribution

The cell-cycle analysis revealed that in closed tubes without any replenishment of fresh medium, the cellular proliferation gradually reduced and the cells "rested" in a quiescent stage (G0/G1) (Fig. 6). The intensity of DNA peak representing G2/M phase (mitotic phase) receded with time, indicating reduction of proliferative activities. The result depicted in Fig. 6 shows that Ped-2E9 cells gradually cease to proliferate and accumulate toward G0/G1 resting phase. A progressive decrease in G2/M peak can be

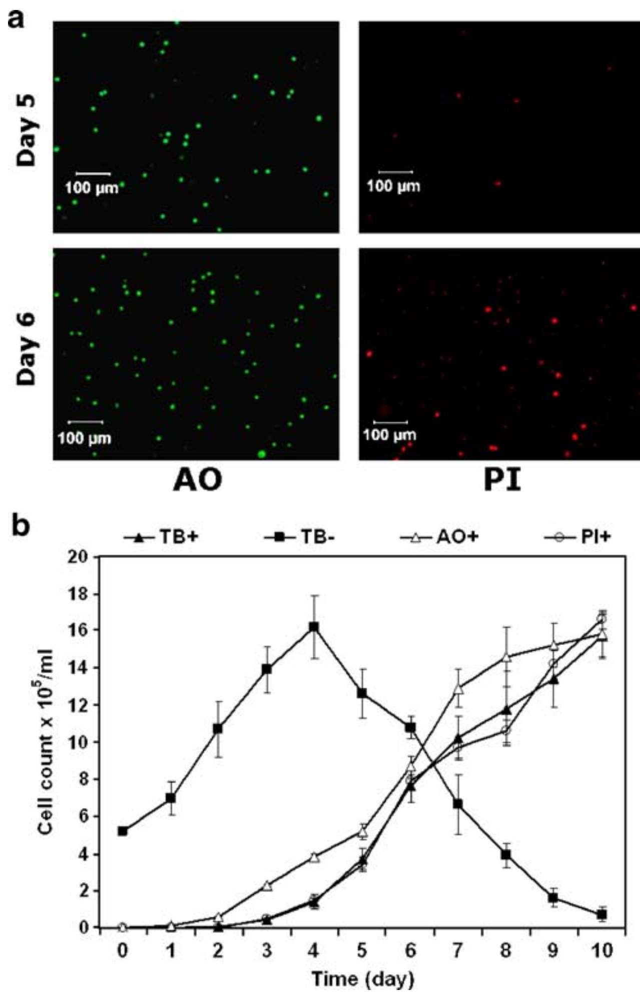

Fig. 5 Quantitative analysis of apoptotic and necrotic Ped-2E9 cells as depicted by intake of fluorescence dyes AO (*green*) and PI (*red*). Cells were grown in DMEM-PRF medium in sealed tubes in a non-CO_2 37°C incubator. **a** Fluorescence micrograph images from days 5 to 6. A *green* fluorescence (*AO*+) indicates viable but early-apoptotic cell population, while a *red* fluorescence (*PI*+) indicates dead cells (necrotic or apoptotic). **b** Relative population of Ped-2E9 cells by intake of fluorescence dyes AO and PI in comparison with trypan blue (*TB*). *Positive* or *negative* sign indicates whether the hybridoma cells have taken up or excluded these dyes, respectively. Values are presented as mean±SEM of two experiments done in duplicate

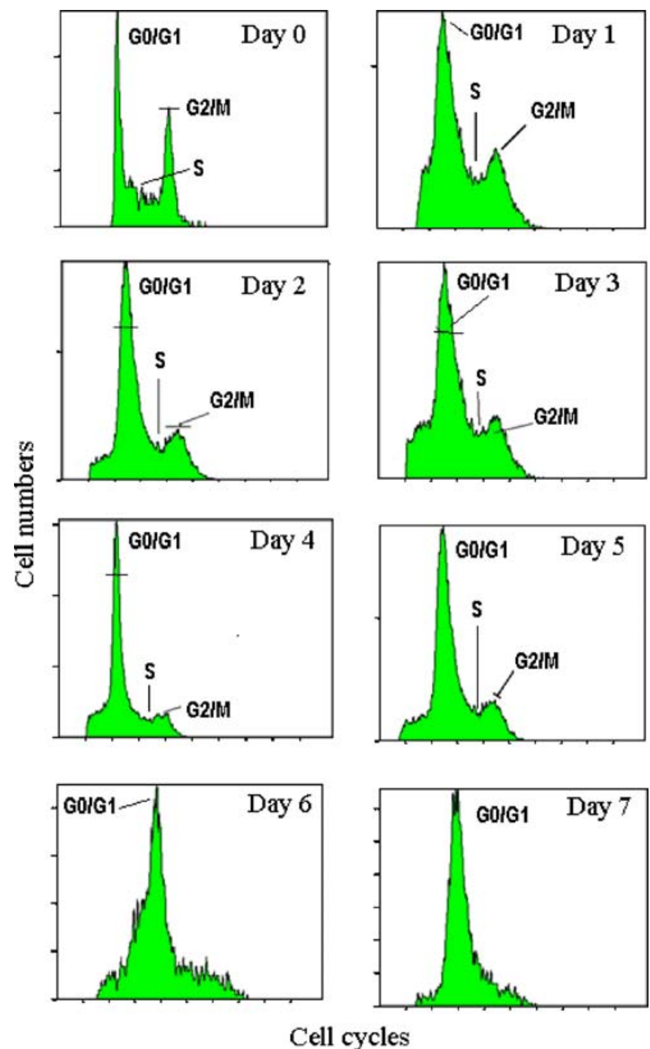

Fig. 6 Cell cycle distribution and DNA content of Ped-2E9 cells in closed tubes at different days. G0/G1, S, and G2/M represent the population of cells in resting phase, synthesis state, and proliferating phase, respectively

observed from day 2 and by day 6 almost 85% of the cells were in G0/G1 phase.

Discussion

The primary focus of this study was to evaluate the potential use of lymphocyte origin murine Ped-2E9 hybridoma cells in a cell-based biosensor device under development for onsite product testing. To achieve this goal, it is imperative to minimize the use of typical cell-culture facilities such as the CO_2 incubator. Furthermore, elimination of routine cell culture maintenance was also a critical consideration in our study.

Serum is a key regulatory factor for viability and growth of hybridoma cells (Froud 1999; Lubiniecki 1999). Our study with Ped-2E9 cells (Fig. 1) indicated that DMEM supplemented with 2.5 or 5% serum supported better Ped-2E9 cell growth than when 10% FBS or 1% FBS serum was used. This result is consistent with several previous studies, which suggested that depending on hybridoma cell types, reduction in serum concentration (Borth et al. 1992; Lubiniecki 1999) or total depletion of serum (serum-free) from hybridoma culture media improved the growth and viability of hybridoma cells (Baker et al. 1985; Froud 1999; Jayme and Blackman 1985; Kovar and Franek 1984). Reduction of the serum component from the cell culture media would also reduce the overall expense of the CBA system.

Our results show that the viability of Ped-2E9 cells was comparable when grown in closed polystyrene tubes

without external supply of CO_2 or in the presence of 7% CO_2 supply (Fig. 2). CO_2 is essential for the growth of hybridoma cells (deZengotita et al. 2002; Zanghi et al. 1999). Although no external CO_2 was provided to the cells in the closed tubes, Ped-2E9 cells remained viable in sufficient numbers for 6 days to allow the cytotoxicity-based bioassay to work, and after which the viability started to decline. We infer that the CO_2 concentration inside the headspace was sufficient to maintain the viability for the given window of time (6 days). This result indicates that the endogenously produced CO_2 (presumably by cellular respiration) may be enough for media buffering and cell survival and therefore a need for external CO_2 supply may be eliminated for a certain period of time.

We found that the cell growth was also dependent on initial cell densities. When the initial cell density was about 5×10^5 the cells tended to increase in number until the fifth day (Fig. 1). On the contrary, when the initial cell density was higher (~1.6×10^6) the number of viable cells decreased over time (Figs. 2 and 4a). By modulating the initial cell density, a desirable concentration of viable cells can be achieved.

These findings also have implications for the design boundaries of the sensor device. This result indicates that Ped-2E9 cells are well suited for use in a closed device for a fairly long period of time without replenishing with fresh medium. The viable vs nonviable cell population was found to be time-dependent (Fig. 3) in closed tubes. Until the fourth day, viable cell number outnumbered nonviable cell counts. After the sixth day the ratio of dead cells outnumbered live cells, indicating that media and the environment inside the tube was less favorable for cell growth or survival after this period of time.

Further, our result reveals that pH drop is minimal in sealed tubes (reduction of pH was only by 1 unit in 10 days). When high initial inoculum (1.6×10^6 viable cells/ml) was used in the experiments; apparently the population of live cells went down with a decrease in pH (Fig. 4a). But when a low (5×10^5 viable cells/ml) initial inoculum was used, the number of live cells tended to increase even if there was a slight drop in pH (0–4 day, Fig. 4a). The pH drop is most likely due to metabolic activity resulting in the production of acidic byproducts and due to production of CO_2 resulting from cellular respiration. These data indicate that in the sealed tubes minimal change in pH has an effect on cell death, but the pH drop was minimized, which in turn did not harshly affect the viability of the Ped-2E9 cells (Fig. 4).

Because the present assay is a colorimetry-based assay, we investigated if phenol red, known to quench color, could be eliminated from the growth media. To reduce color quenching during the colorimetric detection of ALP we used phenol red-free media. A comparative study revealed that Ped-2E9 cell growth was identical in DMEM with or without phenol red (data not shown), indicating that phenol red-free media can be used to grow Ped-2E9 cells in closed tubes.

We showed that Ped-2E9 cells, held in a closed system without replenishment of fresh media, can be effectively used to detect *Listeria*-mediated cytotoxicity. Data show that Ped-2E9 cells as old as 6 days can release detectable ALP (measured as percent cytotoxicity) in response to virulent *Listeria* infection. After day 6, ALP release and number of nonviable cells is not a good predictor of *Listeria*-mediated cytotoxicity (Table 1). This may be due to the fact that Ped-2E9 cells of 7–8 days old are more vulnerable to environmental shock, such as medium osmolality and pH. As a result of these conditions a portion of the cells die naturally during the incubation period of 2 h. There are fewer numbers of cells in the population for *Listeria* to kill, and the release of ALP is less. This is evident from the data of nonpathogenic “induced” cell death of *L. innocua* on days 7 and 8.

In the present study, our data of apoptosis of Ped-2E9 cells reveal that prolonged storage of these cells may first induce apoptosis and subsequently both apoptosis and necrosis ensue. The level of apoptosis of Ped-2E9 cells grown in closed conditions remains at a lower level until day 6. Many reports have suggested the involvement of genetic factors, such as p53, *bax*, and *bcl-2,* or environmental factors (like endoplasmic reticulum stressors and serum starvation) in the induction of apoptosis in hybridoma grown in culture (al-Rubeai et al. 1992a, Fukuda et al. 2006; Simpson et al. 1998; Singh and al-Rubeai 1998). Our flow cytometry data in addition to our apoptosis data also indicated that the Ped-2E9 cells grown for a longer period of time remained in a resting phase at G0/G1. In this stage cellular proliferation is much lower and other cellular metabolisms remained at a minimal stage (Ishaque and al-Rubeai 1998) This resting phase or G0/G1 arrest of Ped-2E9 cell growth can be attributed to the G1 restriction point (R-point) (Pardee 1974). As described in several reports (Cooper 2003; Dedov et al. 2004), a population of serum-starved or otherwise inhibited cells tend to accumulate at this unique point. In the present study the replenishment of nutrient supplements restored Ped-2E9 cell growth and DNA synthesis (data not shown). Because this type of growth arrest and subsequent restoration of growth is a hallmark of R-point check, we believe that this G1/G0 arrest is related to R-point. As a transformed cell line, however, the association of Ped-2E9 cell growth and arrest and R-point control still need to be verified. Several other cell cycle checks (such as p21, cyclin-dependent kinase, and p53) are also likely contributors to this cessation of Ped-2E9 cell cycle. Moreover, at this resting phase the cells remain metabolically viable, so the sensitivity of the Ped-2E9 cells (resting at G0/G1 phase) to pathogenic or toxigenic exposures remains largely unaltered.

Overall, our data show that Ped-2E9 cells can be maintained in viable state in closed culture tube in DMEM supplemented with 2.5 or 5% FBS without any further replenishment of nutrient media. As the cells are maintained in closed condition, there is no need of a humidified CO_2 incubator for sustaining the viability. This implicates that the hybridoma Ped-2E9 cells can be maintained for duration at a desirable number without any conventional cell culture facility. In addition to this, our results indicate that these cells can be grown in phenol red-free media, allowing for direct use in colorimetry-based cytotoxicity assays for *Listeria* and *Bacillus*. This assay produces satisfactory results with Ped-2E9 cells of different ages, ranging from 3 to 6 days. Taken together, our data indicate that Ped-2E9 cells are potential candidate for use in hand-held cell-based biosensor device.

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