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# Letter to the Editor

## EFFECT OF MEDIUM SERUM CONCENTRATION ON N1E-115 NEUROBLASTOMA MEMBRANE POTENTIAL DEVELOPMENT

Dear Editor:

Differentiating neuroblastoma cells may have potential for use as *in vitro* teratogenesis models. For example, Kisaalita and Bowen (1996) and Hernandez and Kisaalita (1996) have used the murine neuroblastoma, N1E-115 (derived from a spontaneously arising murine neuroblastoma, C1300), to evaluate retinoid and aspirin developmental toxicity. Also, Walum et al. (1987) compared neuroblastoma and fibroblast cells and found neuroblastoma cells to exhibit higher sensitivity to the neurotoxic compound, acrylamide. A number of investigators have used several methods to chemically induce morphological and electrophysiological differentiation in C1300 and/or N1E-115. For example, Seeds et al. (1970) and Peacock et al. (1972) observed that different clonal lines derived from C1300 appeared to differentiate morphologically when maintained in serum-free medium. Also, morphological differentiation was observed when N1E-115 and other C1300-derived clonal cells were grown in medium supplemented with dimethylsulfoxide (DMSO) (Kimhi et al., 1976), cyclic adenosine 5′-monophosphate (AMP) (Chalazonitis and Green, 1974; Kato and Narahashi, 1982) or aminopterin (Tuttle and Richelson, 1975).

Neuronal electrophysiological differentiation is characterized by the ability of the cell to generate repetitive discharges either spontaneously or in response to stimulation (Tuttle and Richelson, 1975; Quandt et al., 1984). This is achieved at elevated resting membrane potential and reflects the development of functional $Na^+$ and $K^+$ channels that underlie the fast action potential and of functional $Ca^{2+}$ and $Ca^{2+}$-activated $K^+$ channels that underlie slow repetitive discharges (Fishman and Spector, 1981). Because a temporal separation of morphological and electrophysiological differentiation of N1E-115 cells has been reported (Cosgove and Cobbett, 1991), we were interested in identifying a differentiation approach that yields the most differentiated cells with respect to resting membrane potential ($V_m$) development. Given that our long-term goal is to demonstrate the suitability of differentiating N1E-115 as an *in vitro* teratogenesis model, use of differentiation-inducing agents previously shown to be teratogenic in animal studies such as DMSO, cyclic AMP, and aminopterin (Mummery et al., 1984) was avoided. Also, we were unsuccessful in differentiating and maintaining the differentiated N1E-115 cells in serum-free medium as previously reported (Seeds et al., 1970; Peacock et al., 1972; Kloog et al., 1983). The purpose of this study was to identify the differentiating medium serum concentration required to induce the most electrophysiologically differentiated population (characterized by $V_m$ development) of N1E-115 cells.

N1E-115 cells of Passage 12 were obtained from Dr. M. Nirenberg, National Institute of Health (Bethesda, MD). Previously published protocols for N1E-115 cell culture (Kimhi et al., 1976; Miyake and Kurihara, 1983) were followed. Briefly, N1E-115 cells were routinely cultured at 37° C in air plus 10% $CO_2$ and at 90% relative humidity. Growth media was composed of Dulbecco's modified Eagle's medium (DMEM) containing 0.12% $NaHCO_3$ and supplemented with 13% fetal bovine serum (FBS), 50 units penicillin/mL, 50 units streptomycin/mL, and 2% glutamine. Cultures were monodispersed gently by flushing the confluent cells from the base of a 75-cm² T-flask (Costar, Cambridge, MA) by a stream of medium ejected from a Pasteur pipette. The suspension was centrifuged (500 ×g; 10 min) and the pellet resuspended in 25-cm² T-flasks (Costar) with fresh growth media at $2.0 \times 10^6$ viable cells (able to exclude trypan blue) per flask, unless otherwise stated. Flasks (in triplicate) were incubated overnight (24 h) to allow cells to settle and adhere to the base of the flask. Cells were then exposed to the following media: (1) DMEM/13% FBS (control), (2) DMEM/13% FBS + 2% DMSO, (3) DMEM/2% FBS, (4) DMEM/1% FBS, and (5) DMEM/0.5% FBS. In an additional experiment, we exposed cells to aminopterin (APT) by supplementing DMEM/13% FBS with APT to a final concentration of 1.0 μ*M*. In all cultures, media were changed every 3 to 4 days. Relative $V_m$ distributions were determined by flow cytometry with a potential-sensitive dye (oxonol) at 2 and 16 days after exposure to the differentiating medium.

The negatively charged oxonol dyes undergo $V_m$-dependent distribution between the cytoplasm and the extracellular medium and are generally less toxic to cells than the carbocyanines (Kohen and Hirschberg, 1989). The anionic oxonol dye, bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4[3]$), was chosen for these experiments because it can reliably indicate $V_m$ without major contributions from the mitochondrial potential, making it superior to carbocyanines for flow cytometry applications (Wilson and Chused, 1985). Cellular loading with oxonol is a function of $V_m$. As cell depolarization occurs, dye loading increases, causing an increase in fluorescence intensity (Brashford et al., 1985). Therefore low intensity reflects high $V_m$ and vice versa. The oxonol used in this study has been reported to exhibit the highest voltage sensitivity of all oxonols (Bräuner and Hülser, 1984).

Before flow cytometry analysis, cells were monodispersed, centrifuged (500 ×g, 10 min), resuspended in a saline solution and counted with a hemocytometer. The saline solution contained (in m*M*): 140 NaCl, 5 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 HEPES, 10 D-glucose, pH 7.3. Cells were washed (500 ×g, 10 min), resuspended at $0.5 \times 10^6$ cells/ml and incubated with 0.4 μ*M* $DiBAC_4[3]$ and propidium iodide (20 μg/ml) dyes for 30 min at 37° C. Flow cytometry analysis of the cell suspension was performed on the University of Georgia Research Services' Coulter EPICS 753 (Hialeah, FL) dual-beam instrument. Forward-angle light scatter (FALS), side-light scatter (SSC), and oxonol and propidium iodide signals/emissions were obtained in response to argon-ion laser excitation (488 nm) at 200-mW power output (Coherent, Palo Alto, CA). Oxonol emissions were determined by $PMT_1$ after passage through a 525-nm band-pass filter.

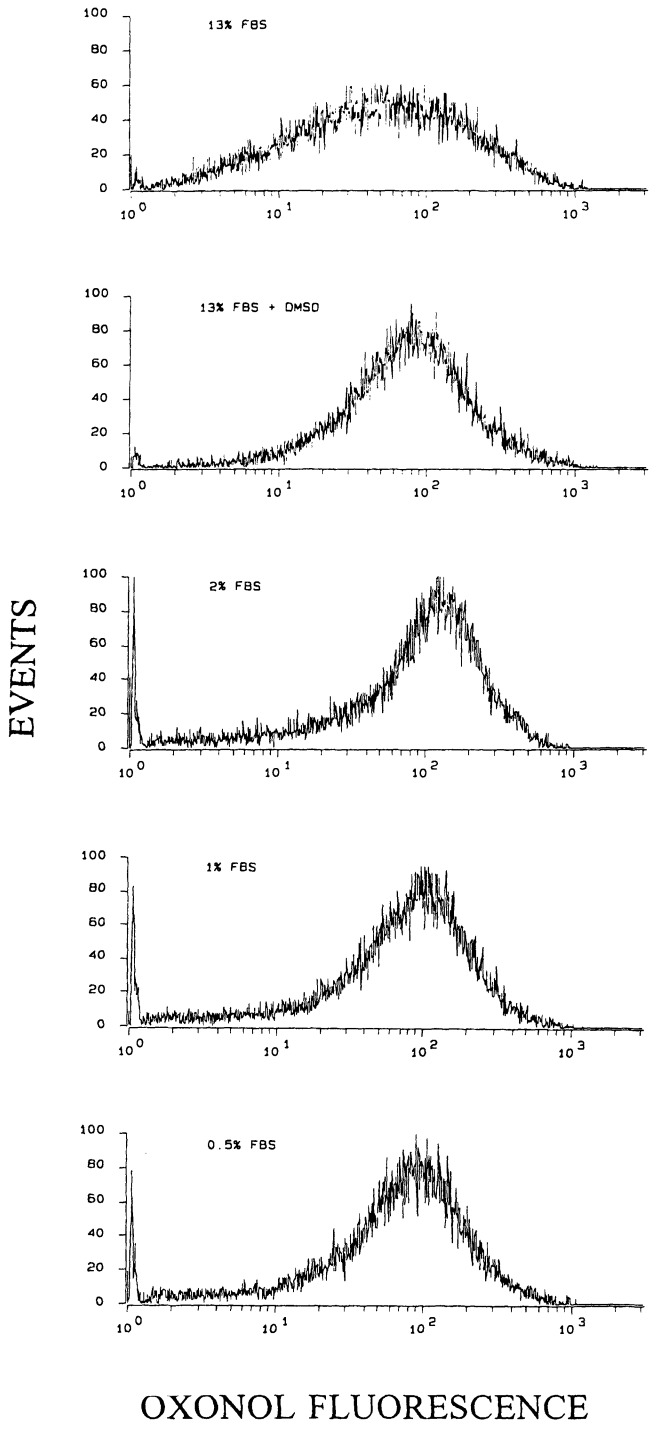


FIG. 1. Relative resting membrane potential distribution in N1E-115 neuroblastoma cells, 2 days after differentiation was initiated by culture in DMEM supplemented with 13% FBS (control—not induced), 13% FBS + 2% DMSO, 2% FBS, 1% FBS, and 0.5% FBS.

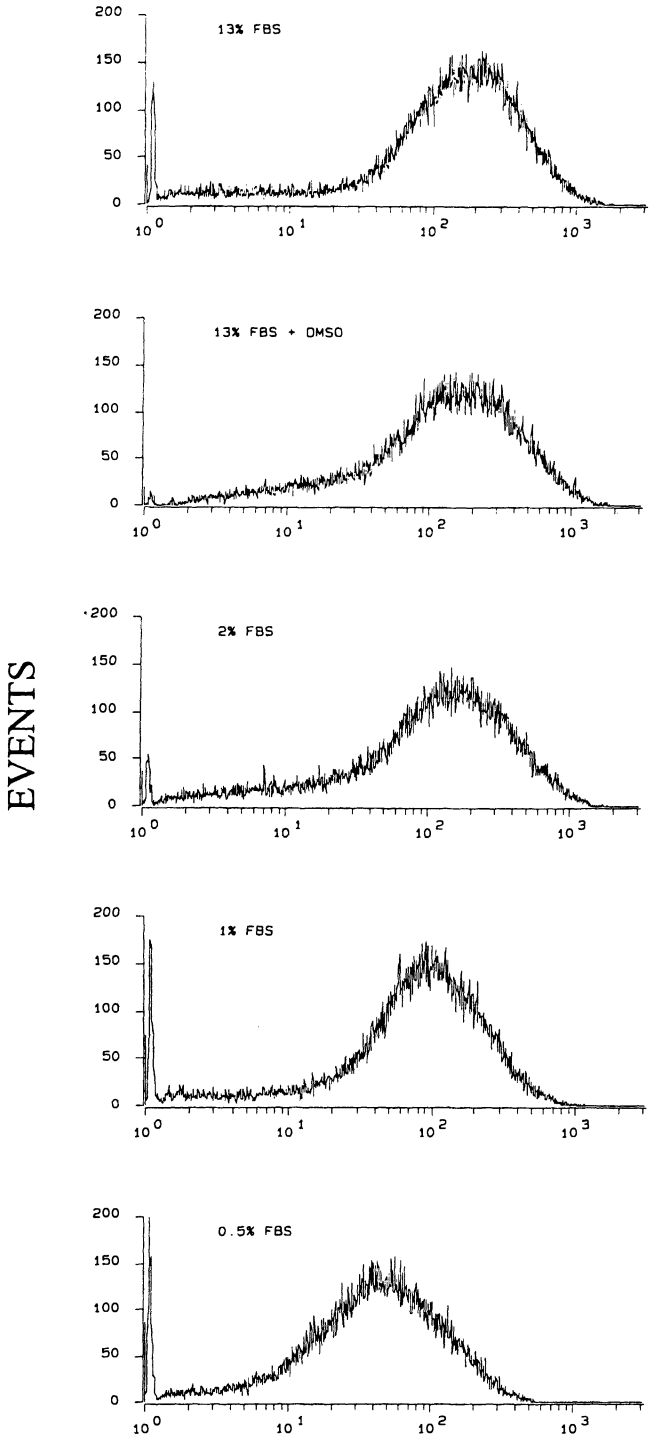


FIG. 2. Relative resting membrane potential distribution in N1E-115 neuroblastoma cells, 16 days after differentiation was initiated by culture in DMEM supplemented with: 13% FBS (control—not induced), 13% FBS + 2% DMSO, 2% FBS, 1% FBS, and 0.5% FBS.

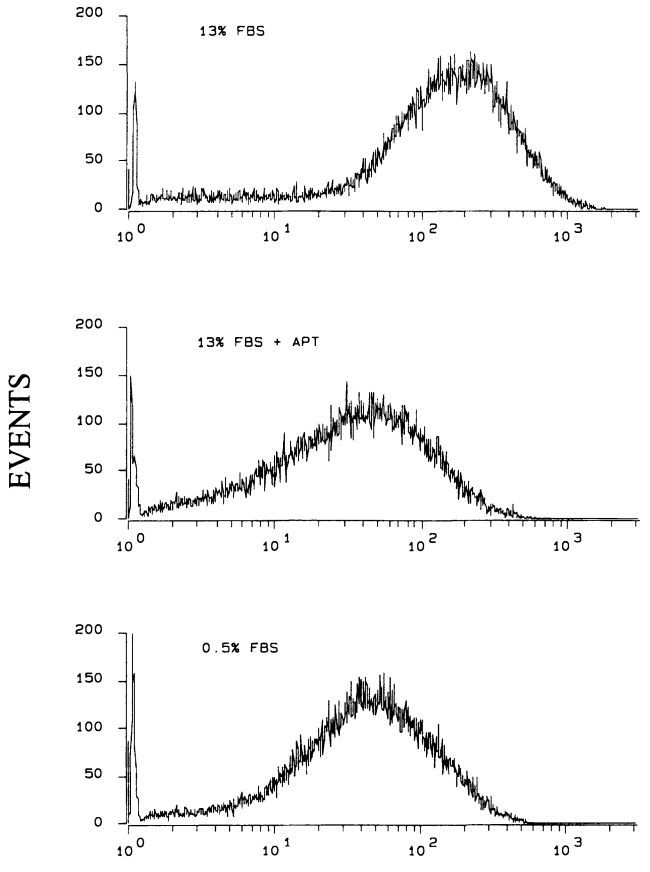


FIG. 3. Comparison of relative resting membrane potential distribution in N1E-115 neuroblastoma cells after 16 days of culture in DMEM supplemented with: 13% FBS (control—not induced), 13% FBS + aminopterin (APT) and 0.5% FBS.

Propidium iodide (PI) emissions were determined by $PMT_2$ after passage through a 610-nm long-pass filter. SSC was determined by $PMT_4$. FALS signals were linearly amplified with a gain of 2. Unless otherwise stated, 50,000 events were counted at an approximate rate of 200 events per second. Because subcellular debris has low FALS, this parameter was used to gate out these particles. Oxonol fluorescence analysis was restricted to events that were PI-negative, since dead or dying cells are stained by PI. To compare results for experiments conducted at different times, polystyrene fluorospheres (Coulter Corporation, Hialeah, FL) were used to set the $PMT_1$ (oxonol signal) and $PMT_4$ (SSC signal) at channel numbers of 35 ± 1 and 100 ± 2, respectively, before each experiment.

DMSO was included in this study as a positive control, because it is considered one of the most potent enhancers of electrical excitability among other chemical differentiating agents (Spector and Baumgold, 1982). Aminopterin was also included as a positive control. Aminopterin blocks the normal biosynthetic pathway by which nucleotides are made (Henderson et al., 1965) and therefore was expected to kill off most if not all the dividing (nondifferentiating) cells and thus yield the most differentiated cells. Previous $V_m$ results obtained by intracellular and patch clamp recording techniques on a few cells have indicated that the transition from low to high $V_m$ occurred between 7 and 10 days (Kimhi et al., 1976; Santone et al., 1986; Baumgold and Spector, 1987; Cosgrove and Cobbett, 1991). On the basis of these observations $V_m$ distribution profiles at 2 and 16 days were considered accurate representation of nondifferentiated and differentiated states, respectively. Although the flow cytometry technique does not provide the exact $V_m$ values, the technique was preferred over the standard microelectrode approach, because the flow cytometry relative results are conducted on a large population of cells, which provides a more accurate indication of the $V_m$ distribution in the culture. Besides, microelectrodes may impale and damage the cell and thus provide compromised $V_m$ data (Hernandez et al., 1996).

$V_m$ frequency distribution after 2 and 16 days in differentiating medium showed that at Day 2, FBS concentration did not influence $V_m$ distribution within the cell population (Fig. 1). However at Day 16, a shift of the $V_m$ distribution profile to the left with decreasing FBS is evident, suggesting an increase in $V_m$ with the maximum obtained at the lowest FBS level of 0.5% (Fig. 2). It is interesting to note that 0.5% FBS treatment yielded cells with much higher $V_m$ than did the 2% DMSO treatment. The $V_m$ distributions from aminopterin-treated and 0.5% FBS-treated cells were similar (Fig. 3), suggesting that maximum $V_m$ in N1E-115 cells can be obtained with DMEM/0.5% FBS. On the basis of these results, DMEM/0.5% FBS was selected as the differentiating medium in subsequent N1E-115 studies addressing the development of an *in vitro* teratogenic screening model (Kisaalita and Bowen, 1996).

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