Electronmicroscopic identification of type C particles in cultured murine neuroblastoma[1]

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Summary. Monolayer of murine neuroblastoma were treated with dexamethasone and examined by electronmicroscopy. Most of the treated cells were morphologically differentiated and exhibited type C virus particles which were budding from the cell surface. This in vitro system may be of great value for exploring the oncogenic potential of the virus, and its possible role in cell differentiation.

Substantial evidence implicates viruses in the oncogenesis of a wide variety of animal tumors[2,3]. Nearly all known oncogenic RNA viruses display type C morphology. Recently, biochemical evidence has shown that the release of type C virus is correlated with the capability of myeloid leukemia cells to differentiate morphologically[4]. In the case of neuroblastoma, we need to ask whether type C virus has the ability to initiate tumor formation and/or to influence normal differentiation of these cell lines of neural crest origin. Although type C virus has been demonstrated serologically[5] and biochemically[6] in cultured murine neuroblastoma, there has been no morphological demonstration of its existence. Since biochemical evidence suggests that type C virus production increases up to 10fold after neuroblastoma cells are treated with dexamethasone[6], we used this steroid in the hope of causing proliferation and release of the virus, thereby improving our chance of visualizing this virus in cultured cells.

Materials and methods. The clone NBP_2, derived from C-1300 murine neuroblastoma, was maintained by the procedure of Prasad and Hsie[7]. Cells were grown in monolayer culture in Falcon flasks containing F-12 medium. The medium was supplemented with 10% GG-free newborn calf serum and antibiotics. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. For experiments, cells were plated at 50,000/60 mm Falcon dish

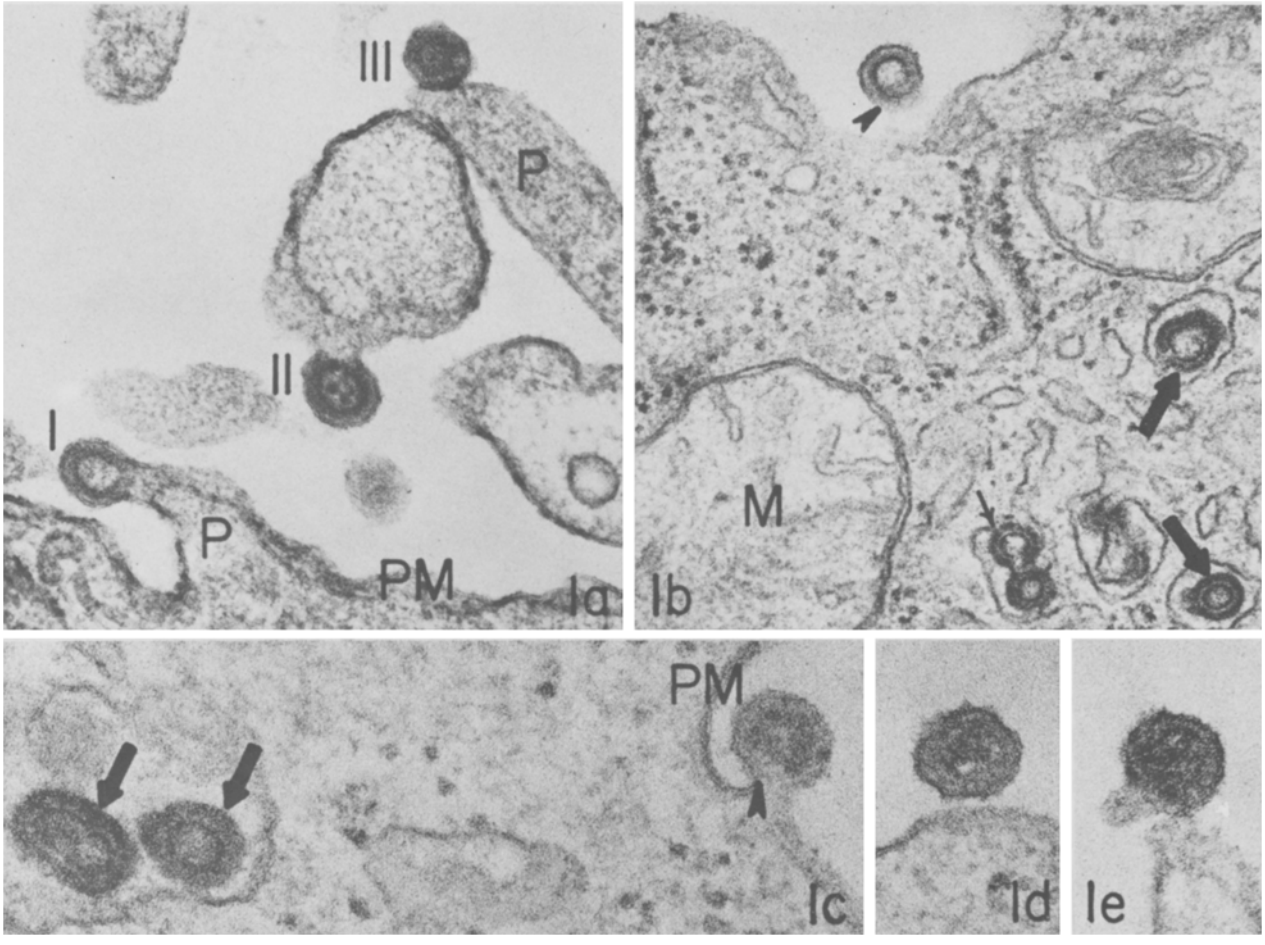

Fig. 1. Murine neuroblastoma cells treated with dexamethasone 10 µg/ml. *a* 3 type C virus particles (I, II and III) budding from or adjacent to the cell surface. Note the variable density of their cores (nucleoids). P: microprojection bearing a virus particle; PM: plasma membrane of the cell. × 81,000. *b* Several intracisternal type A particles (arrows) and an extracellular type C particle (arrowhead). The thinner arrow indicates a zone where there is continuity between the agranular endoplasmic reticulum and the shell of the virus particle. M: mitochondrion. × 81,000. *c* Higher magnification of intracisternal type A (arrows) and a type C (arrowhead) particle budding into the extracellular space. Note that the plasma membrane (PM) of the cell is continuous (at the tip of the arrowhead) with the outer membrane of the particle. × 135,000. *d, e* Mature type C particles containing electron-dense nucleoids. Some 'fuzzy' material adheres to the outer membrane. × 135,000.

and, starting the next day, treated for 4 days with dexamethasone at concentrations of 10 µg/ml or 50 µg/ml (0.125 mM). The dexamethasone was dissolved in 95% ethanol and added as a 0.05 ml aliquot to 5 ml medium in the dish. Cultures left untreated or solvent-treated (1% ethanol) served as controls. Drugs and medium were changed daily. On day 5, cells were fixed in situ with 3% phosphate-buffered glutaraldehyde for 30 min, then rinsed and post-fixed with 1% OsO_4 for 30 min. The fixed cells were dehydrated in graded ethanols and embedded in epon. Ultrathin sections were stained with uranyl acetate and lead citrate, and observed with a Philips 201 electronmicroscope.

Results. Type C virus particles were found in the monolayer cultures of murine neuroblastoma cells. In control cultures, they were seen in very small numbers (usually none, with a maximum of 2 particles per neuroblastoma cell outline as seen in a thin section). In cultures treated with dexamethasone, the particles were more frequently observed (usually 1-4 per cell outline). These results were consistent over the course of several experiments. Type C particles were always associated with the plasma membrane or were found nearby in the extracellular space. The particles were seen in various stages, ranging from budding forms with crescent-shaped prenucleoids to free particles with dense nucleoids. Free particle diameter was 100-120 nm. Ultrastructure of the particles, examples of which are illustrated (figures 1, 2 and 3), closely resembled previously reported particles in other tumors[2]. The morphology of the type C particles appeared the same, whether or not the cultures had received dexamethasone treatment. In all our preparations, budding particles were more numerous than free particles. Contrastingly, an in vivo study showed that budding particles occurred less commonly than extracellular ones[8]. It may be that in preparing the cultures for electronmicroscopy, free particles were washed out of the monolayer.

In addition, perikarya of neuroblastoma cells contained abundant type A particles, 90-100 nm in diameter, either budding into the lumen of the endoplasmic reticulum or apparently free within the dilated cisternae (figure 1b, c). Intracisternal type A particles are an established component of murine neuroblastoma, both in vitro[9,10] and in vivo[8,11-13], but have also been described in normal murine cells[14,15]. Intracisternal type A particles have not been shown to possess biological activity.

Discussion. This study has provided morphological confirmation that type C virus particles are present in neuroblastoma culture. In consideration of the morphological, serological and biochemical evidence, it now seems reasonable to conclude that these particles are a feature not only of this particular clone, but also of other murine neuroblastoma clones. Why have type C particles not been observed in previous ultrastructural studies of cultured neuroblastoma? Certainly, without the use of dexamethasone they appear to be sparse, making their detection difficult. Since type C and type A particles have been observed in tumors generated by s.c. inoculation of cultured neuroblastoma cells[8,12], we now postulate that both of these viruses were introduced with the cultured cells.

A controversy exists concerning the possible viral etiology of murine neuroblastoma. Prasad et al.[11] reported that a cell-free extract of this tumor produced a tumor of the same histologic type, whereas others could not obtain confirmation[5]. Only recently have viruses with hypothesized oncogenic ability (type C RNA viruses) been seen with murine neuroblastoma cells in vivo[8,12,15]. The demonstration that dexamethasone causes proliferation of type C virus particles (including free particles) in culture suggests that this in vitro system could serve as a ready source for viral concentrates, thereby facilitating future study of their oncogenic potential.

As reported elsewhere[16,17], dexamethasone given in the same concentrations used in this study produces both morphological and cytochemical differentiation in neuroblastoma cells from this clone. Increased numbers of type C particles were associated with dexamethasone-treated cells

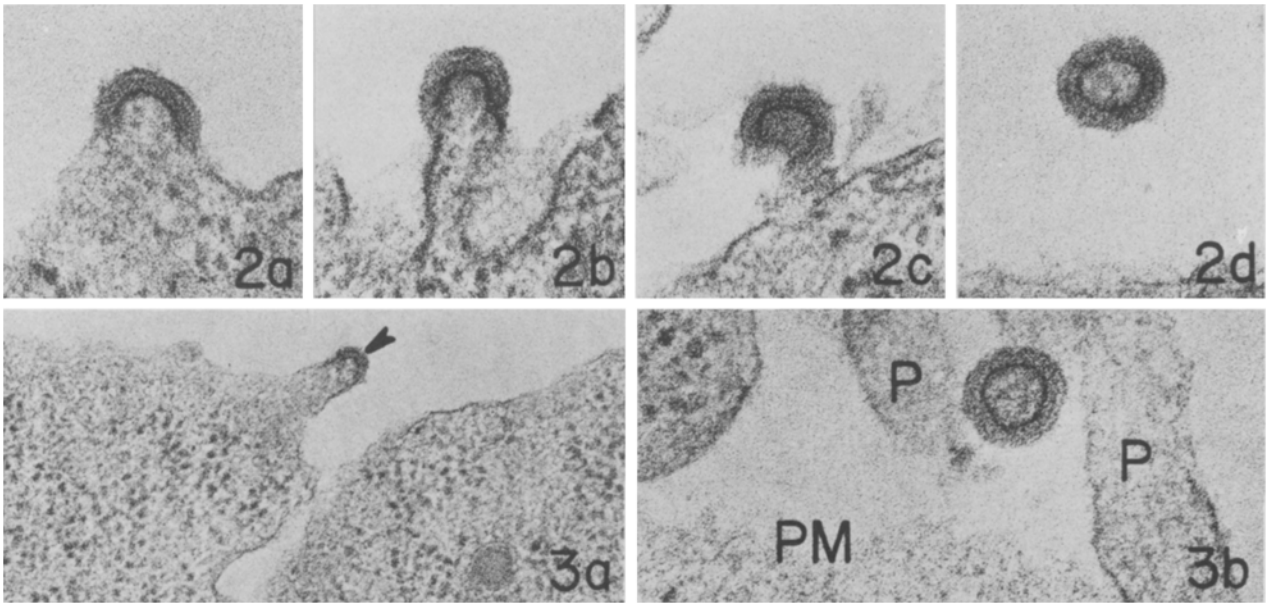

Fig. 2. Cells treated with dexamethasone 50 µg/ml. Stages in the development of immature type C particles are shown: budding (*a, b*), almost pinched-off (*c*) and extracellular (*d*). 3 layers - nucleoid, inner membranelike structure, and outer envelope - are clearly distinguished. × 135,000.

Fig. 3. Untreated cells. *a* Type C particle (arrowhead) budding from the cell surface. × 60,000. *b* Type C particle seen among microprojections (P) of the cell surface. PM: tangentially sectioned plasma membrane. × 120,000.

versus controls; but it is not known whether this viral proliferation was induced by dexamethasone per se, or was the result of the presence of a greater number of differentiated cells, as suggested by an in vivo study[12]. It would be of interest to study the effects, on type C virus production, of other differentiation-inducing agents, especially those which increase intracellular cyclic AMP levels in murine neuroblastoma[18].

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Elevated glucose levels influence in vitro hatching, attachment, trophoblast outgrowth and differentiation of the mouse blastocyst

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Summary. A glucose concentration of 3.5 mg/ml is optimal for in vitro embryo attachment and trophoblastic cell outgrowth. Raising the concentration above 3.5 mg/ml does not improve embryo culture and can at certain concentrations be detrimental to embryo development.

Post-blastocyst development of the mouse embryo to the egg cylinder and/or beating heart stage can be accomplished in vitro[2-4]. Relatively little information is available concerning the metabolic requirements of these embryos during this stage of embryogenesis. Spindle and Pederson[5] have reported that increased amino acids are beneficial while exogenous nucleosides had no influence[6]. We have previously reported that glucose is a necessary factor since reduced glucose levels can delay hatching from the zona pellucida and attachment to the substratum[7]. The basic culture media for post-blastocyst development has been modified tissue culture media. Most media contain a glucose concentration of 1 mg/ml. Little information is available concerning the beneficial or detrimental effects of elevated glucose levels on post-blastocyst embryo development. The objective of this study was to ascertain the affects of elevated glucose levels on the in vitro hatching, attachment, trophoblastic cell outgrowth and differentiation of the mouse blastocyst.

Superovulation was induced in random bred Swiss mice by the method of Gates[8]. Injected female mice were caged overnight with male mice and mating was verified the following morning by the presence of a copulatory plug in the vagina. 4 days postmating blastocysts were recovered by flushing the excised uterus with 0.5 ml of modified Brinster's medium[9]. Recovered blastocysts were pooled in culture medium under silicone oil in an atmosphere of 95% air plus 5% CO_2. Previously sterile 60×30 mm plastic petri dishes were layered with collagen reconstituted from rat tails as described by Ehrmann and Gey[10] and modified by Hsu et al.[11]. Individual petri dishes were equilibrated with 5 ml of the culture media 60 min prior to the introduction of the mouse blastocysts.

The control blastocyst culture media consisted of Eagle's basal medium (BME) supplemented with 10% fetal calf serum (FCS). The fetal calf serum was previously analyzed for glucose content and determined to contain 150 mg/ml. Since BME contains 1.0 mg/ml of glucose, when supple-

Influence of elevated glucose levels on postblastocyst embryo development*

Glucose concentration (mg/ml)	Hatching from the zona pellucida (%)	Attachment to the collagen substratum (%)	Trophoblastic cell outgrowth (%)	Differentiation to the egg cylinder stage (%)
2.5 (control)	58/ 80 (73)[a]	49/ 58 (85)[a]	40/ 49 (82)[a]	2/ 40 (5)[a]
3.5	125/184 (68)[a]	119/125 (95)[b]	109/119 (92)[b]	6/109 (6)[a]
4.5	65/123 (53)[b]	45/ 65 (69)[c]	24/ 45 (53)[c]	0/ 24 (0)[b]
6.5	99/151 (66)[a]	93/ 99 (94)[b]	73/ 93 (76)[a,d]	4/ 73 (6)[a]
11.5	81/105 (77)[a]	76/ 81 (94)[b]	67/ 76 (88)[a,b]	5/ 67 (5)[a]

* Embryos cultured in Eagle's basal medium (BME) supplemented with 10% fetal calf serum. Each concentration of glucose was replicated 3-5 times. Percentages with different superscripts are significant at the $p < 0.05$ level using a statistical analysis which tests a hypothesis between population proportions.