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# 5-Bromodeoxyuridine-Induced Differentiation of a Neuroblastoma

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**Abstract.** 5-Bromodeoxyuridine induces the differentiation of mouse neuroblastoma C1300 to cells that morphologically resemble mature neurons. The induced differentiation can take place in the absence of DNA synthesis. This suggests that the halogenated pyrimidine need not be incorporated into DNA to alter the phenotype of the cell.

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It has been demonstrated that a cloned tissue-culture line of mouse neuroblastoma C1300 can be induced to "differentiate" *in vitro*.[1] When the cells are grown in suspension in Petri dishes, they have an anaplastic round-cell morphology. If, however, cells are transferred to tissue-culture dishes, they attach to the surface and differentiate to cells which are morphologically and physiologically similar to mature sympathetic neurons. The round neuroblastoma cell and its differentiated derivative differ in staining characteristics, presence of microtubules, chromosome number, and spectrum of proteins synthesized.[1,2] In addition, the development of acetylcholine sensitivity is analogous to that observed with parasympathetic neurons *in vivo*.[3] Since the previously described differentiation of these cells was an asynchronous process requiring several days, it was desirable to define conditions that lead to a rapid and synchronous differentiation. The following experiments describe the induced differentiation of the neuroblastoma by 5-bromodeoxyuridine (BrdU).

**Materials and Methods.** The culture conditions, cloning procedures, and morphological and physiological characteristics of mouse neuroblastoma C1300 have been previously described.[1–3] The cells were maintained in Eagle's modified medium containing 10% fetal calf serum at 37°C. Falcon plastic tissue-culture and Petri dishes were used exclusively. Cell number was determined by dissociating the cells with Viokase and counting in a Coulter Counter.[2] Viability was determined by the uptake of fluorescein dibutyrate.[4]

Stock solutions of the halogenated pyrimidines and the various inhibitors were prepared directly before use. All manipulations involving BrdU were done in indirect light and the cultures were maintained in the dark. The effect of inhibitors on DNA, RNA, and protein synthesis were carried out as described previously.[2, 5] Isotopic labeling with [$^3$H]- and [$^{14}$C]leucine, reduction and alkylation, and acrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate were carried out as described previously,[6] except that 8-cm gels were used and electrophoresis was for 7 hr at 6 mA per tube. Cells were labeled with both [$^{14}$C]- and [$^3$H]leucine at a final leucine concentration of $10^{-5}$ M.

The morphological criterion used to define a differentiated cell was the existence of one or more axon-like processes (neurites) extending from the perikaryon that were *at least*

equal to the diameter of the perikaryon, i.e. more than 40 $\mu$m.[1,2] To determine the fraction of differentiated cells in a population, at least 500 individual cells were scored in several fields of two culture dishes. In all of the experiments described here using tissue-culture dishes, more than 90% of the exponentially growing cells were attached to the surface.

**Results. Specificity of induction:** To define the effect of brominated pyrimidines on the growth and differentiation of the neuroblastoma, cells were grown in tissue-culture dishes in the presence of these compounds. As shown in Fig. 1,

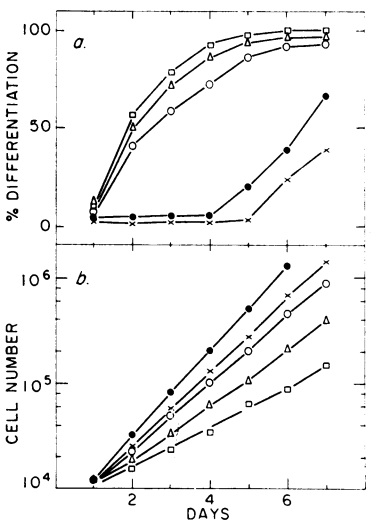


FIG. 1. BrdU-induced differentiation. Exponentially growing cells in Petri dishes were dissociated with Viokase and plated in tissue-culture dishes containing BrdU. Cell number, viability, and the fraction of differentiated cells were determined as described in *Materials and Methods*. (*a*) The percent of differentiated cells plotted as a function of time. (*b*) The number of viable cells per 60-mm culture dish plotted as a function of time. ●, $6.4 \times 10^{-8}$ M BrdU; ○, $1.6 \times 10^{-6}$ M BrdU; △, $8 \times 10^{-6}$ BrdU; □, $4 \times 10^{-5}$ M BrdU; ×, control without BrdU.

BrdU induced the differentiation of the neuroblastoma cells. 5-Bromouridine, 5-bromouracil, 5-fluorodeoxyuridine, and 5-bromocytosine did not produce this effect over a concentration range from $10^{-4}$ to $10^{-13}$ M. 5-Bromodeoxycytidine also induced, but since it is likely that it is converted to BrdU by deoxycytidylate deaminase, only the effect of BrdU will be discussed here.

To define the specificity of the induction process, BrdU was added to exponentially growing cultures in the presence of the different pyrimidines. As shown in Fig. 2, BrdU induced, without lag, the differentiation of the culture. This process was inhibited by thymidine and deoxycytidine; none of the other pyrimidines tested was effective.

There are two alternatives which explain the above observations. Either BrdU exerts its effect directly as a consequence of its incorporation into DNA, or the induction is a result of BrdU acting at a site other than DNA. The former possibility has been proposed to explain the inhibition of myoblast fusion[7] and the synthesis of tissue-specific components in cultured chondrocytes[8] and amnion cells.[9] The following experiments rule out this hypothesis, and show that BrdU exerts its effect at a site other than DNA in the mouse neuroblastoma.

**DNA synthesis and differentiation:** To establish that differentiation could be induced by BrdU in the absence of DNA synthesis, exponentially growing cells

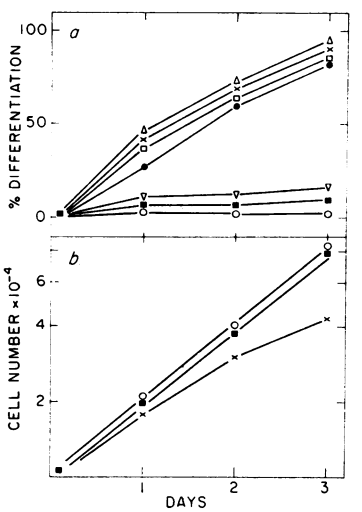


FIG. 2. Inhibition of induced differentiation by pyrimidines. Pyrimidines were added at $4 \times 10^{-4}$ M to cells growing exponentially in tissue-culture dishes; $4 \times 10^{-6}$ M BrdU was then added. Viable cell number and the fraction of differentiated cells were defined as in Fig. 1. Pyrimidines alone affected neither differentiation nor the growth rate at the concentrations employed. (*a*) Per cent differentiation plotted as a function of time. △, uridine + BrdU; x, BrdU alone; □, cytidine + BrdU; ●, deoxyuridine + BrdU; ▽, deoxycytidine + BrdU; ■, thymidine ($4 \times 10^{-5}$ M) + BrdU; ○, control without added pyrimidines. (*b*) Viable cell number plotted as a function of time. ○, control; ■, thymidine ($4 \times 10^{-5}$ M) + BrdU; ×, BrdU alone. BrdU plus the remaining pyrimidines described in (*a*) showed a deviation of less than 8% from the cell number with BrdU alone.

were treated with 1-β-D-arabinofuranosylcytosine (ara-C) and mitomycin C at concentrations that inhibited DNA synthesis as defined by the uptake of [$^{14}$C] thymidine (Fig. 3). At 6 hr and 20 hr after the addition of the inhibitors, BrdU was added at a final concentration of $4 \times 10^{-6}$ M. As shown in Fig. 4, BrdU induced the differentiation of the neuroblastoma in the absence of DNA synthesis; this process was inhibited by thymidine and deoxycytidine as in Fig. 2. The increase in cell number after the addition of the inhibitors (Fig. 4*b*) reflects the division of cells that were in the post-DNA-synthetic period and mitosis at that time. Since differentiation is induced when BrdU is added after 20 hr, it follows that cell division is not mandatory for the differentiation process. Conversely, it has been shown that inhibition of cell division does not induce differentiation.[2]

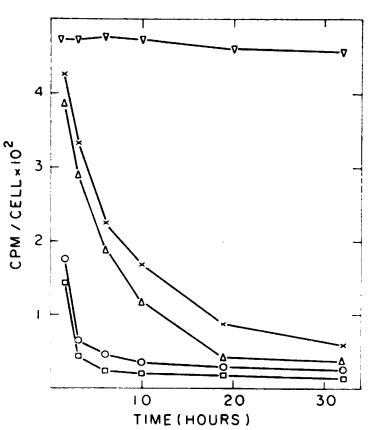


FIG. 3. Inhibition of DNA synthesis by ara-C and mitomycin C. Exponentially growing cells were pulse-labeled for 1 hr with [$^{14}$C]-thymidine at various times after the addition of ara-C and mitomycin C as described in *Materials and Methods*. The uptake of $^{14}$C per cell into trichloroacetic acid-precipitable material is plotted as a function of time. ×, mitomycin C, 0.1 μg/ml; △, mitomycin C, 0.3 μg/ml; ○, ara-C, $5 \times 10^{-8}$ M; □, ara-C, $1 \times 10^{-7}$ M; ▽, control.

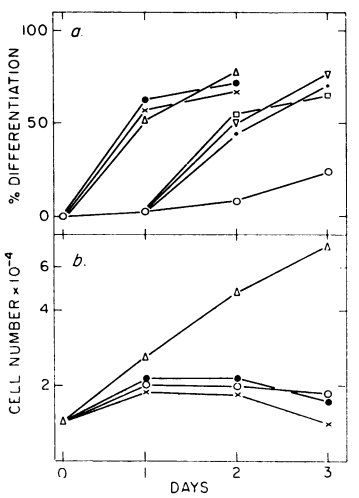


FIG. 4. Induction of differentiation in the absence of DNA synthesis. $1 \times 10^{-7}$ M ara-C or mitomycin C was added to exponentially growing cultures in tissue-culture dishes. 6 or 20 hr later, $4 \times 10^{-6}$ M BrdU was added. To ara-C cultures that received BrdU at 20 hr, additional ara-C was added at this time to bring the final concentration to $2 \times 10^{-7}$ M. (*a*) Percentage differentiation plotted as a function of time. (*b*) Viable cell number plotted as a function of time. O, $1 \times 10^{-7}$ M ara-C or mitomycin C at 0.1 and 0.3 μg/ml; ×, ara-C + BrdU at 6 hr; □, ara-C + BrdU at 20 hr; ●, mitomycin C (0.1 and 0.3 μg/ml) + BrdU at 6 hr; •, mitomycin C (0.1 and 0.3 μg/ml) + BrdU at 20 hr; △, BrdU alone at 6 hr; ▽, BrdU alone at 20 hr. Higher concentrations of ara-C and mitomycin C lysed the cells within 24 hr.

**Mechanism of induction:** Since BrdU does not induce differentiation by virtue of its incorporation into DNA, there are three basic mechanisms which could explain this process. One alternative is that BrdU acts at a nonmetabolic locus by increasing the interaction between the cell and the surface of the culture dish. It has been shown that such interactions induce differentiation under normal growth conditions in media containing 10% serum, and also in low-serum media.[2] The other possibilities are that BrdU alters the metabolism of the cell such that *all* of the molecules necessary for differentiation are induced, or that BrdU causes a limited number of metabolic changes that indirectly induce differentiation. For example, if there were an alteration in membrane metabolism which leads to an increased affinity between the cell and the surface of the culture dish, this interaction could, in turn, stimulate the synthesis of proteins needed for differentiation.

Two experiments were carried out to distinguish between these alternatives. If BrdU induces a metabolic change in the cell, it follows that cells grown in the presence of BrdU in Petri dishes under conditions where they cannot morphologically differentiate should differentiate in the absence of BrdU when transferred to a surface where they can attach. Thus, BrdU was added at a final concentration of $4 \times 10^{-6}$ M to an exponentially growing Petri-dish culture of cells. At various times after the addition of BrdU, cells were washed three times with BrdU-free media. The cell clumps were gently dispersed by pipetting and then plated on tissue culture dishes with $8 \times 10^{-6}$ M BrdU, and without BrdU. Fig. 5 shows that after 48 hr in the presence of BrdU, most of the cells were able to differentiate when plated in the absence of BrdU. It follows that BrdU must modify the metabolism of the cell on which it acts.

If the surface of the culture dish plays a role in directing the synthesis of the macromolecules involved in differentiation, the spectrum of macromolecules

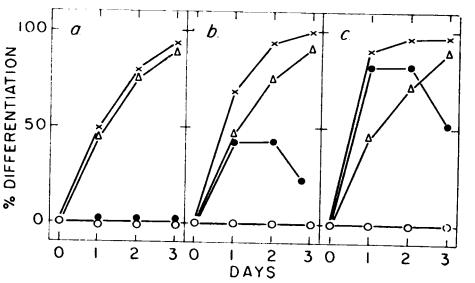


FIG. 5. BrdU transfer experiment. $4 \times 10^{-6}$ M BrdU was added to exponentially growing cells in Petri dishes. At 10 min, 24 hr, and 48-hr cells were centrifuged, washed, and plated on tissue culture dishes in the presence and absence of $8 \times 10^{-6}$ M BrdU. Untreated control cultures were carried through the same procedure. The percentage differentiation is plotted as a function of the days after the transfer to tissue-culture dishes. (*a*) 10 min; (*b*) 24 hr; and (*c*) 48 hr in the presence of BrdU. ×, cells grown in BrdU and plated in BrdU; △, cells grown in the absence of BrdU but plated with BrdU; ●, cells grown in BrdU and plated in the absence of BrdU; ○, cells grown and plated in the absence of BrdU.

synthesized by cells growing on tissue culture dishes in the presence of BrdU should be different from that made by cells growing in Petri dishes in the presence of BrdU. On the other hand, if the surface of the culture dish has only a passive role, as a substrate which allows morphological expression of the differentiated phenotype, the macromolecules made in the morphologically differentiated and undifferentiated cultures should be identical.

To rule out one of these alternatives, an exponentially growing Petri-dish culture was divided into two equal parts. Half of the cells were plated in tissue-culture dishes, and the other half in Petri dishes. BrdU was added to half of the dishes in each set at $4 \times 10^{-6}$ M. After 3 days the cells grown on tissue-culture dishes in the presence of BrdU were completely differentiated (Fig. 2); the cells grown in Petri dishes with BrdU remained in suspension and had a round-cell morphology. None of the cells grown in the absence of BrdU exhibited a differentiated phenotype. [$^3$H]- or [$^{14}$C]leucine was then added to separate sets of cultures, the cultures were incubated at 37°C for 4 hr and then lysed with detergent, and the cytoplasmic fractions were mixed in the manner described in Fig. 6. The mixtures were then lyophilized, fully reduced and alkylated, and electrophoresed on acrylamide gels. Figure 6 shows the ratios

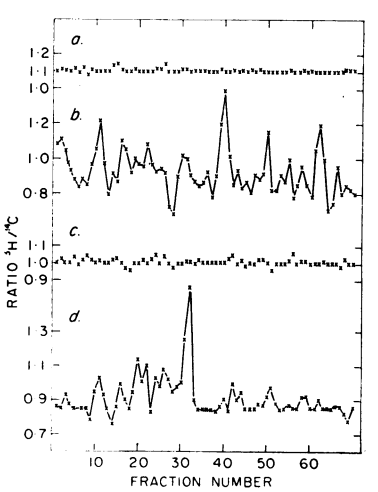


FIG. 6. Ratios of leucine-containing macromolecules. Cells were labeled with [$^{14}$C] or [$^3$H]leucine and then lysed, and the cytoplasms were mixed as indicated below. The mixtures were then reduced, alkylated, and electrophoresed on acrylamide gels as described in *Materials and Methods*. Each sample was counted three times to less than 1% standard error. The data are represented as the numerical average of the $^3$H:$^{14}$C ratios of three determinations. (*a*) $^3$H (Petri) versus $^{14}$C (Petri); (*b*) $^3$H (tissue-culture, $4 \times 10^{-6}$ M BrdU) versus $^{14}$C (Petri); $4 \times 10^{-6}$ M BrdU; (*c*) $^3$H (tissue-culture) versus $^{14}$C (Petri); (*d*) $^3$H (Petri, $4 \times 10^{-6}$ BrdU) versus $^{14}$C (Petri).

of $^3H$ to $^{14}C$ across the gels. Figures 6*a* and *c* show that the macromolecules synthesized in the $^{14}C$ (Petri) and $^3H$ (Petri), and the $^3H$ (tissue-culture) and $^{14}C$ (Petri) cultures were indistinguishable by this criterion. The acrylamide-gel results for the $^3H$ (BrdU, tissue-culture) and $^{14}C$ (BrdU, Petri-dish) cultures shown in Figure 6*b* did, however, indicate that there was a quantitative difference in the spectrum of leucine-containing macromolecules synthesized under these conditions. It follows that the interaction between the cell and the surface of the culture dish must play a role in the control of macromolecular synthesis which is superimposed on the effect of BrdU. It was similarly shown that the macromolecules synthesized in the presence of BrdU were quantitatively different from those made under the same culture conditions in the absence of the pyrimidine (Fig. 6*d*). These results thus rule out the possibility that BrdU alone induces the alterations in macromolecular synthesis required for differentiation. It should be stressed, however, that these results do not demonstrate that there is a change in the protein synthesis between the cells whose leucine-containing macromolecules show variations in specific activity in the acrylamide gels, for it has been shown[10] that changes in the carbohydrate composition of proteins can alter their mobility in acrylamide gels in the presence of sodium dodecylsulfate.

To show that protein synthesis is required for differentiation, cells were grown for three generations in the presence of $4 \times 10^{-6}$ BrdU in Petri dishes. They were then centrifuged, washed, and plated in tissue-culture dishes in the presence of either cycloheximide, puromycin, or actinomycin D at concentrations which inhibit protein and RNA synthesis by more than 95 and 90%, respectively.[2] In addition, cells were plated with colchicine or vinblastine sulfate, two alkaloids which are known to bind to microtubule proteins.[11,12] The data in Table 1 show that cycloheximide, puromycin, and the vinca alkaloids block the

TABLE 1. *Inhibition of neurite formation.*

| Inhibitor | Concentration | % Differentiation | % Viable cells |
|---|---|---|---|
| Puromycin | 10 $\mu$g/ml | 4 | 72 |
| Cycloheximide | 40 $\mu$g/ml | 7 | 80 |
| Actinomycin D | 5 $\mu$g/ml | 73 | 78 |
| Vinblastine sulfate | $4 \times 10^{-6}$ M | 2 | 89 |
| Colchicine | $2 \times 10^{-6}$ M | 6 | 86 |
| Control | ... | 81 | 87 |

Cells were grown in $4 \times 10^{-6}$ M BrdU for three generations in Petri dishes and then plated on tissue culture dishes in the presence of the inhibitors. After 15 hr the percent of differentiated cells and cell viability was determined as described in *Materials and Methods*.

morphological differentiation of the neuroblastoma as defined by the outgrowth of neurites. Actinomycin at 5 $\mu$g/ml does not, however, block differentiation. Although such a result is necessary to define a translational control mechanism, it is clearly not sufficient and requires further study before any conclusions can be reached.

**Discussion.** The following conclusions may be drawn from the above data. (1) BrdU induces the differentiation of mouse neuroblastoma C1300 to cells that morphologically resemble mature neurons. (2) This induced differentiation

can take place in the absence of DNA synthesis, which indicates that the halogenated pyrimidine need not be incorporated into DNA to alter the phenotype of the cell. (3) BrdU causes a metabolic change in the cell which *indirectly* induces the differentiation of the neuroblast, probably by increasing the affinity between the limiting membrane of the cell and the surface of the culture dish. (4) The expression of the differentiated state is dependent on protein and microtubule synthesis.

In contrast to these observations, it has been established that BrdU *inhibits* myoblast fusion and the synthesis of such differentiated cell products as chondroitin sulfate and hyaluronic acid.[7-9] Since BrdU can be incorporated into DNA in place of thymidine, it has been argued that BrdU alters the phenotype of the cell by virtue of its substitution into DNA.[7] The fact that differentiation can be induced in the absence of DNA synthesis excludes this hypothesis for the neuroblastoma and argues against this mechanism in other systems. This is particularly true since the spectrum of halogenated pyrimidines which induces the neuroblastoma to differentiate is the same as that which inhibits the expression of the differentiated phenotype in other cells.[7] In addition, it has been observed that BrdU induces flattening of cells,[7] which suggests an increased affinity between the cell and the surface of the culture dish. This interaction could then alter the synthesis of specialized products.

Since it has been established that the interaction between cell and surface of the culture dish is responsible for the induced differentiation of the neuroblastoma,[2] the most likely explanation for BrdU-induced differentiation is that BrdU increases the affinity between the cell and the surface of the culture dish which in turn induces the synthesis of the specialized cell products required for differentiation. This argument is supported by observation that macromolecular synthesis is quantitatively different when cells are grown on tissue-culture or Petri dishes in the presence of BrdU (Fig. 6) and that protein and microtubule synthesis are required for detectable neurite formation (Table 1).

Although the mechanism of the extrachromosomal effect of BrdU remains to be defined, the most plausible explanation is that BrdU affects the synthesis of carbohydrate moieties associated with the limiting membrane of the cell. It is known that animal cell surfaces contain glycoproteins and mucopolysaccharides, and that the synthesis of the polysaccharide chains involves the formation of nucleotide sugars, usually as uridine diphosphate derivatives.[13] It follows that any alteration in the synthesis of the polysaccharide would change the surface structure of the cell, which would alter its phenotypic behavior.

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Abbreviations: BrdU, 5-bromodeoxyuridine; ara-C, 1-β-D-arabinofuranosylcytosine.

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