The Effect of 5-Bromodeoxyuridine on Interferon Production in Human Cells

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SUMMARY

5-Bromodeoxyuridine (BrdUrd) increased interferon production by the Namalwa line of human lymphoblastoid cells treated with Sendai virus, but inhibited their growth. Thymidine, which also inhibited cell growth had no effect on interferon production, so that growth inhibition per se was not the cause of the stimulation. BrdUrd was incorporated into cellular DNA; 5-chlorodeoxyuridine and 5-iododeoxyuridine (which are also incorporated) increased the interferon yield, but 5-fluorodeoxyuridine (which is not incorporated) did not. Thymidine reduced both the incorporation of BrdUrd and its stimulatory effect on interferon production. Deoxycytidine (which prevents the cytotoxic effects of BrdUrd) had no effect on the stimulation. BrdUrd also stimulated interferon production in response to poly(rI).poly(rC) in growing human diploid fibroblasts but not in SV40 virus-transformed human cells. Since BrdUrd was incorporated into the DNA of all these cells, we concluded that incorporation is necessary, but not sufficient for the stimulation of interferon formation.

INTRODUCTION

The effect of halogenated pyrimidines on gene expression in culture cells has been much studied. Low concentrations of 5-bromodeoxyuridine (BrdUrd) reversibly suppress the expression of differentiation in various cells (Bischoff & Holtzer, 1970; Lasher & Cahn, 1969; Coleman et al. 1970; Rutter et al. 1973); enhance the rate of production of some enzymes and other proteins (Koyama & Ono, 1971, 1972; Bick & Soffer, 1976; Biswas et al. 1977; O’Brien & Stellwagen, 1977); activate murine or avian C-type viruses in latently-infected cells (Lowy et al. 1971; Aaronson et al. 1971; Robinson et al. 1976); and activate the production of Epstein–Barr virus by transformed lymphoid cells (Hampar et al. 1972; Gerber, 1972).

It appears likely that these effects are not all mediated by a common mechanism. BrdUrd is an analogue of thymidine and is readily incorporated into DNA (Davidson & Bick, 1973). It may also cause changes in cell morphology (Davidson & Horn, 1974) and it is toxic at high concentrations. This toxicity, which is reversible, is probably due to inhibition of the enzyme ribonucleotide reductase, so that the cell is effectively starved of deoxycytidine (Meuth & Green, 1974); addition of deoxycytidine to the medium prevents this toxic effect, although the BrdUrd is still incorporated into DNA.

Incorporation of halogenated pyrimidines into DNA is essential for the induction of endogenous C-type viruses (Teich et al. 1973) or of DNA viruses such as lambda and polyoma. Addition of deoxycytidine to murine cells greatly reduces the cytotoxic effect without diminishing C-type virus induction (Besmer et al. 1975). In contrast, the incorporation of BrdUrd into DNA is not the cause of the changes in specialized gene expression in some cases, for example the suppression of specialized differentiated functions in cultured myoblasts (Rogers et al. 1975) and melanoma cells (Davidson & Kaufman, 1977).
It has been postulated that incorporated BrdUrd affects transcription by altering the binding affinities of chromosomal proteins for DNA (Lin & Riggs, 1972; David et al. 1974; Lapeyre & Bekhor, 1974; Gordon et al. 1976; Schwartz, 1977). Altered patterns of transcription have been reported in cells treated with BrdUrd (Kotzin & Baker, 1972; Lykkefeldt & Anderson, 1977).

Cells of the human lymphoblastoid line, Namalwa, derived from a Burkitt's lymphoma and transformed with Epstein-Barr virus, produce high titres of interferon in response to Sendai virus (Strander et al. 1975). Tovey et al. (1977) reported that BrdUrd stimulates interferon production in this and several other lymphoblastoid cell lines. We have determined the optimal conditions for maximum interferon yields from Namalwa cells and have compared the effect of BrdUrd to that of other analogues of thymidine. We have also investigated the effect of BrdUrd on interferon yields from two human fibroblast cell lines.

**METHODS**

**Materials.** BrdUrd, 5-iododeoxyuridine (IdUrd) and 5-fluorodeoxyuridine (FdUrd) were purchased from Sigma, 5-chlorodeoxyuridine (CldUrd) from Calbiochem, 3H-thymidine (dThd, 24 mCi/mmol) and 3H-BrdUrd (3.0 mCi/mmol) from The Radiochemical Centre, Amersham and polyriboinosinic acid—polyribocytidylic acid [poly(rI). poly(rC)] from P-L Biochemicals.

**Cells and viruses.** A subline (CSN 20) of Namalwa cells, obtained from the Wellcome Research Laboratories was grown in suspension in RPMI 1640 medium containing 10% new-born calf serum (NCS; Flow Laboratories), 60 μg penicillin and 100 μg of streptomycin/ml and buffered with 20 mM-Hepes.

Cells were diluted two- to threefold every 3 or 4 days when they reached a cell count of $1.0 \times 10^6$ to $1.5 \times 10^6$/ml. HFF (human foreskin fibroblasts) cells were a gift from Dr T. Merigan, Stanford University, Calif. U.S.A., and a line of SV40 virus-transformed WI-26 cells (WI-26 VA4-FU3) was obtained from Dr L. Diamond, Wistar Institute, Phil., U.S.A. These were both grown in Glasgow Modified Eagle's Medium (GMEM) containing 10% foetal calf serum (FCS; Flow Laboratories). Bovine turbinate (BT) cells, a gift from Dr A. W. McClurkin, National Animal Disease Centre, Ames, Iowa, U.S.A., were grown in GMEM containing 10% horse serum (Flow Laboratories). Sendai virus and Newcastle disease virus (NDV), strain F were grown in the allantoic cavity of 10 day-old eggs and harvested after 60 or 72 h, respectively.

**Interferon induction.** Namalwa cells were centrifuged (2000 g for 5 min), resuspended in RPMI 1640 with 2% NCS at $5 \times 10^6$ cells/ml and incubated at 37 °C in the dark for 48 h with the appropriate concentration of BrdUrd. After centrifugation, the cells were suspended in RPMI 1640 with 2% NCS at $2 \times 10^6$ cells/ml, and 100 haemagglutinating units (HAU) of Sendai virus were added for each $10^6$ cells. After further incubation at 37 °C for 24 h, the cells were centrifuged and the supernatant was dialysed against 50 vol. of pH 2 buffer for 24 h and then against PBS for 8 h before it was assayed for interferon.

Human fibroblasts were incubated with the appropriate concentration of BrdUrd in growth medium. At the required time, the medium was removed and the cells were washed with PBS and induced with either 100 HAU/10^6 cells of NDV or 50 μg/ml of poly(rI). poly(rC) as described by Atherton & Burke (1975). With poly(rI). poly(rC) induction, the cells were primed by pre-treatment overnight with 200 units/ml of human interferon in GMEM with 2% FCS; or treated simultaneously with DEAE-dextran (50 μg/ml); or superinduced with cycloheximide (50 μg/ml), added from 0 to 5 h after induction, and actinomycin D (5 μg/ml) from 5 to 5.5 h after induction, before they were washed at 5.5 h after induction and incubated in GMEM plus 2% FCS.

**Interferon assay.** Interferon was assayed by the RNA-reduction method (Atherton &
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Burke, 1975) with HFF cells for human fibroblast interferon and BT cells for lymphoblastoid interferon. The results are expressed in reference units, based on the human leukocyte interferon research standard (69/19), which is defined as containing 5000 reference research units and which has a titre of 2500 in HFF cells and 50000 in BT cells. The dose-response curves for leukocyte and fibroblast interferons were not parallel but no fibroblast interferon reference standard was available. In 10 consecutive titrations, our laboratory reference standard had a mean of $4.0 \times 10^6$ units with a standard deviation of 0.143.

Radioactive isotope incorporation. Incorporation of $^3$H-BrdUrd or $^3$H-dThd was measured by adding 10 $\mu$Ci in the presence of 25 $\mu$g/ml BrdUrd or 20 $\mu$g/ml dThd (both 81.4 $\mu$M) to $5 \times 10^6$ Namalwa cells in 5 ml RPMI 1640 with 2% NCS or to $2 \times 10^6$ fibroblast cells in GMEM with 10% FCS. Incorporation was stopped by washing the cells with ice-cold PBS, followed by precipitation with 5% ice-cold trichloroacetic acid. Namalwa precipitates were filtered through Whatman GF/C filters and washed and their radioactivity was determined. Fibroblast precipitates were counted after washing with 5% TCA and drying with ethanol.

Measurement of BrdUrd incorporation into cellular DNA. Namalwa cells were grown to $5 \times 10^6$/ml in RPMI 1640 with 2% NCS and with or without 25 $\mu$g/ml BrdUrd for 48 h. The cells were washed once by centrifugation and resuspension in PBS, then centrifuged again and resuspended in a few ml of hypertonic buffer (10 mM-tris, 1.5 mM-MgCl$_2$, 20 mM-KCl, pH 7.2) for 5 min at 20 °C. Then 10% sodium dodecyl sulphate (SDS) in water was added to a final concentration of 1%. Lysis of the cells was checked by light microscopy and occurred within 5 min.

HFF and SV40-transformed WI-26 cells were grown in GMEM with 10% FCS and 25 $\mu$g/ml BrdUrd for 3 and 8 days, respectively and then the monolayers were washed with PBS and lysed by the method described above. The DNA was not further purified before centrifugation.

Buoyant densities of DNA were determined by comparing the equilibrium position in a caesium chloride gradient to that of an E. coli DNA marker. The gradient was established during an overnight run at 65000 g in a Beckman Model E ultracentrifuge and the density of the DNA was calculated according to Schildkraut et al. (1962). The change in density is proportional to the degree of substitution by BrdUrd, and the mole fraction of BrdUrd in the DNA can be determined by established methods (Hackett & Hanawalt, 1966; Round, 1967).

RESULTS

Effect of BrdUrd on interferon production

Namalwa cells produce interferon in large but variable amounts in response to viruses, for example Sendai virus, but very little in response to poly(rI), poly(rC). This variation was largely eliminated by pre-treatment with BrdUrd (data not shown) which increased yields as described by Tovey et al. (1977). Preliminary experiments showed that pre-treatment for 48 h usually gave better results than pre-treatment for shorter or longer periods (data not shown). The decreased yields after longer times were probably due to drug cytotoxicity as shown by an increasing inhibitory effect on cellular RNA and protein synthesis (data not shown). The concentrations of BrdUrd, of cells, of Sendai virus and of calf serum in the medium were then systematically varied. As a result, 48 h pre-treatment with 25 $\mu$g/ml BrdUrd, followed by induction with 100 HAU of Sendai virus/10$^6$ cells at a cell concentration of $2 \times 10^6$/ml in medium containing 2% calf serum was selected as optimal (data not shown). Measurement of the rate of interferon production with and without BrdUrd pre-treatment showed that BrdUrd treatment increased the yield of interferon at all times (Fig. 1a). In this respect the treated cells behaved like primed fibroblasts, but unlike
super-induced fibroblasts where the kinetics of interferon production are greatly changed (Friedman, 1966; Vilcek & Ng, 1971).

The effects of other analogues of thymidine on interferon production by Namalwa cells were next measured in order to test the specificity of the BrdUrd effect. It was found that CldUrd had about the same effect as BrdUrd and that IdUrd was somewhat less effective. On the other hand, FdUrd depressed the interferon yield, although the cells remained viable, as shown by trypan blue exclusion. Thymidine itself had no effect on the interferon yields at a concentration of 25 μg/ml; when present with BrdUrd it prevented this from stimulating interferon production, presumably because of competition for uptake and incorporation of the analogue, an effect also reported by Tovey et al. (1977). Addition of deoxycytidine, along with the BrdUrd, did not prevent the increase in interferon yield (Table 1), although it did reverse the inhibitory effect of BrdUrd on Namalwa cell growth (Table 1 & Fig. 2a) and therefore, presumably, its toxicity.

Interferon yields were also increased when HFF cells were pre-treated for 3 days with BrdUrd (Table 2 and Fig. 1 b). The stimulatory effect of BrdUrd was shown whether the cells were treated with poly(rI).poly(rC) plus DEAE-dextran, or were primed by pre-treatment overnight with homologous interferon, or were superinduced by addition of metabolic inhibitors. It was therefore concluded that BrdUrd was increasing the yield of interferon by a mechanism which was distinct from that involved in either priming or superinduction. There was much less stimulation of virus-induced interferon formation. As with Namalwa cells, addition of thymidine did not stimulate interferon production and the enhancement

Table 1. The effect of BrdUrd and deoxycytidine on interferon production in Namalwa cells induced with Sendai virus

<table>
<thead>
<tr>
<th>BrdUrd (μg/ml)</th>
<th>Deoxycytidine (mM)</th>
<th>Cell density (× 10^4/ml)</th>
<th>Interferon yield (log_10 units/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0.92</td>
<td>2.84</td>
</tr>
<tr>
<td>25</td>
<td>0</td>
<td>0.75</td>
<td>3.62</td>
</tr>
<tr>
<td>25</td>
<td>10</td>
<td>0.98</td>
<td>3.61</td>
</tr>
<tr>
<td>25</td>
<td>100</td>
<td>0.80</td>
<td>3.65</td>
</tr>
<tr>
<td>50</td>
<td>100</td>
<td>0.93</td>
<td>3.36</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>0.85</td>
<td>3.05</td>
</tr>
</tbody>
</table>

* Measured at time of induction.
BrdUrd and interferon production

Fig. 2. The growth of (a) Namalwa, (b) HFF and (c) SV40-transformed WI-26 cells in the presence (●—●) or absence (○—○) of 25 µg/ml BrdUrd, and in the presence of 25 µg/ml BrdUrd and 100 mM-deoxycytidine (□—□). The additions were made on day 0.

Table 2. Interferon yields in human fibroblast cells after treatment with BrdUrd

<table>
<thead>
<tr>
<th>Cells</th>
<th>Inducing treatment*</th>
<th>From control</th>
<th>25 µg/ml</th>
<th>50 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFF (pre-treated for 3 days)</td>
<td>Poly(rI).poly(rC)</td>
<td>From cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+DEAE-dextran</td>
<td>2.5</td>
<td>3.83</td>
<td>3.68</td>
</tr>
<tr>
<td></td>
<td>Polyr(l).poly(rC)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+priming</td>
<td>2.85</td>
<td>3.86</td>
<td>3.00</td>
</tr>
<tr>
<td></td>
<td>Poly(rI).poly(rC)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+superinduction</td>
<td>3.38</td>
<td>3.95</td>
<td>3.87</td>
</tr>
<tr>
<td></td>
<td>NDV</td>
<td>2.78</td>
<td>3.12</td>
<td>3.18</td>
</tr>
<tr>
<td>SV40 WI-26 (pre-treated for 6 days)</td>
<td>Poly(rI).poly(rC)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+DEAE-dextran</td>
<td>2.18</td>
<td>2.48</td>
<td></td>
</tr>
</tbody>
</table>

* See Methods for details.

due to BrdUrd was reduced when thymidine was also present. Addition of deoxycytidine, along with BrdUrd, did not prevent an increase in interferon yield (data not shown) suggesting that in this system too, the increased interferon yield was due not to the cytotoxic effect of BrdUrd, but to its incorporation into cellular DNA. This conclusion was strengthened by the results of an experiment in which interferon was induced in HFF cells which had been treated with BrdUrd for 2 days immediately after the culture became confluent. Under these conditions, where it was shown by ³H-thymidine labelling that DNA synthesis was depressed to about 5% of normal, there was no stimulatory effect of BrdUrd (yield of interferon from control 2.2 log₁₀ units; yield from BrdUrd treated cells 2.17 log₁₀ units) presumably because it was not incorporated into cellular DNA to any significant extent.
Table 3. Buoyant density of DNA from cells grown in 81.4 μM-BrdUrd or thymidine

<table>
<thead>
<tr>
<th>Cells</th>
<th>Additive*</th>
<th>Time (days)</th>
<th>Density</th>
<th>Mole fraction BrdUrd</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFF</td>
<td>dThd</td>
<td>3</td>
<td>1.699</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>BrdUrd</td>
<td>3</td>
<td>1.739</td>
<td>0.12</td>
</tr>
<tr>
<td>Namalwa</td>
<td>dThd</td>
<td>2</td>
<td>1.699</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>BrdUrd</td>
<td>2</td>
<td>1.750</td>
<td>0.15</td>
</tr>
<tr>
<td>SV40-transformed</td>
<td>dThd</td>
<td>6</td>
<td>1.699</td>
<td>0</td>
</tr>
<tr>
<td>WI-26</td>
<td>BrdUrd</td>
<td>6</td>
<td>1.754</td>
<td>0.16</td>
</tr>
</tbody>
</table>

* dThd at 20 μg/ml; BrdUrd at 25 μg/ml.

Pre-treatment of SV40 WI-26 cells with BrdUrd for 6 days had little effect on the amount of interferon formed in response to poly(rI).poly(rC) (see Table 2); the same result was obtained in a further experiment in which the cytotoxic effect of BrdUrd was largely reversed by the addition of deoxycytidine.

Effect of BrdUrd on cell growth and DNA synthesis

The growth of Namalwa and the two fibroblast cell lines was increasingly inhibited by increasing BrdUrd concentrations (data not shown); both HFF and SV40-transformed WI-26 cells were almost completely inhibited by BrdUrd (25 μg/ml) (Fig. 2b, c) but the growth of Namalwa cells was far less affected (Fig. 2a). No changes were seen in the morphology of any of the cell types.

Incorporation of radioactively-labelled BrdUrd (25 μg/ml, i.e. 81 μM) or of dThd (81 μM) was next measured. It was found that there was little incorporation of BrdUrd into either HFF or SV40-transformed WI-26 cells, but substantial incorporation into Namalwa cells (data not shown). However, interpretation of these results was complicated by the possible effects of BrdUrd on pool sizes. The incorporation of BrdUrd into cellular DNA was therefore determined directly by measuring the density of the cellular DNA after the cells had been grown in medium containing BrdUrd for several days. All three cell types incorporated about the same amount of BrdUrd into their cellular DNA (Table 3). We conclude that incorporation of BrdUrd is a necessary but not sufficient cause of the stimulation in interferon yield.

DISCUSSION

We have shown that BrdUrd augments interferon formation and inhibits cell growth of human lymphoblastoid cells and fibroblasts. There is no connection between these phenomena, since BrdUrd inhibited the growth of SV40 virus-transformed fibroblasts without stimulating interferon formation. Conversely, addition of deoxycytidine reversed the inhibitory effect of BrdUrd on the growth of Namalwa cells without affecting the stimulatory effect of interferon production. If incorporation of BrdUrd into human fibroblast DNA was prevented because the cells were no longer dividing, then no stimulation was seen. We conclude that incorporation of BrdUrd into cell DNA is probably necessary for the increased interferon production, a conclusion strengthened by the behaviour of the halogen analogues of BrdUrd. Those that are incorporated into cellular DNA increased the interferon yield, while FdUrd, which was not incorporated, actually depressed the interferon yield. It is known that IdUrd is incorporated into DNA to a lesser extent than BrdUrd because of the size of the substituted atom and the steric preferences of the enzyme DNA polymerase, and that FdUrd is not incorporated into cellular DNA (Cozzarelli, 1977; Santi & McHenry, 1972). However, incorporation by itself was not sufficient since BrdUrd
did not stimulate interferon production in SV40 virus-transformed fibroblasts, even in the presence of deoxycytidine to suppress cytotoxic effects. We do not know why, but these cells can neither be primed nor superinduced, so that some of the control systems that modulate interferon production may be altered and may possibly be unaffected by the BrdUrd. We conclude that incorporation into cellular DNA is essential but not sufficient.

In all three cell lines, the mole fraction of BrdUrd incorporated was between 0.12 and 0.16 and since the mole fraction of thymidine in unsubstituted DNA is about 0.3 this suggests that about 50% of the thymidine residues are replaced by BrdUrd. This would correspond to one round of DNA synthesis using BrdUrd as a base instead of thymidine, after which DNA synthesis presumably stops, and would explain why there was no greater stimulation of interferon production at higher concentrations of BrdUrd and also the cessation of cell growth.

The increased interferon yields from Namalwa cells are not due to a change in membrane permeability, since the intracellular interferon levels are very low and are unaffected by BrdUrd treatment (unpublished data). The stimulation shown by treated Namalwa and fibroblast cells applies to all inducers and conditions tested, including spontaneous production by Namalwa cells (Tovey et al. 1977). This suggests that a common point in the interferon mechanism is affected by BrdUrd treatment. Furthermore, since total protein synthesis is slightly depressed (data not shown) the effect of BrdUrd must be selective.

This effect of BrdUrd on interferon production may be compared to that on C-type virus induction, for in both cases transcription is affected by a process that requires analogue incorporation, but which is not affected by treatment with deoxycytidine. It may be distinguished from the inhibitory effect on the production of specialized proteins by differentiated cells, a process which is reversed by deoxycytidine.

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REFERENCES


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