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DNA double labelling with IdUrd and CldUrd for spatial and temporal analysis of cell proliferation and DNA replication

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Summary

A procedure was developed that very effectively distinguishes between IdUrd and CldUrd incorporated in the DNA of cell nuclei and chromosomes. For double staining we used the rat anti-BrdUrd monoclonal antibody from Sera-lab that binds specifically to CldUrd and BrdUrd but not to IdUrd, in combination with the mouse anti-BrdUrd monoclonal antibody from Becton Dickinson. This antibody binds to all three halogenated deoxyuridines, but when the nuclei are washed in TRIS buffer with a high salt concentration the antibodies linked to CldUrd-labelled DNA are removed. When analysing the effect of the deoxyuridines on the cell cycle we found that the growth kinetics of Chinese hamster cells were not changed by adding IdUrd or CldUrd for 30 min at a concentration of 10 μM, whereas adequate double labelling required only 2 min pulses. The effectiveness of the technique was demonstrated in two model experiments. The first test concerned the assessment of cell recruitment in the central areas of slow-growing clones, after addition of fresh medium. The second experiment focussed on the spatial resolution of the method. Double-labelled metaphase chromosomes showed interspersed green and red replication bands with a spacing corresponding with medium resolution Giemsa banding patterns.

Introduction

Methods for labelling newly synthesized DNA are being used in almost every area of cell biology. Until recently the standard procedure was based on autoradiography of incorporated tritiated thymidine but the range of applications of DNA labelling has increased significantly since the introduction a few years ago of the much less cumbersome BrdUrd technique (Gratzner, 1982; Dolbeare *et al.*, 1983, 1985; Schutte *et al.*, 1987; Beisker *et al.*, 1987; Bakker *et al.*, 1989). This method has proven its usefulness in studies on cell kinetics (Begg *et al.*, 1985), tumour proliferation (Wilson *et al.*, 1988) and DNA replication (Dierendonck *et al.*, 1989; Vogel *et al.*, 1986). However, for a detailed analysis of many processes, in particular those involving time-dependent phenomena such as changes in the dynamics of cell proliferation or synthetic activity at the subcellular level, even this method has severe limitations. Studies of that type can be carried out effectively only by using two or more independent DNA replication labels in the same experiment. One solution to this problem is the application of tritiated thymidine in combination with BrdUrd, but that requires the re-introduction of autoradiography techniques. An additional disadvantage of this approach, in particular for studies at the subcellular level, is the limited spatial resolution of autoradiography. These handicaps could be eliminated by the introduction of a double-labelling procedure based on the application of two non-radioactive DNA precursors that can be incorporated in cells *in vivo*. A significant contribution to the solution of the problems mentioned was made by Shibui *et al.* (1989) who applied a combination of IdUrd and BrdUrd to cells in culture. For the detection of these deoxyuridines they used two monoclonal antibodies developed by Van der Laan & Thomas (1985) and Van der Laan *et al.* (1986), with different specificities: Br-3, which recognizes only BrdUrd, and IU-4 which recognizes both BrdUrd and IdUrd. Their method can distinguish between three groups of cells: unlabelled cells, cells labelled with IdUrd, and a group containing (i) cells which have incorporated BrdUrd only, and (ii) cells which have incorporated both BrdUrd and IdUrd. However, the inability, of their procedure to discriminate between all four populations, in particular between cells labelled with BrdUrd alone and cells labelled with a combination of BrdUrd and IdUrd,

limits its range of applications. A double-labelling procedure, allowing the independent detection of both labels, has many more applications in the study of cell cycle kinetics and in other fields of cell biology. In an attempt to develop a double-labelling procedure with these characteristics that would not require autoradiography, we applied different combinations of halogenated deoxyuridines. After a series of experiments we were able to select a pair of antibodies that could be used for highly specific staining of IdUrd and CldUrd. With this combination, we were able to detect independently IdUrd and CldUrd incorporated in cell nuclei (Bakker *et al.*, 1991). However, before the procedure could be applied effectively as a method for double labelling in cell biological investigations, we had to show that the procedure provides reliable information on the spatial and temporal distribution of DNA replication activity. In the present study we report on investigations concerning these questions. Data are presented on the specificity of various antibodies and on the effect of the labelling on the progression through the cell cycle. In addition we tested the suitability of the method for analysis of changes in cell proliferation activity in clones, and for analysis of the evolution during the S-phase of DNA replication patterns on metaphase chromosomes.

Materials and methods

Cell culture

Cultures of V79 Chinese hamster cells, RUC rat ureter carcinoma cells (Barendsen *et al.*, 1977), and HSF-7 human skin fibroblasts, kindly provided by Dr J. Chen, Los Alamos National Laboratories, were grown as monolayers in Costar tissue culture flasks. The V79 and RUC cells were grown in Eagle's minimum essential medium with Hanks' balanced salt solution, supplemented with 10% fetal calf serum, glutamine and penicillin in a moist atmosphere of 2% CO_2 in air at 37°C. HSF-7 cells were incubated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, glutamine and penicillin in a moist atmosphere of 6% CO_2 in air at 37°C. Under these conditions the cell cycle time of the V79 and RUC cells was 10 h, while that of the HSF-7 cells was 30 h.

Preparation of single- and double-labelled nuclei

Cells from exponentially growing cultures were used for all experiments. In the single-labelling experiments cells were pulse-labelled for 2 min with a iododeoxyuridine(IdUrd)-, bromodeoxyuridine(BrdUrd)-, or chlorodeoxyuridine(CldUrd)-containing medium (final concentration 10 μM) to test the specificity of the monoclonal antibodies. In the double-labelling experiments, cells were incubated for 2 min with IdUrd after which the medium was removed. Cells were then washed three times with prewarmed 'half-and-half' medium, a 1 : 1 mixture of fresh medium and medium that had been conditioned as a result of culturing cells in it for 3 days. During the first washing, thymidine was added at a concentration of 100 μM. Subsequently the cells were cultured for 5 h in prewarmed 'half-and-half' medium and then pulse labelled with CldUrd for 2 min. At the end of the labelling procedure the cells were washed with a buffered solution (8.0 g $NaCl\,l^{-1}$, 0.4 g $KCl\,l^{-1}$, 0.35 g $NaHCO_3\,l^{-1}$, 1.0 g glucose l^{-1} and 2.5 mg Phenol red l^{-1}) at room temperature, harvested by trypsinization, centrifuged at 200 **g** for 5 min and resuspended in 1 ml hypotonic solution containing 37.5 mM KCl, 10 mM $MgSO_4$ and 5mM HEPES (pH 8). After 10 min incubation at room temperature, 0.5 ml of the same solution supplemented with 0.2% Triton X-100, was added. Subsequently the cells were incubated for 5 min and finally syringed twice through a 22 gauge needle to release the nuclei from the cells. The nuclei were spun down by centrifugation at 200 **g** for 5 min on slides and fixed in 70% ethanol for 30 min at room temperature. The slides were air-dried and could be stored at 8°C for up to 3 weeks.

Cell cycle kinetics: effects of halogenated deoxyuridines

The influence of the halogenated deoxyuridines IdUrd and CldUrd on the cell cycle of V79 Chinese hamster cells was measured by flow cytometry and by time-lapse cinematography.

Flow cytometry. Exponentially growing cultures were trypsinized and transferred to Costar tissue culture Petri dishes ($r = 50$ mm), 10^6 cells per dish in 10 ml medium. Twenty-four hours later, corresponding to about two cell generations, the cells were pulse labelled for 30 min with 10 μM IdUrd or CldUrd, or were sham-treated. Cultures were sampled 0 h, 2 h, 4 h and 6 h after labelling. The cells were trypsinized, treated with RNase, and stained with 50 μg mol^{-1} of propidium iodide for DNA content analysis. The stained cell suspensions were assayed with a Cytofluorograph System 30 flow cytometer. The DNA histograms were analysed using an algorithim that fits boundaries between the G1, S and G2/M fractions, taking into acount the CV of the histogram.

Time-lapse cinematography. Exponentially growing cultures were trypsinized and plated in 25 cm^2 Costar tissue culture flasks. After plating, the cultures were replaced in the incubator for 4 h. When the cells had become attached to the bottom of the culture flask and the medium had reached the correct pH the cells were placed under the film unit and filming was started. Every 30 min a frame was exposed. After a period of 24 h, corresponding to about two cell generations, the cells were pulse labelled for 30 min with 10 μM of IdUrd or CldUrd. The field of view was marked on the bottom of the culture flasks in order to allow us to film the same group of cells before, during and after the pulse labelling. Pedigrees were constructed for each cell.

Cell proliferation in clones

For the analysis of cell recruitment in clones of RUC cells the following protocol was used. One-thousand exponentially growing cells were seeded in a Costar Petri dish with 10 ml of medium. After 3 days, half of the medium was replaced with fresh medium, and, at the end of day 5, 9 ml of the medium were replaced by medium without fetal calf serum for 16 h to induce the cells to enter the G_0-phase. The clones were then labelled with 10 μM IdUrd for 30 min, followed by washing with medium. To recruit cells, fresh medium with fetal calf serum was given to the cultures, and, 0 h or 16 h later, CldUrd was added at a final concentration of 10 μM for a period of 30 min. At the end of the second labelling period the cultures were fixed with 70% ethanol and dried. An electrically heated

wire was used to cut rectangular pieces, shaped like microscope slides, from the bottom of the Petri dish for use in microscopic analysis of cell labelling in the RUC clones.

Double labelling of metaphase chromosomes

V79 Chinese hamster cultures were pulse labelled with 10 μM of IdUrd for 30 min, washed, and incubated for 1 h in 'half-and-half' medium; the cultures were labelled for 30 min with CldUrd 2.5 h after the CldUrd pulse vindesine sulphate (Eldisine, EliLilly, The Netherlands) had been added for 1.5 h to a final concentration of 3.5×10^{-8} g ml^{-1} to arrest the cells in mitosis. The mitotic cells were collected by mechanical shake-off, pelleted by centrifugation for 5 min at 200 **g**, and resuspended in 5 ml hypotonic solution containing 37.5 mM KCl and 5 mM

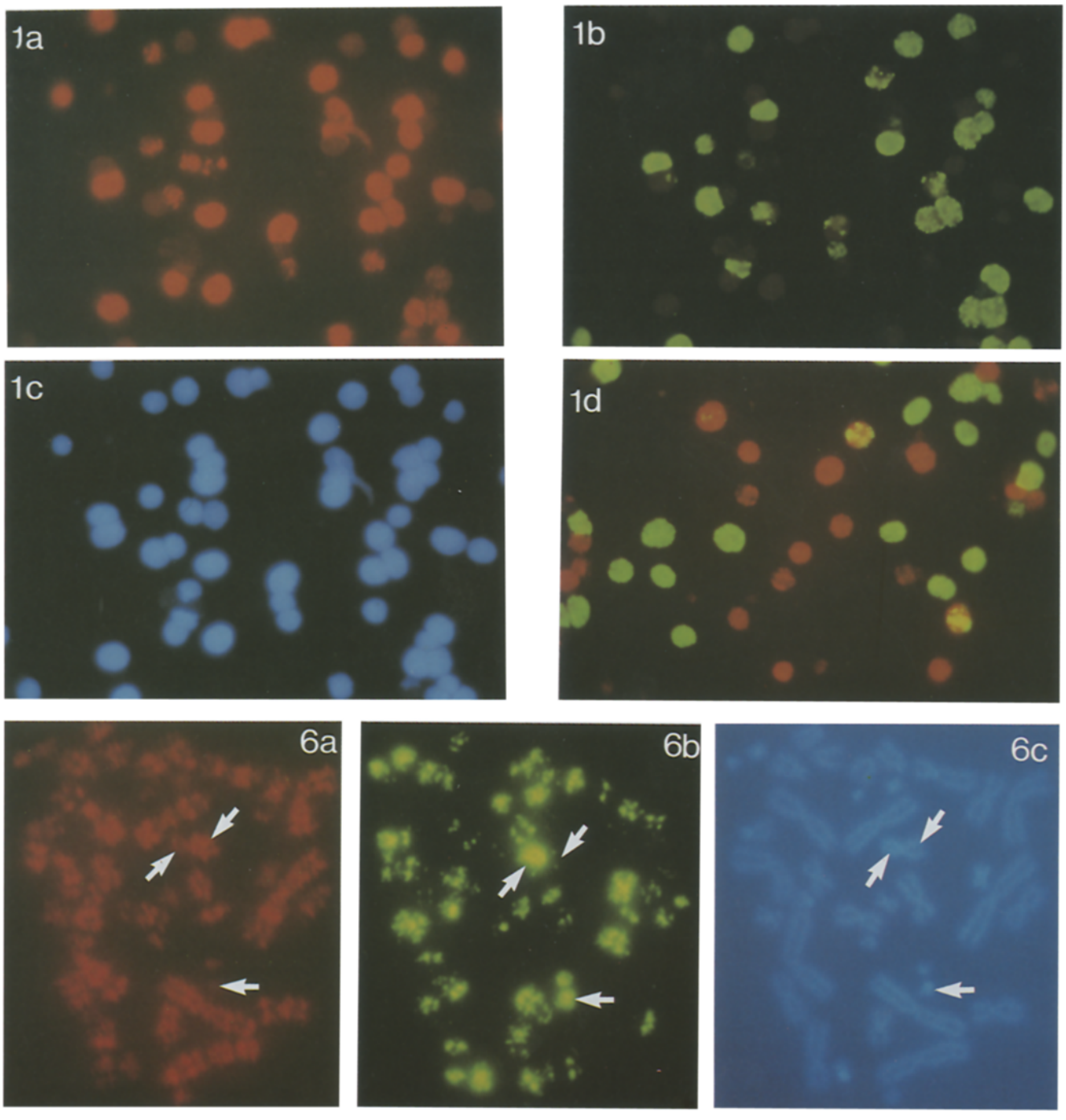

Fig. 1. Double-labelled nuclei of V79 cells. The cells were given a 2 min IdUrd pulse and harvested following a 2 min CldUrd pulse, given 5 h later. (a) IdUrd distribution (Texas Red). (b) CldUrd distribution (FITC). (c) Distribution of nuclear DNA (DAPI) in the same sample. Distributions of IdUrd and CldUrd in a different sample are shown together in (d).

Fig. 6. Metaphase chromosomes of a V79 cell labelled in mid S-phase with IdUrd and in late S-phase with CldUrd. Micrographs of the same metaphase showing (a) IdUrd distribution, (b) CldUrd distribution, and (c) DAPI staining. Arrows indicate regions with only CldUrd or only IdUrd incorporation.

HEPES (pH 8.0). After incubation at 37°C for 8 min, the cells were centrifuged for 5 min at 200 **g** and resuspended in 10 ml fixative (3 : 1 methanol–acetic acid). After centrifuging again for 5 min at 200 **g** the cells were resuspended in 10 ml fixative and kept overnight at 4°C. Finally, after another change of fixative, the cells were dripped onto clean slides. Immediately after dripping the cells, the slides with the metaphases were washed with ca. 2 ml fixative to remove the cytoplasm of the cells. The slides were aged by incubation for 2 days at 60°C in a constant airflow and could be stored for up to 3 weeks before staining.

Staining procedure

Several monoclonal antibodies were tested for their specificity for either IdUrd, BrdUrd or CldUrd : rat anti-BrdUrd (Sera-lab, MAS 250C, clone Bu/75) diluted 1 : 50–1 : 1000; mouse anti-BrdUrd (Becton Dickinson, no. 7580) diluted 1 : 1–1 : 100; mouse anti-BrdUrd (IU4), diluted 1 : 20–1 : 1000 and mouse anti-BrdUrd (Br-3) diluted 1 : 10–1 : 1000, both kindly provided by Dr F. Dolbeare of the Lawrence Livermore National Laboratory; and mouse anti-BrdUrd (Dakopatts M744, clone Bu 20A) diluted 1 : 10–1 : 100. An indirect immunofluorescent staining procedure was used to detect the halogenated nucleosides. All incubations were carried out at room temperature and in a humidified chamber. Partial denaturation of the nuclear DNA was achieved by immersing the slides for 2 min in 0.07 N NaOH. To neutralize the base the slides were washed in 0.1 M $Na_2B_4O_7$ (pH 8.5) for 5 min. After washing the slides twice in PBT buffer (phosphate-buffered saline (PBS) containing 0.05% (v/v) Tween 20, pH 7.4) for 7 min, the nuclei were incubated in a 20% bovine serum albumin (BSA) solution for 10 min (50 µl BSA were used for each slide).

In the single-labelling experiments the nuclei were then incubated for 30 min with a monoclonal anti-BrdUrd antibody, diluted in PBT (50 µl for each slide), and washed twice in PBT buffer for 7 min. After 10 min pre-incubation with normal goat serum (Dakopatts, no. X907) the nuclei were incubated for 30 min with Texas Red-conjugated goat anti-mouse IgG (Jackson, no. 115-075-100) or, in the case of the rat anti-BrdUrd antibody (Sera-lab), with fluorescein-conjugated goat anti-rat IgG (Jackson, no 112-015-102). Both antibodies were diluted 1 : 100 in PBT. After incubation the slides were washed twice in PBT for 7 min, and the nuclei were subsequently mounted with a cover-slip for microscopic examination.

In the double-labelling experiments, rat anti-BrdUrd (Sera-lab) was used in the first step at a 1 : 100 dilution. The slides were then washed twice in PBT buffer for 7 min. After 10 min pre-incubation with normal goat serum (Dakopatts, no. X907), the nuclei were incubated with fluorescein-conjugated goat anti-rat IgG (Jackson, no 112-015-102) diluted 1 : 100 in PBT. Subsequently, after 10 min pre-incubation with BSA, the nuclei were incubated for 30 min with mouse anti-BrdUrd (Becton Dickinson) diluted 1 : 2 in PBT. The slides were washed in TRIS buffer containing 29.2 g NaCl l^{-1}, 4.44 g TRIS-HCl l^{-1} and 0.5% Tween 20 (pH 8.0) for 5–7 min followed by another wash in PBT for 10 min. After 10 min pre-incubation with normal goat serum, the nuclei were incubated for 30 min with Texas Red-conjugated goat anti-mouse IgG (Jackson, no. 115-075-100) at a 1 : 100 dilution in PBT. Finally, after washing the slides twice in PBT for 5 min, the nuclei were mounted with a cover-slip using a mounting medium containing 90% glycerol with 2.3% 1,4-diazabicyclo(2.2.2)octan (DABCO) and 10% 0.2 M TRIS-HCl (with 0.02% NaN_3, pH 8.0, and 0.5 µg 4',6-diamino-2-phenyl-indole ml^{-1} (DAPI)).

Unlabelled cells were used as controls to exclude specific binding of the monoclonal antibodies. To test the specificities of the secondary antibodies the same procedure was used as in the single-labelling experiments. In this procedure IdUrd-labelled cells were incubated with the monoclonal anti-BrdUrd antibody (Becton Dickinson), but instead of Texas Red-conjugated goat anti-mouse IgG, fluorescein-conjugated goat anti-rat IgG was added. CldUrd-labelled cells were incubated with the monoclonal anti-BrdUrd antibody (Sera-lab) and subsequently incubated with Texas Red-conjugated goat anti-mouse IgG.

Microscopy and photography

Microscopic examination was carried out on a Leitz fluorescence microscope (Ortholux II). Photographs were taken on Kodak Ektachrome-200 film using a Leitz Orthomat-W camera attached to the fluorescence microscope, with the photo-ocular adjusted for 10× magnification and a Leitz 40 × /1.30 NPL fluotar oil immersion objective.

Results

Double staining

Information on the binding of various monoclonal antibodies to nuclear DNA labelled with IdUrd, BrdUrd or CldUrd is summarized in Table 1. In the double-staining procedure the Sera-lab rat monoclonal antibody was used at a dilution of 1 : 100 to detect nuclei labelled with CldUrd, and the Becton Dickinson antibody was used at a dilution of 1 : 2 to detect nuclei labelled with IdUrd. Optimal results were obtained when the Sera-lab antibody was applied first. The procedure was tested with V79, RUC and HSF cells. In Fig. 1 samples of V79 nuclei are displayed in four fluorescence micrographs obtained using different filter combinations. Figure 1a, excitation between 520 and 550 nm, shows the Texas–Red fluorescence of the IdUrd-labelled DNA. In Fig. 1b the same area illuminated at 420–530 nm shows the FITC fluorescence of the CldUrd-labelled DNA. The relative position of the cells can be derived from the DAPI fluorescence image in Fig. 1c. In Fig. 1d, a different sample of nuclei, excited at both wavelengths, shows both labels. The micrographs in Fig. 1 demonstrate that the double-staining procedure very effectively distinguishes between IdUrd and CldUrd incorporated into cell nuclei. The same results were obtained with the other cell types used.

Cell cycle kinetics: effects of IdUrd and CldUrd

Changes in cell cycle kinetics of exponentially growing V79 Chinese hamster cells, caused by 30 min pulse labelling with 10 µM of IdUrd or CldUrd, were measured by flow cytometry and by time-lapse cinematography. Flow cytometry provided detailed information on the transition of the cells through the cell cycle during the

Table 1. Test results for several commercially available monoclonal antibodies assayed for their binding to different halogenated deoxyuridines

		Halogenated deoxyuridines		
Anti-BrdUrd monoclonal antibodies	*Species*	*CldUrd*	*BrdUrd*	*IdUrd*
IU-4 (Caltag MD 5000)	Mouse	+	+	+
Br-3 (Caltag MD 5200)	Mouse	+	+	−
Dakopatts (M 744, Bu 20A)	Mouse	+	+	+
Becton Dickinson (no. 758)	Mouse	−*	+	+
Sera-lab (Mas 250c clone Bu/75)	Rat	+	+	−

* After washing with TRIS buffer with a high salt concentration the antibody could be removed.

first 6 h after the treatment (cf. Fig. 2). During this period no differences in cell kinetics were observed between the pulse-labelled and the sham-treated cultures. The only detectable effect on the cell cycle was caused by changing the culture medium. The duration of the cell cycle was measured by time-lapse cinematography. We started filming one cell cycle before the cells were pulse labelled. This allowed us to determine the changes in cell cycles times induced by the addition of nucleosides. The time-lapse data in Figs 3 and 4 show that the cell cycle time was not increased by addition of the labels.

Cell proliferation in clones

RUC cells were grown in Petri dishes to form large clones. The maximum culture time was 7 days; 8 days after plating, the clones started disintegrating. When, at day 6, after 16 h progression without fetal calf serum, the cells were labelled for the first time, proliferation activity was still observed at the edge as well as at the centre of the clones. The average frequency of IdUrd-labelled cells at the centre of the larger clones, however, was significantly lower than at the edge. When CldUrd was added 16 h after the cells had been reactivated by incubation with fresh medium, an increase in the fraction of labelled cells was observed in the central region of the larger clones ($n > 2000$) (Table 2). No increase was observed at the edge of the clones. The smaller clones ($n < 1000$) did not show this increase in labelling index at the centre. The difference in labelling patterns, before and after reactivation of clonal proliferation by addition of fresh medium, is also illustrated in the micrographs in Fig. 5. Other micrographs (not shown) demonstrated that the addition

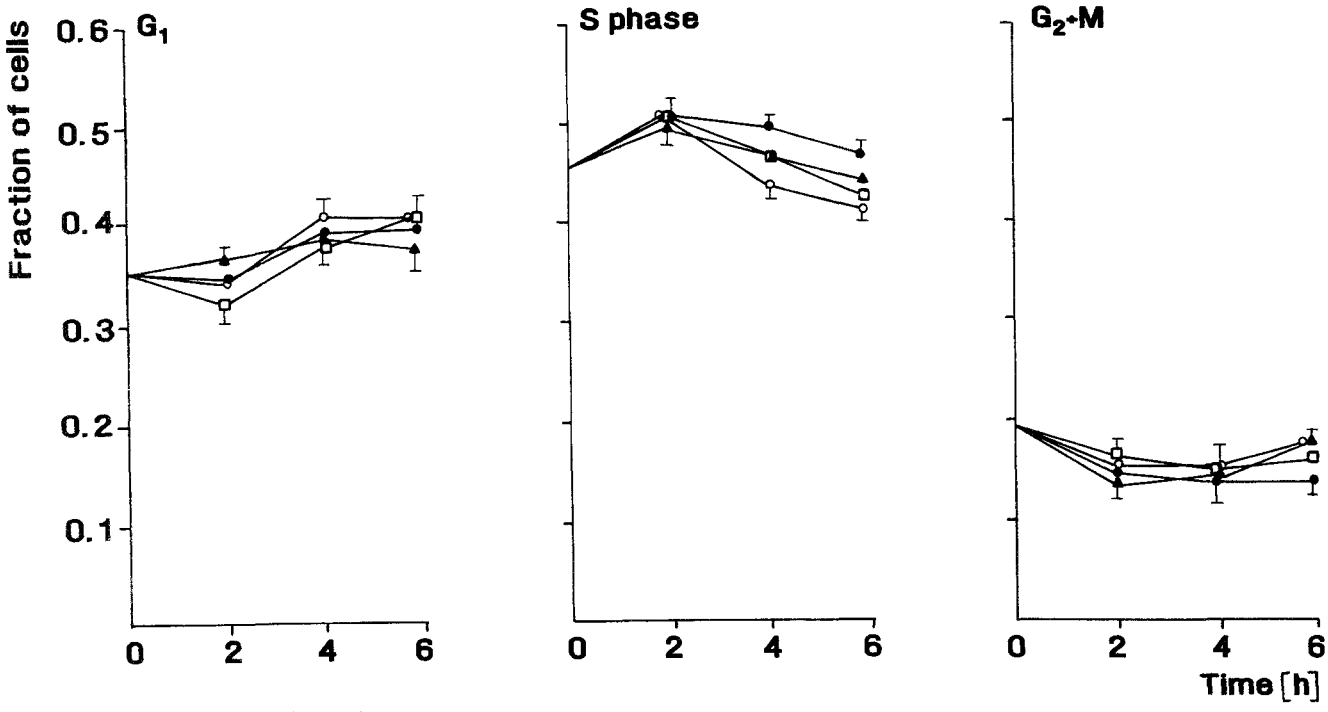

Fig. 2. Flow cytometry analysis of cell kinetics after pulse labelling. Fractions of V79 cells in G_1-, S-, and G_{2+}M-phase are presented for the first 6 h after treatment. Cells labelled with IdUrd ▲, and with CldUrd □; sham-treated cells (medium change) ○, and untreated cells ●. The bars represent the average standard error of the mean for each time point, obtained from three experiments for each treatment modality.

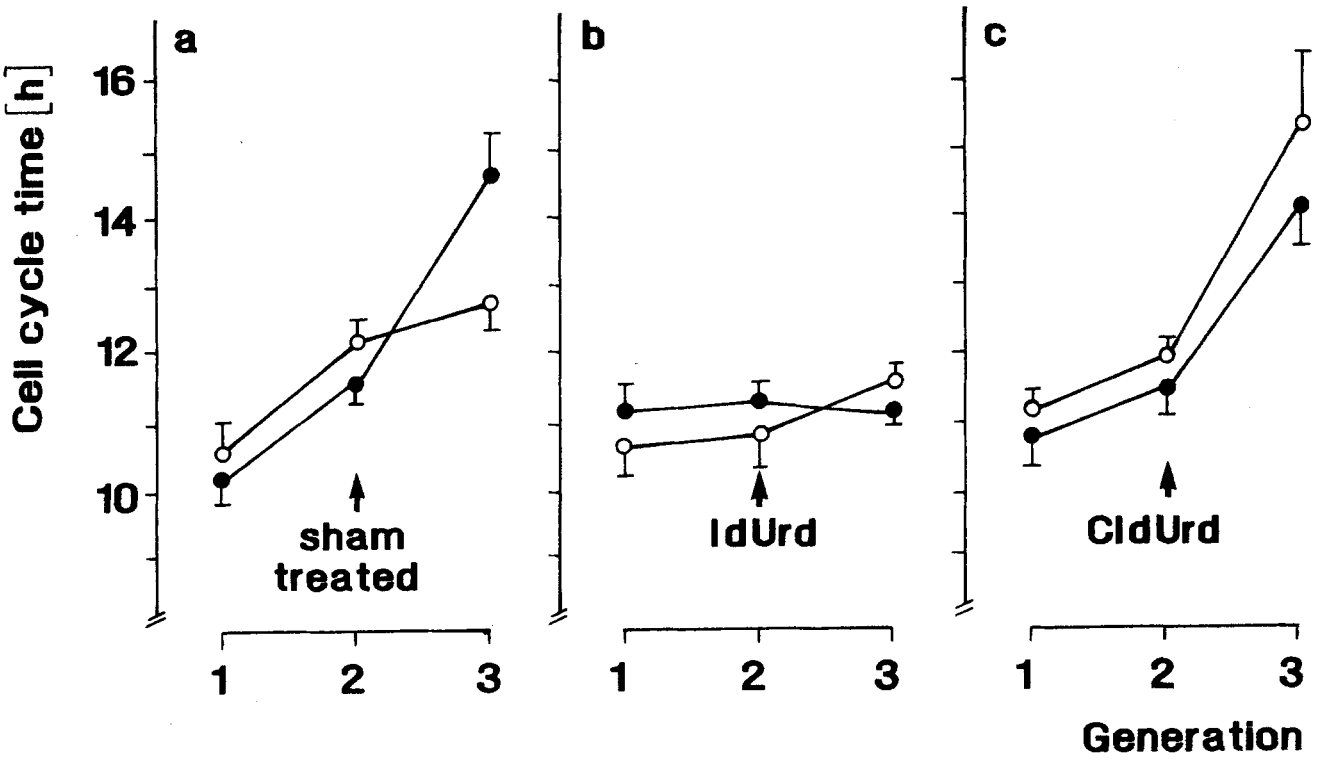

Fig. 3. Time-lapse cinematography analysis of pulse-labelled cells (30 min). Cell cycle times are presented for three generations: before, during and after treatment (the arrow indicates period of treatment). For every treatment the cells were divided in two subpopulations: cells in S-phase that incorporated the label (●) and cells in other phases of the cell cycle (○). Data are presented for (a) sham-labelled cells (change of culture medium only), (b) IdUrd-treated cells, and (c) CldUrd-treated cells. The cell cycle times were determined from the intervals between two successive cell divisions. For each treatment modality data were used from 20 cells in three experiments. The bars represent the standard error of the mean.

of CldUrd immediately after changing the medium resulted in an overlap of IdUrd and CldUrd label in about 90% of the labelled cells.

DNA replication in metaphase chromosomes

Figure 6 illustrates the application of the double-staining procedure to metaphase spreads from a cell culture double labelled with an interval of 1 h. The micrographs clearly show the IdUrd (Fig. 6a) and CldUrd (Fig. 6b) labels distributed in bands over the V79 Chinese hamster chromosomes. The relative position of the chromosomes can be derived from the DAPI fluorescence image in Fig. 6c. All the possible combinations of labelling classes can be observed: regions showing only red or green fluorescence (see arrows), regions with both fluorochromes, and dark regions.

Discussion

The data in Table 1 indicate that, in a DNA double-labelling experiment, independent detection of the two labelling pulses can be achieved when the nucleosides IdUrd and CldUrd are used as markers. IdUrd can then be detected with the Becton Dickinson mouse antibody. For the detection of CldUrd two monoclonal antibodies could be used, the Br-3 mouse and the Sera-lab antibody. The Br-3 antibody, in our hands, however, showed a much higher level of background staining than did the Sera-lab antibody. In the double-staining procedure we therefore used the Sera-lab/Becton Dickinson antibody combination, also described in our paper by Bakker *et al.* (1991). The difference in genetic backgrounds of these two antibodies evidently facilitates the second step in the staining procedure. The micrographs in Fig. 1 show that the procedure yields highly specific results. All four labelling classes can clearly be distinguished: cells with both labels, cells with only IdUrd or CldUrd, and unlabelled cells. The quality of the double staining of the labelled nuclei did not depend on the cell type used. The procedure worked equally well with the Chinese hamster V79 cells, the rat RUC cells and the human HSF cells.

Before the combination IdUrd with CldUrd could be used reliably for the analysis of cell kinetics, the possible effects of these compounds on the progression through the cell cycle had to be determined. This is particularly important with respect to CldUrd on which little information is available. We therefore studied the effects of 30 min pulses of 10 μM IdUrd and CldUrd on the proliferation of V79 cells by two methods. The perturbation of the cell cycle during the 6 hours immediately following the pulse label was analysed by flow

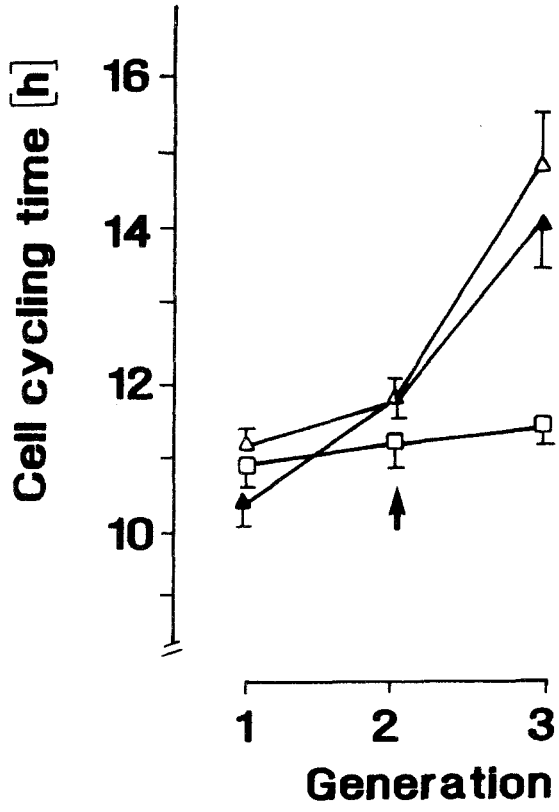

Fig. 4. Time-lapse cinematography analysis of pulse-labelled cells. Comparison of cell cycle times after different treatments. Sham-labelled cells (change of culture medium) ▲, IdUrd-labelled cells □, CldUrd-labelled cells △. The bars represent the standard error of the mean.

cytometry. The data in Fig. 2 show that the cell cycle parameters of the pulse-labelled and of the sham-treated cultures were identical to within an experimental variation of 3%.

Analysis of the effects during the entire cell cycle after treatment was performed by time-lapse cinematography. This yielded information on two different types of responses. A strong correlation was observed between the cell cycle times of cells having incorporated the label in S-phase, and of other cells from the same culture, also registered on the film, but not in S-phase during incubation with the label (cf. Fig. 3). This indicates that incorporation into the nuclear DNA of IdUrd or CldUrd, applied at a concentration of 10 μM for 30 min, does not hinder cell progression during the first cell cycle. Moreover, comparison of the data from pulse-labelled and sham-treated cultures shows that metabolic effects other than incorporation do not reduce the rate of proliferation either (Fig. 4). While differences in sensitivity between cell types cannot be excluded, these results indicate that short pulse labels of halogenated deoxyuridines applied at moderate concentrations should not disturb cell proliferation. Further experiments showed that even with five-fold lower concentrations, or with pulses as short as 2 min, the same staining results were obtained. Thus, if cells are expected to be very sensitive to one of the halogenated deoxyuridines, lower concentrations or shorter pulses may be used. The standard pulse length selected for these *in vitro* experiments was 30 min because that approximately equals the half-lifetime of halogenated deoxyuridines in the serum of mammals.

Once the reliability of the method had been established through these preparatory experiments, two studies were performed to test the effectiveness of the double-labelling procedure. We wanted, in particular, to investigate its suitability for the assessment of changes in DNA replication activity.

The first test concerned the reactivation of cell proliferation in slow-growing clones. The radial distributions of cells labelled with IdUrd just before, and with CldUrd at 0 or 16 h after, addition of fresh medium with fetal calf serum, were analysed in 'small' and 'large' clones, respectively. Before and immediately after reactivation of cell growth, proliferation in the central part of the 'larger' clones was considerably reduced. However, 16 h after

Table 2. Changes in fractions of labelled cells

	Fractions of labelled cells							
	Clone-centre				*Clone edge*			
	IdUrd		*CldUrd*		*IdUrd*		*CldUrd*	
t	*f*	SD	*f*	SD	*f*	SD	*f*	SD
0 h	0.14	(0.06)	(0.15)	(0.04)	0.34	(0.12)	(0.33)	(0.12)
16 h	0.17†	(0.06)	0.38†	(0.08)	0.50	(0.11)	0.32	(0.10)

* Large clones of RUC cells ($n > 2000$) cultured in medium without serum, were pulse labelled just before changing the medium. At 0 h or 16 h after changing to medium containing fetal calf serum, a pulse label of CldUrd was given and the cultures were fixed.

† Fractions of cells labelled with IdUrd and CldUrd were significantly different ($p < 0.01$, Mann–Whitney test).

t = time after changing the culture medium:

f = average fraction of cells labelled.

SD = standard deviation; f and SD were obtained from 10 or more clones.

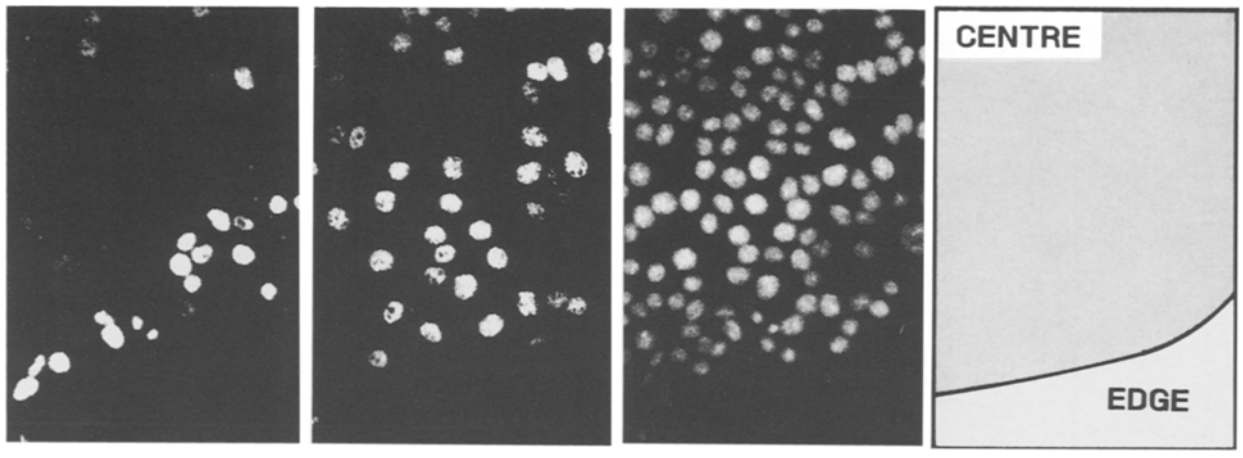

Fig. 5. Reactivation of cell proliferation in a slow-growing clone, visualized by double labelling. The IdUrd label was given before reactivation. The CldUrd label was given 16 h after providing the culture with fresh medium. The centre and the edge of the clone are located near the upper left and in the lower part of the micrograph, respectively.

medium renewal the S-phase cells were distributed more or less equally between the centre and the periphery of these 'large' clones (Table 2 and Fig. 5). In the 'smaller' clones, the radial distributions of the frequencies of both the IdUrd- and CldUrd-labelled cells were flat from the centre to the periphery. These results indicate that cell proliferation was suppressed in the central regions of the 'larger' clones only, and that recovery took place within 16 h after growth reactivation. This experiment demonstrates that, using the IdUrd/CldUrd double-labelling method, cell recruitment after treatment can be monitored by taking a single tissue sample. To our knowledge no simple procedure is available for the analysis of cell recruitment by other methods.

The spatial resolution of the method was determined in experiments on the double labelling of chromosome replication. The V79 metaphase cell in Fig. 6 shows a distribution obtained by applying two 15 min pulses given with an interval of 1 h. The first label, IdUrd, incorporated in mid S-phase, shows a wide distribution of replication bands. The second label, CldUrd, incorporated in late S-phase, shows a more compact replication pattern. All the four possible combinations of labels – red, green, both labels and no labels – can be distinguished in this photograph. In other experiments the duration of the pulses was reduced to 2 min, which is shorter than the replication time of a single replicon, $\simeq 20$ min. Metaphases from these experiments showed closely interspersed red and green replication bands with a resolution corresponding with a medium resolution Giemsa banding pattern. By varying the duration of the pulses and the interval between them, and by selecting cells labelled at different positions in the S-phase, detailed information on the evolution of replication patterns can be obtained. This double-labelling approach eliminates the cell-to-cell variation that seriously reduces the resolution of chromosome replication studies based on single-labelling experiments.

Conclusion

The procedure described here could be of considerable interest as it provides the first method for the specific double labelling of DNA synthesis, based on non-radioactive precursors. In an earlier publication Shibui *et al.* (1989) described a procedure for the double staining of cells labelled with BrdUrd and IdUrd. They were able to distinguish cells labelled with IdUrd only, from double-labelled cells, but they could not discriminate between double-labelled cells and cells labelled with BrdUrd only. This limits the application of their method to the analysis of the S-phase transit time and the turnover time of a cell population. The evaluation of more complex cell cycle parameters, such as the fraction of non-cycling cells or the rate of re-entry of resting cells into the cell cycle, requires a method such as that presented here, i.e., a method which can discriminate between all four labelling combinations.

The specificity and sensitivity of the double-labelling method and its high resolution in time and space, are qualities important for many types of kinetics studies in both cell and tumour biology. Using this procedure we are now studying the spatial and temporal development of replication patterns in interphase nuclei. Moreover, the doses of IdUrd and CldUrd required for *in vivo* labelling are well below the toxic levels, making possible a detailed analysis of cell proliferation in tumours *in situ*. The fraction of cycling cells in tumours, and the recruitment of resting tumour cells into the cell cycle, after treatment with drugs or radiation, can be assessed only by highly specific *in vivo* DNA double-labelling methods.

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