**102. Differential Staining of Sister Chromatids in
Bloom Syndrome Cells under Very Low
Bromodeoxyuridine (BrdU)
Concentrations**

Monoclonal Anti BrdU Antibody

By Yukimasa SHIRAIISHI, Masako NOHZU-JINZENJI, Takayo SANO,
Sayuri MORITA, Shoko KOMATSU, Michiyo TAKECHI,
and Michiyo OZAWA

Laboratory of Human Cytogenetics, Department of Anatomy,
Kochi Medical School, Nankoku-City, Kochi 781-51

(Communicated by Sajiro MAKINO, M. J. A., Dec. 12, 1986)

Introduction. 5-Bromodeoxyuridine (BrdU) is an analogue of thymidine and can be incorporated specifically into the DNA during the S phase of the cell cycle. An immunological method based on monoclonal antibodies specific for BrdU and 5-iododeoxyuridine (IrdU) was developed by Gratzner *et al.*¹⁾ and applied to the study of the sister chromatid exchange (SCE)⁴⁾ because of the specificity of the immunological reaction. However, this antibody technique combined with the fluorescence has not been stable and permanent in the sister chromatid differential staining (SCD). By combining the immunoperoxidase reaction with the DAB (3,3'-diaminobenzidine) reaction, we have established stable and permanent SCD procedures, which enable the detection of SCE in cells labeled with very low BrdU concentrations. In order to observe SCD at very low BrdU concentrations it is necessary to avoid the use of flame drying during the preparation of the slides. SCD was detected at 0.1 $\mu\text{g/ml}$ BrdU with the antibody technique, though with fluorescence plus Giemsa staining (FPG) using nonflame dried slides, the minimum concentration, at which SCD was detectable, was 0.2 $\mu\text{g/ml}$ of BrdU. The antibody technique was applied to evaluate SCE frequencies in diploid BS cells under very low BrdU concentrations.

Materials and methods. *Cell lines and cell culture:* Permanent BS B-lymphoblastoid cell lines (LCLs; BS₁₋₂, BS-SY₂) and a normal B-LCL (KS82), previously established^{6),7)} were used in the present study. As previously described, the BS₁₋₂ and BS-SY₂ were found to have the characteristically elevated rate of SCE (average SCE value; 71.6 ± 2.31 in BS₁₋₂, 70.6 ± 2.06 in BS-SY₂) in the cell labeled with 10 $\mu\text{g/ml}$ BrdU for two cell cycles. BS₁₋₂ cells also have abnormal karyotypes; BS₁₋₂, 46,XY,7p+,12q+,+14,t(15;15),(p11;q12), while BS-SY₂ cells had a normal karyotype. These cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) at 37°C in a 5% CO₂ humidified incubator. In all the cell lines established with EBV, tests of cell surface markers, such as clumping of lymphoid cells, the negative spontaneous rosette formation, and positive surface immunoglobulines, revealed the lymphocytes to be of the B-cell origin.^{2),3)}

Differential staining for sister chromatids: For the observation of the sister chromatid differentiation (SCD), the cells were labeled with 5-bromodeoxy-

uridine (BrdU) at 0.1, 0.2, 0.3, 0.5, 1.0, 2.0, 3.0, 5.0 and 10.0 $\mu\text{g/ml}$ for two rounds of the cell replication, each in the replication culture. Second mitoses were observed at 35 hrs with BrdU concentrations of 0.1–1.0 $\mu\text{g/ml}$, although they were obtained at 44 hrs with BrdU concentrations of 2.0–10.0 $\mu\text{g/ml}$. Colcemid at a concentration of 0.1 $\mu\text{g/ml}$ was added for the last 2 hr before the fixation in order to arrest the cells in metaphase. Mitotic cells were fixed by the routine hypotonic method with 0.075 M KCl, and chromosomes were spread by the flame drying procedure and by air-drying over 37°C warm water (humidified conditions). Even though the flame dried chromosome preparations were stained by the routine fluorescence plus Giemsa (FPG) technique⁸⁾ for the SCD over the 2.0 $\mu\text{g/ml}$ BrdU condition, they were not suitable for SCD staining below 2.0 $\mu\text{g/ml}$ BrdU concentrations.

Immunoperoxidase procedure for SCD: The flame dried chromosome preparations were not usable for this staining procedure. The chromosome preparations, which were prepared with air-drying over 37°C warm water and aged for 3–4 days at the room temperature, were suitable for this staining procedure. Chromosome preparations, prepared from the cells prelabeled with BrdU at 0.1, 0.2, 0.3, 0.5 and 1.0 $\mu\text{g/ml}$ for 35 hrs and at 2.0, 3.0, 5.0 and 10.0 $\mu\text{g/ml}$ for 44 hrs, were used for this staining procedure.

The procedures were as follows:

- (1) Immerse the slides in 0.07N NaOH for 3–5 min at the room temperature.
- (2) Wash in 0.02N phosphate buffer (PBS) for 5 min and dry at the room temperature.
- (3) Immerse the slides in 0.3% H_2O_2 -methanol (diluted one part of 3% H_2O_2 with nine parts of 100% methanol) for 15 min at the room temperature.
- (4) Wash in 0.02N PBS for 15 min with shaking and dry at the room temperature.
- (5) Draw a circle with a "magic ink" marker (on the back of slides) about 15–20 mm diameter and add 100–500 μl of normal goat serum (non diluted) to each slide and let stand for 15 min at the room temperature.
- (6) Wash in 0.02N PBS for 30 min with shaking and dry at the room temperature.
- (7) Add 100 μl of diluted unconjugated anti-BrdU antibody (Becton Dickinson, Inc.; No. 7580, Lot No. G0712) diluted 1:10 with 0.02N PBS to each slide in the circled area and let stand for 30 min in a 5% CO_2 humidified incubator (37°C) as the second antibody treatment.
- (8) Wash in 0.02N PBS for 30 min with shaking and dry at the room temperature.
- (9) Add 100 μl of peroxidase conjugated goat anti mouse IgG (CAPEL) diluted 1:20 with 0.02N PBS in the circled area and let stand for 30 min in a 5% CO_2 humidified incubator (37°C) as the second antibody treatment.
- (10) Wash in 0.02N PBS for 30 min with shaking and dry at the room temperature.
- (11) Stain with DAB reaction by immersing the slides in 0.02% 3,3'-diaminobenzidine-4HCl including 100 μl of 5% H_2O_2 , diluted with Trizma (Sigma, PH 7.4) for 10–20 min in the complete darkness.
- (12) Wash in 0.02N PBS briefly and in tap water.
- (13) Air-dry at the room temperature and observe under an ordinary microscope.

Result and discussion. For the observation of SCD, chromosome preparations were evaluated on both flame dried slides and air dried ones (on a metal screen) over 37°C warm water. When chromosome slides were prepared with flame drying, SCD was not usually observed below 2.0 $\mu\text{g/ml}$ BrdU. SCD was observable even at low BrdU concentrations (0.1, 0.2, 0.3, 0.5, 1.0, 2.0, 5.0 and 10.0 $\mu\text{g/ml}$) in air-dried slides. SCD could be seen at 0.1 $\mu\text{g/ml}$ BrdU with the antibody technique, whereas it was not possible to see SCD at this concentration using FPG technique (Fig. 1). Therefore, the antibody technique seems more

effective for SCD staining than the FPG procedure. Fig. 1 shows mean SCE values at various BrdU doses. In the FPG technique with flame dried slides, the difference of SCE values between 2.0 and 10.0 $\mu\text{g/ml}$ was not remarkable in BS₁₋₂ and BS-SY₂ cells; mean SCE values retained about 70 SCE per cell level.

Fig. 1. SCE frequencies in normal (KS82) and BS (BS₁₋₂, BS-SY₂) cells under various BrdU concentrations. Mean SCE values were compared between FPG and antibody techniques. Note that SCE frequencies were highly decreased in BS cells labeled with BrdU below 0.5 $\mu\text{g/ml}$.

In the antibody and FPG techniques with air-dried slides, SCE frequencies decreased with the dose dependency in BS as well as in normal cells, though the reduction rates in BS cells were remarkably higher than those in normal cells. Mean SCE values began to drop at 1.0 $\mu\text{g/ml}$ and further decreased to a low level at 0.1 and 0.2 $\mu\text{g/ml}$. At 0.1 $\mu\text{g/ml}$, the lowest concentration giving an adequate contrast for SCD, BS₁₋₂ and BS-SY₂ cells averaged 19.6 and 22.4 SCE per cell. Below the 0.2 $\mu\text{g/ml}$ BrdU condition, cells with the normal SCE level were often involved in the BS cell population counted. The difference of SCE values between antibody and FPG techniques at BrdU concentrations of 0.2, 0.3, 0.5, 1.0, 2.0, 5.0 and 10.0 $\mu\text{g/ml}$, respectively were not statistically significant ($P>0.1$). Fig. 2 shows SCE at 0.5 $\mu\text{g/ml}$ BrdU doses in BS₁₋₂ cells. As seen from this figure, BS SCE frequencies highly decreased with BrdU doses at 0.5 $\mu\text{g/ml}$ compared to original SCE level at 10 $\mu\text{g/ml}$. Fig. 3 shows almost a normal level of SCE seen in BS-SY₂ at 0.1 $\mu\text{g/ml}$ BrdU.

The method described here was basically for permanent staining in which one of the chromatids containing the BT strand was stained in dark brown, while the other chromatid with the BB strand is brightly stained. The most important point for SCD staining under low BrdU concentrations is the drying condition for the chromosome preparations; slides prepared with flame drying were not suitable. When chromosome slides were prepared with flame drying, SCD was not usually observable below 2.0 $\mu\text{g/ml}$ in human B-LCLs labeled with BrdU. However, SCD was detected at 10.0, 5.0, 2.0, 1.0, 0.5, 0.3 and 0.2 $\mu\text{g/ml}$ BrdU with FPG in non-flame drying preparation and was possible even at 0.1 $\mu\text{g/ml}$ BrdU with the antibody technique. Even though previous studies⁵⁾⁻⁷⁾ have shown that BS SCE was highly increased and that most of BS SCEs were caused by BrdU, SCE levels in those studies were usually evaluated at the high BrdU concen-

trations between 10 and 2 $\mu\text{g}/\text{ml}$ and had not been examined below the 2.0 $\mu\text{g}/\text{ml}$ level of BrdU, under which SCD was not detectable with flame dried slides. Therefore, the correlation of the decreasing concentrations of BrdU and the

Figs. 2-3. 2: A metaphases from a BS-SY₂ cell labeled with 0.5 $\mu\text{g}/\text{ml}$ BrdU for 48 hrs and stained with antibody technique. About 50 SCEs are counted. 3: A metaphase from a BS-SY₂ cell labeled with 0.1 $\mu\text{g}/\text{ml}$ BrdU for 48 hrs and stained with antibody technique. Note that almost normal level of SCE can be seen.

decreasing levels of BS SCE is of special interest. When BrdU concentrations decreased from 2.0 $\mu\text{g}/\text{ml}$ to 0.2 and 0.1 $\mu\text{g}/\text{ml}$, which were the minimum concentrations for SCD with the FPG and the antibody techniques, respectively, BS SCE values decreased with BrdU concentrations reaching about 20 average SCE per cell though the SCE value was not estimated in the absence of BrdU. The finding that the SCE level in some of the BS cells (even though not in all of the cells) was reduced to the normal level (about 10 SCE/cell) and that the average SCE values decreased with dependence on the amount of BrdU incorporated strongly supported our previous notion⁵⁾ that most of the BS SCEs were caused by BrdU. The average slope of the curve in Fig. 1 suggested that a near normal level of SCE may exist in BS cells without BrdU incorporated into DNA, though the SCE values below 0.1 $\mu\text{g}/\text{ml}$ were not measurable. The exact SCE value in BS cells below 0.1 $\mu\text{g}/\text{ml}$ BrdU will be evaluated elsewhere using endomitotic cells labeled with low BrdU doses.

Acknowledgements. This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture, Japan, and the Nissan Science Foundation. The authors are grateful to Professor Emeritus Dr. Sajiro Makino, M. J. A., the Hokkaido University for reading through the manuscript.

References

- 1) Gratzner, H. G. (1982): *Science*, **218**, 474.
- 2) Jondal, J., and Klein, G. (1975): *J. Exp. Med.*, **138**, 1365.
- 3) Platts-Mills, T. A. E., and Ishizaka, K. (1975): *J. Immunol.*, **114**, 1058.
- 4) Raza, A. *et al.* (1985): *Cancer Genet. Cytogenet.*, **15**, 187.
- 5) Shiraishi, Y. *et al.* (1983a): *Proc. Natl. Acad. Sci. USA.*, **80**, 4369.
- 6) Shiraishi, Y. *et al.* (1983b): *Cancer Genet. Cytogenet.*, **56**, 129.
- 7) Shiraishi, Y. (1985): *The EMBO J.*, **4**, 2553.
- 8) Wolff, S., and Perry, P. (1974): *Chromosoma (Berl.)*, **48**, 341.
- 9) — (1975): *Exp. Cell Res.*, **94**, 23.