AN ANALYSIS OF FACTORS FOR THE DIFFERENTIAL STAINING OF SISTER CHROMATIDS IN HUMAN CHROMOSOMES USING GIEMSA

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Various reagents were tested for the purpose of developing an improved Giemsa staining technique for the differential staining of sister chromatids in human chromosomes. Reagents like acids, bases, buffers, protein denaturants and proteolytic enzymes were all potent inducers of differential staining. The best results were obtained by brief trypsinization followed by extraction of nucleic acids by incubation in hot HCl. There was poor contrast between unifilarly and bifilarly BrdU substituted chromatids in slides from which trypsin treatment was omitted. The method of slide preparation as they affect the spreads of BrdU substituted metaphases were also evaluated. The results support the role of these reagents in the conformational changes and structural lesions of chromosomal protein leading to differential staining.

INTRODUCTION

The differential labelling of sister chromatids using autoradiography by Taylor (1958) has been reproduced by Zakharov and Egolina (1972). They observed that when the Chinese hamster chromosomes are grown in the presence of 5-bromodeoxyuridine (BrdU) for two rounds of replication and subsequently stained with Giemsa, the chromatids which are bifilarly substituted (BB) stains weakly as compared to unifilarly substituted (TB) chromatids which are darkly stained. Staining of BrdU substituted chromosomes with Hoechst 33258 also resulted in differential staining of sister chromatids (Latt 1973). Fluorescent dyes like acridine orange (Kato 1974) and 4,6-diamidine-2-phenylindole (Lin and Alfi 1976) showed similar results. This differential staining was enhanced to produce " harlequin " chromosomes by the fluorescent plus Giemsa (FPG) technique (Perry and Wolff 1974, Wolff and Perry 1974, Kim 1974). Treatments like incubation in hot alkaline salt solution (Korenberg and Freedlender 1974), digestion with proteolytic enzyme-trypsin (Pathak et al. 1975) produced differential staining of sister chromatids without recourse to fluorescence technique. These techniques were modified to produce " reverse " differential staining using basic fuchsin (Scheres et al. 1977) and extraction of proteins by acid treatment followed by Giemsa staining (Takayama and Sakanishi 1977). In the present paper, we wish to report the analysis of technical variables which are critical in the production of differential staining of sister chromatids in human chromosomes using Giemsa, in order to develop a technique which could be used routinely.

* Aided by C.S.I.R. Grant No. 7/45 (1052/77) EMR, I.

CODEN: AGMGAK 27 81 (1978) — ISSN: 0001-5660
Acta Genet. Med. Gemellol., 27: 81-87

https://doi.org/10.1017/S1120962300792183 Published online by Cambridge University Press

MATERIAL AND METHODS

Cell Culture, Harvest and Slide Preparation

Peripheral lymphocytes separated from the plasma layer and buffy coat of heparinized human venous blood by gravity sedimentation for 2 hours at 4°C were inoculated into a culture bottle supplemented with 8 ml TC 199, 2 ml fetal calf serum and 0.2 ml PHA-M. 5-bromodeoxyuridine (SIGMA) was added 24 hours after initiation at a concentration of 10 μg/ml and the cultures reincubated for an additional 48 hours at 37°C. Colcemid was added to a final concentration of 0.1 μg/ml, two hours prior to harvest. Following hypotonic treatment with 0.075 M KCl at 37°C for 15 minutes, cells were fixed with 3 changes of 3:1 methyl alcohol acetic acid fixative. Two drops of the cell suspension were dropped onto a clean glass slide and spreading was accomplished by blowing vigorously at right angle to the slide surface and passing the slide through a flame.

Staining and Photography

The slides, within 24 hours of preparation, were treated with solutions of various reagents like acids, bases, buffers, protein denaturants, proteolytic enzymes, etc. Their concentrations, together with temperature of treatment and pH, are given in Table 1.

The treated slides were washed thoroughly and stained for 20 minutes in 5% Giemsa (Gurr RPM1)-buffer solution (Sörensen, pH 6.8). The stained slides were rinsed briefly in water, dried, passed through Xylene and mounted in DPX. Photographs were taken using bright field optics and green interference band filter (Leitz No. S 542-19).

RESULTS

The yield of metaphases in the BrdU substituted cultures was in general greatly reduced compared to that in untreated cultures. Mitotic index was minimum in those cases where BrdU was added at the time of setting of the cultures, though all the chromosomes were BrdU substituted, whereas not all the metaphases incorporated BrdU in their DNA in one day substituted cultures. Introduction of BrdU after 24 hours of culture initiation and allowing it to grow for another 48 hours produced an optimal number of BrdU substituted metaphases. Air-dried preparations nearly always resulted in overlapped spreads. This difficulty was overcome by vigorously blowing and passing the slide through a flame.

1. Effects of Acids

Of the various acids tested, only perchloric acid and HCl were able to induce differential staining of sister chromatids. Concentrations of 1, 2, 3, 4 and 5N HCl and 10, 20, 30, 40 and 50% perchloric acid were each tested for 10, 15, 20 and 25 minutes at 55, 60 and 65°C. Satisfactory differential staining was induced by 3N HCl and 20% perchloric acid at 55°C for 15 minutes. At higher concentration and temperature, there was either loss of cells from the slide's surface or the chromosomes lost their affinity for stains completely. At lower temperatures and concentrations, no differential staining was observed.

2. Effects of Bases

Almost all the bases employed were able to induce differential staining at lower temperatures and concentrations. However, in general the staining was not satisfactory. At higher concentration and temperature, there was considerable chromosomal swelling and often loss of cells from the surface of the slide. There was marked improvement in staining with weak bases like $Ba(OH)_2$. In addition to the differential staining, banding patterns were induced

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Table 1. *Induction of differential staining of sister chromatids by various reagents*

Reagents	Concentration	pH	Time	Temp. (°C)	Differential staining of sister chromatids
Deionized distilled water		7.0	60 min	60	—
Acids:					
HCl	1N, 2N, 4N, 5N		15 min	55	—
HCl	3N		15 min	55	+
Perchloric acid	10%, 30%, 40%, 50%		15 min	55	—
Perchloric acid	20%		15 min	55	+
H_2SO_4	1N, 2N, 3N		15 min	55	—
Bases:					
NaOH	0.1N	13.1	3 sec	20	+
KOH	0.1N	12.6	3 sec	20	+
LiOH	0.1N	12.2	3 sec	20	+
NH_4OH	0.1N	11.3	3 sec	20	+
$Ba(OH)_2$	0.1N	13.0	10 sec	25	+
Buffers:					
Sörensen's phosphate buffer	($M/15\ Na_2HPO_4$—$M/15\ KH_2PO_4$)	7.0	20 min	60	—
		8.5	10 min	89	+
Mc Ilvaine's buffer	($M/5\ Na_2HPO_4$—M/10 citric acid)	7.0	20 min	60	—
		8.5	10 min	89	+
Na_2HPO_4	1M	8.0	10 min	89	+
KH_2PO_4	1M	8.0	10 min	89	+
2 × SSC		7.0	60 min	60	+
Protein denaturants:					
Urea	4M	8.5	20 min	37	+
Guanidine HCl	3M	5.5	20 min	37	+
Sodium dodecyl sulphate in 2 × SSC		7.0	10 sec	20	+
Proteolytic enzymes:					
Trypsin	2%		12 sec	0	+
Alphachymotrypsin	2%		15 sec	4	+
Pepsin	2%		15 sec	4	+
Pronase	2%		15 sec	4	+
Collagenase	2%		15 sec	4	+

in the unifilarly substituted or bifilarly substituted chromatid depending on the intensity of treatment. At lower concentration only the lighter chromatids showed banded appearance, whereas at higher concentration bands were induced in both the lightly and darkly stained chromatids.

3. *Effects of Buffer Solutions*

Out of the various buffer solutions used, only 2 × SSC was able to induce differential staining at 60°C. Other buffers like Na_2HPO_4 and KH_2PO_4 were able to induce differential staining only at higher pH and temperature (pH 8.0 and temperature 89°C). Sörensen's phosphate

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buffer and Mc Ilvaine's buffer induced differential chromatid staining readily at alkaline pH and higher temperatures but occasionally induction of G-bands also resulted.

4. Effects of Protein Denaturants

Protein denaturants like urea, guanidine-HCl and sodium dodecyl sulphate, induced differential staining of sister chromatids. At higher temperature, G-bands could sometimes be seen.

5. Effects of Proteolytic Enzymes

Brief digestion with proteolytic enzymes of the chromosomes was sufficient to induce differential staining. The enzymes tested consisted of trypsin, alpha-chymotrypsin, pepsin, pronase, and collagenase. The enzymes were diluted and the slides were treated at lower temperatures. Of all the proteolytic enzymes, trypsin gave the best result. The treatment time of trypsin varied with ageing of the slides. Older slides needed longer treatment as compared to freshly prepared slides. The time taken for trypsinization of chromosomal material, from preparation of various ages, are detailed in Table 2. The results show that trypsin treatment has to be controlled carefully since overaction often results in the degradation of chromatid component and final loss of differential staining (Fig. 1).

Table 2. *Optimum digestion time by trypsin of the slides of different ages*

Age of the slides (days)	Treatment time (seconds)	Preferred temperature (°C)
1	12	0
2-4	15	0
5-10	18	0
11-20	22	0
21-30	30	0
31-40	45	4
41-60	65	4
61-90	115	4

6. Modified Giemsa Method for the Differential Staining of Sister Chromatids

The modified Giemsa method now used routinely in this laboratory for the differential staining of sister chromatids is given in Table 3. It has been observed that brief pretreatment with trypsin greatly increases the contrast between the chromatids. HCl treatment without incorporating trypsin action resulted in poor contrast between TB and BB chromatids. The best results were however obtained by brief trypsinization followed by extraction of nucleic acids by hot HCl treatment (Fig. 2*a* and 2*b*).

Table 3. *The modified Giemsa technique for the differential staining of sister chromatids*

1. Place one-day-old slides in trypsin solution (Difco 1:50, diluted in deionized water) at 0°C for 12 seconds.
2. Rinse in distilled water at 8-10°C for 5 minutes.
3. Incubate in 3N HCl at 55°C for 15 minutes.
4. Rinse the slides in 70% ethanol at 20°C for 5 minutes.
5. Rinse slides in 95% ethanol at 20°C for 5 minutes.
6. Stain for 20 minutes with 5% buffered Giemsa, pH = 7.0.

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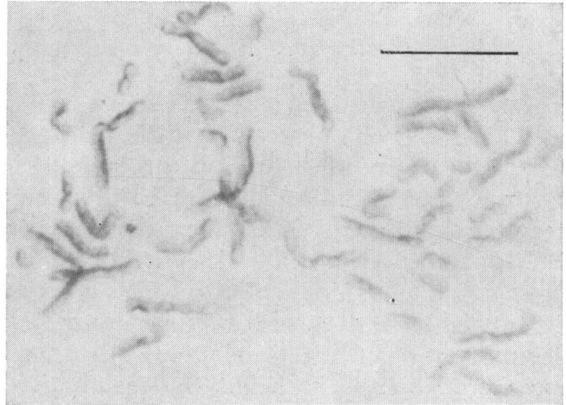

Fig. 1. Human metaphase chromosomes showing overaction of trypsin resulting in the degradation of chromatid component and final loss of differential staining. Scale denotes 10 μm.

Fig. 2*a*.

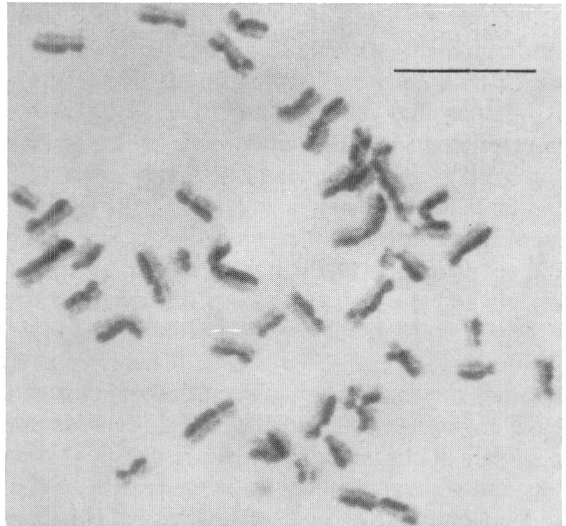

Fig. 2*b*.

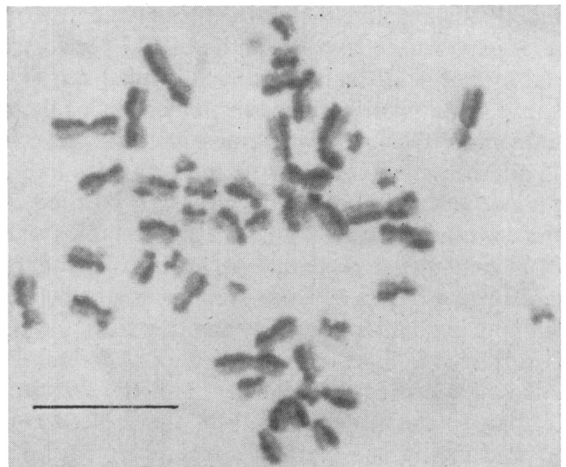

Fig. 2*a* and *b*. Human lymphocyte chromosomes grown for two generations in BrdU and sequentially treated with trypsin and hot HCl resulting in differential staining of sister chromatids. Scale denotes 10 μm.

https://doi.org/10.1017/S1120962300792183 Published online by Cambridge University Press

DISCUSSION

The mechanism involved in the differential staining of sister chromatids is not clear. Zakharov and Egolina (1972) observed that the morphology of the chromosome is modified due to contraction delay when BrdU is incorporated into the DNA. They also showed that the lesser contracted chromatid appears pale as compared to its sister chromatid. Latt (1973) reported that Hoechst 33258 fluorescence was partially quenched in BrdU containing DNA. Ikushima and Wolff (1974) attributed this differential staining to a differential bindings of protein to the DNA of chromatin. This is because proteins are more tightly bound to DNA substituted by BrdU than to unsubstituted DNA (David et al. 1974). Goto et al. (1975) showed that differential staining was a function of concentration of Hoechst 33258 and the amount of exposure to light. Harlequinization of chromosomes also resulted when BrdU substituted chromosomal preparations were exposed to light (Wolff and Bodycote 1977) leading to breakage of disulfide bonds in protein (Mousseron-Canet and Moni 1972). These studies indicate the role of differential compaction of chromosomes in the differential staining which are influenced by the binding of non-histone proteins. These results are supported by Korenberg and Ris (cf. Wolff 1977) who showed that differentially substituted chromatids have different densities observable by both phase and electron microscopy. BrdU incorporation into chromosomes has primary effect at the level of packing of the 25 nm fiber into the larger chromosomal unit. The bifilarly substituted chromatid is more open with looser gyres than is the unifilarly substituted chromatid. Thus the non-histone proteins affecting the condensation of the chromosomes play a role in the differential staining of sister chromatids and staining seem to reflect merely an underlying structural differences between the chromatids (Korenberg and Freedlender 1974).
Contrary to these observations, Scheres et al. (1977) and Takayama and Sakanishi (1977) induced the " reverse " differential staining of sister chromatids where the BB chromatids were darkly stained and the TB chromatids showed pale staining. The method employed routinely in our laboratory also showed the bifilarly substituted chromatids to have contracted less and stained strongly as compared to unifilarly substituted chromatid. This may be due to the fact that as a result of higher BrdU content, the bifilarly substituted chromatids become more resistant and therefore disintegrate less readily than unifilarly substituted chromatid after exposure to trypsin and hot HCl. Nucleic acids are extracted from chromosomal material by hot acid treatment and it seems likely that differential extractability of DNA and probably some proteins between the BB and TB chromatids is the principle mechanism of the differential Giemsa staining observed in " reverse " staining of differential sister chromatids. A number of solutions of various reagents like acids, bases, buffer solutions, protein denaturants, and proteolytic enzymes, also induced differential staining of sister chromatids, but the contrast between TB and BB chromatids was poor unless there was sequential treatment of chromosomal preparations by trypsin and HCl. Thus, it seems that trypsin and HCl treatment prior to Giemsa staining is not necessarily indispensable for inducing differential staining but rather plays a promoting role, and that structural lesion of chromosomal proteins is responsible for the production of differential staining. Biochemical, electron and phase-contrast microscopic studies of the BrdU substituted metaphases treated with various reagents leading to the understanding of the molecular architecture of the chromosome might help to understand the mechanism of differential staining of sister chromatid.

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Acknowledgement

Thanks are due to Dr. R.P. Sharma, Div. Genet., Indian Agricultural Research Institute, New Delhi, for going through the manuscript.

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