

DYE-NUCLEOPROTEIN INTERACTIONS IN GIEMSA BANDING

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INTRODUCTION

Fixed chromosome preparations subjected to treatments such as pH 9 (1) or trypsin hydrolysis (2) and stained with Giemsa's demonstrate similar banding patterns irrespective of the pre-treatments used. It has been shown that this

phenomenon is the result of modifications of DNA-protein and protein-protein associations which alter dye interaction (3-5). It appears that only Giemsa's or related staining solutions can produce the characteristic banding after appropriate treatment (6), and therefore, the present study was designed to identify those com-

ponents of Giemsa stain which are essential for banding.

MATERIALS AND METHODS

Human leukocyte cultures were grown for 72 h, using the method of Moorhead et al. (7) with modifications. After 5 min in 0.75 M KCl for the hypotonic pretreatment, the cells were fixed in methanol-acetic acid (3:1) and spread on cold wet slides (7). The slides were then subjected to the following banding treatments: (a) 5–15 min in a 0.2 M solution of cesium chloride in a 65°C water bath (8), (b) 10–30 min in a pH 9.2 solution (isotonic KCl or phosphate buffer) at room temperature, and (c) 45–65 s in a 0.025% trypsin solution at room temperature. After the first two of these banding techniques, the slides were immediately immersed in various stains without rinsing; trypsin-treated slides were first rinsed in Hanks' basic buffer solution before staining.

The different stains used include Giemsa (a 10% solution of no. 620, Harleco, Philadelphia, Pa.) and Leishmann stain, as well as the thiazine chlorides (azure A, B, C, toluidine, and methylene blue), thiazine-eosinates (azure A-, azure B-, and azure C-eosinates), hematoxylin, cresyl violet, eosin Y, and quinacrine (Gurr's Atebrin-HCl). Azure A, toluidine, and methylene blue, cresyl violet, and eosin Y were obtained from Allied Chemical Corp., National Aniline Division, Morristown, N. J., and other stains were obtained from Schmid and Company of Stuttgart, Germany, through the Roboz Surgical Instrument Co. Inc., Washington, D. C.

The slides were stained separately, sequentially, and in combinations of the above stains at concentrations of 1 or 2% in double-distilled water (with the exceptions noted) for 1–10 min, with optimal staining for the thiazines usually obtained at 3–5 min. The pH of the staining solution (except for eosin) was approximately 6.5–7.2. Due to the insolubility of the thiazine-eosinates, the azure-eosinate solution was prepared by dissolving 0.5 g of azure B-eosinate in 4 ml methanol and 1 ml glycerine, with the filtrate added to 50 cc of distilled water (with the final concentration approximately 0.5% as determined by desiccation dye recovery studies).

Controls consisted of untreated slides stained with all of the above stains, as well as CsCl- and pH 9.2-treated slides which were destained by immersion in 70% alcohol and were then subjected to different treatments and/or stains. Metaphase spreads were examined visually at $\times 1,000$ and scored as to the presence and quality of banding; photographs were taken on a Zeiss photomicroscope (Carl Zeiss, Inc., New York, N. Y.) and enlarged to $\times 3,000$ for analysis.

In an effort to explore the role of the fixative in

Giemsa (G) banding, the following agents were used in place of acetic acid-methanol: 4% formaldehyde, absolute ethanol, absolute methanol, 50% acetic acid (with H₂O), as well as solutions of 1 part saturated tartaric, oxalic, or citric acid to 1 part water.

RESULTS

On the basis of the various fixatives used, it was found that acetic acid-methanol fixation was necessary for banding with the techniques employed. None of the other fixatives yielded chromosomes of adequate morphology to demonstrate banding, as spreading tended to be very poor. Therefore, all of the banding experiments were performed using acetic acid-methanol fixation.

After treating slides with cesium chloride or pH 9.2 as described, it was found that a brief staining exposure (1–2 min) with azure A, B, or C produced faint bands, while longer staining produced no banding (see Table I). Good bands were seen only with staining solutions prepared from azure-eosinate powder or with Giemsa and Leishmann stains, both of which contain thiazine-eosinate. Such banding occurred in adequately stained as well as in understained slides, unlike the situation with azure. None of the other single stains produced banding, although a mixture of methylene blue and eosin Y, as well as a mixture of azure B and eosin Y, both produced poor banding (Table I). No attempt was made to determine the exact proportions of thiazine and eosin which produced optional banding.

Persistence or stability of bands also seems to be dependent on the use of a stain containing thiazine-eosinate (see Table II). When banded chromosomes stained with azure-eosinate were counterstained with azure or destained and restained with azure, the original bands were still visible. However, the faint bands originally seen with azure staining did not persist after destaining and restaining, even when azure-eosinate was used as the second stain.

Similarly, slides treated with CsCl or pH 9 which were first stained with quinacrine and then counterstained with azure-eosinate did not show bands, even if the quinacrine was removed by destaining before azure-eosinate treatment. Slides which had been treated with trypsin showed bands with either azure or azure-eosinate.

DISCUSSION

Although control slides stained with azure for 1–2 s showed uniform dye uptake, azure staining of

TABLE I
Staining Reactions with Slides Treated with CsCl or pH 9.2

Stain*	Counterstain*	Result
Azure A, B, or C (1-2 min)	—	Faint bands
Azure A, B, or C (5-10 min)	—	No bands (uniform staining)
Azure A-, B-, or C-eosinates‡ (1-5 min)	—	Good bands
Giemsa§ (1-3 min)	—	Good bands
Leishman (1-3 min)	—	Good bands
Toluidine blue (3-5 min)	—	Uniform staining (pale)
Methylene blue (3-5 min)	—	Uniform staining (pale)
Hematoxylin (3-5 min)	—	Uniform staining (pale)
Eosin Y (3-5 min)	—	Uniform staining (faint)
Cresyl violet (3-5 min)	—	Uniform staining (pale)
Methylene blue (5 min)	Eosin Y (5 min)	Uniform staining (pale)
Eosin Y (5 min)	Methylene blue (5 min)	Uniform staining (pale)
Azure B (2-5 min)	Eosin Y (2-5 min)	Uniform staining (pale)
Eosin Y (2-5 min)	Azure B (2-5 min)	Uniform staining (pale)
Mixture of methylene blue + eosin Y	—	Poor banding
Mixture of azure B + eosin Y	—	Poor banding

* All stains 1-2% in distilled water, except as noted.

‡ 0.5%

§ 10% solution of Harleco Giemsa no. 620.

TABLE II
Persistence of G Bands Produced by CsCl or pH 9.2

Original stain	Second stain	Result
Azure B (1-2 min)	Destain	No bands
Azure B (1-2 min)	Destain	No bands
Azure B (1-2 min)	Counterstain	No bands
A-E*	Destain	Pale bands
A-E	Destain	Good bands
A-E	Counterstain	Good bands
A-E	Destain	Sharper Q bands
Quinacrine	Destain	No bands
Quinacrine	Counterstain	No bands

* Azure B-eosinate

cesium-treated slides for up to 2 min produced faint bands, while longer staining did not. These findings can be explained on the basis that the lack of stain uptake is related to the presence of cesium, so that after 2 min cesium is washed out, and those regions previously prevented from staining can now take up the dye, and banding is no longer observed. This mechanism is consistent with recent studies with electron beam microanalysis using the scanning electron microscope which demonstrated the presence of cesium in interband areas, while no cesium was detected in banded regions (9).

In contrast to azure, staining with azure-eosinate, Giemsa's, or Leishmann's produced

relatively persistent bands, as noted by the observations that bands remained after destaining and restaining with azure. This finding, and the fact that it was not possible to counterstain interbands with azure after staining with azure-eosinate, suggests that either: (a) thiazine-eosinate staining involves a nonequilibrium reaction in which there has been a dye nucleoprotein (DNA + protein) interaction resulting in a relatively stable conformational change, or (b) that material has been extracted and/or masked in "interband" areas. Recent studies suggest that protein extraction is insignificant in G banding (5), and this may explain the finding that azure can give bands or uniformly stained chromosomes de-

pending on staining time after CsCl or pH 9.2 treatment. Therefore, it seems that a conformational change which is maintained or produced by the thiazine-eosinate complex affects dye affinity in adjacent chromosome regions (interbands). This conformational change is not necessarily related to protein extraction, but rather may reflect condensation changes in chromatin induced by the various pretreatments (10, 11). That condensation can be reversibly or irreversibly altered by different salt solutions and pH is readily observed using phase microscopy with unfixed chromosomes (12). The probability that conformational changes occur in G banding is further supported by the fact that the bands induced in chromosomes in the G_2 banding techniques are visible under phase microscopy before staining (13). Furthermore, it has been demonstrated that destained, trypsin-banded chromosomes maintain bands visible with phase microscopy (10), and such bands can also be visualized by Feulgen staining (13). Also, obvious condensation differences between bands and interbands have been reported using Nomarski phase and the scanning electron microscope (6, 14), as well as the transmission electron microscope (15).

Since no banding was observed when CsCl or pH 9.2 treatments were followed by quinacrine and then staining with azure-eosinate (even when the quinacrine was removed by destaining), it appears that the necessary condensation changes are either not produced or not maintained unless azure-eosinate follows immediately after these banding pretreatments. However, the fact that azure shows bands with staining times under 2 min suggests that such bands have been produced by the treatments used, but that it is the stabilization of the condensation changes which is dependent on thiazine-eosinate. Therefore, both banding treatment and dye interaction appear necessary for postfixation banding techniques (10, 11, 13).

In view of the fact that quinacrine has been shown to intercalate on DNA (16), and an intercalation mechanism has also been established for toluidine blue (17), a thiazine dye similar to azure, it appears that the mechanism of dye reaction may involve intercalation on DNA in addition to chromosomal protein interaction.

Although a specific dye-nucleoprotein interaction appears essential for G banding with CsCl and pH 9.2, banding with trypsin does not appear to be as dependent on the stain, perhaps because

such enzymatic digestion produces irreversible protein degradation (13, 18), unlike the milder CsCl and pH 9.2 pretreatments. Yet the fact that trypsin G bands are so similar to those produced by the other two techniques suggests that all three are acting on a common labile site to produce poorly staining regions (i.e., interbands). Since trypsin is known to act by cleaving the peptide bonds on the free carboxyl side of arginine or lysine (19), it would appear that those sites containing unbound lysine-arginine-rich proteins would be most susceptible to tryptic digestion. The ability of pH 9.2 to affect the same sites to produce identical interbands supports the hypothesis that these regions are characterized by the presence of proteins whose associations with DNA can be altered by changes in pH. In general, physical-chemical interactions are at a minimum when the pH of the solution is equal to the pK_i of a protein (isoelectric point) (20), and this includes electrostatic dye complexing (21). Such pH changes may also lead to disruption of ionic bonds maintaining chromosome conformation. However, since it is possible to band using Giemsa stain at pH 9 (1), this implies either that the eosin interacts with those positively charged (i.e., basic) proteins which are capable of reacting at this pH, or that the eosin interacts with intercalated thiazine molecules kept in close proximity by the maintenance of conformation (chromosome packing or coiling) by alkaline resistant proteins (i.e., histones).

Some investigators have contended that proteins, particularly histones, play no role in G banding, since histochemical staining of acetic-acid/methanol-fixed chromosomes for protein appears to be negative (22, 23). Although fixation may remove some proteins from metaphase chromosomes, significant protein must remain since metaphase morphology is largely a function of protein-protein and protein-DNA interactions (19, 24, 25). Removal of all proteins would, by definition, result in near naked DNA and destruction of chromosome morphology. Also, fixed metaphase chromosomes are trypsin-sensitive, and this implies the presence of susceptible proteins (18). Furthermore, although data obtained on isolated (purified) metaphase chromosomes are lacking, it has been reported that ethanol-acetic acid (3:1) extracted only 7-8% of the total histones from isolated chromatin (26), and one would expect nonisolated, intracellular, condensed chromatin (i.e., metaphase chromo-

somes) to be equally or more resistant. Indeed, the persistence of histones following acetic acid-methanol fixation has been demonstrated using radioisotope labeling (27) and even localized on metaphase chromosomes using histone antibody (28).

These findings with pH 9.2 and trypsin implicate alteration of DNA-histone associations as a major factor in the nucleoprotein modifications which form the basis of Giemsa bands (13). This possibility is supported by studies on purified chromatin which suggest that DNA is composed of regions which differ in efficiency of histone binding, and that regions characterized by the most efficient binding are maximally resistant to thermal denaturation as well as nuclease attack (29). This hypothesis is not negated by the fact that attempted histone extractions with HCl after acetic acid-methanol fixation do not interfere with G banding (22, 23), since the efficacy of such extractions in this *in situ* system is questionable (3, 13, 30). The fact that CsCl also produced such bands suggests that cesium interacts maximally with regions where protein binding is least efficient, resulting directly or indirectly in inhibition of dye molecule attachment, and this produces an interband. Since the bright bands produced by quinacrine fluorescence have been shown to be composed of uninterrupted long sequence A-T (16, 31), the fact that these correspond to Giemsa dark bands suggests that A-T bases are more efficient at binding histone than are G-C bases. This base-related difference in protein binding could explain the apparent base-related banding pattern observed with Giemsa banding techniques, and its similarity to quinacrine fluorescence, where histone associations have also been shown to be a contributing factor (32).

SUMMARY

Chromosome preparations from human peripheral blood prepared by standard techniques were subjected to a variety of treatments and stains to elucidate the role of the Giemsa stain in G banding. Thiazine-eosinate, a component of Giemsa and other stains that produce G bands, was found to be directly and uniquely related to formation and maintenance of G bands produced by CsCl and pH 9.2 treatments. The mechanism appears to involve formation of a thiazine-eosinate nucleoprotein complex which augments chromosomal condensation changes produced by these two

banding pretreatments. This study supports the hypothesis that Giemsa banding is related to alterations in DNA-protein associations and is not dependent on DNA strandedness or extraction.

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