Fluorescent G- and C-bands in mammalian chromosomes by using early BrdU incorporation simultaneous to methotrexate treatment

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Fluorescent G- and C-bands were obtained in human and cattle chromosomes of lymphocytes grown at 37.5°C for 72–76 hours. 24 hours before the completion of the culture, 5-bromodeoxyuridine (BrdU) in a final concentration of 20 μg/ml and increasing doses of methotrexate (MTX) were added. After 17 hours the cells were washed and allowed to recover for 6 hours in a medium containing thymidine. Colcemid treatment lasted 1.5 hours. The air dried slides were stained with acridine orange and observed under fluorescence microscopy. Compared to the control (without MTX), it was possible by increasing the MTX doses to increase the number of cells in the first cycle of replication in the presence of BrdU (G-bands in both chromatids) with a concomitant reduction of the number of cells in the second cycle of replication (G-bands in one chromatid), which also allows demonstration of SCEs. The advantages of this technique and the different cellular responses between the two species are discussed.

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Since CASPERSSON et al. (1970) obtained the first banding patterns in human chromosomes by using quinacrine staining (Q-bands), several banding techniques have been used to improve the banding pattern resolution. As is known, Q- and G-banding techniques stain the heterochromatic regions (late replicating) while the R-banding technique stains the euchromatic ones (early replicating). G-bands are the most widely used in several laboratories and several techniques are currently applied to obtain them, but almost all of these require a pre-treatment of the cytological preparation, either with proteolytic enzymes (SEABRIGHT 1971; WANG and FEDOROFF 1972), alkalies (SCHNEDL 1971), or salt solution at high temperature (SUMNER et al. 1971). By the use of cell synchronization techniques including methotrexate (MTX), thymidine and 5-bromo-deoxyuridine (BrdU) as S-blocking agents (YUNIS

1976, 1980; VIEGAS-PEQUIGNOT and DUTRILLAUX 1978; CAMARGO and CERVENKA 1980; PAI and THOMAS 1980) it has been possible to obtain high percentages of prometaphases with high resolution G-and R-bands. DUTRILLAUX and VIEGAS-PEQUIGNOT (1981) achieved early and late incorporation, resulting in G- and R-bands, respectively, in the same preparation by using high doses of BrdU.

The present paper shows that it is possible to have early BrdU incorporation in the presence of MTX, achieving, besides a good cell synchronization, also the demonstration of good G-and C-bands in one and in both chromatids in the same cytological preparation of human and cattle chromosomes, the frequency of the two types of cells depending upon the MTX concentration. Furthermore, when G-bands are present in one chromatid, also sister chromatid exchanges (SCEs) can be demonstrated.

Material and methods

Culture techniques. — Peripheral blood, drawn from three humans and two cattle, was cultured for 72–76 h at 37.5°C in 25 ml sterile bottles containing 7 ml TC McCoy's SA modified medium (GIBCO), 2 ml FCS (foetal calf serum) or autologous plasma of human and cattle, respectively, and phytohemagglutinin (0.2 ml), without antibiotics. 24 h before completion of the cultures, 20 μg/ml of BrdU (Sigma) was added to all cultures, whereas MTX (Lederle) was added at the following doses: 0, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 μg/ml in cattle and 0, 0.05, 0.2, 0.8, 3.2, 12.8 and 25.6 μg/ml in human culture. After 17 h the cells were washed in sterile Puck's saline and allowed to recover for 6 h in the same medium with thymidine (7 μg/ml). 1.5 h before harvest, colcemid was added (0.1 and 0.03 μg/ml in human and cattle, respectively). The cells were centrifuged and treated with KCl (0.075 M) for 20 min at 37.5°C and fixed in methanol-acetic acid (3:1) two times (the first overnight).

Acridine orange staining was performed as for fluorescent RBA-bands previously reported by Dɪ Bᴇʀᴀʀᴅɪɴᴏ and Iᴀɴɴᴜᴢᴢɪ (1982).

Sequential C+G-bands procedure. — This procedure was successful only in cattle chromosomes, and only after reducing the recovery time to 5–5.5 h and fixing the cells for 3–4 days. After staining with acridine orange, the best metaphase plates, showing only C-bands, were selected and microphotographed; the day after or after a long exposure to U.V. light (3–4 h), the G-bands appeared.

Giemsa counterstaining. — Plates observed under fluorescent light can be counterstained with Giemsa (6 % for 15 min) as follows: after the coverslip has been removed, the slide is dipped in fixative (methanol-acetic acid, 5:1) for 20s, washed in deionized water, and dehydrated in 30, 70, 96 % ethanol. With this method slides can be observed under bright field and made permanent, but the C+G-banding pattern will be visible only in those metaphases which have been exposed for at least 1–2 min in microscope U.V. light.

Recording of the metaphase and coding. — For each dose of MTX used, 50 metaphases and prometaphases were analysed, and the best microphotographed under fluorescence and in bright field with Kodak film 2415. The following code was used: M1 and M2 to indicate cells at first (G+C-bands in both

chromatids) and at second (G+C-bands in one chromatid = SCE) replication cycle, respectively, in the presence of BrdU; S1 and S2 to indicate the first and second S-phase; C1 and C2 for cattle 1 and cattle 2; H1, H2, H3 for human 1, human 2 and human 3. The human and cattle chromosomes nomenclature accords with the ISCN (1978) and the READING CONFERENCE (Proceedings of the First International Conference for the Standardization of Banded Karyotypes of Domestic Animals 1980).

Results

Cellular response curves

The fraction of M1 cells of the five subjects examined as well as the average of each species are demonstrated in Fig. 1. In cattle (C1 and C2) the fraction of M1 cells, compared to cells not treated with MTX, remained unvaried when the minimum dose of MTX (0.05 μg/ml) was used, while it increased remarkably with higher doses, reaching 96 and 98 % in C1 and C2, respectively, when the maximum dose (3.2 μg/ml) was employed. In humans (H1, H2, H3) a different behaviour was found. In fact, compared to the cells not treated with MTX, by using the minimum dose of MTX (0.05 μg/ml) there was a significant increase of M1 cells (37, 56 and 62 %, respectively) in H3, H1 and H2. By increasing the dosage of MTX further, there was a slight reduction followed by a constant behaviour (around 50 %) in H1, an increase from 62 to 80 % followed by a slight reduction to 74 % in H2 and a steady increase of M1 cells from 37 to 80 % in H3. By examining the average data in cattle (section line) and humans (broken line) comprising 800 and 1050 metaphases examined, respectively, the following facts could be observed: (a) in the cells not treated with MTX the fraction of M1 cells was 15 % in humans and 22 % in cattle; (b) at the minimum dose of MTX (0.05 μg/ml) M1 cells increased with 27 % (3.3 times the control), while in cattle they were almost unvaried (1 % increase); (c) at subsequent doses the increase of M1 cells was greater in cattle (63 % or 3.7 times the control at 0.3 μg/ml), compared to humans where the response was found to be much lower, even when high doses (25.6 μg/ml) of MTX were used. There appeared to be no significant cytotoxic effect of MTX even at high doses, since no significant differences in cell growth between cells simultaneously treated with MTX and untreated with BrdU could be observed.
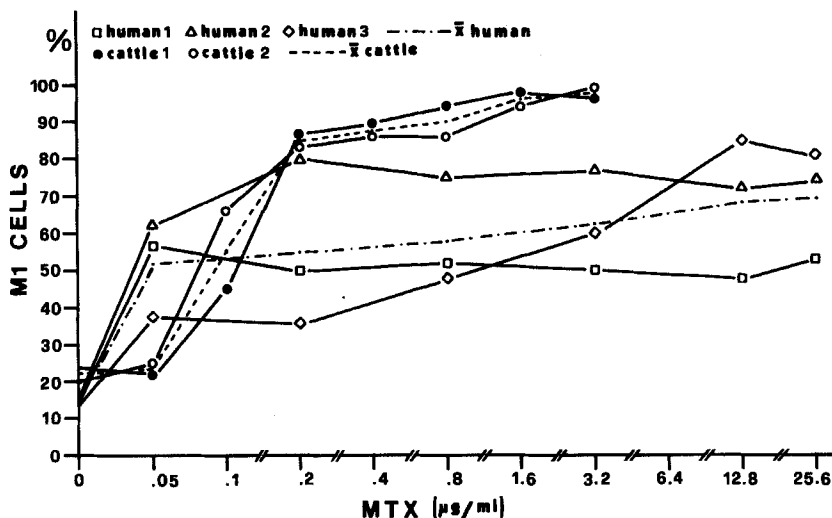
Fig. 1. Cellular responses in the three humans and the two cattle after 24 h incorporation of BrdU (20 μg/ml) and MTX at increasing doses.

Exemplification of the banding patterns obtained

Typical banding patterns of the human chromosomes are demonstrated in Fig. 2 and 3. Fig. 2 shows the G+C banding pattern obtained in M1 male cells at metaphase (A) and prometaphase (B) and in a M1 prometaphase female cell, under fluorescence (C) and Giemsa counterstaining (D). Notice the intense staining of the Y chromosome, of the late replicating X and of the constitutive heterochromatin areas (C-bands), as well as the C-band heteromorphism in chromosome pair 16. Fig. 3A shows an M2 cell in which SCEs are visible in chromosomes 1, 7, 8, 9 and 10, respectively, and localized at the interbands 1q42–43, 7q22–31, 8q22–23, 9q12–13 and 10q25–26 (Fig. 3B). Fig. 3C shows two partials of prometaphase of the same subject in which two homologous 'break-exchange-sites' have been found in chromosome 1 (interbands 1p32–33).

Typical banding patterns of the cattle chromosomes are demonstrated in Fig. 4, 5 and 6. Fig. 4 shows the fluorescent G+C-banding pattern in an M1 prometaphase cell of a male (A) and a female (B). As in humans, notice the intense staining of the Y chromosome, the late replicating X and the constitutive heterochromatin (C-bands). Fig. 5A-B shows the results of the sequential C+G-bands procedure in the same cell. Notice the C-band heteromorphism in chromosome pairs 1 and 14. Fig. 6 shows an M2 cell with SCEs in chromosomes 4, 6, 10, 14 and, probably, 20.

Discussion

As known, the MTX is used to reduce cell growth in some kinds of cancer, because it inhibits DNA synthesis (SKEEL et al. 1976). For this reason it has been used as cell synchronizing agent to achieve high percentages of prometaphases with high resolution G-bands (YUNIS 1976, 1980; CAMARGO and CERVENKA 1980) and R-bands (PAI and THOMAS 1980). After simultaneous treatment with MTX and BrdU the cells are allowed to recover in the presence of thymidine; high quality G+C-bands can be obtained in both chromatids and in one chromatid in metaphase as well as in prometaphase cells of the same preparation, simply by staining with acridine orange and/or counterstaining with Giemsa. The data shown in Fig. 1, even if limited to five subjects (three humans and two cattle), show a 3–5 times increase of M1 cells compared to the control values. It is difficult to explain the differences observed in the responses between the two species and between the three human subjects. In the latter, in fact, after the first dose of MTX (0.05 μg/ml), which is a little more than the dose normally used in the synchronization techniques (YUNIS 1976), we have different cell responses which, however, do not bring M1 cells far beside the average value of 70 % when the highest dose is applied. We could hypothesize a variable cellular resistance to MTX for the three humans examined. In fact, by increasing the doses of MTX in a cell line it is possible to achieve MTX-re-
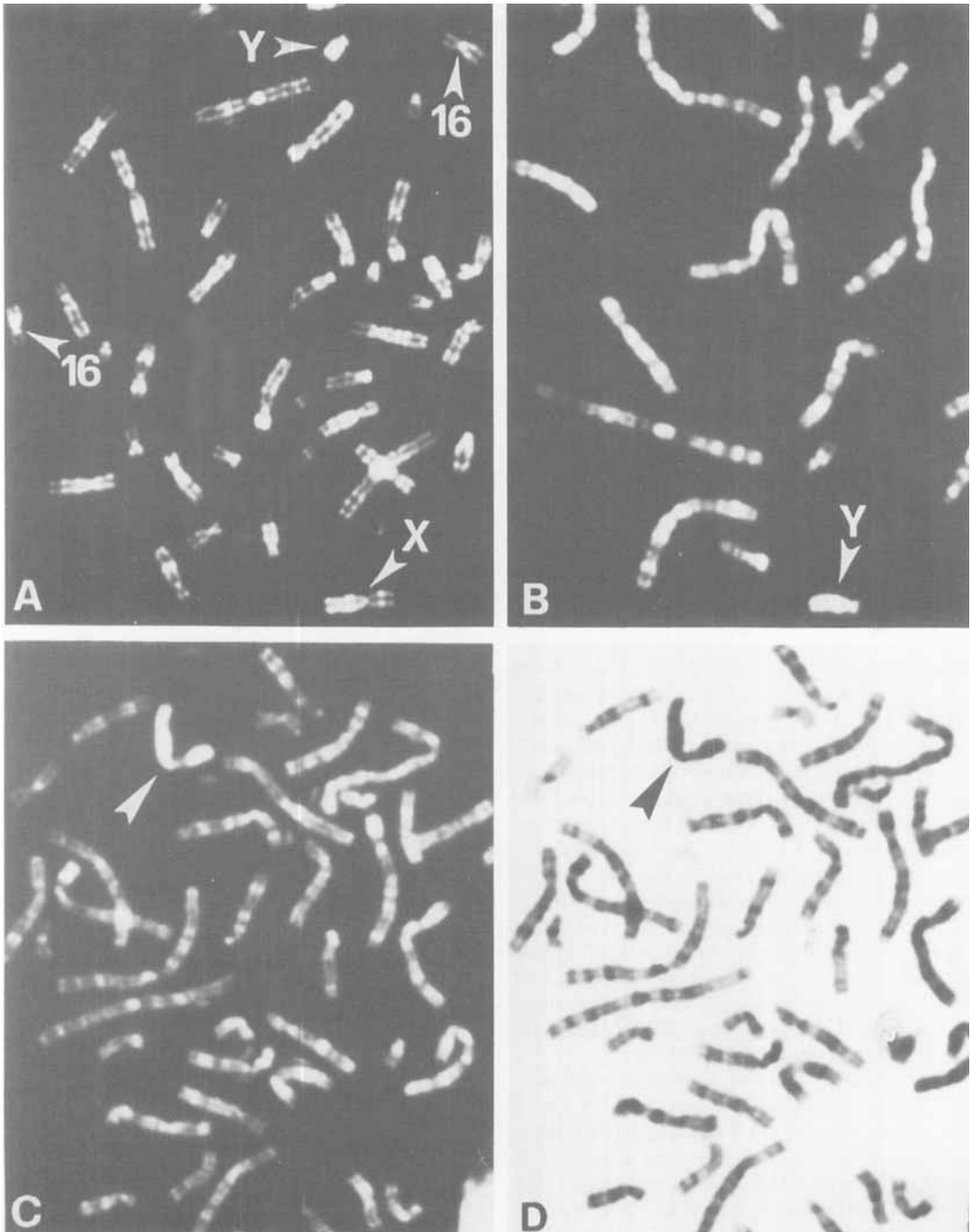
Fig. 2A–D. G+C-bands in human chromosomes. Notice the good resolution of G-bands even in contracted chromosomes. Partial metaphase **(A)** and prometaphase **(B)** cells of a male with C-band heteromorphism in chromosome pair 16, and sex chromosomes indicated. Partial prometaphase female cell in fluorescence **(C)** and after Giemsa counterstaining **(D)**. The late replicating X is indicated.
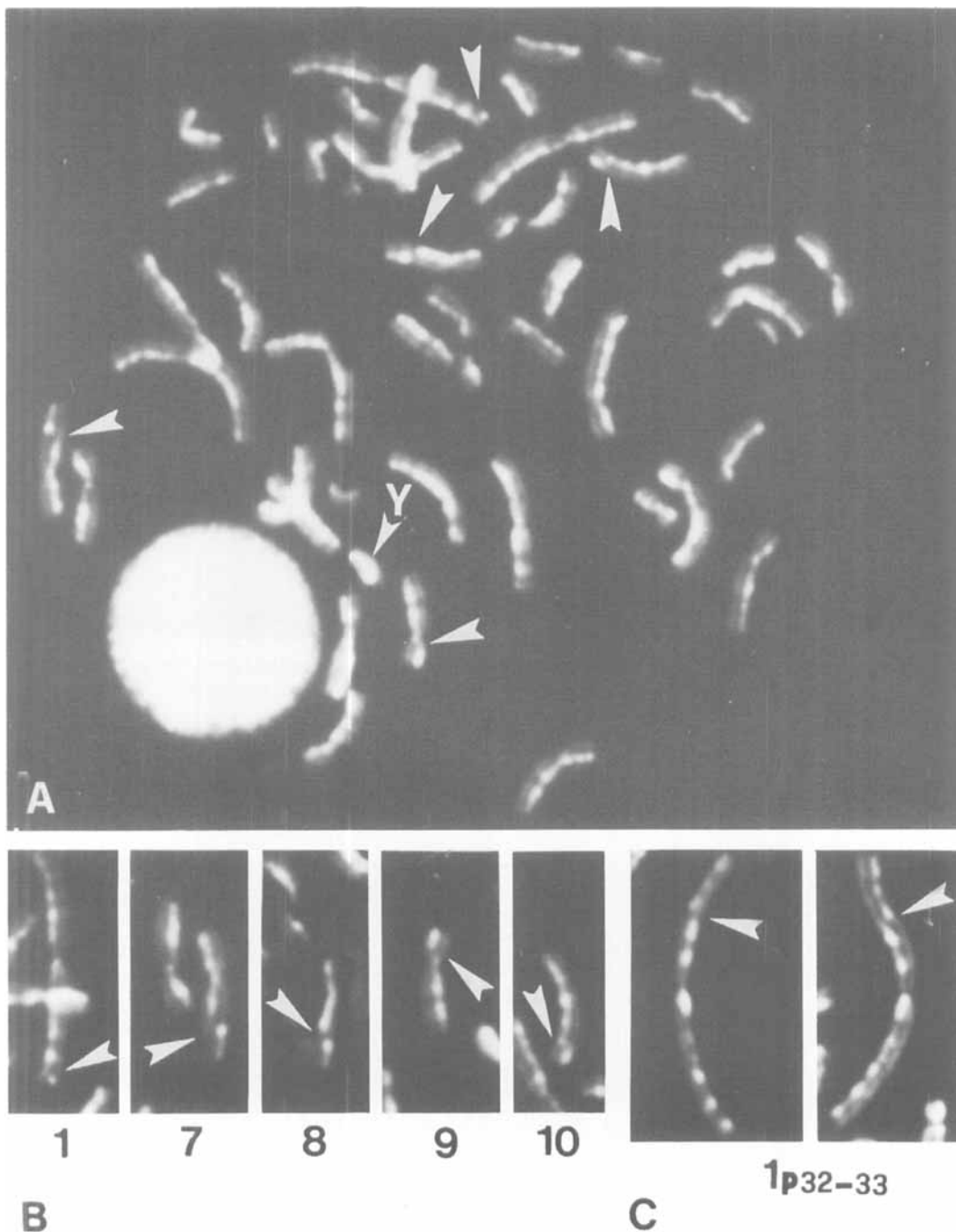
Fig. 3A–C. G+C-bands in one chromatid demonstrated in a human metaphase **(A)** with the five chromosomes contain-ing SCEs (arrows) cut out **(B).** The SCEs are located in chromosomes 1, 7, 8, 9 and 10 at the interbands 1q 42–43, 7q 22–31, 8q 22–23, 9q 12–13 and 10q 25–26, respectively. Two homologous 'break-exchange-sites' **(C)** were localized on chromosome 1 (interbands 1p 32–33) in the same subject (arrows).
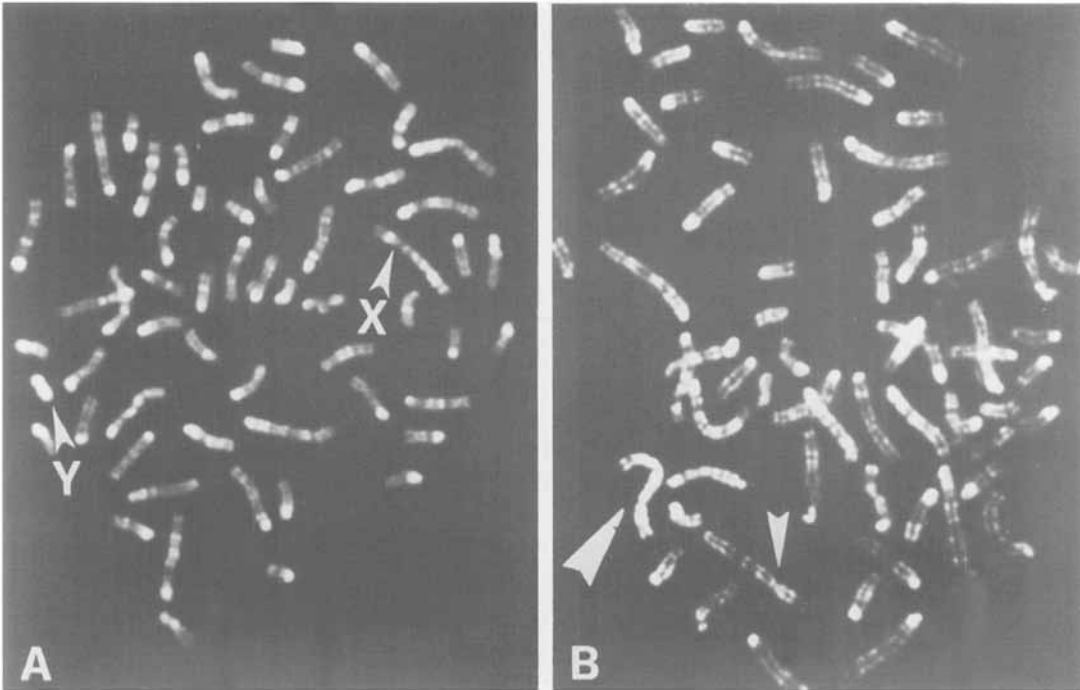
Fig. 4A–B. G+C-bands in cattle chromosomes in a male **(A)** and a female **(B)** prometaphase cell. The male sex chromosomes and the early replicating X of the female are indicated with small arrows, the late replicating X of the female is indicated with a large arrow.
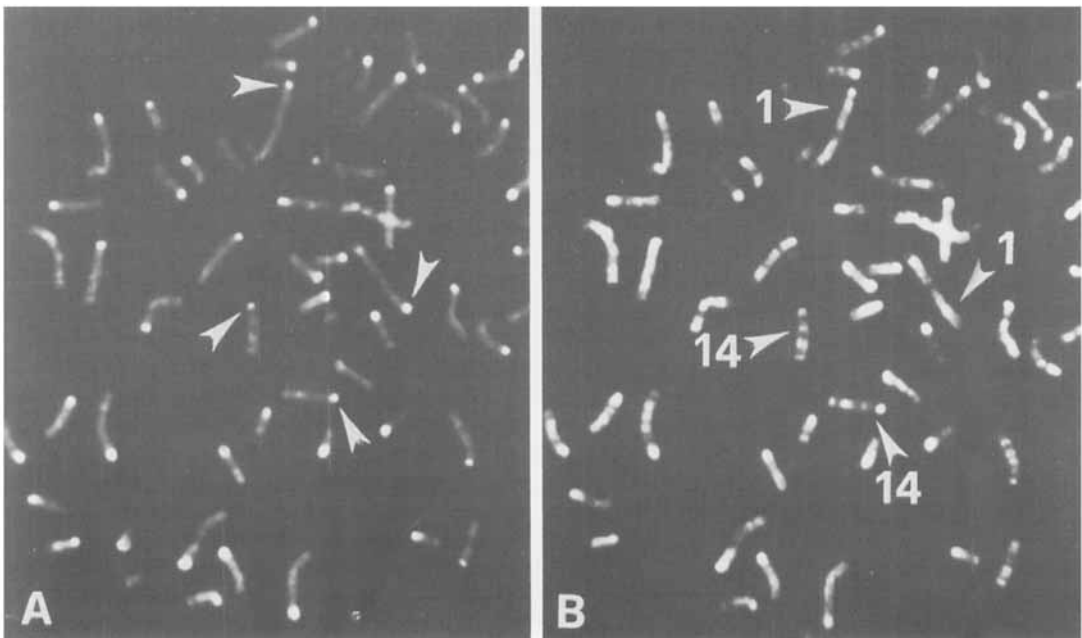

Fig. 5A–B. The sequential C+G-banding procedure applied on cattle chromosomes of the same cell demonstrating the C-bands **(A)** and the G-bands **(B)**. Notice the C-band heteromorphism in chromosome pairs 1 and 14 (arrows).
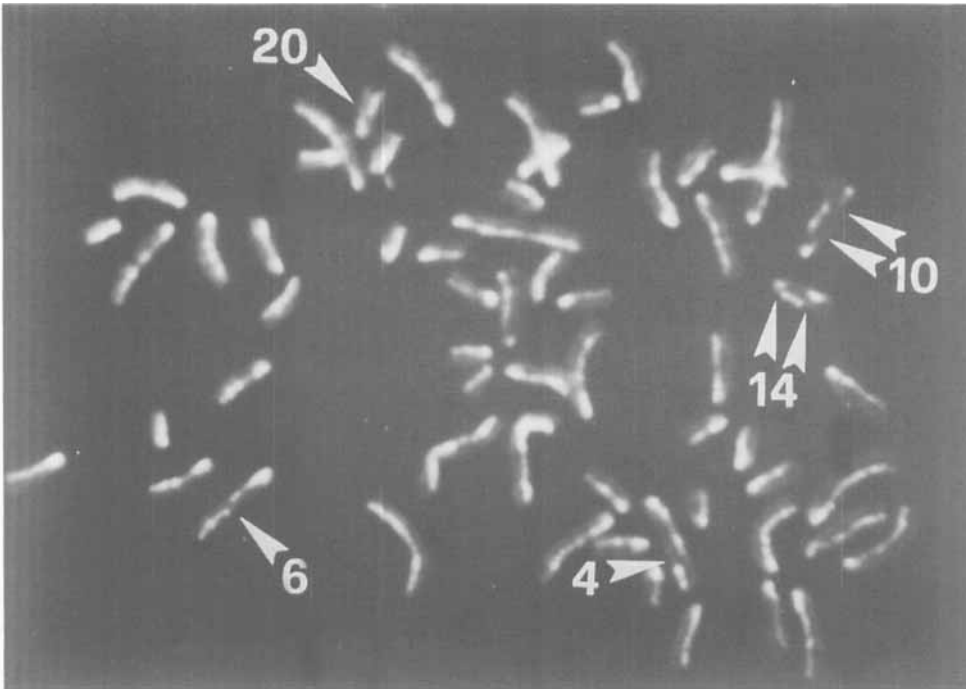
Fig. 6. Simultaneous demonstration of G+C-bands and SCEs (arrows) in cattle chromosomes. The exchange occurred in chromosomes 4, 6, 10, 14 and, probably, 20.

sistance. This has been explained by amplification of dihydrofolate reductase genes localized in the homogeneously staining regions of a single chromosome (JACK et al. 1978) or as being associated with the occurrence of double minute chromosomes (RANDAL et al. 1979). Cattle lymphocytes appeared to be less sensitive to 0.05 μg/ml. With regard to the average data obtained in humans and in cattle, the doses of MTX to be used in this technique range from 0.05 to 0.8 μg/ml in humans and from 0.1 to 0.4 μg/ml in cattle, to ensure, in both species, an increase of M1 cells 3–4 times the control.

It is reasonable to think that the presence of BrdU (pyrimidine base analog) reduces the MTX action, but while in cattle the increase of MTX blocks almost all the DNA synthesis, in humans, even by increasing the MTX, a fraction of cells will synthetize DNA with help of the large amount of BrdU contained in the medium. Furthermore, the MTX block and the presence of BrdU may stimulate cells to utilize all the purines to synthesize DNA by the alternative pathway. Another factor which might account for the presence of M2 cells could be that the MTX block is somewhat subsequent to BrdU incor-

poration, so that a fraction of cells escapes the MTX block, completes the S-phase and returns to replicate in the presence of BrdU in the next cell cycle but only in the S1-phase because in S2 they will take up thymidine.

More work is necessary for a better understanding of the latter phenomenon. However, by the technique presented in this paper, it is possible to achieve: (a) G+C-bands in one and both chromatids in the same cytological preparation, with different frequencies in relation to the MTX doses and without alteration of the cytological preparations; (b) high mitotic index; (c) easy demonstration of the late replicating X; (d) knowledge of distribution and localization of SCEs in individual chromosomes; (e) increased information of the centromere region in relation to the polymorphism of constitutive heterochromatin; (f) high resolution G+C-bands; (g) utilization of the same preparations for application of other cytological techniques.

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