Proc. Natl. Acad. Sci. USA
Vol. 78, No. 12, pp. 7727–7731, December 1981
Medical Sciences

High-resolution analysis of human peripheral lymphocyte chromosomes by flow cytometry

(metaphase chromosomes/peripheral blood cultures/autosomal polymorphism)

B. D. YOUNG*, M. A. FERGUSON-SMITH†, R. SILLAR*, AND E. BOYD†

*Beatson Institute for Cancer Research, Garscube Estate, Switchback Road, Glasgow G61 1BD, Scotland; and †Duncan Guthrie Institute of Medical Genetics, Yorkhill, Glasgow G3 8SJ, Scotland

Communicated by Victor A. McKusick, July 20, 1981

ABSTRACT **A method for high-resolution analysis of the human karyotype by flow cytometry has been developed. Metaphase chromosomes are prepared from short-term peripheral blood cultures, stained with ethidium bromide, and analyzed on a standard fluorescence-activated cell sorter (FACS-II). Flow karyotypes with up to 20 peaks can be obtained with coefficients of variation in the range 1–2%. At this level of resolution the contribution of many of the human chromosomes can be evaluated separately. Significant and reproducible differences between normal individuals have been detected and have been correlated with differences in the centric heterochromatin of certain chromosomes as revealed in their C-banded karyotypes.**

In recent years, flow cytometry has shown great potential for automated analysis of the human karyotype. In principle, the development of such an approach could offer the clinician an objective assessment of chromosome abnormalities, thus complementing the more traditional forms of karyotype analysis. However, there have been two main obstacles to the development of flow cytometry as a cytogenetic tool. First, conventional methods of chromosome preparation (1) may damage interphase nuclei, producing fragments of chromatin which cannot be distinguished from chromosomes by a flow cytometer. For this reason, most flow karyotypes have been derived from cells grown in monolayer (2, 3) and it has not been possible to use peripheral blood lymphocytes as a source. The second limitation is that commercially available flow cytometers have not been shown to produce adequate flow karyotypes. Only specially built, and therefore expensive, systems have been used (4, 5).

We report here an extension of a previously developed procedure (6) which yields high-resolution flow karyotypes from short-term peripheral blood cultures with a standard fluorescence-activated cell sorter (FACS-II). At this level of resolution it is possible to detect some of the commonly occuring centric, satellite, and Y polymorphisms (7).

MATERIALS AND METHODS

Short-Term Lymphocyte Culture. A 20-ml sample of heparinized blood was obtained from each subject and diluted with an equal volume of phosphate-buffered saline. This was divided equally into four sterile tubes and each aliquot was carefully underlayered with 7.5 ml of Lymphoprep (Nyegaard, Oslo, Norway). Centrifugation (400 × *g* at the interface) for 30 min at 20°C gave a well-separated band of lymphocytes which were removed and washed twice in medium to dilute out any remaining Lymphoprep which might inhibit stimulation. The cells in an aliquot were counted in a hemocytometer, and the lymphocyte concentration was adjusted to between 0.3×10^6 and 0.4×10^6 cells per ml, typical culture volumes being between 20 and 30 ml in sterile glass bottles with a 5-cm-diameter base. The culture medium was chromosome medium 1A (GIBCO) containing phytohemagglutinin.

After 24 hr at 37°C, leuko-agglutination could be observed, with clumps of approximately 20 cell diameters increasing in size to about 50 cell diameters after 48 hr. Cells were disaggregated by gentle inversion of the culture flask, and a 2-ml aliquot was removed for cell-cycle analysis. The remaining culture was centrifuged and resuspended in 1.5 times the initial volume of fresh medium.

For cell-cycle analysis, cells were washed twice in phosphate-buffered saline at 4°C and the resulting pellet was fixed by dropwise addition of ice-cold 70% methanol. Cells were stained in chromomycin A3 (20 μg/ml) containing $MgCl_2$ at 3 mg/ml and analyzed on a FACS-II flow cytometer with excitation at 457.9 nm.

Dual-parameter analysis of the scatter and fluorescence signals was used to estimate how well the cultures had responded to the stimulation. The initial resonse to phytohemagglutinin was an increase in scatter signal which occurred over the first 24 hr; this was followed by the onset of DNA synthesis. If the proportion of cells that had entered S phase was 20% or greater, Colcemid was added to the culture after 50 hr (at 0.1 μg/ml) for 17 hr. If stimulation could not be observed, the culture was continued and sampled at 72 hr and, if necessary, at 96 hr. Once stimulation was observed, Colcemid was added as above.

Chromosome Preparation. Chromosomes were prepared by using a slight modification of a described method (6). Lymphocyte cultures were harvested by centrifugation at 900 rpm for 10 min. The cell pellet was resuspended in 0.075 M KCl and placed on ice for 5 min. This suspension of swollen mitotic cells was centrifuged at 750 rpm for 10 min and the pellet resuspended in 5 ml of polyamine buffer (15 mM Tris·HCl/2 mM EDTA/0.5 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid/80 mM KCl/20 mM NaCl/0.2 mM spermine/0.5 mM spermidine/14 mM 2-mercaptoethanol, pH 7.2 at 25°C) at 4°C. At this stage, mitotic cells were clearly visible by fluorescence microscopy. The cells were centrifuged at 1500 rpm for 2 min and resuspended in 1–2 ml of polyamine buffer containing 0.1% digitonin. This suspension was held on a vortex mixer for 30 sec at high speed to lyse the mitotic cells and then was stored on ice.

Flow Cytometry. Samples were stained in suspension with ethidium bromide (final concentration, 100 μg/ml) and analyzed on a Becton Dickinson FACS-II. A Spectra Physics 164-05 laser producing 1.5 W of output power at 514.5 nm was used to excite chromosomal fluorescence, and a Schott OG550 emission filter blocked scattered laser light.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Dual-parameter analysis of the scatter and fluorescence signals allowed discrimination of chromosomes from certain background signals as described (6). Relative flow rate estimations indicated that perhaps only 25% of all fluorescent signals were due to chromosomes, and these were selected when flow histograms were compiled. The flow karyotypes were analyzed by least squares fitting of the data with a series of Gaussian distributions, each of which was allowed variable position, height, and coefficient of variation and superimposed on a variable exponential or horizontal background.

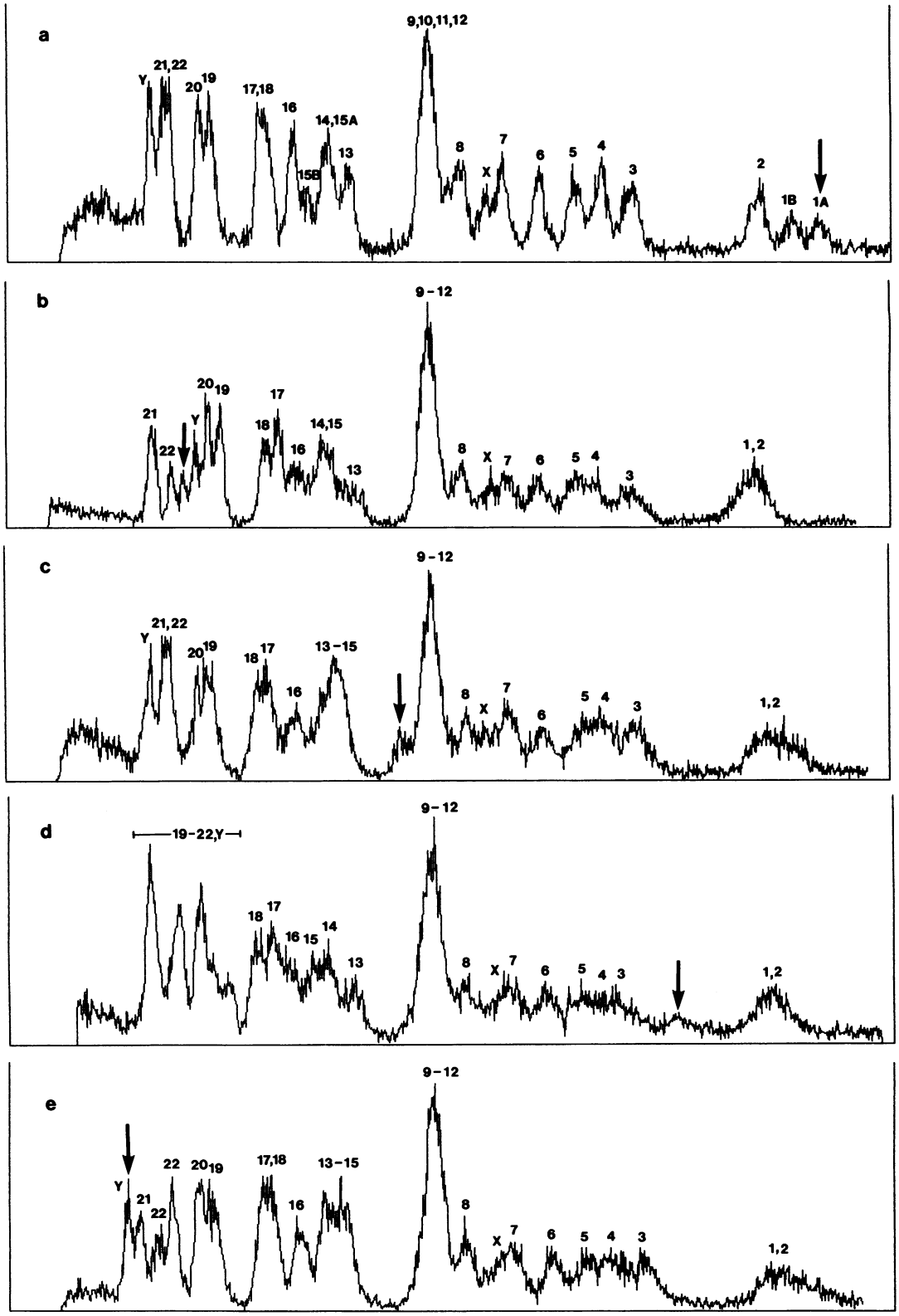

FIG. 1. Flow karyotypes obtained from peripheral blood samples. Between 20,000 and 30,000 chromosomes were analyzed from each sample and the number of chromosomes (abscissa) is plotted versus relative fluorescence (ordinate). The peaks are labeled to indicate the chromosomes or groups of chromosomes they contain. The arrows indicate the presence of polymorphisms and correspond to the C-banded chromosomes arrowed in Fig. 2.

Karyotype Analysis. Peripheral blood lymphocytes were stimulated for 72 hr with phytohemagglutinin and slides were prepared and stained by standard techniques (8). For G banding, slides were incubated in 0.30 M NaCl/0.03 M sodium citrate, pH 7, at 60°C for 2 hr, treated with 0.25% trypsin for 1–1.5 min, and stained for 3.5 min with Leishman's stain diluted with 3 vol of pH 6.8 buffer. For C banding, slides were pretreated with 0.1 M HCl for 1 hr, incubated at 60°C with 5% barium hydroxide for 10 sec, and with 0.30 M NaCl/0.03 M sodium citrate, pH 7, for 1 hr, and finally stained for 1 hr in Giemsa diluted 1:50 with pH 6.8 buffer.

RESULTS AND DISCUSSION

The time required for phytohemagglutinin-stimulated lymphocytes to enter the S phase of the cell cycle is known to vary considerably (9). Conventional cytogenetic analysis does not require full stimulation because only a small number of metaphase cells are analyzed. The flow karyotype, on the other hand, depends on having a greater number of cells in metaphase and may be poorly resolved and have a high background if an insufficient proportion of cells have entered S phase. If each culture is monitored by flow cytometry, prior to and during treatment with Colcemid, to ensure maximal stimulation, high-resolution flow karyotypes with low background can be obtained reproducibly. The only limitation with this approach is that the lengthy treatment with Colcemid, combined with our preparative technique, effectively destroys the banding structure of the chromosomes (6).

The results from five male donors are shown in Fig. 1. Despite the overall similarity in the profiles, it is clear that each karyotype has characteristic features that distinguish it from the others. The data in Fig. 1*a* are resolved best and therefore we have applied full mathematical analysis to this karyotype (Table 1). A second flow karyotype was obtained from this individual 6 months later (Fig. 2) and the peak positions are also presented in Table 1. For both sets of data the peaks are almost identical and hence the flow karyotype of this individual is quite reproducible. Flow karyotypes from other individuals have also been shown to be reproducible. The relative fluorescence values (normalized to make the peak due to chromosome 2 equal 1000) agree quite closely with previously published (3) chromosomal DNA contents. This information, combined with the computed relative areas of the peaks, has allowed identification of the contribution of each chromosome or group of chromosomes to the overall pattern. It can be seen from Table 1 that the larger chromosomes are slightly underrepresented whereas the smaller ones are overrepresented. We often observe this anomaly which may be due either to slightly greater degradation of larger chromosomes or to a hydrodynamic effect in which the smaller chromosomes are more efficiently passed through the flow system.

Table 1. Results of computer analysis of flow karyotype in Fig. 1*a*

Chromosome	Relative fluorescence*	Coefficient of variation	No./cell†
1A	1082;1074	0.85	0.79 (1)
1B	1045;1044	0.90	0.85 (1)
2	1000;1000	1.07	1.81 (2)
3	831;828	1.28	2.00 (2)
4	791;787	1.09	2.09 (2)
5	757;758	1.33	1.98 (2)
6	709;708	1.14	1.78 (2)
7	661;653‡	1.25	2.00 (2)
X	636;653‡	1.15	1.00 (1)
8	601;603	1.59	3.01 (2)
9, 10, 11, 12	560;561	1.93	6.86 (8)§
13	455;453	1.61	1.80 (2)
14, 15A	430;425	2.27	3.10 (3)
15B	400;398	1.27	0.76 (1)
16	382;381	1.74	2.20 (2)
17, 18	338;342	2.61	3.96 (4)
19	270;269	2.26	2.39 (2)
20	255;255	2.01	2.05 (2)
21, 22	211;211	3.80	3.78 (4)
Y	191;191	2.31	1.79 (1)

* Values from first flow karyotype and from sample obtained from same individual 6 months later.
† Values in parentheses are the expected values.
‡ Peaks due to chromosomes 7 and X could not be distinguished in second flow karyotype.
§ Data suggest a polymorphism in one of the 9–12 group, moving an unidentified chromosome into the peak normally due to chromosome 8 alone.

The most striking feature of the flow karyotype in Fig. 1*a* in comparison to the others is the existence of three peaks in the region where chromosomes 1 and 2 normally yield a single peak. Examination of the G-banded karyotype (not shown) of this individual revealed a heteromorphism of the centric heterochromatin of chromosome 1, and this is confirmed in the C-banded preparation in Fig. 3*a*. Hence this slight polymorphism can be detected in the flow karyotype as a difference of about 3% in the fluorescence of the autosomes. The data in Table 1 also suggest that there is a difference of about 7.5% in the fluorescence of the chromosome 15 homologues, although such a difference in the C- or G-banded karyotypes could not be observed. The Y chromosome appears to yield a peak that is considerably larger than expected. This may be due to errors in fitting the baseline at this part of the flow karyotype.

In the flow karyotype in Fig. 1*b* there are small peaks lying in the region that normally separates chromosomes 22 and 20. The G-banded karyotype showed an enlarged satellite on one

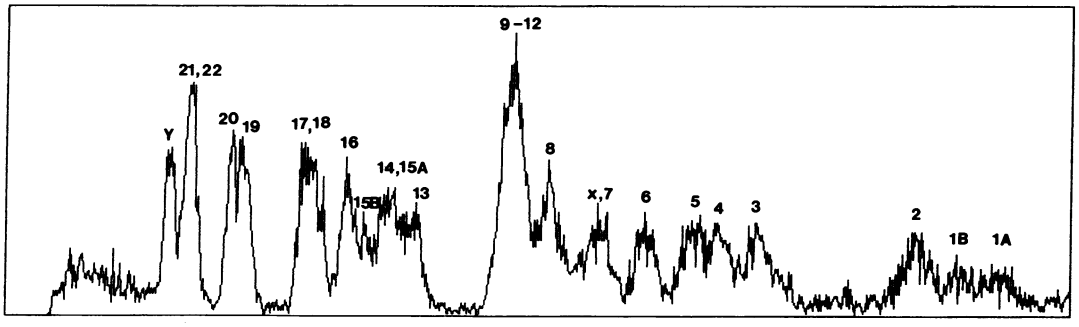

FIG. 2. Repeat of the flow karyotype shown in Fig. 1*a*, obtained from the same individual 6 months later.

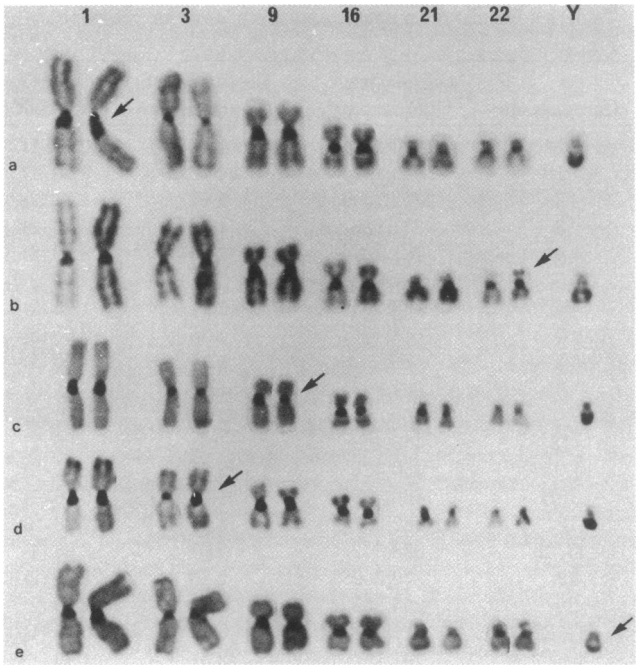

FIG. 3. C-banded karyotype analysis, illustrating polymorphisms in the centric heterochromatin (arrows) corresponding to those indicated in Fig. 1.

homologue of chromosome 22 and this was confirmed in the C-banded preparation shown in Fig. 3*b*. Hence, two of the three small peaks are probably due to separation of chromosomes 22 while the third peak has been tentatively identified as the Y chromosome.

Having established correlations between flow karyotypes and conventional karyotypes, it was decided to examine samples from individuals known to have certain other polymorphisms. A sample was obtained from an individual whose G-banded karyotype had previously revealed a reduction in the centric heterochromatin of one chromosome 9. The flow karyotype (Fig. 1*c*) showed a small peak (arrow) lying to the left of the main peak which is due to chromosomes 9–12. The C-banded karyotype (Fig. 3*c*) confirms that there is a reduction in the centric heterochromatin of one chromosome 9. Computer analysis indicated that this peak is due to a single chromosome from the 9–12 group because the relative areas are approximately 7:1.

A sample was obtained from an individual whose G-banded karyotype had previously indicated increased centric heterochromatin in one chromosome 3. The flow karyotype (Fig. 1*d*) indicates a peak in the region that normally separates chromosome 3 and the peak containing chromosomes 1 and 2. The C-banded karyotype (Fig. 3*d*) confirms that this extra peak is due to one chromosome 3 bearing extra centric heterochromatin.

Finally, a blood sample was obtained from an individual whose G-banded karyotype had previously shown an unusually small Y chromosome. The corresponding flow karyotype (Fig. 1*e*) indicates a peak lying to the left of chromosome 21 which normally contains no peaks. The presence of an unusually small Y chromosome is confirmed in the C-banded karyotype (Fig. 3*e*). It is also apparent from Fig. 1*e* that there is a polymorphism on chromosome 22, and this is confirmed in Fig. 3*e*.

The most obvious differences between flow karyotypes can thus be explained as autosomal polymorphisms as revealed in the C-banded karyotypes. The peaks due to chromosomes 13–16 also show a high degree of variability between individuals (Fig. 1), due in part at least to variation in the ribosomal RNA genes (10) which have been mapped to the satellite regions of chromosomes 13–15. However, at this stage in our investigation it is difficult to correlate precisely the differences in this region with particular autosomal polymorphisms because of the proximity of these peaks to each other. The Y chromosome is also known to be very variable and it is therefore quite difficult to be certain of its position in the flow karyotype. It is known that chromosomes 1, 3, 4, 9, 13, 14, 15, 16, 21, 22, and Y all exhibit a high frequency of polymorphism (7). However, because flow cytometry can resolve chromosomes with a coefficient of variation of 1–2%, in the future it may be possible to detect polymorphisms in chromosomes that previously have been thought to be invariant.

An autosomal polymorphism is most readily detected when it results in a new peak. If the polymorphism moves a chromosome to the position of another chromosome, the only observable change will be in the relative areas of the peaks. The computer analysis (Table 1) has revealed such a "hidden" polymorphism in that the relative areas for the peaks due to chromosomes 9–12 and 8 are calculated to be 7:3 instead of 8:2, the expected value. This was a consistent feature as shown by the second analysis performed 6 months later (Fig. 2). Examination of the G-banded or C-banded karyotype does not clearly reveal the origin of this feature and therefore it seems quite possible that the flow karyotype can provide information that is not available from the conventional analysis.

Our main aim in developing this approach is to provide the cytogeneticist with an objective analysis that can be related to the conventional karyotype of an individual. In this regard, the flow karyotype offers several advantages over slide-based cytophotometry. First, because the chromosomes are in suspension and are probably more uniformly stained, much greater resolution can be obtained with coefficients of variation routinely around 2%. Second, a large number of chromosomes from each sample can be analyzed rapidly (50,000 in 10 min). The simplicity of the preparative technique means that this approach may find application in the screening of large numbers of patients. We can anticipate that aneuploidy will be detected as an increase or decrease in the corresponding peak. A translocation will be observed as increased or decreased fluorescence of the chromosomes receiving or losing the fragment of DNA, respectively. In fact, both phenomena have been observed in flow karyotypes from the lymphoblastoid cell lines GM1416 which contains four X chromosomes and GM3197 which contains a 17;22 translocation (unpublished data).

With continuing development we expect that abnormalities that are undetectable by conventional banding techniques could be detected and quantitated by flow cytometry. However, before this method can be used as a means of diagnosis of true abnormalities it will be necessary to quantitate the large number of autosomal polymorphisms that occur in about 30% of the normal population (7). Such polymorphisms are easily disregarded by the trained cytogeneticist but may confuse the interpretation of a flow karyotype.

This work was supported by grants from the Medical Research Council, the Cancer Research Campaign, and Action Research for the Crippled Child.

1. Wray, W. & Stubblefield, E. (1970) *Exp. Cell Res.* **59**, 469–478.
2. Gray, J. W., Carrano, A. V., Steinmetz, L. L., Van Dilla, M. A., Moore, D. H., II, Mayall, B. H. & Mendelsohn, M. L. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 1231–1234.

3. Carrano, A. V., Gray, J. W., Langlois, R. G., Burkhart-Schultz, K. J. & Van Dilla, M. A. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 1382–1384.
4. Gray, J. W., Peters, D., Merrill, J. T., Martin, R. & Van Dilla, M. A. (1979) *J. Histochem. Cytochem.* **27**, 441–444.
5. Cram, L. S., Arndt-Jovin, D. J., Grimwade, B. S. & Jovin, T. M. (1979) *J. Histochem. Cytochem.* **27**, 445–453.
6. Sillar, R. & Young, B. D. (1981) *J. Histochem. Cytochem.* **29**, 74–78.
7. Ferguson-Smith, M. A. (1973) in *Chromosomes Today*, eds. Wahrman, J. & Lewis, K. R. (Wiley, New York), Vol. 4, pp. 235–246.
8. Ferguson-Smith, M. A. (1974) *La Ric. Clin. Lab.* **4**, 297–335.
9. Soren, L. (1973) *Exp. Cell Res.* **78**, 201–208.
10. Miller, D. A., Tantravahi, R., Dev, V. G. & Miller, O. J. (1977) *Am. J. Hum. Genet.* **29**, 490–502.