J. Tiss. Cult. Meth. 14:125–132, 1992

0271-8057/92 $01.50+0.00

IMMUNOFLUORESCENT STAINING OF KINETOCHORES IN MICRONUCLEUS FOR DETECTION OF ANEUPLOIDY INDUCING AGENTS

Channarayappa, J. Nath and T. Ong[1]

Division of Plant and Soil Sciences, College of Agriculture and Forestry, West Virginia University, Morgantown, West Virginia 26506 (C., J. N., T. O.), and National Institute for Occupational Safety and Health, Division of Respiratory Disease Studies, Morgantown, West Virginia 26505 (T. O.)

SUMMARY: Immunofluorescent staining of kinetochores in the micronucleus was used for distinguishing between clastogenic and aneuploidogenic effects of various chemicals. The technique consists of culturing cells on glass slides, chemical treatment, fixing cells in situ, and fluorescent staining. Anti-kinetochore antibody was used as a primary antibody with fluoresceinated goat anti-human IgG as a secondary antibody to locate kinetochores, and 4,6-diamidino-2-phenyl-indole (DAPI) or propidium iodide was used for identifying the nucleus, micronucleus, and cytoplasm. Kinetochores appear yellow/green against the orange/red nuclear material and the cytoplasm appears faint green in slides counterstained with propidium iodide. In DAPI counterstained slides, kinetochores appear apple green and the nucleus and micronucleus appear light blue. Thus, location of kinetochores and nuclear material within the cell boundary can be achieved. The presence/absence of kinetochore(s) in a micronucleus is used as an indicator to determine the origin of the micronucleus. The proportion of kinetochore-positive and kinetochore-negative micronuclei is used in the determination of the clastogenic and aneuploidogenic properties of different genotoxic agents. This method is simple and applicable to a variety of cells.

Key words: kinetochores; antikinetochore antibody; clastogen and aneuploidogen; micronucleus assay; immunofluorescence.

I. INTRODUCTION

Development of tests for assessing the ability of chemicals to induce aneuploidy is one of the major goals in genetic toxicology. Detection of aneuploidy in mammalian cells is based primarily on cytogenetic assays such as metaphase chromosome counting or analysis of lagging chromosomes in anaphase, multipolar spindles, or C-mitotic effects. None of these methods, however, has been sufficiently validated, and there is a need for development and validation of assay procedures (6,15). Some investigators have proposed using micronuclei induction as an assay for aneuploidy (15,16). A major limitation in this approach has been that micronuclei can arise either from acentric chromosome fragments or from centric fragments or whole chromosomes, and therefore the assay fails to discriminate between aneuploidogens and clastogens. Several attempts have been made to distinguish between micronuclei derived from broken chromosomes and those from lagging chromosomes resulting from spindle malfunction (10,12,14,17,20,21). Direct evidence for a micronucleus containing whole chromosome(s) is only obtained when techniques for identifying centromeres and associated materials are used.

Application of immunofluorescence technique using an anti-kinetochore antibody facilitates localization of kinetochores in metaphase and interphase cells (13). Recently, several investigators have reported on the usefulness of this assay for the detection of clastogenic and aneuploidogenic effects of various agents (2,5,8,11,18). Micronuclei induced by clastogenic agents contain a majority of cells with kinetochore-negative micronuclei while spindle poisons induced micronuclei containing predominantly kinetochore-positive micronuclei. Thus, this assay has been used to understand the mechanism behind the induction of micronuclei by different genotoxic agents.

This report describes a simple and reproducible technique for the differentiation of clastogens from aneuploidogens by studying kinetochores in micronuclei. The presence of kinetochore(s) in the micronucleus is determined by using anti-centromere antibodies in in situ fixed V79 cells. This procedure could be used for other cell types and for both in vitro and partial in vivo genotoxicity assays.

II. MATERIALS

A. Equipment

Balance, top loading, Metler, model PC 440, no. 01-913-351, Fisher[1]

Centrifuge, IEC table top, model HN-SII, no. 05-111, Sigma[2]

[1] To whom correspondence should be addressed.

Hood, Biological safety cabinet 800 series, Bellco[3]
Incubator, model 3326 S/N 32334-3979, Forma Scientific[4]
Microscopes
Inverted, Reichert series 1820 Biostar inverted Biological Microscope, American Scientific[5]
Light, AO Microstar, General Lab[5]
Fluorescence, Zeiss Standard microscope vertical illuminator IV FL, Carl Zeiss Instruments Inc.[6]
Epi-fluorescence condenser IV FL with 2 filter set
HBO 50 high-pressure mercury lamp
100 × (N. A., 1.25) fluorescence objective (no. 461905-9901)
Differential counter (Clay Adams), Cell counter, no. B 4120-4[5]
pH Meter, model 630 Accumet with standard glass electrode, no. 13-639-90[1]
Pipet-aid, filler and dispenser, no. 13-681-15[1]
Refrigerator with freezer (−20° C), Laboratory model 812, no. R 4040-1[1]

B. Plastics and glassware
Plastics
Centrifuge tubes
Falcon 2095, conical graduated, 15 ml, screw cap, no. 2610-L 35, Thomas Scientific[7]
Falcon 2098, conical graduated, 50 ml, screw cap, no. 2610-L 54[7]
Filters, membrane
Nalgene 120-0020, 0.20 μm, no. 09-740[1]
Nalgene 245-0045, 0.45 μm, no. 09-740-5[1]
Tissue culture flasks
Lab-Tek 4021, 100 × 15-mm square petri dishes[5]
25116-75, 75 cm^2, tissue culture flasks, Corning[8]
Pipettes
Serological, cotton plugged, individually wrapped
1 ml no. 7503, Falcon[9]
5 ml no. 7529[9]
10 ml no. 7530[9]
Vials, polyethylene, no. 03-338-1C[1]
Glassware
Beakers, 500 ml Pyrex, no. 1000[5]
Bottles, 100 and 500 ml, screw cap, GIBCO[10]
Couplin staining jars, no. S 7655-1[5]
Microslides, frosted, no. 12-552[5]
Cover glasses, 22 × 50 mm, no. M6045-6[5]
Pasteur pipettes, no. 13-678-208[1]

C. Chemicals and culture media
Absolute methanol (reagent grade), no. M3641[2]
Cytochalasin-B, no. C 6762[2]
4,6-Diamidino-2-phenyl-indole (DAPI), no. D 1388[2]
Dimethyl sulfoxide (DMSO), no. 9224-1, J. T. Baker[11]
Ethylenediaminetetraacetic acid (EDTA), no. E 2004[2]
Fetal bovine serum-heat inactivated (FBS-HI), no. 220-6300[10]
Glycerol, no. G 9012[2]
L-Glutamine-200 m*M*, no. 320-5030[10]
Hanks' balanced salt solution (HBSS), no. 310-4170AJ[10]
Minimum essential medium (MEM), liquid with Earl's salts without L-glutamine, no. 320-1090 AJ[10]
Penicillin-streptomycin, no. 16-700-46[10]
Photo-Flo 200 solution, no. 1464502, Kodak[12]
p-Phenylenediamine, no. P-6001[2]
Potassium chloride, KCl, no. P 4504[2]
Potassium phosphate, monobasic, KH_2PO_4, no. P 5379[2]
Propidium iodide (PI), no. P 4170[2]
Sodium borate, $Na_2B_4O_7.10\ H_2O$, no. S 9640[2]
Sodium chloride, NaCl, no. P 5379[2]
Sodium phosphate, dibasic, Na_2HPO_4, no. S 0876[2]
Trypsin (2.5%), lyophilized, 610-5095 AE[10]
Tween-20, practical, no. 9005-64-5[11]
V79 cell line (or any other cell line)

D. Antibodies
Anti-kinetochore antibody (Anti-centromere antibody), no. 5134, Antibodies Incorporated[13]
Flourescein isothiocyanate (FITC)-conjugated goat anti-human IgG, no. 2240[13]

E. Miscellaneous
Blotting paper
Desiccator, dry seal safety desiccator, no. D 1350-100[5]
Drierite ($CaSO_4$)
Slide container (plastic), no. M 6270-10[5]
Forceps, extra fine, no. 13-812-42[1]
Gloves, latex, nonsterile disposable, no. 46-320, Edmont[14]
Hemocytometer, bright line, no. 5972-H 15[2]
Ice bucket, PVC, no. 6106-A 02[5]
Kodak, Ektachrome, day light, 400 ASA, print or slide film[12]
Lamp, laboratory, no. 36554-004, VWR[15]
Microslide holder, no. 6707-G 20[5]
Nitrogen gas
Pop-up slide holder[5]
Putative chemical mutagens of interest
Rubber cement
Rubber bulbs, no. 1951-B 10[7]
Sterile distilled water
Syringe, lock tip, 3 cc, no. S 9520-3[5]
Syringe needle, 25 G × 5/8 in., no. S 9549-25 D[5]
Timer, 2 h interval, GE, no. 9371-J 70[7]

III. PROCEDURE

A. Prepare the stock solutions
1. Cytochalasin-B solution
a. Dissolve 10 mg of cytochalasin-B in 5 ml of DMSO (2 mg/ml).
b. Sterilize by using 0.20-μm membrane filter.
c. Store at −20° C.
2. Fixative
Prechill absolute methanol at −20° C before use.
3. Hypotonic solution (0.075 *M* KCl)
a. Dissolve 2.8 g of potassium chloride in 500 ml distilled water.
b. Store at room temperature.
4. Phosphate buffered saline solution (PBS, pH 7.4)
a. Dissolve 8 g NaCl, 0.2 g KCl, 0.12 g KH_2PO_4, and 0.91 g Na_2HPO_4 in enough distilled water to make 1 liter.
b. Sterilize by autoclaving.
5. Carbonate-bicarbonate buffer (pH 9)

a. Dissolve 0.42 g $NaHCO_3$ in 10 ml distilled water.
b. Adjust pH to 9 with 1 *N* NaOH.

6. Borate buffer (pH 8.3)
   a. Dissolve 190.7 g of sodium borate in 1 liter of water.
   b. Measure pH (8.3).
7. PBS with 0.1 % Tween-20
   a. Add 1 ml of Tween-20 into 500 ml PBS buffer (pH 7.2).
   b. Store at room temperature.
8. Photo-Flo 200 solution (0.4%)
   a. Add 0.8 ml of Photo-Flo solution into 200 ml of 0.5 *M* borate buffer (pH 8.3).
   b. Mix well and store at room temperature.
9. Trypsin/EDTA solution (T/E)
   a. Dissolve 0.02 g EDTA in 100 ml HBSS and sterilize by vaccum filteration through a 0.22-μm Nalgene filter unit.
   b. Add 20 ml of sterile distilled water to the bottle containing trypsin powder to constitute 2.5% of trypsin stock solution.
   c. Add 10 ml of trypsin (2.5%) solution into 90 ml of EDTA solution to give 0.025% of T/E solution.
   d. Filter sterilize (0.2 μm).
   e. Transfer 10 ml aliquots to sterile 17 × 100-mm plastic snap-top tubes.
   f. Freeze at −20° C for up to 1 yr. Each aliquot should be thawed just before use and remaining solution should be refrozen.
10. Antifade formulation
    a. Dissolve 50 mg of *p*-phenylenediamine in 5 ml of PBS.
    b. Remove the undissolved chemical with 0.22 μm filter.
    c. Adjust pH to 8 with 0.5 *M* carbonate-bicarbonate buffer.
    d. Mix with 45 ml glycerol in a brown bottle.
    e. Store in the dark at −20° C.

B. Prepare complete culture medium
   1. To 440 ml of MEM liquid with Earl's salts without L-glutamine add:
      a. 50 ml of FBS-HI,
      b. 5 ml of 0.2 *M* L-glutamine in distilled water,
      c. 5 ml of penicillin-streptomycin stock; filter sterilized stock solution of 10 000 U of penicillin/ml and 10 000 μg of streptomycin/ml of water.
      d. Mix well and keep at 37° C for immediate use or store at 4° C for later use.

C. Setting up the cultures
   1. During the course of the experiments, the cell cultures were maintained in MEM supplemented with 10% FBS-HI, L-glutamine (final concentration 2 m*M*), and 1% penicillin-streptomycin. Cell cultures were grown at 37° C in a humidified atmosphere containing 5% CO_2.
   2. Use sterile conditions while setting up the cultures.
   3. Wash cultures (about 80% confluent) with HBSS.
   4. Add 2 ml of T/E solution to 75-cm^2 dish and gently rotate the dish for even distribution.
   5. Incubate at 37° C for 2 to 4 min and continue to incubate at 37° C while monitoring the rate of release of cells from the monolayer with an inverted microscope. When all of the cells have rounded-up and are beginning to come free of the monolayer, add 1 ml of complete culture medium to each dish.
   6. Gently pipette the cell suspension twice over the surface of the culture dish to detach as many cells as possible.
   7. Count the number of cells with a hemacytometer.
   8. Place precleaned, sterile slides in a culture dish, three per dish.
   9. Seed the trypsinized cells onto precleaned and sterile glass slides (0.5×10^5 cells in one ml of growth medium per slide) in square petri dishes (100 × 15 mm, Lab-Tek) and allow to adhere for 2 h.
   10. Add 12 ml of growth medium to each dish (final volume 15 ml).
   11. Incubate cultures at 37° C in a humidified atmosphere containing 5% CO_2.
   12. Perform chemical treatment after 24 h of initial incubation.
   13. Add cytochalasin-B (4 μg/ml) to cultures 4 h after chemical treatment.
   14. Incubate for further 18 h in the presence of cytochalasin-B to block cytokinesis.

D. Slide preparations
   1. Terminate the cultures 18 h after cytochalasin-B addition.
   2. Wash slides once with PBS by adding 15 ml PBS directly into the petri dishes and aspirate after 5 min.
   3. Perform hypotonic treatment by slowly adding 15 ml of hypotonic solution (0.075 *M* KCl) to the petri dishes.
   4. Keep petri dishes at room temperature for 10 min and aspirate hypotonic solution from the petri dishes.
   5. Remove slides from petri dishes and label.
   6. Arrange slides in a slide holder and fix them in situ by placing in prechilled (−20° C) absolute methanol.
   7. Incubate slides for 15 min at −20° C.
   8. Dry slides for a few minutes by blowing air gently.
   9. Store slides at −20° C in plastic bags containing N_2 gas or in a desiccator containing silicon granules.
   10. For immunofluorescence staining, wash slides twice with PBS (pH 7.4) after fixation and lay horizontally in a petri dish until antibody staining.

E. Antibody staining
   1. Place slides in PBS containing 0.1% Tween-20 for 5 min. This improves permeability of cell membranes and enhances antibody staining.
   2. Add 40 to 50 μl of anti-kinetochore antibody solution on each slide and place a cover slip over the top, making sure that no air bubbles are present.
   3. Incubate slides in a humidified box at 37° C for 1 h. Precaution should be taken to prevent drying off from this point onwards.
   4. After 1 h, remove cover slips from the slides and wash with PBS from a wash bottle then place slides in coplin jars for 5 min and drain out excess fluid.
   6. Add 50 to 60 μl of fluoresceinated goat anti-human IgG (secondary antibody) solution on each slide.

7. Carefully mix the second antibody and the residual PBS by raising and lowering one end of the cover slip while holding the other end in contact with the slide.
8. Incubate slides in a humidified box at 37° C for 1 h in the dark.
9. After 1-h incubation, remove cover slips and rinse off secondary antibody once with PBS from wash bottle then in coplin jars for 3 × 5 min as mentioned above.

F. Counter-staining
1. Using a Pasteur pipette, add 2 to 3 drops of DAPI (0.25 μg/ml) onto the slides for 10 min.
2. Mix the residual fluid and DAPI solution as described for the second antibody. Or
1. Slides to be stained with DAPI may be used for propidium iodide staining. Using a Pasteur pipet add 2 to 3 drops of propidium iodide (5 μg/ml) onto slides for 5 min.
2. Mix the residual fluid and propidium iodide solution as described for DAPI staining.
3. Rinse slides briefly with distilled water.

G. Flattening cells
1. Dip slides in 0.4 % Photo-Flo 200 solution in 0.5 m*M* borate buffer pH 8.3 for 10 s.
2. Dry slides in darkness until the fluorescence analysis is made. The Photo-Flo 200 emulsion treatment flattens the cells/nuclei to two dimensions allowing the slides to be stored for 2 to 3 wk.

H. Storing slides
1. Keep slides in five-slide containers and enclose in a plastic bag.
2. Fill the plastic bags containing slides with nitrogen gas and store in refrigerator (4° C) until the fluorescence analysis.

I. Mounting slides
1. Rinse the preparations briefly with distilled water and remove excess water.
2. Add 1 to 2 drops of antifade solution in the middle of the slide, place 24 × 50-mm cover slip on mounting medium (antifade solution) and allow surface tension to draw down.
3. Seal cover slips with rubber cement to prevent evaporation.
4. Slides may be stored for several days at 4° C without loss of quality.

J. Fluorescent microscope and the optical set-up
1. Use Epi-fluorescence condenser IV F1 for fluorescence analysis.
2. Optical set-up: DAPI fluorochromes excite maximally at 355 nm and emit 450 nm (DAPI filter settings; ultraviolet excitation at 365 nm, band width 11 nm, and a barrier filter at 395 nm). Fluorescein excites maximally at 488 nm and emits at 520 nm (fluorescein filter settings; blue excitation at 485 nm, band width 20 nm, and a barrier filter at 520 nm).

K. Scoring procedure for immunofluorescence stained micronucleated cells
1. Randomize and code slides before scoring. Replicate cultures for each experiment and determine the number of micronucleated cells at each dose level by scoring 1000 binucleated cells per culture and a total of 2000 cells per treatment at ×1000.
2. Score micronuclei in binucleated cells (8) by using DAPI excitation first to identify the micronuclus in a cell along with the main nucleus. This allows simultaneous identification of both the nucleus and the cell membrane. After the location of a micronucleated cell, determine the presence of kinetochores in the micronucleus using fluorescein filter settings.
3. In slides stained with propidium iodide and FITC, identify kinetochores by using the same optical set-up. The kinetochores appear yellow/green against the orange/red nuclear material, while the cytoplasm appears faint green.

L. Photomicrographs

Immunofluorescence of kinetochores can be easily examined by epifluorescence microscope using appropriate filter settings. For examining kinetochores in immunofluorescent stained cells a 100× (oil) objective lens is suitable. Ektachrome (daylight) color slide or print film, ASA 400, gives good photographic results.

IV. DISCUSSION

The immunofluorescent staining of the micronuclei in cytokinesis-blocked binucleated cells (3,7) seems to have considerable potential as a rapid assay to discriminate between micronuclei containing acentric chromosome fragments and those containing centric fragments or whole chromosomes. In this assay, scoring of micronucleus is restricted only to the binucleated cells, thus the effect of cell division on micronucleus ratios is corrected (2,7). In untreated V79 cell cultures more than 80% binucleated cells could be obtained. However, in treated cultures the number of binucleated cells varies with cytotoxicity of the chemical. The method described here relies on cells fixed and stained in situ, so that technical artifacts due to chromosome loss or destruction of cell membrane are avoided. Also, established cells have several advantages over peripheral lymphocytes such as short cell cycle, easy maintenance, large number of dividing cells, ease in growing on cover slips or slides, and elimination of variability from donor to donor. It has been reported that micronuclei induced by clastogens are likely to originate from acentric chromosome/chromatid fragments and those from the aneuploidogens from centric fragments or whole chromosomes (8,14,19). However, it has been reported that alkylating agents, although principally clastogenic, may also exhibit some degree of aneuploidogenic properties (1,8). In contrast, many aneuploidogens, in addition to causing major damage to spindle apparatus, may also induce chromosomal breaks (4,9). Thus the proportion of kinetochore-positive and kinetochore-negative micronuclei could be used to quantify or differentiate between clastogenic and aneuploidogenic effects of various genotoxicants.

Results reported by earlier investigators (2,3,8,18,19) indicate that this assay can distinguish clastogens from aneuploidogens. The frequency of cells with one or more

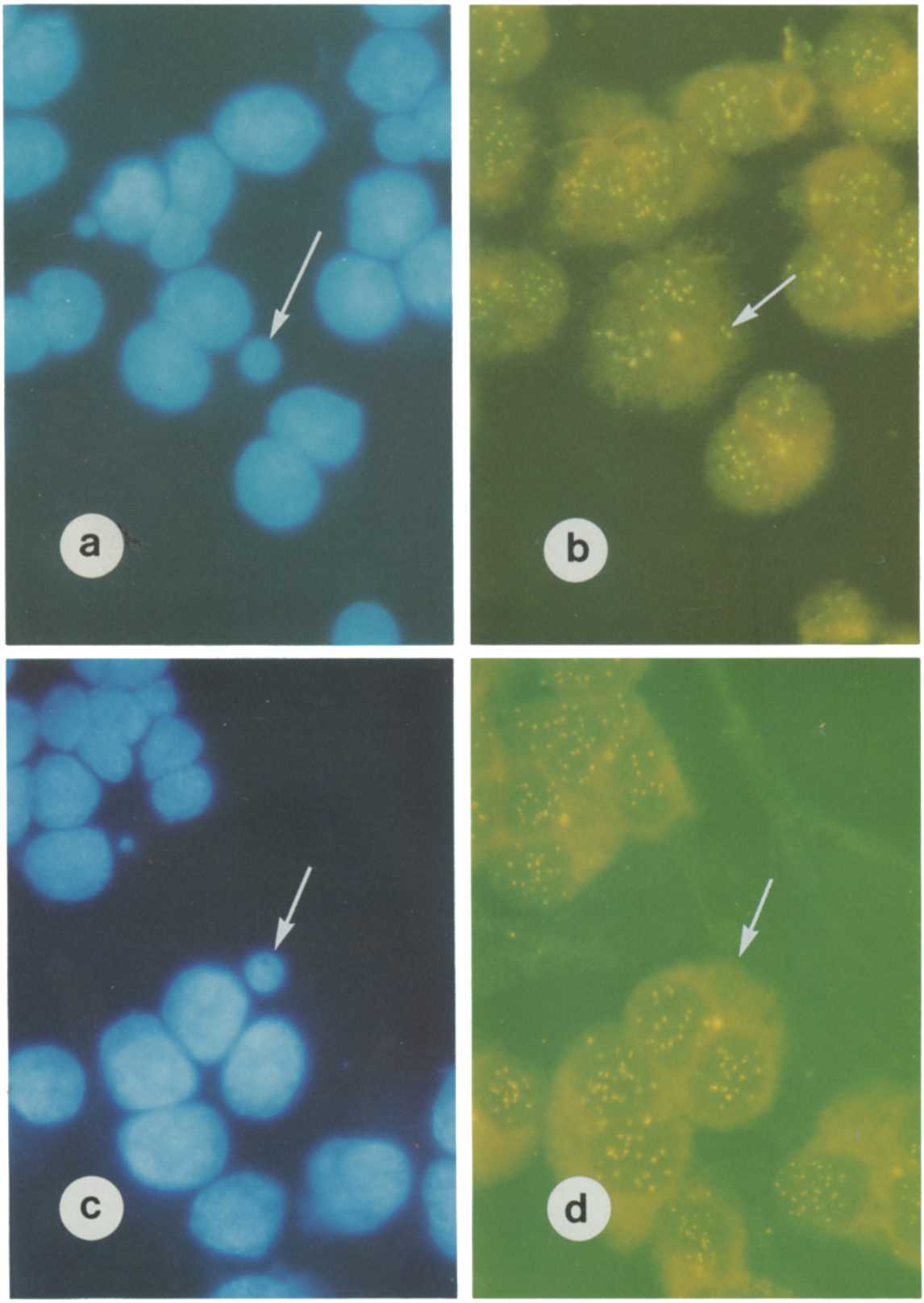

Fig. 1. Photomicrographs of cytokinesis-blocked V79 cells stained with anti-kinetochore antibody followed by FITC-conjugated anti-human IgG and counter-stained with DAPI dye. *Left panel* (*a* and *c*) show the binucleated cells with two nuclei and a micronucleus (*arrow*); *right panel* (*b* and *d*) shows the fluorescence of kinetochores in nucleus, kinetochore-positive MN (*small arrow*), and kinetochore-negative MN (*big arrow*). ×1000.

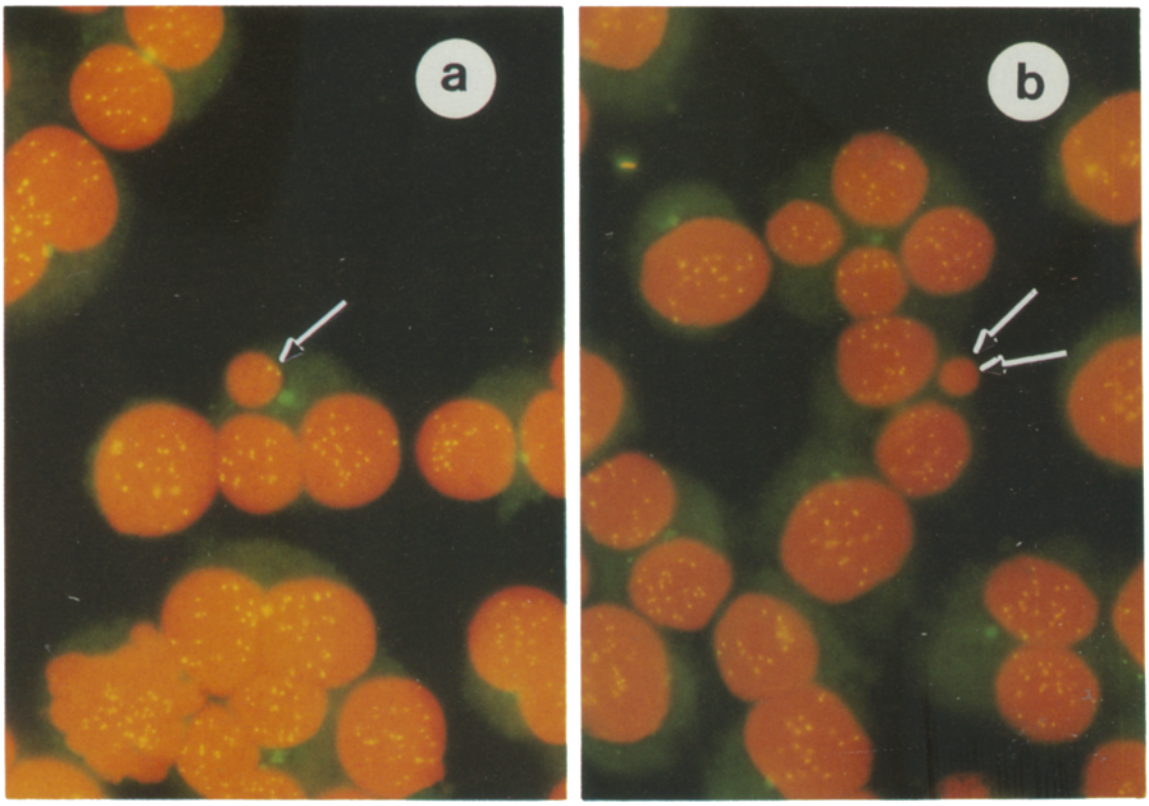

Fig. 2. Cytokinesis-blocked V79 cells stained with anti-kinetochore antibody followed by FITC-conjugated anti-human IgG and counter-stained with PI dye. *a*, Binucleated cell with kinetochore-positive micronucleus (*arrow*); *b*, with kinetochore-negative micronucleus (*double arrows*). ×1000.

kinetochore-positive micronuclei are substantially higher in cells treated with aneuploidogens than clastogens. Untreated cultures of V79 cells contain about 2% micronucleated binucleated cells and within the total micronucleated cells 50 to 60% contain kinetochore-positive micronuclei, indicating that their origin is from both the chromosome/chromatid fragment(s) and whole chromosome(s) (5,8). In propidium iodide counter-stained slides, kinetochores appear yellow/green against the orange/red nuclear material, whereas the cytoplasm appears faint green (Fig. 1). In DAPI counter-stained slides, nuclear material appears light blue and kinetochores appear apple green (Fig. 2). This method is simple, reliable, and applicable to various cell types.

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Results of cooperative investigation of West Virginia Agricultural and Forestry Experiment Station, and NIOSH. Published with the approval of the Director of West Virginia Agricultural and Forestry Station as Scientific paper No. 2261.

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[1] Fisher Scientific, Pittsburgh, PA
[2] Sigma Chemical Company, St. Louis, MO
[3] Bellco Glass, Inc., Vineland, NJ
[4] Forma Scientific, Inc., Marietta, OH
[5] American Scientific Products, Philadelphia, PA
[6] Carl Zeiss, D-7082 Oberkochen, Germany
[7] Thomas Scientific, Swedesboro, NJ
[8] Corning Glass Works, Corning, NY
[9] Falcon, Oxnard, CA
[10] Grand Island Biological Co., Grand Island, NY
[11] J. T. Baker Chemical Co., Phillipsburg, NJ
[12] Eastman Kodak Company, Rochester, NY
[13] Antibodies Incorporated, Davis, CA
[14] Edmont, Coshocton, OH
[15] VWR Scientific, Pittsburgh, PA