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Brief Communication

Anti-Kinetochore Antibodies: Use as Probes for Inactive Centromeres

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SUMMARY

Application of a modified immunofluorescence technique using an anti-kinetochore serum enables cytogeneticists to obtain quality metaphase spreads and to localize kinetochores. In a patient with a 45, XX, −9, −11, tdic (9p;11p) constitution, we found that the dicentric marker chromosome has an intensely fluorescent kinetochore (no. 11), the functional centromere, and a less intensely fluorescent kinetochore (no. 9), the inactive centromere. The data suggest that in the process of tandem fusion (telomere-telomere between 11p and 9p), the centromere of chromosome 9 was not deleted, but, rather, inactivated.

INTRODUCTION

Dicentric chromosomes occur in humans as well as in many other mammalian species in the course of chromosomal evolution. Many cases of spontaneous and induced Robertsonian fusion events have been shown to result in dicentric chromosome products [1, 2]. Most of these chromosomes are stable, behaving as monocentric chromosomes, presumably due to the close proximity of the

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two centromeres. Another explanation for the stability of these chromosomes maintains that one centromere becomes inactivated, allowing the chromosome to behave as a monocentric chromosome. Non-Robertsonian-type fusions such as centromere-telomere and telomere-telomere fusions [3–5], which result in stable dicentric chromosomes, can best be explained by centromere inactivation. Centromere inactivation has been postulated in almost all of the spontaneously occurring tandem fusions found in humans, providing an explanation for the viability of an otherwise unstable translocation product. The mechanism of centromere inactivation has not yet been elucidated, nor have structural or molecular differences between active and inactive centromeres been determined.

In the past, few cytological or molecular techniques have been available to probe this problem. Molecular studies of mammalian centromeres have been more recent [6–8] but have not addressed the phenomenon of the regulation of centromeric activity. Cytological techniques such as G-banding, Cd-banding, and Kt-staining lack the specificity to localize the actual kinetochore in mammalian metaphase chromosomes, and their mechanisms of action are poorly understood; therefore, these techniques are ineffective in studying centromere regulation.

Much interest has recently been directed at the association of autoimmune disease with the production of antibodies against various cellular components. More specifically, individuals with the CREST syndrome of scleroderma (calcinosis, Raynaud phenomenon, esophageal dysmobility sclerodactyly, telangiectasia) have been shown to produce autoantibodies against the kinetochore [9], and several investigators have thoroughly studied the cell biology of this immunological phenomenon [6, 8, 10]. Brenner et al. [10] showed the antibody to bind to the actual kinetochore plates seen at the electron microscopic level; this has been recently confirmed by Earnshaw et al. [8], who showed that even after extensive digestion of the DNA and extraction of the bulk of chromosomal protein, the anti-kinetochore antiserum still bound to the kinetochore.

Most of these studies have used interphase cells and molecular biological approaches to more precisely define the binding of the antiserum and to study the antigenic nature of the kinetochore. However, they have involved the use of techniques that do not require or allow for optimal metaphase chromosome preparations (i.e., standard chromosome spreading and morphology required for cytogenetic analysis). Techniques that are routinely used by cytogeneticists generally result in the dissolution of the antigenic components of the kinetochore that bind scleroderma antibodies.

In the present study, we employed an anti-kinetochore immunofluorescence procedure [11] that enabled us to stain kinetochores while maintaining acceptable metaphase chromosome spreads. Using this approach, we have been able to detect differences between active and inactive centromeres not previously seen by conventional staining techniques.

MATERIALS AND METHODS

Cell line TCH #3049 was derived from a skin biopsy taken from an infant with multiple congenital anomalies and obtained from G. S. Sekhon, Department of Pediat-

rics, Washington University School of Medicine, St. Louis, Missouri. Fibroblasts were maintained in our laboratory in Hamm's F-10 medium supplemented with 20% fetal bovine serum. This cell line had previously been characterized cytogenetically [12] as having a karyotype of 45, XX, −9, −11, tdic (9;11). To confirm this karyotype, G-banding was performed using a modification of the trypsin-Giemsa technique of Seabright [13]. In addition, C-banding [14] and Cd-staining [15] were performed in order to study centromeric activity of the so-called dicentric chromosome.

For analysis by indirect immunofluorescence, actively growing fibroblasts of this cell line were treated with Colcemid (0.04 μg/ml) for 1 hr prior to harvest. At the time of harvest, cells were removed from flasks by a mild trypsinization (0.01%) and centrifuged for 5 min at 1,000 rpm. The pellet was resuspended in 10 ml of a Tris-buffered hypotonic solution (10 mM Tris-HCl, pH 7.4, 40 mM glycerol, 20 mM NaCl, 5 mM KCl, 1.0 mM $CaCl_2$, and 0.5 mM $MgCl_2$) [11] and incubated at 4°C for 15 min. An aliquot of 0.2–0.3 ml of the cell suspension was then placed in cytocentrifuge cups, and the cells were centrifuged onto clean glass slides at 1,000 rpm for 8 min. Slides were removed and allowed to air dry for 15–30 seconds and then placed in chilled 80% ethanol (−20°C) for 30 min. After this time, the slides were placed in Dulbecco's phosphate-buffered saline (PBS) for 10 min. They were then laid horizontally in Petri dishes; the cells were overlaid with 10 μl of anti-kinetochore antiserum (diluted 1:100 in PBS), according to Brenner et al. [10], and incubated at 37°C for 30 min. After being rinsed in PBS at room temperature for 30 min, the cells were overlaid with 10 μl of fluorescein-conjugated goat antihuman IgG (Miles-Yeda, Elkhart, Ind.), and incubated at 37°C for 45 min. Slides were rinsed in PBS for 1 hr, counterstained with propidium iodide (1 μg/ml) for 1 min, rinsed in water for 5–10 min, and mounted in glycerol:PBS mounting medium (9:1) containing p-phenylenediamine (1 mg/ml) [16]. Immunofluorescence was observed with a Leitz Ortholux II microscope equipped with epifluorescence capability. Kodak Ektachrome film, ASA 400, was used for making color photomicrographs.

RESULTS

In conventional Giemsa-stained preparations, the marker chromosome tdic (9;11) appeared to be a long submetacentric with an unequal arm ratio. There was very little indication of its dicentricity (fig. 1*a*, small arrow). This chromosome was present and unaltered in 100% of the metaphase cells analyzed. C-banding revealed a small amount of constitutive heterochromatin at the centromeric region of this chromosome, and a large block of intercalary

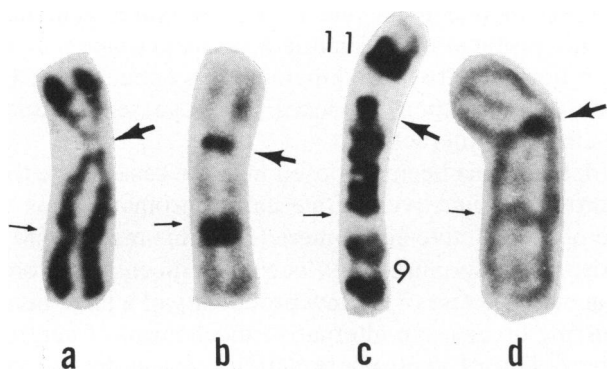

FIG. 1.—The compound chromosome resulting from a fusion of 11p and 9p. *a*, Conventionally Giemsa-stained; *b*, C-banded; *c*, G-banded; and *d*, Cd-banded patterns. The active centromere of chromosome 11 is marked by *thick arrows*, and the inactive centromere of chromosome 9, by *thin arrows*.

heterochromatin in the middle of its long arm (fig. 1*b*). G-banding showed a compound chromosome composed of chromosomes 11 and 9 fused at the telomeres of 11p and 9p with no apparent loss of any material from either chromosome (fig. 1*c*). Thus, the short arm of this compound chromosome was actually 11q. The large block of intercalary heterochromatin stained very lightly with the G-banding technique; this is an inherent characteristic of human chromosome 9. Cd-banding showed a positive reaction with the centromere of only chromosome 11 (fig. 1*d*, large arrow).

In metaphases stained with anti-kinetochore antiserum, each chromosome showed a brightly fluorescent spot at its centromere location. In most chromosomes, a fluorescent spot was seen on each chromatid. The tdic (9;11) compound chromosome exhibited two fluorescent centromeres: a bright one at chromosome 11 and a less intensely fluorescent one at chromosome 9 (fig. 2).

DISCUSSION

Identification of centromeres in preparations for light microscopy is often equivocal because of the inability of conventional procedures to identify the structural kinetochore observed in electron micrographs. G-banding is not useful for identification of centromeres; C-banding reveals only the pericentric heterochromatin and not the actual centromere, and conventional Giemsa-staining reveals only an achromatic constriction. The Cd-banding technique is purported to be a centromere-specific stain and has been used to differentiate active (stained) and inactive (unstained) centromeres [17, 18]. However, Cd-banding does not indicate whether an inactive kinetochore is absent or modified in some manner. Electron microscopic studies of inactive centromeres have not been done because of the formidable task of locating such a centromere in a metaphase cell for electron microscopic examination. Our observation that the anti-kinetochore antisera isolated from CREST scleroderma patients bound to the inactive centromere of the present case is the first positive identification of an antigenic kinetochore at the location of the inactive centromere.

The prevailing immunofluorescence technique does not allow observations on chromosomes of sufficient quality for cytogenetic analysis (viz., well-spread chromosomes lying in one focal plane). Furthermore, acid fixation, which would correct this problem and which is a critical component in routine cytogenetic preparations, destroys the kinetochore's antigenicity. The procedure described in the present report produces good cytogenetic quality and preserves antigen-antibody interactions.

Centromere deletion has been proposed in some cases to be the mechanism rendering a centromere inactive; in one case, chromatid gaps were actually seen at the site of the inactive centromere [19]. This mechanism is unlikely to account for most of the spontaneously occurring dicentric chromosomes with inactive centromeres because of the low probability of a large number of simultaneously occurring breaks. An alternative mechanism of centromere regulation is the suppression of centromere activity, by molecular or mechanical means, without loss of material [4]. Our data show that two centromeres are indeed present in the compound chromosome. In most cells, the inactive cen-

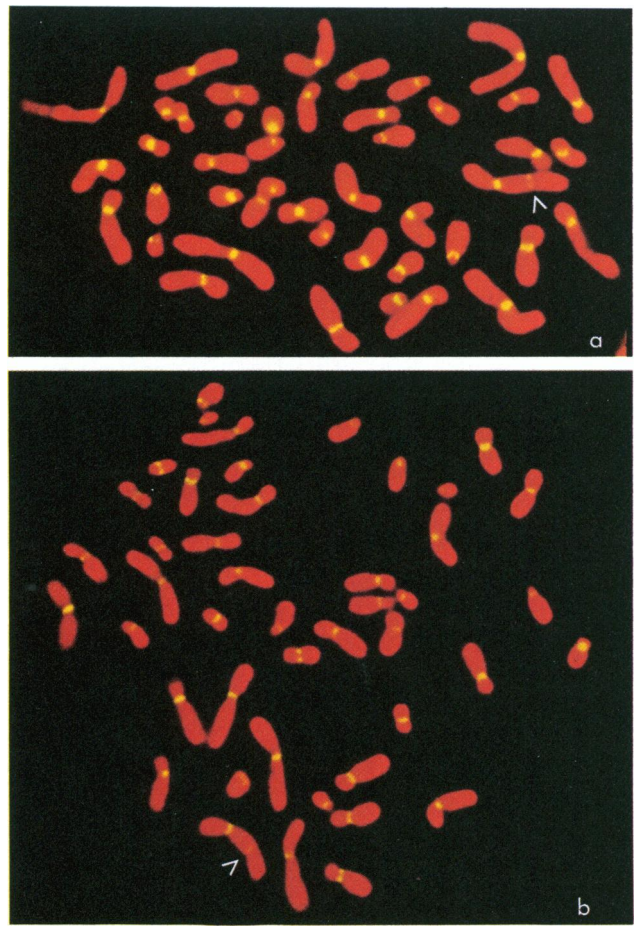

FIG. 2.—Two metaphase plates (*a* and *b*) stained with anti-kinetochore antiserum and counterstained with propidium iodide. The centromeres of most chromosomes are brightly fluorescing with fluorescein while the rest of the chromosomes are stained red. Note the presence of less intense fluorescence in the inactive centromere of chromosome 9 (*open arrow heads*).

tromere appears to be less intensely fluorescent than all other centromeres; however, experiments to show that this is an inherent characteristic of the inactive centromere and is not simply due to chromatid separation are still needed. Searching for molecular or structural differences between active and inactive centromeres may yield insight into the control mechanism for centromere activity. In the past, a means for positively identifying inactive centromeres was not available. With the findings of the present study, however, detailed structural (by electron microscopy) and molecular analyses of inactive centromeres can now be performed. Recently, Nakagome et al. [18] proposed the "loss" of centromeres in X chromosomes of aged women. It is quite possible that in such cases the centromere is also rendered inactive and is not lost.

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REFERENCES

1. NIEBUHR E: Dicentric and monocentric Robertsonian translocations in man. *Humangenetik* 16:217–226, 1972
2. HSU TC, PATHAK S, BASEN BM, STARK GJ: Induction of Robertsonian fusions and tandem translocations in mammalian cell cultures. *Cytogenet Cell Genet* 21:86–98, 1978
3. THERMAN E, SARTO GE, PATAU K: Apparently isodicentric but functionally monocentric X chromosome in man. *Am J Hum Genet* 26:83–92, 1974
4. HSU TC, PATHAK S, CHEN TR: The possibility of latent centromeres and a proposed nomenclature system for total chromosome and whole arm translocations. *Cytogenet Cell Genet* 15:41–49, 1975.
5. SINHA AK, PATHAK S, NORA JJ: Fusion of two apparently intact human X chromosomes. *Hum Genet* 32:295–300, 1976
6. COX JV, SCHENK EA, OLMSTEAD JB: Human anticentromere antibodies: distribution, characterization of antigens, and effect on microtubule organization. *Cell* 35:331–339, 1983
7. LICA L, HAMKALO B: Preparation of centromeric heterochromatin by restriction endonuclease digestion of mouse L929 cells. *Chromosoma* 88:42–49, 1983
8. EARNSHAW WC, HALLIGAN N, COOKE C, ROTHFIELD N: The kinetochore is part of the metaphase chromosome scaffold. *J Cell Biol* 98:352–357, 1984
9. MOROI Y, PEEBLES C, FRITZLER M, STEIGERWALD J, TAN EM: Autoantibody to centromere (kinetochore) in scleroderma sera. *Proc Natl Acad Sci USA* 77:1627–1631, 1980
10. BRENNER S, PEPPER D, BERNS MW, TAN E, BRINKLEY BR: Kinetochore structure, duplication, and distribution in mammalian cells: analysis by human autoantibodies from scleroderma patients. *J Cell Biol* 91:95–102, 1981
11. STENMAN S, ROSENQUIST M, RINGERTZ NR: Preparation and spread of unfixed metaphase chromosomes for immunofluorescence staining of nuclear antigens. *Exp Cell Res* 90:87–94, 1975
12. SEKHON GS, MONTELEONE P, VOLK SLR, SINGH H: A stable dicentric chromosome due to an unusual fusion. *Mammalian Chromosomes Newsl* 18:46, 1977
13. SEABRIGHT M: A rapid banding technique for human chromosomes. *Lancet* ii:971–972, 1971
14. HSU TC: Constitutive heterochromatin (C-band) technique, in *Chromosome Identification-Technique and Application in Biology and Medicine,* edited by CASPERSSON T, ZECH L, *Nobel Symposia,* vol 23, New York and London, Academic Press, 1973, pp 32–33
15. EIBERG H: New selective Giemsa technique for human chromosomes, Cd staining. *Nature* 248:55, 1974
16. JOHNSON GD, NOGUEIRA ARAUJO GMDC: A simple method of reducing the fading of immunofluorescence during microscopy. *J Immuno Methods* 42:349–350, 1981
17. MARASCHIO P, ZUFFARDI O, CURTO FL: Cd bands and centromeric function in dicentric chromosomes. *Hum Genet* 54:265–267, 1980
18. NAKAGOME Y, ABE T, MISAWA S, TAKESHITA T, IINUMA K: The "loss" of centromeres from chromosomes of aged women. *Am J Hum Genet* 36:398–404, 1984
19. DEWALD GW, BOROS SJ, CONROY MM, DAHL RJ, SPURBECK JL, VITEK HA: A tdic (5;15) (p13;p11) chromosome showing variation for constriction in the centromeric regions in a patient with the Cri du chat syndrome. *Cytogenet Cell Genet* 24:15–26, 1979