Injection of Anticentromere Antibodies in Interphase Disrupts Events Required for Chromosome Movement at Mitosis

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Abstract. We have used autoantibodies to probe the function of three human centromere proteins in mitosis. These antibodies recognize three human polypeptides in immunoblots: CENP-A (17 kD), CENP-B (80 kD), and CENP-C (140 kD). Purified anticentromere antibodies (ACA-IgG) disrupt mitosis when introduced into tissue culture cells during interphase. We have identified two execution points for antibody inhibition. Antibodies injected into the nucleus ≥ 3 h before mitosis prevent the chromosomes from undergoing normal prometaphase movements in the subsequent mitosis. Antibodies injected in the nucleus during late G_2 cause cells to arrest in metaphase. Surprisingly, antibodies introduced subsequent to the beginning of prophase do not block mitosis. These results suggest that the CENP antigens are involved in two essential interphase events that are required for centromere action in mitosis. These may include centromere assembly coordinate with the replication of α-satellite DNA at the end of S phase and the structural maturation of the kinetochore that begins at prophase.

T HE dramatic movements that chromosomes undergo during mitosis have held the interest of cell biologists for over a century, and yet the underlying mechanisms remain unsolved (19, 52). These movements begin at prometaphase, when the nuclear envelope first breaks down and chromosomes come into contact with microtubules. The progressive attachment of microtubules to first one sister centromere, then the other during prometaphase results in a balanced production of force that eventually poises the chromosome midway between the spindle poles. Anaphase is initiated after all of the chromosomes have moved to the midzone forming a metaphase plate. During anaphase the disjoined sister chromatids move in concert towards the spindle poles. The locations and identity of the mechanochemical motors involved in these movements are currently the subject of intense investigation.

One of the most dramatic aspects of mitotic movements is the extent to which they are held under strict regulatory control. Each mitosis involves a crucial "decision" point, at which the metaphase cell abruptly disjoins the sister chromatids and initiates their concerted movement towards the spindle poles. If this process is initiated before all chromosomes are aligned at the midplane of the mitotic spindle the progeny will be aneuploid. This checkpoint is one of a number of points where feedback networks regulate the progress of the cell cycle (16). Cultured cells in metaphase will wait for extended periods for a single monooriented chromosome to achieve its proper position on the metaphase plate (35, 53).

The kinetochore appears to be a key player in mitotic chromosome movements. Observations of kinetochore microtubules during anaphase have indicated that they themselves do not move, but rather are consumed by the chromosomes during anaphase A movement (13, 14). Two observations suggest that the mechanochemical motor responsible for chromosome movements along microtubules is located close to the kinetochore. First, kinetochores detached from prematurely condensed chromosomes can undergo mitotic movements (4). Second, chromosomes can move to within 1 μm of the amputated edge of a cut spindle (28).

The kinetochore develops into a disk-shaped structure at the surface of the centromere during early mitosis. It is composed of four layers (for reviews see [5, 31, 34]); an outermost fibrous corona, an outer dense disk (in which microtubules normally terminate), an unstained zone, and an inner disk that is embedded in the surface of the centromeric chromatin. The structure is pleiomorphic. The corona is not readily seen when there are microtubules bound to the kinetochore, and the inner plate is not seen when cells are arrested in prometaphase with colcemid (37). A recent study suggests that the mechanochemical motor responsible for the earliest poleward movements observed during prometaphase may be located in the fibrous corona (36).

Clearly, the identification of kinetochore proteins is a major priority, and yet little is known about kinetochore composition. The only polypeptide that has been convincingly mapped to this region is tubulin, which is found in the fibrous corona in colcemid-arrested cells (26). Beyond this, the protein composition of the kinetochore is controversial.

A major breakthrough in our understanding of this region

came about in 1980 when it was shown that sera from patients with the CREST variant of progressive systemic sclerosis often contain autoantibodies that bind to the centromere regions of mitotic chromosomes (27). These are referred to as anticentromere antibodies (ACAs)[1]. We and others have used a combination of immunoblotting and affinity purification experiments to identify three structurally related target antigens in human cells that are recognized by these autoantibodies (7, 15, 47). We designated these the centromere protein (CENP) antigens, CENP-A (17-kD), CENP-B (80 kD), and CENP-C (140 kD) (7). A subsequent study indicated that a 50-kD antigen first recognized in rodent cells (7) could also be detected in human cells, and designated this antigen CENP-D (21).

CENP-B is the best characterized of these proteins. It has been cloned and sequenced, and specific polyclonal and monoclonal antibodies have been raised to bacterial fusion proteins (10). We have used these antibodies together with a newly developed series of ultra-small colloidal gold probes to examine the distribution of CENP-B within the centromere by immunoelectron microscopy (6). We found that CENP-B is broadly distributed throughout the centromeric heterochromatin beneath the kinetochore, and is not observed in the kinetochore itself (6). This is consistent with our detection of CENP-B at the inactive centromere of a stable dicentric chromosome (11) (such inactive centromeres are thought to lack kinetochores [48]). The immunolocalization results are also consistent with the recent demonstration that CENP-B binds to a specific 17-bp sequence present in some α-satellite DNA monomers (23). α-satellite DNA is a family of highly repetitive DNA sequences that comprises ~5% of the human genome and is localized exclusively at the centromeres (41). Both CENP-B and α-satellite DNA have been shown to vary in amount from chromosome to chromosome, strengthening the argument that these two centromere elements are associated with each other in vivo. Together, these studies suggest that the majority of the CENP-B protein is distributed throughout the centromeric heterochromatin, with little or none at the kinetochore itself. These results are less consistent with the suggestion that CENP-B binds directly to microtubules, which was proposed based on a crosslinking study showing that an 80-kD Chinese hamster protein can be crosslinked to exogenous tubulin in vitro (1).

The other three CENP antigens are less well characterized. CENP-C may be closely associated with the kinetochore, since this antigen is found only at the active centromere of stable dicentric chromosomes (11). Affinity-purified anti-CENP-C antibodies stain the centromere region of all chromosomes uniformly, suggesting that this antigen may be associated with an invariant structure such as the kinetochore (7, 11). Little is known about the properties of the CENP-C polypeptide, except that it, like CENP-B, is a component of the mitotic chromosome scaffold (8). Nothing is known about the distribution of CENP-A or CENP-D in chromosomes. All antibodies affinity purified from CENP-A and CENP-D that we have obtained to date cross-react with CENP-B (17). Biochemically, CENP-A behaves in many ways like a histone, and it cofractionates with nucleosomes (30). The recent purification of CENP-A should lead to major ad-

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1. *Abbreviations used in this paper:* ACA, anticentromere antibody; CENP, centromere protein.

vances in our understanding of this antigen (D. Palmer and R. Margolis, personal communication).

The ultrastructural distribution of CENP-C and/or CENP-A within the centromere has been deduced by comparison of the staining obtained with monospecific anti-CENP-B antibodies to that obtained with an autoimmune serum that recognizes all three CENP antigens. The only difference between the two patterns is found in the chromatin lateral to the kinetochore plates. This region contains only 5% of the gold grains when anti-CENP-B is used, but contains 18% of the grains when the autoantibody is employed (6). This suggests that CENP-C and/or CENP-A are concentrated in a ring of chromatin that closely encircles the kinetochore, while the kinetochore structure itself rests on a foundation of heterochromatin rich in CENP-B.

The purpose of the present study was to begin to determine whether the CENP antigens perform essential mitotic functions in vivo. We have purified IgG from sera of patients with anticentromere antibodies (ACA-IgG) and injected these into cultured cells. We find that ACA-IgG strongly disrupt the course of mitotic events, thus, demonstrating that the CENP antigens are essential for mitosis. Surprisingly, for the antibodies to inhibit mitosis, they must contact their target antigens during the preceding interphase. Furthermore, two different phenotypes are observed after antibody injection. These depend on the time in the cell cycle at which injection takes place. We are able to define two execution points for antibody disruption of centromere function at mitosis. We use this terminology by analogy to its usage in cell cycle analysis (32), to refer to that point in the cell cycle after which a given mitotic process is no longer sensitive to inhibition by antibody.

Our results begin to provide some clues as to the role of the CENP antigens in the structure/function of the centromere. We suggest that these antigens are required for prometaphase chromosome movements and for the timely initiation of anaphase.

Materials and Methods

Cell Culture

HeLa cells were grown in RPMI (Gibco Laboratories, Grand Island, NY) with 5% calf serum (Hyclone Laboratories, Logan, UT). LLC-PK cells were grown in DMEM (Gibco Laboratories) with 10% FBS. For scrape loading experiments, HeLa cells were grown on 35-mm petri dishes. For microinjection experiments, cells were grown on photoetched glass cover slips (Bellco Glass, Inc., Vineland, NJ).

Scrape Loading

HeLa cells were scrape loaded by the method of McNeil et al. (25). Cells were trypsinized from culture flasks and replated onto 35-mm petri dishes at nearly confluent density. 4–8 h after replating, cells were scraped from the dish in the presence of 50 μl serum or purified IgG. Cells were washed once in cold serum-free medium and replated onto coverslips in complete medium. Cells were fixed and processed for microscopy from 4 h to 5 d after replating.

Loaded cells were identified directly by staining with labeled secondary antibody. 20–40% of cells were found to retain levels of antibody detectable by immunofluorescence. The loaded antibody was found not only in the cytoplasm, but bound to the centromeres as well. Cells were also processed in parallel with antitubulin antibody and DAPI to simultaneously examine microtubule and chromosome morphology.
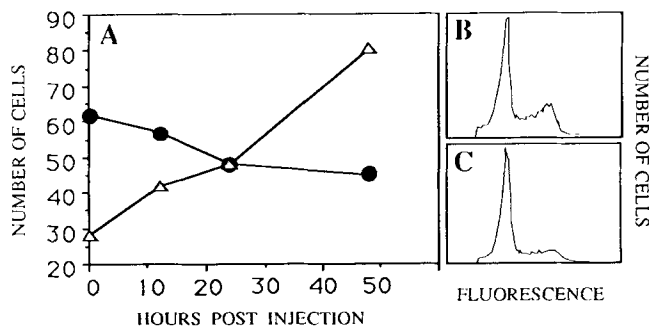
Figure 1. Anticentromere antibodies block mitosis. (*A*) HeLa cells on indexed coverslips were grown into microcolonies of 20–60 cells. At time 0 all cells in a colony were injected with ACA IgG (●) or with control IgG (△), and the number of cells in the colony was noted. The number of cells in each microcolony was then monitored periodically for the next 48 h. (*B*) Flow cytometry profile of a population of HeLa cells 17 h after scrape loading with ACA IgG. Note that in such experiments, only 20–40% of the cells were loaded with antibody. (*C*) Flow cytometry profile of HeLa cells 17 h after loading with normal human IgG.

Microinjection

Either HeLa or LLC-PK cells were plated onto photo-etched coverslips (Bellco Glass, Inc.) at a low density and grown for at least 2 d. Clearly isolated patches of 10–30 cells were identified and all the cells within one patch were injected using a Leitz micromanipulator. A video print of each patch of injected cells was taken for reidentification of cells later. Microinjection was carried out at 37°C with 20 mM HEPES added to the medium. Cells were then fed fresh medium and returned to the incubator.

Observation of Injected Cells

Cells were observed from 1–48 h after injection. Injected cells were identified by their position on locator grids and examined hourly by phase-contrast microscopy. Cells were noted as they entered mitosis and observation was continued as they proceeded through mitosis. At different times after injection, cells were fixed and stained as described below.

We determined by flow cytometry (FACS) analysis that the lengths of the cell cycle phases for HeLa cells growing under these conditions were: G_1, 13 h; S, 4 h; and G_2, 3 h. Injection of either control or anticentromere IgG into the cells did not appear to significantly affect their progress through the interphase portion of the cell cycle, as judged by the frequency with which members of injected colonies were observed to enter mitosis.

Immunofluorescence

Cells on glass coverslips were fixed with 3.5% formaldehyde in PBS-azide (10 mM $NaPO_4$, pH 7.4, 0.15 M NaCl, 1 mM EGTA, 0.01% NaN_3) for 5 min at room temperature. Cells were washed three times for 3 min each at room temperature in lysis/wash buffer (150 mM NaCl, 10 mM Tris pH 7.7, 0.1% Triton X-100, 0.1% BSA). Biotinylated antihuman antibody (Vector Laboratories, Burlingame, CA) at 1:1,000 and mouse anti-β tubulin antibody (Amersham Corp., Arlington Heights, IL) or mouse anti-α tubulin (Accurate Chemical & Scientific Corp., Westbury, NY) at 1:50 was applied for 30 min at 37°C. Cells were again washed three times more in buffer followed by incubation in streptavidin: Texas red (Bethesda Research Labs, Grand Island, NY) at 1:800 and fluoresceinated antimouse antibody (DAKO) at 1:50. Cells were washed three times with buffer. DAPI at 1 μg/ml was added in the second wash (51). Coverslips were mounted onto glass slides in Mowiol prepared with PBS (29). Slides were examined on an Olympus Vanox microscope using either a 100× S plan apochromat lens with an NA of 1.4, or (when optical sectioning was desired) a 100× achromat, NA 1.3. In some cases, images were photographed directly on Kodak T-max 400 film, or Microfluor Hypertech film. Typically, images were obtained with a DAGE SIT camera driven by a Perceptics Hyperscope image processing program. These images were subjected to a Fourier sharpening algorithm and photographed directly from the monitor on Kodak T-max 400 film.

Antibody Purification

All sera came from the collection of the Division of Rheumatic Diseases, Farmington, CT. The following sera were used: ACA(+) that inhibited mitosis either as serum or IgG—J-1311, A-1219, KG, SN, GS, AP, F-2248, J-3051, H-345, G-2016, J-31; ACA(+) that failed to inhibit mitosis either as serum or IgG—G-1942, BM, E-186, H-2870; antitopo I (+)—NM, K-2432, K-782, K-724; antitopo II (+)—K-1618; other ACA(−) patient control—K-1137; normal sera—WE, SE, TA.

Antibodies were purified from whole human serum using Protein A sepharose columns as suggested by the manufacturer (Repligen, Cambridge, MA). 1 ml of Protein A sepharose per ml of whole serum was used. Serum was run over the column and washed with 5 mM KPO_4, pH 8. Antibodies were eluted from the column with 0.2 M glycine:HCl, pH 2.5, at room temperature. Alternatively, small volumes of serum were batch incubated and eluted. Pooled fractions containing antibody were concentrated and dialyzed into PBS using Centricon concentrators (Amicon Corp., Danvers, MA) to ∼5 mg/ml.

Preparation of LLC-PK and HeLa Nuclei and Chromosomes

LLC-PK cells were grown in T-150 tissue culture flasks (Bellco Glass, Inc.) and nuclei were prepared as described (38). HeLa nuclei were prepared from cells grown in spinner culture. Approximately 1×10^8 Hela cells were used per nuclei preparation. Cells were centrifuged at 900 g for 3 min and resuspended in 50 ml RSB (10 mM Tris:HCl, pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$) plus 10 KIU/ml trasylol (aprotinin; Mobay Chemical Corp., Pittsburgh, PA) and 0.1 mM PMSF at room temperature. Cells were swollen for 5 min and centrifuged as above and the supernatant was removed by aspiration. 10 ml of ice cold lysis buffer (15 mM Tris-HCl, pH 7.4, 0.3 mM spermine, 0.75 mM spermidine, 2 mM K-EDTA, pH 7.4, 80 mM KCl, 0.1% digitonin, plus trasylol and PMSF) were added to the cells and transferred to a 15-ml glass dounce homogenizer. Cells were homogenized on ice and
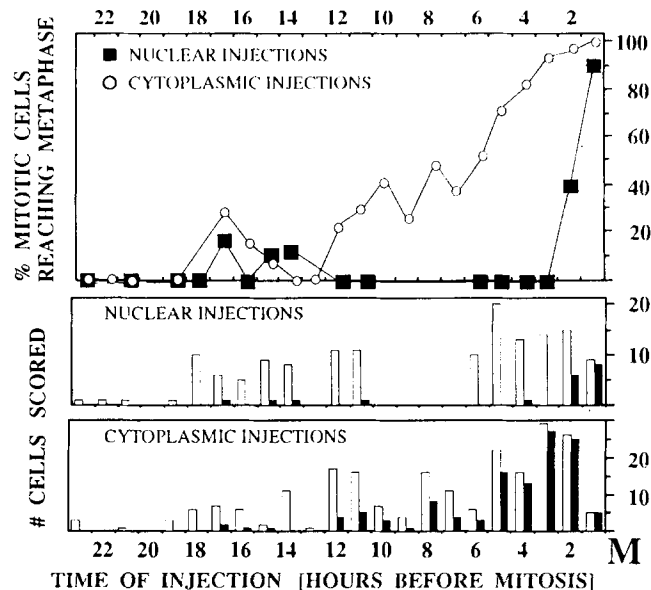

Figure 2. Percentage of mitotic-injected cells reaching metaphase as a function of the time of injection. The upper panel summarizes the data for cytoplasmic (○) and nuclear (■) injections of ACA-IgG. Mitotic cells that did not reach metaphase were arrested in pseudo-prometaphase. Note that it takes ∼8 h for antibodies injected into the cytoplasm to produce an affect comparable to nuclear injections. This corresponds well with the rate of movement of ACA-IgG into the nucleus determined as described in the text. The lower two panels present the data used to calculate the percentage figures. The total injected cells observed to enter mitosis at the indicated time after injection are shown by the open bars. Those injected cells that were observed to progress to metaphase are indicated by the solid bars.
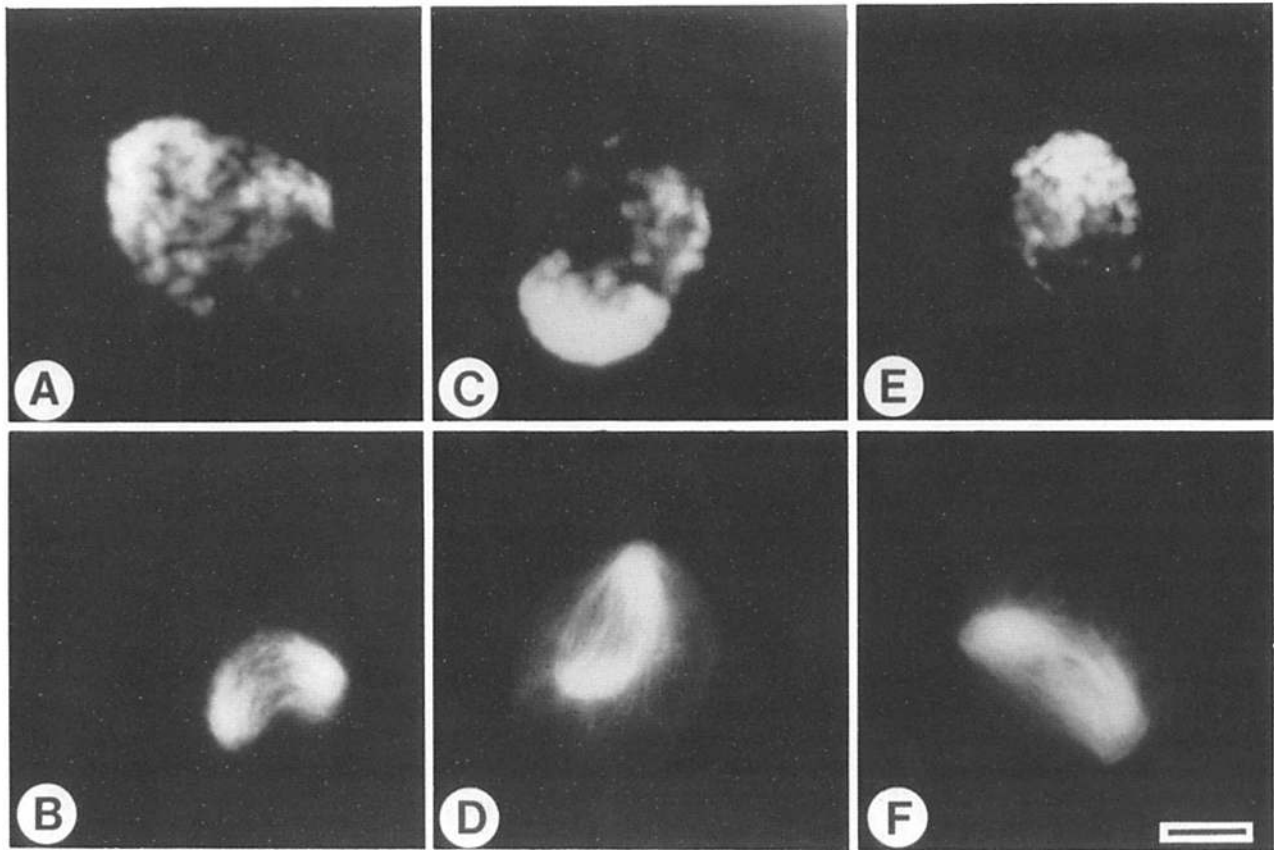
Figure 3. HeLa cells arrested in pseudo-prometaphase at time of fixation. All cells were injected in the nucleus. *A*, *C*, and *E* show a DAPI stain for DNA. *B*, *D*, and *F* show indirect immunofluorescence staining for tubulin. The cells shown in the various panels had been in mitosis for the following times before fixation: (*A* and *B*) 1.5 h; (*C–F*) 2 h. Bar, (*F*) 1 μm.

transferred to a 50-ml round bottom centrifuge tube (Sorvall Instruments Div., Newton, CT). Buffer 3 (5 mM Tris:HCl, pH 7.4, 2 mM KCl, 2 mM K-EDTA, pH 7.4, 0.375 mM spermidine, 0.1% digitonin, plus trasylol and PMSF) was added to a total volume of 50 ml and the nuclei were centrifuged for 10 min at 1,000 g. Nuclei were washed once with the same buffer and centrifuged for 10 min at 1,000 g. Purified nuclei were resuspended in 1 ml buffer 4 (5 mM Tris:HCl, pH 7.4, 2 mM KCl, 0.375 Spermidine, plus trasylol). After addition of Ca^{2+} to 2 mM, DNA was digested with 40 μg/ml micrococcal nuclease (Worthington Biochemical Corp., Freehold, NJ) for 30 min on ice. Nuclei were aliquoted and frozen at −70°C.

HeLa cells growing in RPMI 1640 plus 5% calf serum (Hyclone Laboratories) were blocked in mitosis with 100 ng per ml colcemid for 20 h. Cells were harvested as described above, and crude chromosomes were prepared as described above for interphase nuclei except that centrifugation was for 3,000 g for 20 min in all steps subsequent to cell lysis.

Electrophoresis and immunoblotting were performed as previously described (8).

Results

IgG purified from the sera of patients with anticentromere antibodies (ACA-IgG) were introduced into cells by either of two methods. Direct needle microinjection was used when we wished to monitor the effects of the antibody on the progress of individual cells through mitosis. ACA-IgG were injected into either LLC-PK (pig kidney epithelial) or HeLa cells. LLC-PK cells were used for some experiments since they remain flattened during mitosis, enabling chromosome movement to be observed more clearly in vivo. Immunoblotting of LLC-PK nuclei confirmed that these cells express an-

tigens that comigrate with CENP-C and CENP-B (see Fig. 10).

Certain experiments utilized the scrape loading method for the simultaneous introduction of antibodies into the cytoplasm of a large number of cells (25). This enabled us to examine the effect of the antibody on populations of cells over longer periods of time.

Microinjection of ACA-IgG Disrupts Mitosis

Cells were plated sparsely on locator coverslips and allowed to grow into microcolonies of 10–30 individuals. All cells in a colony were injected with either control IgG or ACA-IgG and the cell number was monitored over a period of several days. In colonies of HeLa or LLC-PK cells injected with anticentromere antibodies, cell number began to decrease as cells attempted to go through mitosis (Fig. 1 *A*). Most of the surviving cells appeared micronucleated when examined by phase microscopy and few of them continued to grow normally. Cells injected with control antibodies continued to grow normally.

The disruption of mitotic events by ACA-IgG was confirmed at a population level by fluorescence activated cell sorting analysis of scrape-loaded cultures. Fig. 1 shows such an analysis of two cultures 17 h after loading with antibody. In this experiment, 18% of the cells in the ACA-IgG-loaded culture had a G_2/M DNA content (Fig. 1 *B*). This compares with 11% in the culture loaded with control antibody
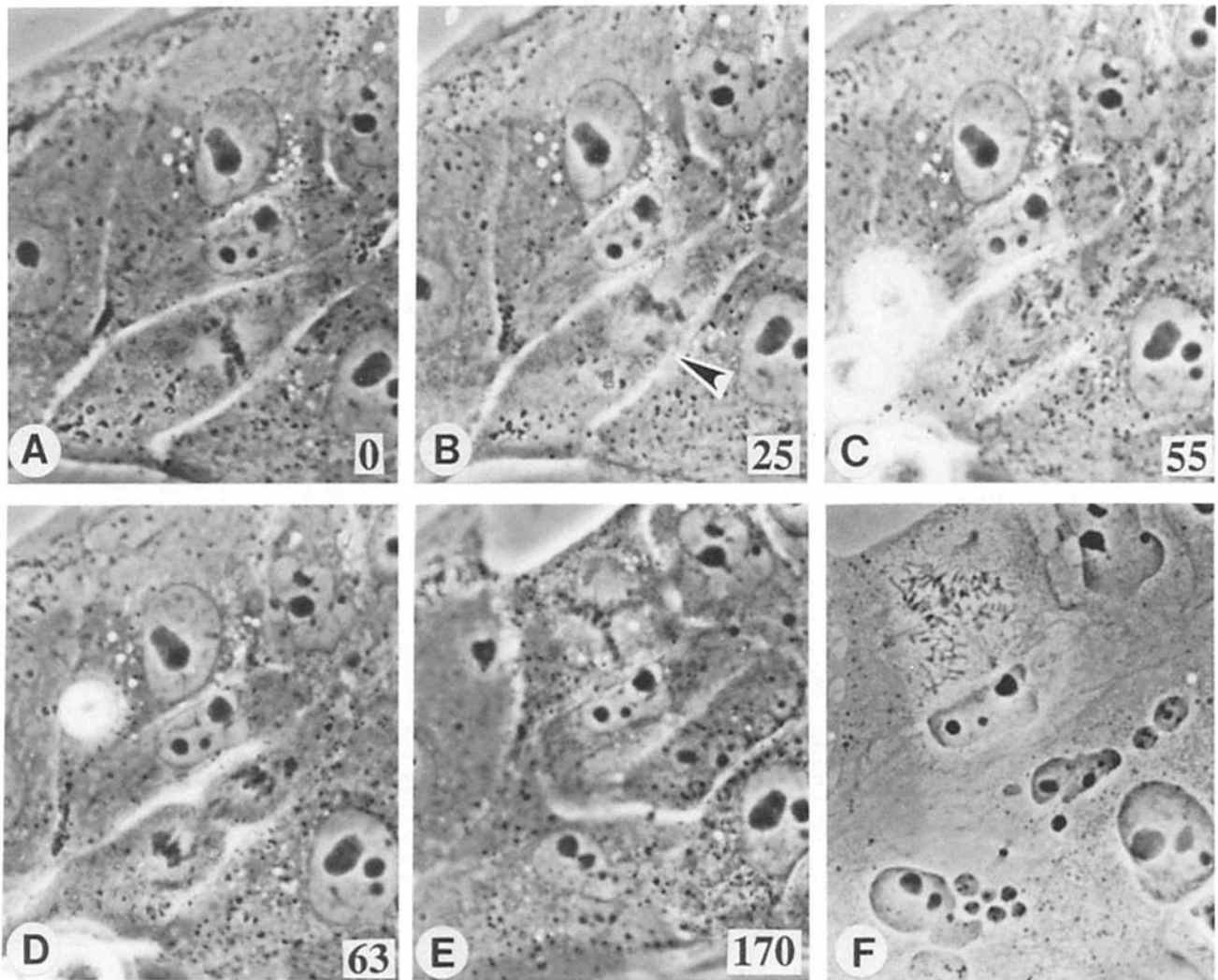
Figure 4. Injected LLC-PK cell observed in vivo during mitosis. The panels show the following stages of mitosis (with the time in minutes indicated at the lower right of each): (*A*) metaphase; (*B*) metaphase with chromosomes falling off the plate; (*C*) anaphase; (*D*) cytokinesis; (*E*) divided micronucleated daughter cells; (*F*) same cells after fixation. Note the chromosomes trapped in the cleavage furrow in *D*. Micronuclei are visible in *E*, and more clearly after fixation in *F*. Note second ACA-IgG-injected cell undergoing tripolar anaphase (*F, top*).

(Fig. 1 *C*). This difference is significant, since in a typical loading experiment, only 20–40% of cells retain levels of antibody detectable by immunofluorescence. The cessation of colony growth and shift towards a G_2 DNA content are both consistent with the inactivation of centromere function by the injected antibody, leading to a disruption of mitotic events.

Antibody Injection into Mitotic Cells Does Not Disrupt Mitosis

It might be expected that anticentromere antibodies would disrupt mitosis by directly interfering with the interaction between chromosomes and microtubules. To test this possibility, purified antibodies were injected into LLC-PK cells during mitosis and subsequent mitotic events were observed by phase-contrast microscopy. Surprisingly, antibodies injected into cells during any stage of mitosis, from prophase through telophase, had no effect on that mitosis other than a slight prolongation of the process. To determine whether this was simply a matter of competition between antibodies

and microtubules for binding sites on the chromosome, cells cultured in the presence of 40 ng/ml nocodazole were injected with antibody as they entered prophase. Under these conditions preexisting microtubules depolymerize and new ones do not assemble. These injected cells were then held for 2 h in prometaphase in the presence of antibody. Upon removal of the drug, the injected cells proceeded normally through mitosis, even after being exposed to the antibodies for several hours. We conclude that the antibody must bind to its target antigen at some point before mitosis in order to exert its inhibitory effect in these cells.

Antibody Injection into Interphase Cells Disrupts Mitosis

It proved to be necessary to inject the antibodies into cells during interphase in order to observe an inhibitory effect on the subsequent mitosis. The microinjection protocol provided a convenient way to determine whether the effects of antibody injection were dependent on the specific cell cycle phase
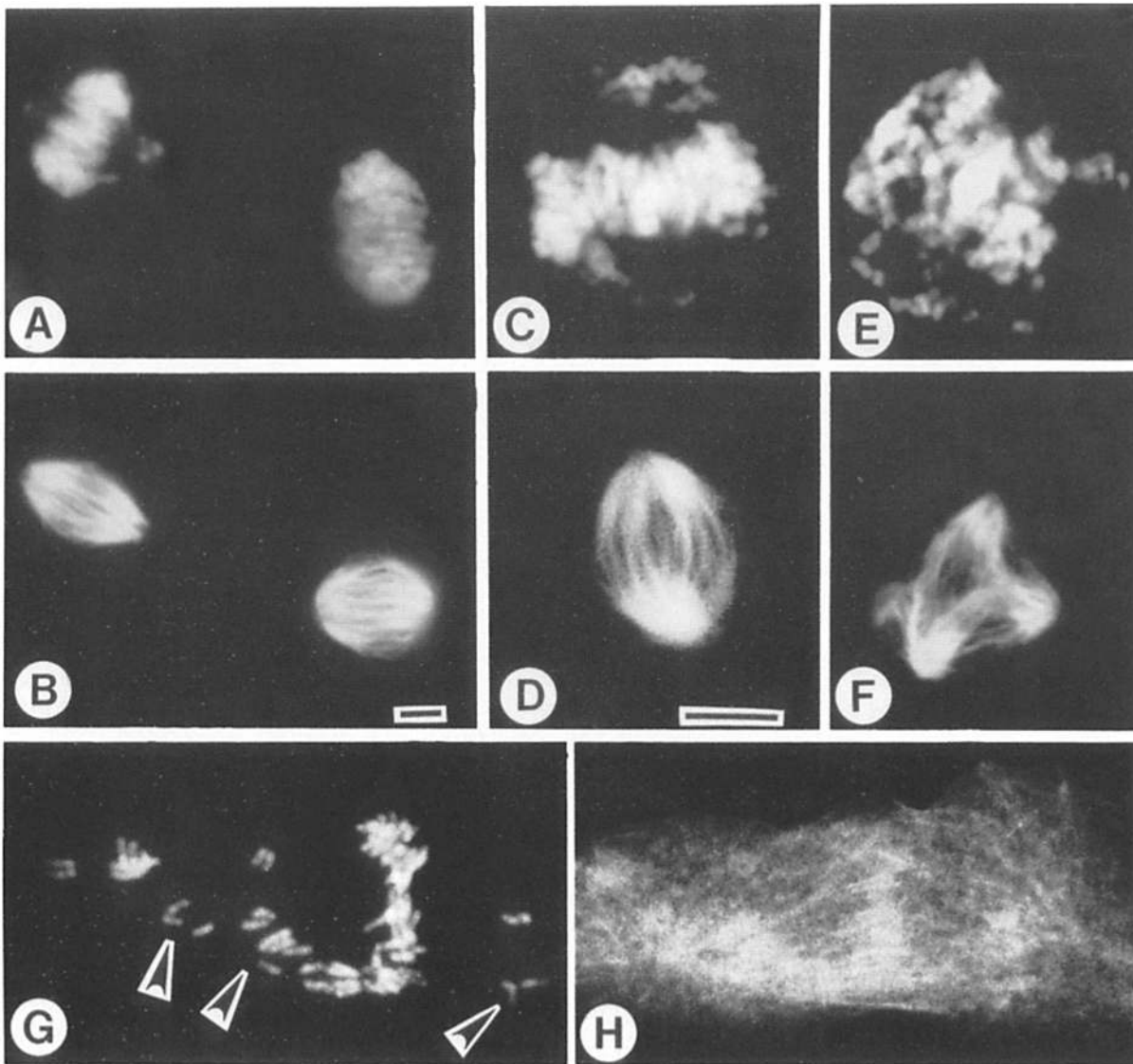
Figure 5. Cells undergoing various stages of degeneration of the metaphase configuration during the course of mitotic arrest. All of these cells had visibly attained metaphase at some time during mitosis as observed by phase microscopy before fixation. *A–F* show HeLa cells stained as follows: (*A, C,* and *E*) DAPI stain for DNA; and (*B, D,* and *F*) indirect immunofluorescence staining for tubulin. The cells shown in the various panels had been in mitosis for the following times before fixation: (*A* and *B*, left) 5 h; (*A* and *B*, right) 4 h; (*C* and *D*) 4 h; (*E* and *F*) 6 h. *G* and *H* show an LLC-PK cell that has initiated anaphase separation of sister chromatids (*arrowheads*). This cell had not been followed in vivo before fixation. Bars: (*B*) 1 μm (applies to *A, B,* and *E–H*); (*D*) 1 μm (applies to *C* and *D*).

of the recipient cell. Colonies were monitored once an hour for cells entering mitosis, beginning as soon as injection was completed. Those that entered mitosis within 3 h of injection are inferred to have been injected in G_2. Cells that entered mitosis >3 h after injection were probably injected in either S or G_1. (Although quantitative data on the length of the cell cycle phases could not be obtained in injected cells, we do not believe that the injections exert a significant effect on the passage of these cells through interphase; see Materials and Methods.) A video print was taken of each colony just subsequent to injection so that it was possible to keep a detailed history for every member of the microcolony. Stages of mitosis observed for each cell were noted for as long as it remained in mitosis (until it either divided or was fixed and processed for immunofluorescence).

Cells injected with ACA-IgG during interphase showed dramatic defects as they attempted to traverse the subsequent mitosis. A complicated pattern of mitotic disruption was observed with a range of abnormalities that depended on the site of injection (nucleus or cytoplasm) and cell cycle phase of the injected cell.

Identification of an Execution Point Required for the Successful Completion of Prometaphase Chromosome Movements

If ACA-IgG was injected into the nucleus three or more hours before mitosis, the recipient cells did not successfully complete prometaphase chromosome congression and were unable to form a mature spindle with the chromosomes aligned on a metaphase plate. Cells injected after this time
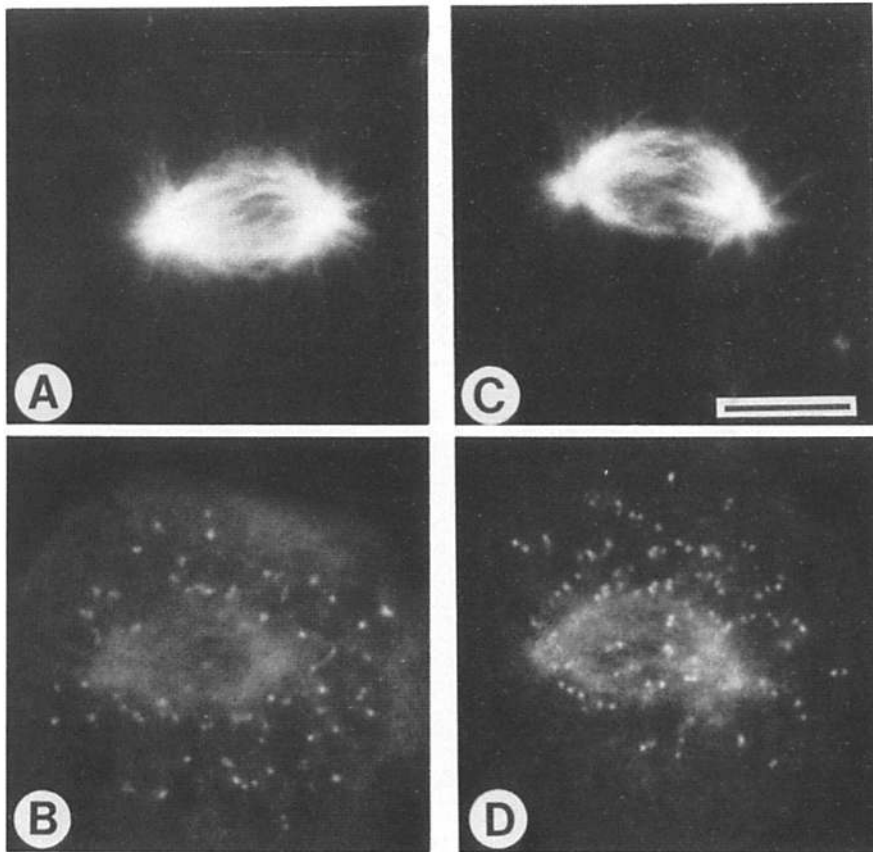
Figure 6. The injected antibody binds to centromeres in vivo. *A* and *C* show indirect immunofluorescence staining for tubulin. (*B* and *D*) show the detection of the injected ACA-IgG bound to centromeres of chromosomes surrounding the spindle with rhodamine-conjugated antihuman antibody. ACA were introduced into these cells by scrape loading, and the cells were examined 18 h later after staining with antihuman antibody to detect the loaded antibodies. DAPI staining of the DNA (not shown) indicated that the ACA staining was limited to the centromere regions of the chromosomes, which were apparently not associated with the mitotic spindle. Staining with antitubulin was as described in Materials and Methods. Bar, (*C*) 1 μm.

successfuly completed prometaphase, but arrested at a later stage of mitosis (Fig. 2). Flow cytometric analysis indicated that the length of G_2 in these cells is ∼3 h. These results lead us to conclude that there is an execution point for antibody inhibition at or near the S-G_2 boundary.

Cells receiving intranuclear injections of ACA-IgG ≥3 h before mitosis remain arrested in mitosis for 2–8 h in a novel state that we will refer to as pseudo-prometaphase. (Normal mitosis lasts ∼≤1 h in these cells.) Pseudo-prometaphase cells typically have a single clump of condensed chromosomes that apparently do not move from their original locations following nuclear envelope breakdown (Fig. 3). In cells observed after fixation within 1–2 h of entry into mitosis there is often a thin bipolar spindle on one side of the cell, beside the cluster of condensed chromosomes. Often these spindles are bent in a banana shape with the outer curve protruding toward the chromosomes. In such instances, microtubules are observed to emanate from the spindle into the cluster of chromosomes. The bent appearance may arise from microtubules that are attached to chromosomes and pull the spindle in that direction. From these observations it appears that chromosomes are able to attach to microtubules, but are unable to move once attached. We also occasionally observe spindles that appear to point away from the chromosome mass (Fig. 3, *C* and *D*). Spindles in arrested cells are not as robust as normal HeLa spindles, which are short and nearly round at metaphase.

An identical pseudo-prometaphase arrest phenotype was observed for cells injected in the cytoplasm 14–20 h before mitosis. This apparent shift in execution point observed for

the cytoplasmic injections may reflect a delay in transport of the ACA-IgG into the nucleus. Alternatively, ACA-IgG might remain in the cytoplasm and disrupt mitosis by a different mechanism. To distinguish between these two explanations, we conducted the following scrape-loading experiment to examine whether antibodies introduced into the cytoplasm enter the nucleus during interphase before nuclear envelope breakdown.

Mitotic cells were obtained from nocodazole-synchronized cultures by selective detachment and replated onto petri dishes. After cells had reattached (3 h after shake off), they were scrape loaded with ACA and replated in the presence of colcemid. With colcemid present, any cells that enter mitosis would be blocked there, and any cells still in interphase must not yet have reentered mitosis. The fate of the loaded antibody was tracked by staining cells with anti–human IgG at various times after replating. The antibody began to be detectable on centromeres within the nucleus of cells 4 h after loading, and was readily visible in most cells after 8 h. At this point, none of the cells had yet entered mitosis. Thus, ACA-IgG enter the nucleus before nuclear envelope breakdown at mitosis.

Identification of an Execution Point Required for Timely Completion of the Metaphase-Anaphase Transition

Cells receiving nuclear injections of ACA-IgG during G_2 (1–2 h before mitosis) successfully complete prometaphase, but remain blocked in metaphase for a number of hours. These experiments thus reveal the existence of a second exe-
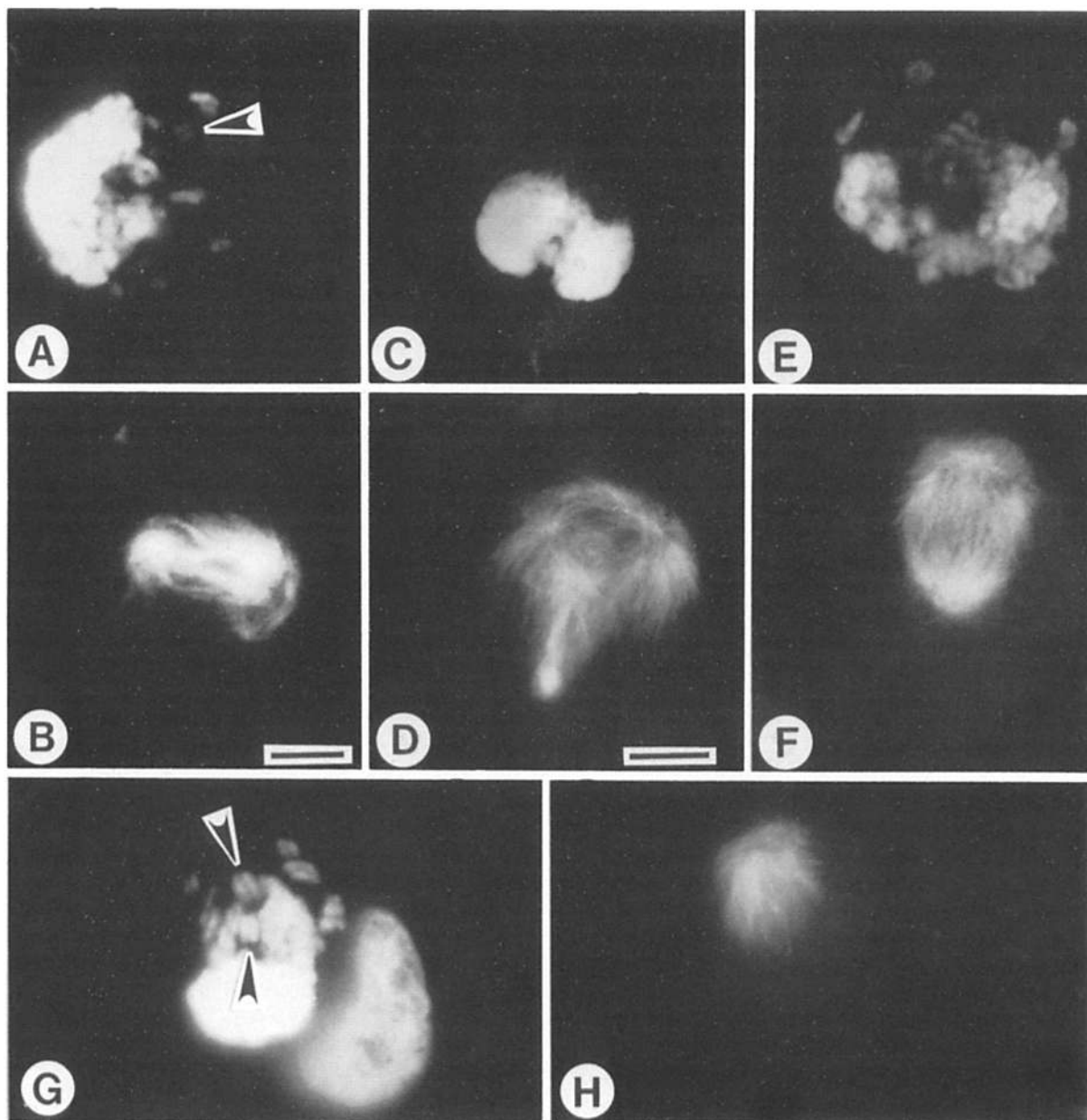
Figure 7. Pseudo-prometaphase-arrested cells undergoing spindle degeneration. Examination of the sister chromatids (where they can be resolved) suggests that these cells have entered anaphase. *A, C, E,* and *G* show DAPI stain for DNA. *B, D, F,* and *H* show indirect immunofluorescence staining for tubulin. The cells shown in the various panels had been in mitosis for the following times before fixation: (*A* and *B*) 5 h; (*C* and *D*) 3 h; (*E* and *F*) 3.5 h; (*G* and *H*) 5 h. Separated chromatids are clearly visible in both *A* and *G* (*arrowheads*). Bars: (*B*) 1 μm (applies to *A, B,* and *E–H*); (*D*) 1 μm (applies to *C* and *D*).

cution point for antibody inhibition just before mitosis. This execution point appears to affect events required for the transition from metaphase to anaphase.

These metaphase-arrested cells were unusual, in that the metaphase plate appeared to undergo a progressive degeneration over time. This was most obvious while monitoring LLC-PK cells in vivo as they remained arrested at metaphase. Chromosomes that had been at the plate were observed to detach and drift into the peripheral cytoplasm (Fig. 4, *A* and *B*). Examination of HeLa cells that had been arrested in metaphase for varying lengths of time confirmed the observations of living cells. The spindle often appeared normal in cells fixed and stained soon after the achievement of meta-

phase, although in some instances a few detached chromosomes could be seen (Fig. 5, *A* and *B*). Cells fixed after longer intervals spent at metaphase often had increasingly disorganized spindles and chromosomes distributed throughout the cytoplasm. Tripolar and quadripolar spindles were also observed (Fig. 5, *E* and *F*). The stability of the chromosomal attachment to the spindle appeared to correlate with the cell cycle time of injection. Cells injected at later points in the cycle exhibit a more stable metaphase conformation.

This dissolution of the metaphase plate is paradoxical. If chromosomes are once able to attach to microtubules and undergo prometaphase movements, why do they fall off and
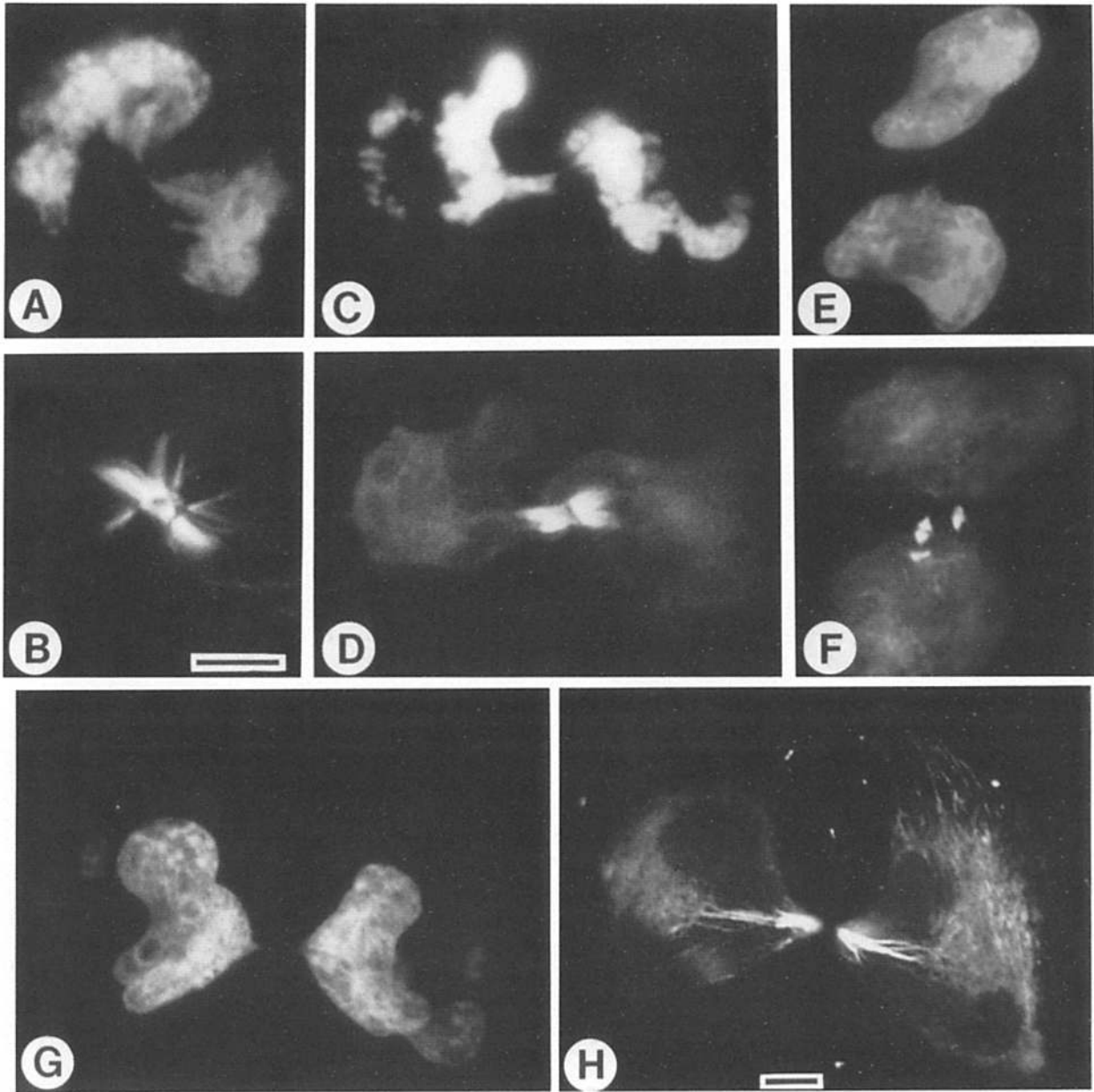
Figure 8. Cells undergoing abnormal cytokinesis after pseudo-prometaphase block in mitosis. *A, C, E,* and *G* show DAPI stain for DNA. *B, D, F,* and *H* show indirect immunofluorescence staining for tubulin. The cells shown in the various panels had been in mitosis for the following times before division: (*A* and *B*) 6 h; (*C* and *D*) 4 h; (*E* and *F*) 1 h; (*G* and *H*) 3 h. In every instance these cells have DNA trapped in the midbody. In most cases the midbody structures are clearly aberrant. Note the presence of two midbodies side by side in *F*. Bars: (*B*) 1 μm (applies to *A–F*); (*H*) 1 μm (applies to *G* and *H*).

remain detached from the spindle during metaphase arrest? While the detachment might result from kinetochore breakage, this seems unlikely, since the injected antibody can be visualized directly on the detached chromosomes and not on the microtubules (Fig. 6). Alternatively, the antibody might interact with centromeres after they detach from the spindle, preventing them from reattaching.

To test this idea, we examined whether metaphase-arrested cells could reestablish a metaphase plate in the presence of injected antibody after a brief exposure to the microtubule depolymerizing drug, nocodazole. Microcolonies of HeLa cells were injected with ACA-IgG under condi-

tions giving a metaphase arrest and periodically monitored for the appearance of mitotic cells. After cells had been blocked in metaphase for 2–5 h, nocodazole (40 nM) was added to the dish. Metaphase plates were no longer visible after 1 h in the drug, and the chromosomes appeared to be dispersed. At this point cells were released from nocodazole and allowed to proceed through mitosis.

Most uninjected cells completed mitosis within 1 h of release from the drug. After another 2 h, cells were fixed and stained with DAPI to visualize the chromosomes. All stages of mitosis were seen in uninjected mitotic cells. One-third (7/21) of the ACA-IgG-injected cells were able to reform
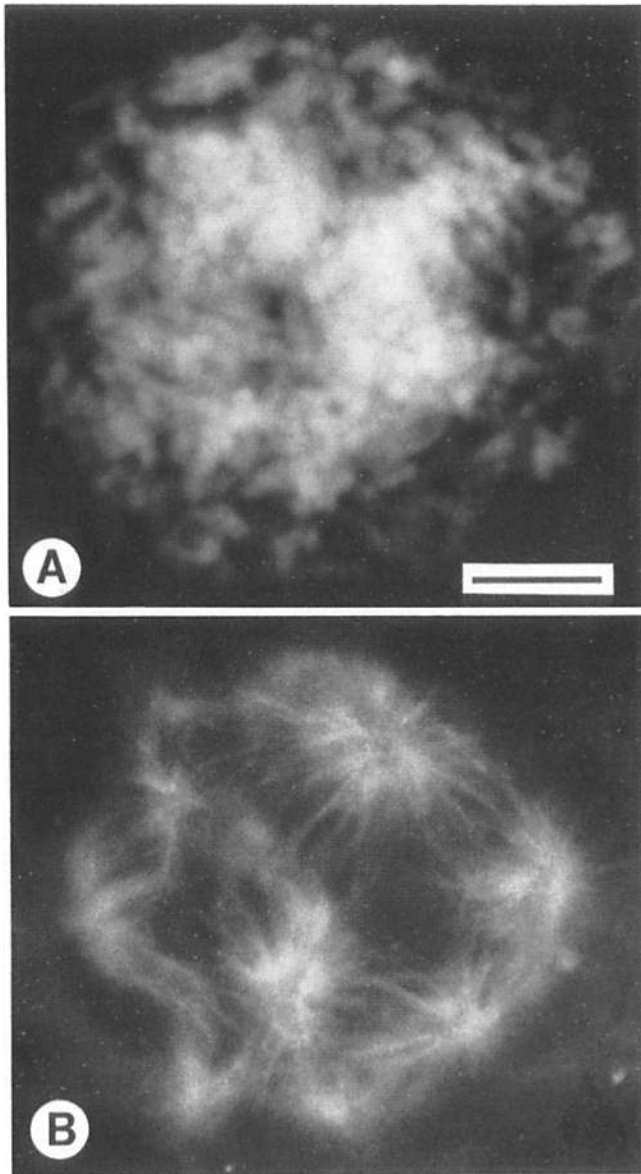
Figure 9. Giant mitotic HeLa cell observed in culture 5 d after scrape loading with ACA-IgG. (*A*) DAPI staining of DNA. (*B*) Indirect immunofluorescence staining for tubulin. This cell has at least eight spindle poles. Bar, (*A*) 1 μm.

metaphase plates. The remaining two-thirds of the metaphase-arrested cells (14 cells) were unable to achieve metaphase twice. In these cells chromosomes were distributed randomly, from the middle of the spindle to the poles. Thus, any inhibitory effect of the antibody on reattachment to the spindle is not absolute.

Terminal Phases of the Affected Mitosis

It is an unusual feature of these experiments that both pseudo-prometaphase-arrested and metaphase-arrested cells eventually attempt cytokinesis.

The onset of anaphase is difficult to detect in pseudo-prometaphase-arrested cells, since the chromosomes are concentrated in a single large cluster. In general, pseudo-prometaphase-arrested cells delayed in mitosis for longer periods exhibit an altered spindle phenotype that becomes highly variable (Fig. 7). In some cells only a disorganized patch of microtubules is observed. Although resolution of individual chromosomes within the cluster is difficult, adjacent sister chromatids appear to have disjoined in a significant percentage of cells where they can be resolved. We interpret such observations to indicate that these cells eventually enter anaphase. The sister chromatids show no sign of poleward movement in these cells.

Metaphase-arrested cells also eventually enter anaphase. In those that enter anaphase while their metaphase plates are still somewhat intact, chromosomes at the plate appear to disjoin normally and undergo anaphase A movement towards the spindle poles (Fig. 4, *C* and *D*). The stray chromosomes scattered throughout the cytoplasm also disjoin, but do not appear to move (Fig. 5, *G* and *H*).

A cleavage furrow forms and division is attempted in the majority of cells, regardless of whether or not a metaphase configuration had been achieved. These attempts at cytokinesis are often unsuccessful. The progeny of such cleavages are typically micronucleated (Fig. 4, *E* and *F*), and are connected by a midbody that contains trapped DNA (Fig. 8, *A*, *C*, *E*, and *G*). Cytokinesis is often reversed in these cells, presumably because the process is incapable of proceeding to completion when DNA is trapped in the cleavage furrow.

The detailed aspects of cytokinesis and the midbody structures produced during attempted cleavage of pseudo-prometaphase cells are often grossly abnormal. This may be explained by the abnormal condition of the spindle, whose morphology is widely believed to dictate the location of the cleavage furrow (33). We often note the initiation and regression of several cleavage furrows in different orientations during in vivo observation of such cells attempting cytokinesis. The ultimate attempt of these cells to divide often results in production of disorganized intracellular bridges with abnormal midbody structures (Fig. 8). We have observed daughter cells with two midbodies side by side and cells that have segregated portions of their cytoplasm as anucleate karyoplasts.

Injected Cells Continue to Cycle but Fail to Divide

Even though these cells are unable to divide normally, many of them continue to grow and traverse the cell cycle. This was most evident in scrape loading experiments, where many large micronucleated cells were observed in populations of cells 48 h after loading with ACA-IgG (data not shown). That these cells continued to cycle was evidenced by the observation of large cells in mitosis, with multipolar spindles and extra sets of chromosomes. When cultures were examined 5 d after loading, these large cells appeared to have persisted and grown even larger, still continuing their unsuccessful rounds of mitosis (Fig. 9). Such cells were not observed after scrape loading with control antibodies.

Attempts to Identify the Target Antigens

21 different autoimmune sera were tested for their ability to disrupt mitosis. While whole serum was used for initial scrape-loading experiments, all microinjections were performed with IgG purified by chromatography on protein A sepharose. Most (11/15) sera with ACA disrupted mitosis. IgG from 5/7 ACA-positive sera disrupted mitosis by microinjection, and 9/15 disrupted mitosis by scrape loading. The
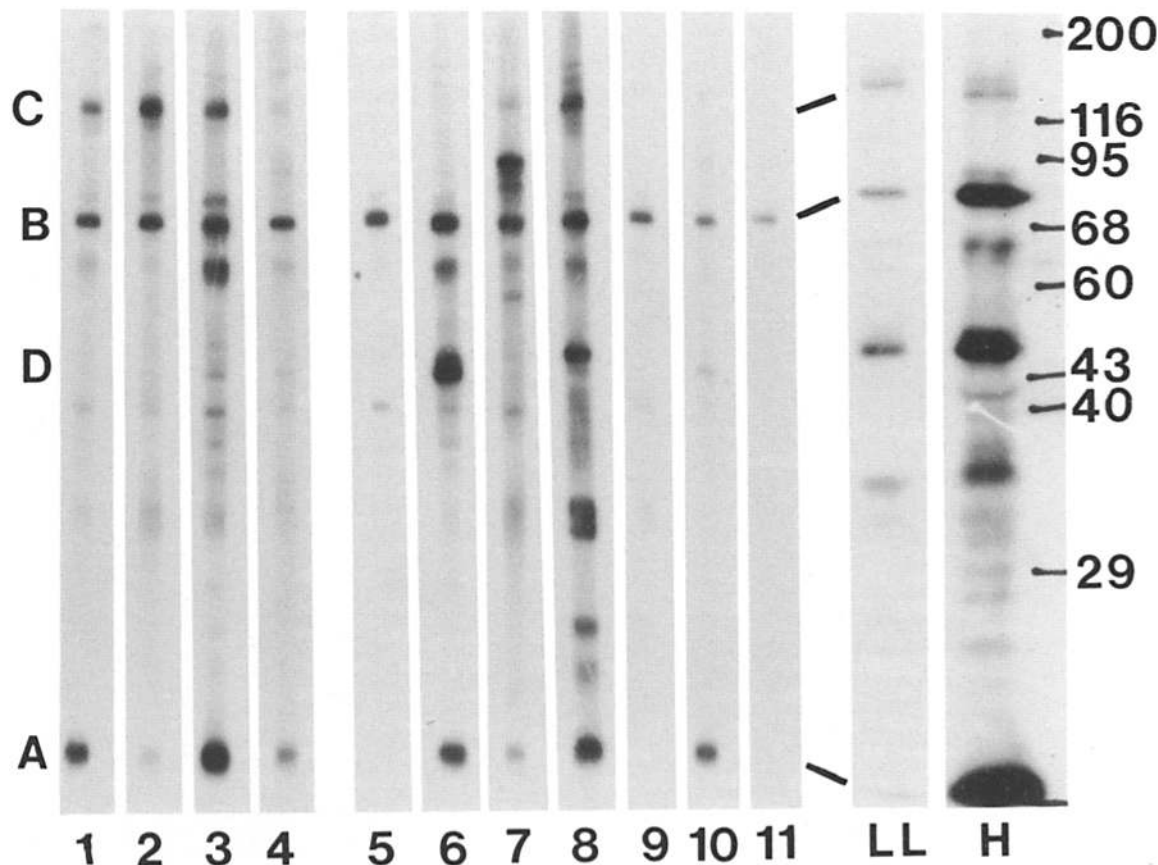
Figure 10. Different ACA(+) sera and ACA-IgG used for microinjection recognize different combinations of the CENP antigens in immunoblots of HeLa chromosomal proteins. Lanes *1–4* show immunoblots with four ACA(+) sera that disrupted mitosis by scrape loading. The sera are: lane *1*, J-1311; lane *2*, A-1219 (lower level of anti-CENP-A); lane *3*, KG; lane *4*, SN (lower level of anti-CENP-C). Lanes *5–11* show immunoblots with ACA-IgG purified from seven sera used in microinjection experiments. ACA-IgG shown in lanes *5–9* disrupted mitosis after microinjection, while those in lanes *10* and *11* did not. The sera from which these IgG were isolated are: lane *5*, GS (low anti-CENP-A and anti-CENP-C); lane *6*, AP (low anti-CENP-C); lane *7*, F-2248; lane *8*, J-3051; lane *9*, H-345 (low anti-CENP-A and anti-CENP-C); lane *10*, G-2016 (low levels of all antibodies); lane *11*, G-1942 (low levels of all three antibodies). Lanes *LL* and *H* show immunoblots of nuclei isolated from LLC-PK and HeLa, respectively, with serum AP. (This was a different gel from that shown in lanes *1–11*, and the position of the molecular weight markers is indicated at the right.) All sera and IgG were diluted 1:1,000. The positions of CENPs *A–D* are indicated at the left. Note that only serum AP consistently recognized CENP-D.

higher percentage in microinjection experiments presumably reflects the introduction of larger amounts of antibody into the cells by this method. In controls, four sera containing antitopoisomerase I, one with antitopoisomerase II, one with non-ACA autoantibodies, and three normal sera, had no effect by either scrape loading or microinjection (as purified IgG). The fact that only ACA-positive sera caused the mitotic disruption suggests strongly that antibodies to at least one of the CENP antigens are responsible for the effect (although we cannot rule out the possibility that antibodies recognizing a hitherto undetected centromere antigen are responsible).

Although we have shown previously that ≥90% of ACA-positive sera recognize three chromosomal antigens, CENPs A, B, and C; by screening the bank of 185 ACA-positive sera available to us, we were able to select 8 sera with reduced levels of anti-CENP-C and/or anti-CENP-A. All sera that inhibited mitosis had high levels of anti-CENP-B antibodies, but variable levels of anti-CENP-C or A by immunoblotting analysis (Fig. 10). In some instances, anti-CENP-C and/or anti-CENP-A were virtually undetectable, particularly after purification of the IgG on protein A columns. This is consis-

tent with earlier observations that anti-CENP-C antibodies are predominately of the IgM subclass (24). Those sera that did not disrupt mitosis appeared to have relatively lower titers of all of the antibodies, including anti-CENP-B.

The results of screening the panel of autoimmune sera suggested strongly that CENP-B may be the target antigen for mitotic disruption. However, three rabbit polyclonal and two murine mAbs that were raised against two different CENP-B fusion proteins had no effect on mitosis when injected into HeLa cells, even though these antibodies were shown by indirect immunofluorescence to bind to centromeres in vivo (data not shown). These results do not rule out CENP-B as the target antigen, however. Rabbit antibodies raised against CENP-B fusion proteins recognize only CENP-B, both when used as crude serum and after affinity purification from nitrocellulose strips of chromosomal CENP-B. However, autoantibodies affinity purified the same way cross-react with CENP-A and/or CENP-C. This suggests that the experimental antibodies and autoantibodies recognize different epitopes on CENP-B (9, 10). The inhibitory autoantibodies may recognize, for example, a post-translational modification pres-
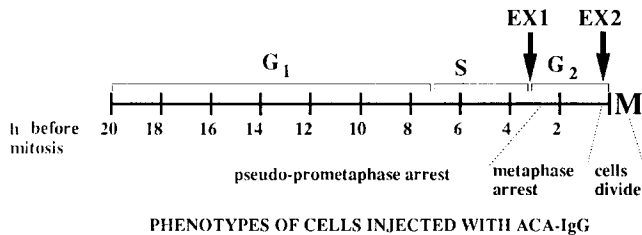
PHENOTYPES OF CELLS INJECTED WITH ACA-IgG

Figure 11. Diagram showing the mitotic phenotypes of cells after nuclear injection with ACA-IgG. The positions of the two execution points are indicated. Cytoplasmic injections yield identical phenotypes for cells injected ~8 h earlier in the cell cycle.

ent on chromosomal CENP-B that is absent from fusion proteins, and is therefore not recognized by the experimental antibodies.

The fact that 4 of the 11 ACA-positive sera failed to cause a mitotic block may simply reflect the presence of a slightly lowered titer of certain critical antibodies. Purified ACA-IgG were typically injected at a concentration of 5 mg/ml. With an estimated injection volume of 0.1 pl, this is ~2 × 10⁶ IgGs injected per cell. Since we have injected total IgG, only 3.8% of the injected antibodies will be directed against CENP-B (for serum AP; N. F. Rothfield and W. C. Earnshaw, unpublished results of quantitative ELISA). We have quantitated CENP-B and found it to be present at 30,000–50,000 copies per cell (R. L. Bernat and W. C. Earnshaw, unpublished results). Thus, the antibody against CENP-B is present in nearly stoichiometric amounts (~76,000 anti-CENP-B IgG molecules). Consistent with this, we noted that a three-fold dilution of our strongest IgG was typically sufficient to abolish the mitotic inhibition. The levels of antibodies to CENP-A and CENP-C have not yet been determined, but examination of immunoblots suggests that the titers of these antibodies are lower than those of anti-CENP-B (9).

One way to identify the target antigen directly would be to affinity purify sufficient amounts of autoantibody from each CENP antigen using immunoblots of the chromosomal proteins as substrate. We have attempted this experiment, but such affinity-purified antibodies have been inactive in mitotic disruption. This is not surprising, since the CENP antigens are present in HeLa cells in extremely small amounts, and the methods required for affinity purification of these antibodies are extremely harsh (7, 20). The failure of the affinity-purified antibodies to disrupt mitosis is probably due to a failure to obtain sufficient amounts of active antibody (which requires IgG concentrations in the 5–10 mg/ml range).

Discussion

ACA-IgG Disrupt Both the Timing and the Pathway of Mitotic Events When Injected into Cultured Cells

Surprisingly, this mitotic disruption requires interaction of the antibody with centromeres during interphase. ACA-IgG interfere with at least two interphase processes with different "execution points," one ~3 h before mitosis and the other just before prophase (Fig. 11). (By analogy to cell cycle mutant analysis, we use "execution point" to refer to that time in the cycle after which an essential function performed by the tar-

get antigen is no longer sensitive to antibody disruption [32].) When ACA-IgG were injected into the nucleus ≥3 h before mitosis, cells were subsequently arrested in pseudo-prometaphase with the spindle and the condensed chromosomes lying side by side in the cytoplasm. Antibodies injected <3 h before mitosis no longer inhibit prometaphase function of the centromere, apparently because the required interphase events sensitive to antibody inhibition have already been completed. Instead, such injections cause cells to arrest in metaphase. Antibodies introduced into cells after the onset of prophase do not disrupt the ongoing mitosis, even if the injected cells are held in prometaphase for several hours in the presence of nocodazole. This, therefore, defines a second execution point for antibody inhibition, just before the initiation of prophase (Fig. 11).

The relationship between the two execution points is not known. The two phenotypes could represent differences in sensitivity of a single epitope at different times in the cell cycle. Alternatively, the two execution points may reflect interaction of the antibodies with different epitopes affecting distinct aspects of centromere function.

Antibodies injected either into the nucleus or the cytoplasm produce identical phenotypes, although cytoplasmic injections must be performed at earlier times in the cell cycle to give a comparable effect (see Fig. 2). The block in prometaphase becomes progressively stronger at earlier times of cytoplasmic injection, presumably reflecting the rate of antibody transport into the nucleus. Immunoglobulins are too large to pass the nuclear pore passively (3, 12, 43), and yet antibodies directed against nuclear antigens have been observed to accumulate in the nucleus, presumably being transported there by newly translated antigen (2, 3, 22, 44, 45). We have shown in this study that anticentromere antibodies introduced into the cytoplasm enter the nucleus during interphase with kinetics that could explain the observed delay.

Although we have thus far been unable to identify the target antigens responsible for the disruption of mitosis, a variety of circumstantial evidence based on our analysis of the effects of 15 different ACA-positive patient sera suggests that at least some of the inhibitory effects may be due to autoantibodies recognizing CENP-B.

The Pseudo-prometaphase Arrest May Result from a Disruption of Centromere Assembly

The execution point for pseudo-prometaphase arrest appears to correspond roughly to the S/G₂ transition (Fig. 11). The end of the S phase is the time of replication of α-satellite DNA, one of the major structural components of the human centromere (20). It has recently been shown that CENP-B binds to a 17-bp sequence found in many α-satellite DNA repeats (23). It is likely that this binding occurs in vivo either coordinate with, or soon after α-satellite DNA replication. The position of the execution point for prometaphase arrest at roughly the same time in the cell cycle thus suggests that the antibody may cause the improper assembly of CENP-B onto α-satellite DNA (or prevent other centromeric proteins from assembling correctly onto the CENP-B:α-satellite DNA complex). Staining with anti-CENP-B reveals that this protein is present at the centromeres of the blocked cells (data not shown), but it is not possible with currently available antibodies to tell whether the localization of CENP-A and CENP-C is normal in these cells.

Immunofluorescence observations frequently revealed the presence of microtubules that appeared to be associated with the centromeres in pseudo-prometaphase–arrested cells. This suggests that the centromere defect induced by ACA-IgG produces a structure capable of binding microtubules but incapable of moving along them. This is consistent with our immunoelectron microscopy experiments in which we have been unable to detect these antigens in the outer kinetochore plate, where the majority of the microtubules are known to bind (6, 34). The inhibition of chromosome movement may be a consequence of the failure to assemble a functional kinetochore structure. Alternatively, the antibody may directly inactivate some component of the mechano-chemical motor responsible for chromosome movement. Future EM studies should reveal whether the antibodies cause any observable defects in kinetochore morphology.

The inhibition of prometaphase movements by injected ACA-IgG has interesting implications for the structure/function of the centromere, since our recent immunoelectron microscopy results indicate that the antigens targeted by these antibodies are found in the chromatin surrounding the kinetochore (6). Thus, this chromatin might be more interesting than had previously been thought. It is worth noting that a recent study has shown that early prometaphase movements in the newt lung cell involve the lateral interaction of microtubules with the corona region of the kinetochore (36). The relationship between the corona material and the CENP antigen-rich chromatin surrounding the kinetochore plate is unknown.

The presence of a bipolar spindle in pseudo-prometaphase cells was unexpected, and suggests that the formation of a bipolar spindle may not be strictly dependent on the presence of bioriented chromosomes at the spindle equator. Apparently two adjacent asters will eventually generate a bipolar spindle between them even in the absence of the normal capping structures such as kinetochores.

Injection of ACA-IgG into the Nucleus during G₂ Disrupts the Timing of the Metaphase–Anaphase Transition

This defines a second execution point for antibody inhibition just before the beginning of prophase. The kinetochore begins to undergo a series of morphological changes at the onset of prophase, culminating in the appearance of a trilaminar plaque structure in prometaphase (37). The metaphase arrest may require antibody binding to the centromere before the onset of these morphological changes.

The reason for the antibody-induced metaphase arrest is unknown. Normal initiation of the metaphase–anaphase transition requires passage of the metaphase cell cycle checkpoint, a process that senses completion of the bipolar attachment of the chromosomes to the spindle (35, 53). ACA-IgG injection may disrupt kinetochore structure, resulting in defective attachment of the chromosomes to the spindle so that even though the chromosomes achieve a bipolar orientation that appears normal, the resulting structure is aberrant. Alternatively, ACA-IgG may disrupt the signaling network directly by binding to a centromere component involved in the pathway. It is unlikely that the metaphase delay arises from an inability of the chromosomes to disjoin, since separation of sister chromatids can occur in these cells, albeit in an altered temporal and spatial context.

Chromosomes begin to detach from the spindle and become randomly distributed throughout the cell as cells remain blocked in metaphase. Our present data do not allow us to determine whether this chromosome detachment is a cause or a consequence of the metaphase arrest. A similar phenotype has been observed in a hamster cell cycle mutant, which undergoes a similar metaphase arrest, followed by the eventual dispersion of chromosomes throughout the cell (49, 50).

Detachment of chromosomes could be a consequence of kinetochore breakage similar to that induced by caffeine treatment of hydroxyurea-arrested cells (39). In such cells, the kinetochores apparently detach from the chromosomes and participate in the formation of a bipolar spindle (4). Any kinetochore detachment that occurs as a consequence of antibody injection must be highly localized, however, since we have shown that the detached chromosomes stain normally with anticentromere antibodies.

Alternatively, the normal oscillations of chromosomes at the metaphase plate might result in transient detachment of the chromosome–spindle connections, which may be unable to reform normally in the presence of structural abnormalities induced by the injected antibody. This seems unlikely since cells arrested in metaphase by injection of ACA-IgG are in many instances able to reform metaphase plates after a transient exposure to nocodazole.

Although Cells Injected with ACA-IgG Are Arrested in Mitosis for Up to Eight Hours, Most Eventually Attempt Cytokinesis

These attempts are often unsuccessful, presumably because the process is incapable of proceeding to completion when DNA is trapped in the cleavage furrow. DNA entrapment apparently results from the failure of some of the chromosomes to move during anaphase. This is most extreme in the pseudo-prometaphase-arrested cells, where we are unable to detect chromosome movement at any time during mitosis (Fig. 8). The effect is less pronounced in cells that had formed metaphase plates. Those chromosomes still at the spindle equator at the onset of anaphase successfully disjoin and move to the spindle poles (Fig. 4). Chromosomes that had detached from the plate apparently do not move and become trapped in the cleavage furrow.

The ultimate result of this abortive cytokinesis bears a striking resemblance to the cut phenotype observed in topoisomerase II mutants of S. cerevisiae and S. pombe (18, 46) as well as in the S. pombe cut mutants (17). The ACA-IgG-induced cut phenotype is distinct from that induced by topoisomerase II mutants, however, since we often observe disjunction of sister chromatids. It is possible that one or more of the S. pombe cut mutants affects a centromere protein related to one or more of the CENP antigens.

That pseudo-prometaphase-arrested cells eventually attempt cytokinesis is surprising, particularly in HeLa cells, which are stably arrested in prometaphase by colcemid treatment, and do not progress through mitosis even after 12–20 h in the drug. Since the only obvious difference between the injected cells and cells blocked with colcemid is the presence of microtubules, this suggests that the cellular mechanism responsible for initiation of cytokinesis requires microtubules. This is consistent with the earlier demonstration that cells such as sea urchin eggs that undergo successive cell

cycles in the presence of colcemid do so without formation of cleavage furrows or other signs of cytokinesis (42).

While these experiments were in progress, Simerly et al. (40) conducted similar experiments on the effect of anticentromere antibodies microinjected into mouse oocytes. Like our study, they found that injection into interphase cells interfered with chromosome congression at the subsequent mitosis. However, unlike our results, antibody injected into nocodazole-arrested cells also interfered with congression after drug removal. They concluded that the effect was not due to a disruption of kinetochore maturation but, rather, was on the congression mechanism itself. The basis for the difference in results in the two systems is not known at present, although one obvious possibility is differences between man and mouse in the organization of the centromeric repetitive DNAs that bind CENP-B.

Conclusions

Our experiments reveal the existence of antibody-sensitive events during interphase that are essential for the ability of the centromere to undergo microtubule-based movements during mitosis. These events, which may include essential stages in the assembly of the centromere, occur at a time of the cell cycle when centromere structure cannot be resolved directly. The availability of cDNA clones and specific antibodies for the CENP antigens should permit their analysis in the future.

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