Antibodies to a Nucleolar Protein Are Localized in the Nucleolus after Red Blood Cell-mediated Microinjection

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ABSTRACT   To determine whether red blood cell-mediated microinjection of antibodies can be used to study nuclear protein localization and function, we microinjected antibodies that have been shown to react specifically with nucleolar acidic phosphoprotein C23 into Walker 256 cells. The intracellular distribution of microinjected anti-C23 antibodies and preimmune immunoglobulins were determined by immunofluorescence. At 3 h after microinjection, affinity-purified anti-C23 antibodies were localized in the cytoplasm and nucleolus. At 17 h after microinjection, the affinity-purified antibody was localized to those nucleolar structures previously shown to contain protein C23. Furthermore, the antibody remained localized in the nucleolus for at least 36 h after microinjection. In contrast to the results obtained with specific antibodies, preimmune immunoglobulins remained in the cytoplasm 36 h after microinjection. These results indicate that red blood cell-mediated microinjection of antibodies can be used to study nucleolar and nuclear antigens.

The introduction of individual proteins into cells by microinjection is a useful procedure for determining their functions in living cells (4, 6, 9, 12, 17, 18, 31, 38). Two methods have been used to microinject proteins into living cells: direct microneedle microinjection (8, 14) and red blood cell (RBC)[1]-mediated microinjection (13, 22, 34). The advantages and disadvantages of these techniques have been reviewed (5, 30). An alternative approach to microinjection of the protein of interest is microinjection of specific antibodies, with the objective of inhibiting the function of the protein (1, 20, 23, 25, 26, 28, 32, 33, 39).

In previous studies nuclear proteins were microinjected into the cytoplasm of cells either by the microneedle method or by RBC-mediated fusion. Subsequently, the injected proteins passed through the nuclear envelope into the cell nucleus (2, 3, 7, 9, 11, 31, 38, 40). The movement of non-nuclear proteins from the cytoplasm to the nucleus appears to be regulated in part by size (2). Thus, small proteins such as myoglobin (17,000 mol wt) distribute evenly between the nucleus and cytoplasm, whereas larger proteins such as BSA (67,000 mol wt) or immunoglobulins (150,000 mol wt) enter the nucleus slowly, or not at all (2). However, size does not totally limit the ability of proteins to penetrate the nuclear envelope since nucleus, nonimmune immunoglobulins and antibodies di-

the nucleus is known to contain many high molecular weight nuclear proteins (3).

Although the results of previous studies would suggest that the nuclear envelope excludes immunoglobulins from the cell nucleus, nonimmune immunoglobulins and antibodies directed against non-nuclear antigens have been used to evaluate nuclear exclusion (2, 25). This approach would not, however, detect uptake and retention of small but possibly meaningful amounts of antinuclear antibodies. Therefore, to further evaluate the applicability of RBC-mediated microinjection of antinuclear antibodies, we have investigated whether specific immunoglobulins can penetrate the nuclear membrane and remain localized at sites that contain their antigens. Antibodies to the nucleolar specific phosphoprotein C23 (21, 24) and preimmune immunoglobulins were microinjected into Walker 256 cells, a rat mammary carcinoma. The localization of the microinjected antibodies was determined by immunofluorescence. Possible mechanisms for the entry of nucleolar-specific antibodies into the cell nucleus, and their subsequent retention are discussed.

MATERIALS AND METHODS

Preparation of Antibodies: Protein C23 was isolated from rat Novikoff hepatoma nucleoli as previously described (21). The purified protein was used to produce rabbit polyclonal antibodies (21). The serum Ig fraction was prepared by standard ammonium sulfate precipitation. For microinjection

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[1] *Abbreviation used in the paper:*   RBC, red blood cell.

experiments, the Ig fraction was dialyzed against 10 mM Tris HCl pH 7.4 and concentrated to 16 mg/ml. The specificity of antibodies was determined by Ouchterlony immunodiffusion analysis (21), enzyme-linked immunoadsorbent assay (10), and immunoblotting (36) as described below.

Affinity-purified antibodies were prepared from immunized rabbit serum. Purified protein C23 was coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). Antiserum to protein C23 was applied to the column and the column was washed with 0.5 M NaCl/0.1 M NaHCO₃, pH 8.3. The bound antibody was eluted with 4 M MgCl₂, dialyzed into PBS (150 mM NaCl/10 mM sodium phosphate, pH 7.4), and precipitated with 50% saturated ammonium sulfate. The antibody was redissolved in 10 mM Tris HCl, pH 7.4, to a final concentration of 5 mg/ml.

Monoclonal antibodies to rat nucleolar protein C23 were produced by standard techniques (16, 29). BALB/c mice were immunized by subcutaneous injection with 300–500 μg of antigen mixed in Freund's complete adjuvant six times over a period of 12 wk. Spleen cells were fused with NS1 myeloma cells using 30% polyethylene glycol. Positive clones were identified by enzyme-linked immunoadsorbent assay and subsequently by immunoblotting as described below.

Immunoblot Assays: Immunoblots were performed using whole Novikoff hepatoma nucleoli as the source of the antigen. Nucleoli were suspended in Laemmli sample buffer (19), heated for 5 min at 100°C, and an aliquot containing 150 μg of protein was applied to either an 8 or 11% SDS polyacrylamide gel (19). After electrophoresis the proteins were transferred to nitrocellulose paper (36) and the paper was cut into strips corresponding to the gel lanes. These were stained with amido black or with antiprotein C23 antibodies (36). The immunoreactions were performed on the nitrocellulose paper as previously described (27). Rabbit antibodies were diluted 1:2,500 and mouse monoclonal antibody to protein C23 was diluted 1:50.

Cells and Cell Culture: The Walker 256 rat mammary carcinoma cell line was obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in Dulbecco's modified Eagle's minimum essential medium containing 4.5 g glucose/liter (Gibco Laboratories, Grand Island, NY) supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM minimum essential medium nonessential amino acids, and 5% fetal calf serum (M. A. Bioproducts, Walkersville, MD).

RBC-mediated Microinjection: Stock cultures of Sendai virus were a gift from Dr. Martin Rechsteiner (University of Utah, Salt Lake City). Loading of human red blood cells and fusion of loaded RBC to Walker 256 cells were performed as described by Schlegel and Rechsteiner (35). The fusion mixture contained 10⁹ loaded RBC, 10⁷ Walker 256 cells, and 1,000–2,000 hemagglutinating units of UV inactivated Sendai virus. The efficiency of microinjection, determined by immunofluorescence, ranged from 40 to 80%. Unfused RBC were separated from rat carcinoma cells by layering the cell suspension over 10 ml of 50% fetal calf serum followed by centrifugation at 250 g for 3.5 min. This procedure was repeated once. Cells were plated either in two chamber microscope slides (Lab-Tek Products, Naperville, IL) or on 22 × 22-mm cover slips in 35-mm petri dishes.

Immunofluorescence: Cells attached to microscope slides or cover slips were washed twice in PBS and fixed for 20 min in 2% methanol-free paraformaldehyde (Polysciences Inc., Warrington, PA). The cells were permeabilized with acetone (−20°C) for 3.5 min and washed again in PBS for 20 min. For indirect immunofluorescence, cells were incubated with the primary antibody diluted in Dulbecco's modified Eagle's minimum essential medium containing 5% fetal calf serum for 1 h at 37°C in a humidified chamber. The cells were washed in several changes of PBS for 30 min, followed by an incubation for 1 h with either a 1:100 dilution of fluorescein isothiocyanate-conjugated goat anti-rabbit IgG, fluorescein isothiocyanate-conjugated goat anti-mouse Ig or a 1:50 dilution of rhodamine-conjugated goat anti-rabbit IgG (Cappel Laboratories, Inc., Cochranville, PA). Cells were then incubated in four changes of PBS, 15 min each, at room temperature, then for 16 h at 4°C. The cover slips were mounted with glycerol/PBS containing p-phenylenediamine (Sigma Chemical Co., St. Louis, MO) as described by Johnson et al. (15). Antibodies microinjected into cells were localized by immunofluorescence as described above, except that the fixation time with paraformaldehyde was reduced to 15 min and incubation with primary antibodies was omitted.

RESULTS

Antibody Specificity

The specificities of the rabbit polyclonal antibody and mouse monoclonal antibody for protein C23 are demonstrated in Fig. 1. Lanes *A* and *C* are the amido black stains of total nucleolar proteins transferred to nitrocellulose paper
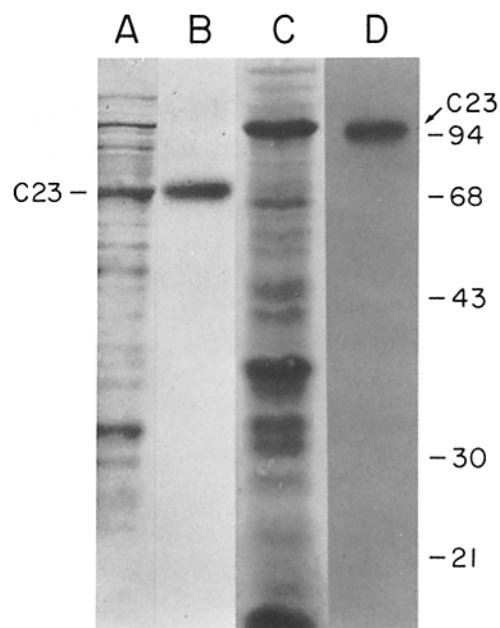

FIGURE 1   Immunoblot analysis of antibody specificity. Total nucleolar proteins were separated on either 8 (lanes *A* and *B*) or 11% (lanes *C* and *D*) SDS polyacrylamide gels and electrophoretically transferred to nitrocellulose paper (36). The nitrocellulose strips were stained with amido black (lanes *A* and *C*) or reacted with antibodies and ¹²⁵I-protein A (lanes *B* and *D*) as described in the Materials and Methods. Lanes *B* and *D* were reacted with rabbit antiprotein C23 serum and mouse antiprotein C23 monoclonal antibody respectively. The molecular weight ordinate applies only to lanes *C* and *D*.

from 8 and 11% polyacrylamide gels, respectively. In lane *B* an autoradiograph of the immunoreaction of the rabbit polyclonal antibody with total nucleolar proteins demonstrates the specificity of the antibody for protein C23. The corresponding immunoreaction of the mouse monoclonal antibody demonstrates that protein C23 is also the only protein recognized by this antibody (lane *D*).

The phase contrast and corresponding fluorescent images demonstrating the localization of protein C23 in rat carcinoma cells by indirect immunofluorescence are shown in Fig. 2. Preimmune immunoglobulins used for microinjection failed to exhibit specific staining of cellular structures (Fig. 2*b*). Both the rabbit polyclonal and mouse monoclonal antibodies specifically reacted with nucleolar structures (Fig. 2, *d* and *f*), demonstrating that protein C23 is a nucleolar specific protein in these cells. Furthermore, the nucleolar staining was eliminated if the rabbit serum was preincubated with protein C23 (21), again demonstrating the specificity of the rabbit antibody for protein C23. These results agree with the localization of protein C23 in other cell types (21, 27). It should be noted that the nucleoli of Walker 256 cells are variable in morphology and often contain multiple nucleoli (Fig. 2, *a*, *c*, and *e*).

Microinjection of Antibodies

To determine whether RBC-mediated microinjection of antibodies could be used to study nuclear protein localization and function, antibodies to protein C23 were microinjected into Walker 256 cells. As a control, cells were also microinjected with an Ig fraction of preimmune rabbit serum. Cells
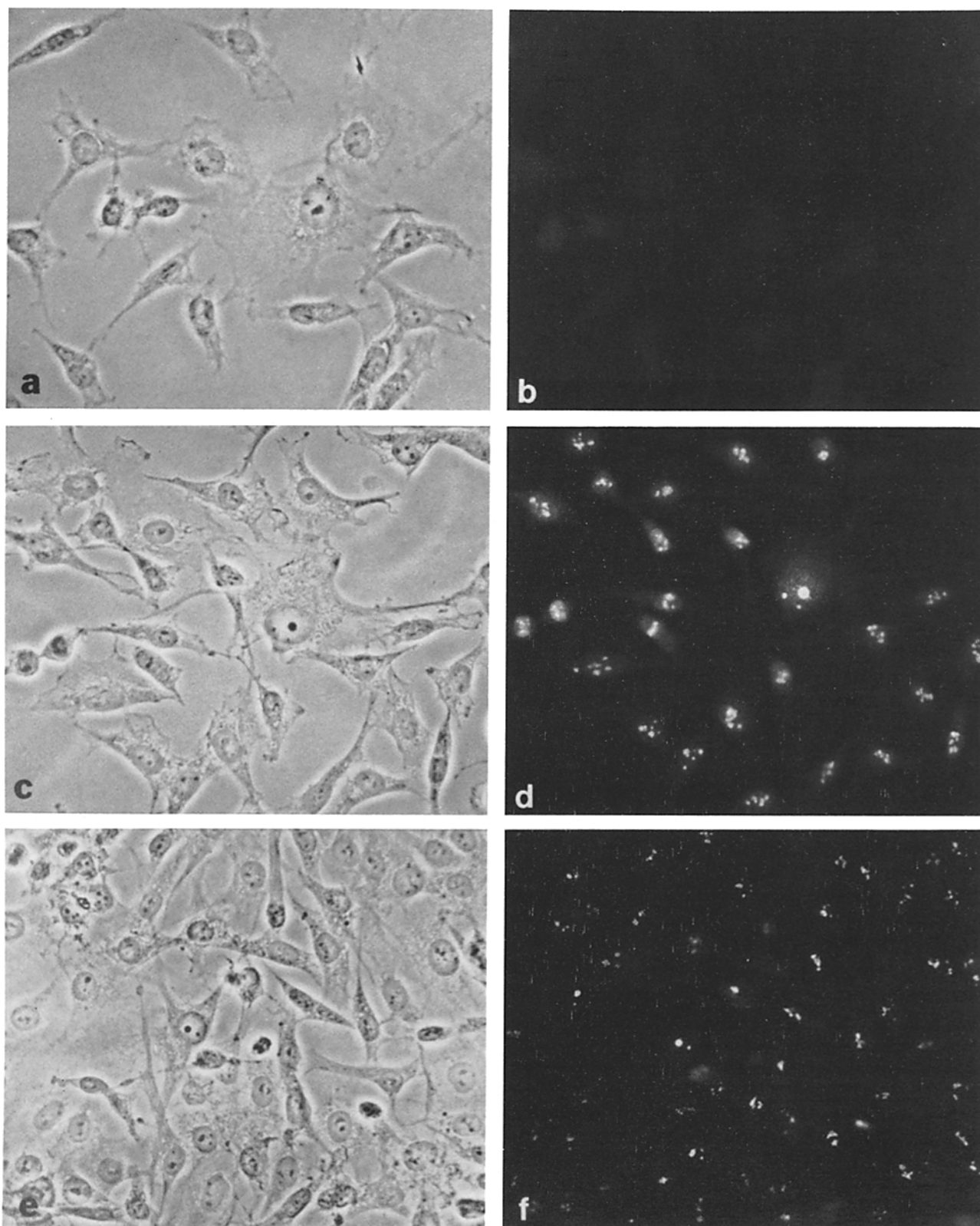
FIGURE 2 Localization of nucleolar protein C23 in rat carcinoma cells by indirect immunofluorescence. Phase contrast (a, c, and e) and corresponding indirect immunofluorescence (b, d, and f) images of cells labeled with preimmune immunoglobulins (b), affinity-purified rabbit antiprotein C23 antibodies (d), or antiprotein C23 mouse monoclonal antibodies (f), as described in Materials and Methods. × 300.

microinjected with antibodies were fixed at 3, 17, or 36 h after microinjection, and the immunoglobulins were localized with fluorescein-conjugated goat anti-rabbit IgG. At 3 (data

not shown) and 17 h (Fig. 3a) preimmune IgG molecules were localized in the cytoplasm. Weak fluorescence was observed in the nuclei of some cells, which could reflect low-
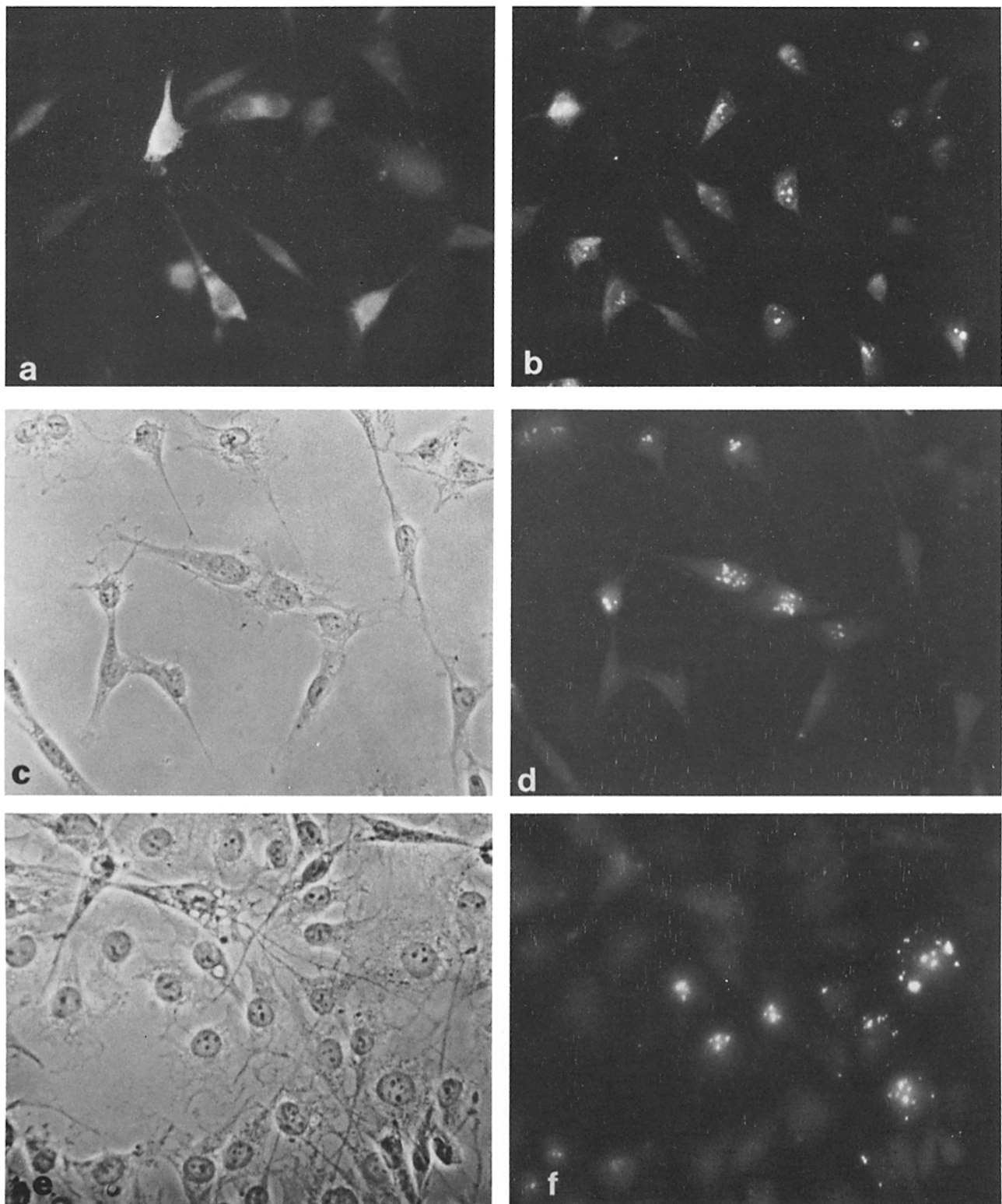
FIGURE 3 Localization of microinjected antibodies. Antibodies were microinjected into rat carcinoma cells and subsequently localized by immunofluorescence as described in the Materials and Methods. (a) Preimmune immunoglobulins 17 h after microinjection; (b) affinity-purified antiprotein C23 antibodies 3 h after microinjection; (c) phase contrast, and (d) immunofluorescence of affinity-purified antibodies 17 h after microinjection; and (e) phase and (f) immunofluorescence of affinity-purified antibodies 36 h after microinjection.

level entry of IgG into the nucleus or IgG in the cytoplasm directly over the nucleus. By 36 h after microinjection, fluorescence was greatly diminished, probably due to dilution of the immunoglobulins with the cytoplasmic material when the cells divide (data not shown); however, the fluorescence remained cytoplasmic. These results confirm those results previously reported by Bonner (2) and McGarry et al. (25).

Localization of microinjected antiprotein C23 antibodies

was markedly different from that observed for preimmune immunoglobulins (Fig. 3a). Immunofluorescence localization of the microinjected antibody resulted in cytoplasmic and nucleolar fluorescence with little nucleoplasmic fluorescence, both at 3 and at 17 h after microinjection (data not shown). To determine how much cytoplasmic fluorescence was due to nonspecific immunoglobulins, protein C23 antibodies were affinity-purified as described in Materials and Methods. 3 h after microinjection, the affinity-purified antibody was localized in the cytoplasm and nucleolus of the cell (Fig. 3b), similar to the result obtained for the Ig fraction of antiserum against protein C23. However, by 17 h the affinity-purified antibody was localized almost exclusively to the nucleolus (Fig. 3d), producing a fluorescence staining pattern in those cells that were microinjected similar to that obtained by indirect immunofluorescence (Fig. 2d). Furthermore, the antibodies remained in the nucleolus for at least 36 h after microinjection (Fig. 3f). Because we have observed nucleolar fluorescence in 95% of the cells microinjected with affinity-purified antibody at 3 h after microinjection, it appears that IgG molecules were able to penetrate the nuclear envelope. Their rate of entry into the nucleus, however, was apparently limited because it took several hours to achieve maximum fluorescence. Our data suggest that once antibodies are bound to nucleolar structures, they remain associated for at least 36 h.

To determine whether the localization of the microinjected antibodies corresponded to those structures that contain protein C23, the following experiment was performed. Affinity-purified antibodies to protein C23 were microinjected into cells; the cells were fixed 17 h after microinjection and subsequently incubated with mouse monoclonal antibody to protein C23. The cells were then double-labeled with fluorescein isothiocyanate-conjugated goat anti-mouse and rhodamine-conjugated goat anti-rabbit antibodies. The immunofluorescent staining observed with monoclonal antiprotein C23 antibody showed, as in Fig. 2f, that protein C23 was localized in nucleoli (Fig. 4b), including small juxtanucleolar structures (arrows). In those cells that were microinjected with affinity-purified antiprotein C23 antibodies (Fig. 4a), the antibodies localized in the same nucleolar and juxtanucleolar structures. The three cells in Fig. 4a that do not show nucleolar fluorescence were not microinjected. The three microinjected cells in Fig. 4b are labeled with the mouse monoclonal antibody to the same extent as noninjected cells, suggesting that the microinjected antibody is substoichiometric to protein C23 in the nucleolus. We have not attempted to microinject the mouse monoclonal antibody to protein C23.

To determine whether the microinjected antibodies pass through the nuclear envelope complexed with newly synthesized protein C23, we microinjected affinity-purified antibodies into cells in which >90% of protein synthesis was inhibited with 1.0 μg/ml cycloheximide. Cells were preincubated for 30 min in the presence of 1.0 μg/ml cycloheximide,
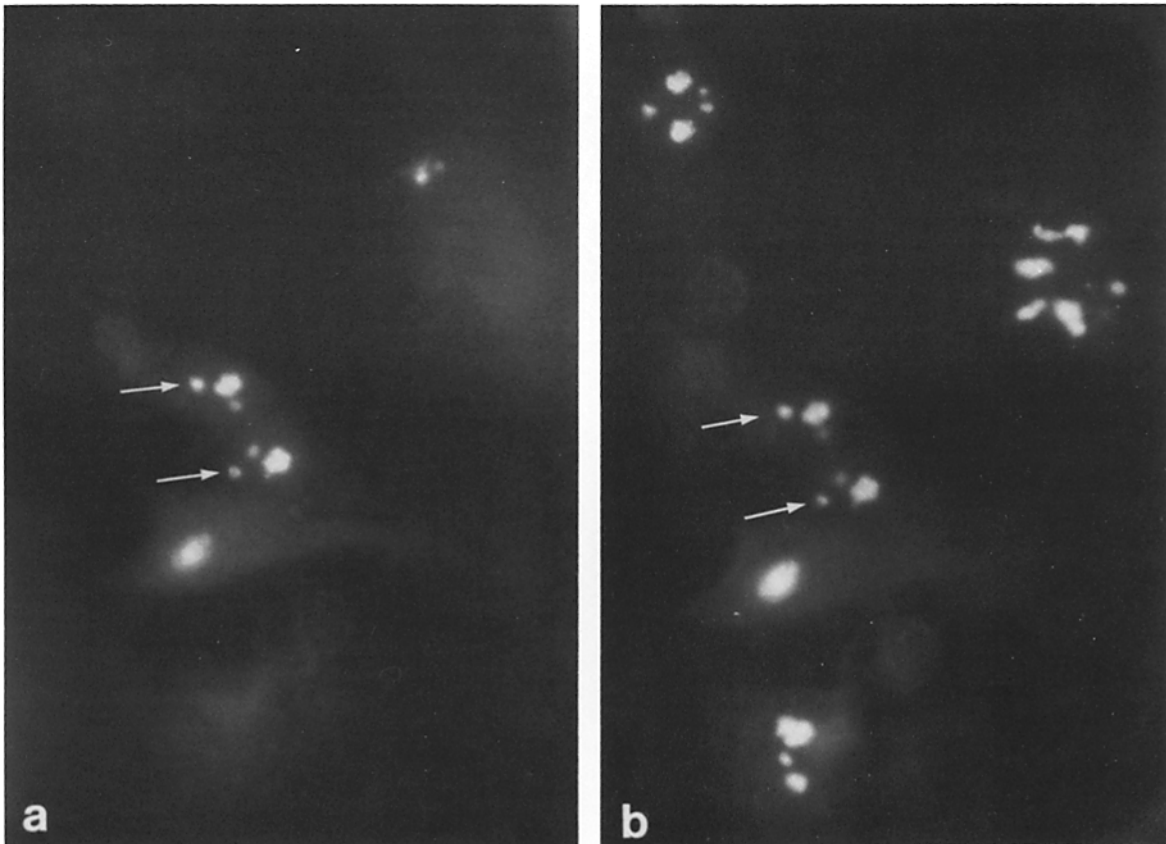

FIGURE 4 Localization of protein C23 in cells microinjected with antiprotein C23 antibodies. Rat carcinoma cells were microinjected with affinity-purified rabbit antiprotein C23 antibodies. Cells were fixed 17 h after microinjection and incubated with mouse monoclonal antibody as described in Materials and Methods. Antibodies were localized by double indirect immunofluorescence using rhodamine-conjugated goat anti-rabbit IgG and fluorescein-conjugated goat anti-mouse Ig. (a) Rhodamine fluorescence demonstrating localization of microinjected antibodies; (b) fluorescein fluorescence demonstrating localization of endogenous protein C23 in microinjected cells, as well as noninjected cells. × 1,030.
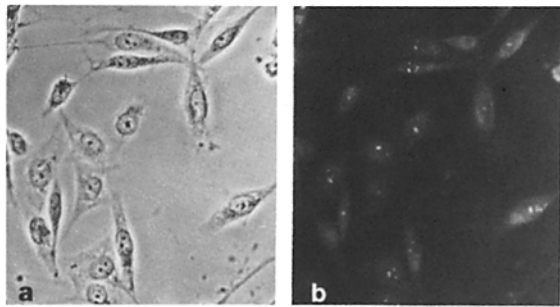
FIGURE 5 Localization of microinjected antibodies in rat carcinoma cells incubated with cycloheximide. Cells were preincubated for 30 min with 1.0 µg/ml cycloheximide, microinjected with affinity-purified protein C23 antibodies, and further incubated in the presence of cycloheximide for 17 h as described in the text. Antibodies were localized by immunofluorescence as described in Materials and Methods. Phase contrast (a) and immunofluorescent image (b) of cells 17 h after microinjection. × 185.

microinjected with affinity-purified anti-C23 antibody, then incubated for either 3 or 17 h in the presence of cycloheximide. To insure that protein synthesis was inhibited during the microinjection procedure, all buffers contained 1.0 µg/ml cycloheximide. The localization of the antibody was similar in control and cycloheximide-treated cells at both time points. By 17 h, the antibody was localized primarily in the nucleolus as shown in Fig. 5. Thus, it does not seem likely that antibodies enter the nucleus complexed to newly synthesized proteins.

DISCUSSION

This study shows that preimmune IgG molecules, microinjected into rat carcinoma cells by RBC-mediated microinjection, remain localized in the cytoplasm up to 36 h after microinjection, in agreement with previous reports (2, 25). It has been reported that bovine IgG molecules microinjected into Friend erythroleukemia cells associate with nuclei (37), but it is not clear whether these molecules actually entered the nucleus or bound to the nuclear membrane during cell fractionation (37).

In contrast to the results obtained with preimmune IgG molecules, we found that antibodies to nucleolar protein C23 penetrated the nuclear envelope. Affinity-purified antibodies were found in the cytoplasm and nucleolus 3 h after microinjection. By 17 h the antibodies were localized almost exclusively in the nucleolus, with little antibody in the cytoplasm or nucleoplasm. Several possibilities could account for the appearance of the protein C23 antibodies in the nucleus while preimmune immunoglobulins were excluded. First, if the fixation of microinjected cells was insufficient, it is possible that, when the cells were permeabilized with acetone, free antibodies could diffuse into the nucleus and bind protein C23 in the nucleolus. Arguing against this possibility is that there was a shift in the localization of the antibody from the cytoplasm to the nucleolus with time following microinjection (Fig. 3, b and d). Because the cells were fixed identically at all times points, this shift in localization can not be attributed to the fixation conditions. Furthermore, increasing fixation times from 15 to 30 min did not change the distribution of microinjected antibodies (data not shown).

A second possibility is that protein C23 antibodies bind to protein C23 during mitosis, when the nuclear envelope is absent, and remain bound to protein C23 when the nucleus reforms. This possibility is also ruled out because >95% of those cells microinjected with protein C23 antibodies exhibit nucleolar fluorescence 3 h after microinjection (Fig. 3b). During this time period <18% of the cells would have gone through mitosis.

A third mechanism that could account for the nucleolar localization of microinjected protein C23 antibodies is that the antibodies bind to newly-synthesized protein C23 and are transported to the nucleolus complexed with protein C23. We excluded this possibility by microinjecting affinity-purified antibodies into cells in which >90% of the protein synthesis was inhibited with cycloheximide. The distribution of the antibody in control and cycloheximide-treated cells was similar at 3 h and, as is shown in Fig. 5b, at 17 h after microinjection. Thus, entry of microinjected antibodies into the nucleus does not appear to depend on protein synthesis.

We consider more likely the possibility that antibodies can penetrate the nuclear envelope but are limited in their rate of entry by their size (2, 25). Nonimmune immunoglobulins do not bind nuclear structures but apparently equilibrate between the nucleus and cytoplasm, perhaps as a function of their molecular size. Antibodies specific for nuclear proteins enter the nucleus like nonspecific immunoglobins, but they bind nuclear proteins with the affinity typical of antigen-antibody reactions. Thus, we interpret the localization of antibodies to protein C23 in the nucleolus to reflect binding to nondiffusible nucleolar components. We do not know whether the accumulation of specific antibodies in the nucleolus is diffusion-limited or due to a nuclear transport system. Feldherr et al. (11) have recently reported that the nuclear uptake of a 148,000-mol wt nuclear protein in frog oocytes occurs at a rate greater than can be accounted for by simple diffusion. Accordingly, there may be a transport system responsible for the nuclear accumulation of this and perhaps other high molecular weight proteins.

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