Optimal Loading of Scraped HeLa Cells with Monoclonal Antibodies to the Proliferation-associated M, 120,000 Nucleolar Antigen

James W. Freeman, Jeffrey E. Hazlewood, Patricia Auerbach, and Harris Busch

Department of Pharmacology, Baylor College of Medicine, Houston, Texas 77030

ABSTRACT

Our laboratory has reported (J. W. Freeman et al., Cancer Res., 48: 1244-1251, 1988) a proliferation-associated M, 120,000 nucleolar antigen (p120), that was found in human tumors but was not detectable in most normal resting tissues and in benign tumors. To study further the function and the localization of this protein, we have investigated various methods of microinjecting p120 monoclonal antibodies into cells. For comparison, we have used a monoclonal antibody to protein C23, a nucleolar protein found in high levels in most cells.

To determine optimal conditions for loading of antibodies to nucleolar antigens into mechanically disrupted HeLa cells, we studied the effects of ionic strengths of loading buffers, various antibody concentrations, and optimal time for loading and antibody localization. With ascites fluids in isotonic buffer containing antibodies to nucleolar proteins p120 and C23, a maximum number of cells, 86 and 84%, respectively, were loaded following a 20-min incubation. Hypertonic buffers decreased the percentage of cells loaded (22%); hypertonie buffers reduced cell viability.

The optimal concentration of purified antibody yielding a maximum number of loaded cells (81%) was 2.5 mg/ml. Higher concentrations of antibody resulted in residual cytoplasmic staining without increasing the percentage of loaded cells. With antibody concentrations less than 2.5 mg/ml, a linear decrease was noted in the percentage of cells loaded with a decrease in intensity of fluorescence.

Following antibody loading, nucleolar fluorescence was observed by 12 h and the intensity increased at 24 h. Localization of the p120 antibody was followed through mitosis where it was perichromosomal and equally divided between the chromosomes at metaphase. A decrease of nucleolar immunofluorescence intensity and percentage of cells labeled were observed in successive cell generations.

INTRODUCTION

The direct injection of cells with specific proteins or their antibodies has proven to be a useful technique in functional analysis (1-9). Microinjection of antibodies into cells also provides means for determining antigen localization and for following the cellular distribution of the antigen during the cell cycle.

Methods which have been developed to microinject antibodies into cells are microneedle injection (10, 11), RBC-mediated microinjection (12), and scrape loading of macromolecules into the cytoplasm of cells (13). Microneedle injection is tedious and only small numbers of cells can be injected at any one time. RBC-mediated microinjection requires loading of RBC with antibody and then fusing the loaded cell with the target cell with the use of Sendai virus. Scrape loading of antibodies into cells is a simple and rapid method of introducing macromolecules into cells (14). It requires the mechanical scraping of adherent cells so as to create "micro" tears in the cell membrane; this allows penetration of the molecules being loaded.

Received 2/1/88; revised 5/23/88; accepted 6/17/88.

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

1 These studies were supported by the Cancer Research Center Grant CA-10893, P1, awarded by The National Cancer Institute, Department of Health and Human Services; the DeBakey Medical Foundation; the Davidson Fund; the Pauline Sterne Wolff Memorial Foundation; H. Leland Kaplan Cancer Research Endowment; Linda and Ronny Finger Cancer Research Endowment Fund; and The William S. Farish Fund.

2 To whom requests for reprints should be addressed.

Studies (15, 16) suggested that proteins microinjected into the cytoplasm might be excluded from the nuclear compartment on the basis of size. However, this appears not to be the case for IgG molecules. Bennett et al. (17) previously showed that antibodies to nucleolar protein C23 could be microinjected into the cytoplasm of cells. The antibodies localized to the nucleolus within 17 h in the presence or absence of cycloheximide. These studies indicated that the nuclear envelope does not exclude entry of antibody molecules. The entry of the antibody into the nuclear component was not dependent on binding of the antibody to antigen in the cytoplasm for active transport through nuclear pores.

In this study, we determined the optimal conditions for maximum loading and specific localization of antibodies to nucleolar proteins C23 and p120 (17, 18) into mechanically torn cells. We also present data suggesting that the redistribution of the p120 nucleolar antigen is associated with the redistribution of chromosomes during mitosis. This method provides a simple procedure for introducing nuclear antibodies into cells for the purpose of assessing function and antigen distribution during the cell cycle.

MATERIALS AND METHODS

Antibody Preparation and Purification. Monoclonal antibodies to nucleolar proteins C23 and p120 were obtained from mouse ascites. The ascites fluid was centrifuged at 1000 × g for 20 min, divided into three equal aliquots, and dialyzed separately against an isotonic buffer (PBS, 0.01 M phosphate, 0.15 M NaCl, pH 7.2), a hypertonic buffer (0.01 M phosphate, 0.50 M NaCl, pH 7.2), or a hypotonie buffer (0.01 M phosphate, 0.01 M NaCl, pH 7.2). When purified monoclonal antibodies were used, antibody was purified from mouse ascites on Pierce MonoPure Protein A columns according to the manufacturer's directions (Pierce Chemical Co., Rockford, IL). Purified antibodies were dialyzed as above and concentrated by ultrafiltration to a final concentration of 5 mg/ml.

Scrape Loading. HeLa cells were plated in 24-well culture plates (2.5 × 10^5 cells/well in 0.5 ml medium) in DMEM containing 10% fetal calf serum. Cells were allowed to attach for 24 h before the medium was aspirated from the culture wells. 100 μl of antibody solution were added to the well and each well was scraped by 10 complete rotations with a plastic cell scraper (Costar, Cambridge, MA). The tip of the cell scraper was first cut with a sterile razor blade to match the circumference of the well. The scraped cells suspended in the antibody solution were then incubated for various times (see "Results") at 37°C. Cells were removed from the culture wells and placed in a 15-ml conical tube; 10 ml of DMEM were added and gently mixed with a pipet. The suspension was centrifuged at 250 × g for 10 min and medium was decanted. Cells were resuspended in 0.5 ml DMEM containing 10% fetal calf serum and replated in 24-well plates.

Antibody Localization. Antibody localization was determined at 5, 12, 24, 36, 48, and 96 h by indirect immunofluorescence. Briefly, cells were removed from wells by trypsinization and washed in 10 ml of DMEM. Following centrifugation cells were diluted in DMEM containing 10% fetal calf serum to a final concentration of 5.0 × 10^5 cells/ml and 100-μl aliquots were attached to slides by cytocentrifugation (19). Immunofluorescence localization was determined using fluorescein-conjugated goat anti-mouse IgG (Abbott Laboratories, North Chicago, IL) at 1:50 dilution. Three equal aliquots of antibody were incubated with cells on slides and then analyzed for staining using epifluorescence microscopy. The optimal concentration of purified antibody yielding a maximum loading and specific localization of antibodies to nucleolar proteins C23 and p120 into mechanically torn cells was 2.5 mg/ml.

The abbreviations used are: p120, M, 120,000 protein; PBS, phosphate-buffered saline (0.01 M phosphate, 0.15 M NaCl, pH 7.2); DMEM, Dulbecco's modified Eagle's minimal essential medium.
SCRAPE LOADING OF NUCLEOLAR ANTIBODIES

Table 1 Comparison of conditions for intracellular loading of monoclonal antibodies to nucleolar antigens p120 and C23 following mechanical disruption of HeLa cells

<table>
<thead>
<tr>
<th>Loading time (min)</th>
<th>% of cells loaded</th>
<th>% of viable cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anti-p120</td>
<td>Anti-C23</td>
</tr>
<tr>
<td>0.5</td>
<td>6.5</td>
<td>4.0</td>
</tr>
<tr>
<td>2</td>
<td>8.6</td>
<td>ND*</td>
</tr>
<tr>
<td>5</td>
<td>12.6</td>
<td>ND</td>
</tr>
<tr>
<td>10</td>
<td>12.2</td>
<td>ND</td>
</tr>
<tr>
<td>20</td>
<td>22.8</td>
<td>17.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antibody buffer</th>
<th>% of cells loaded</th>
<th>% of viable cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypotonic</td>
<td>Anti-p120</td>
<td>Anti-C23</td>
</tr>
<tr>
<td>5.0</td>
<td>23.1</td>
<td>94</td>
</tr>
<tr>
<td>9.4</td>
<td>20.1</td>
<td>98</td>
</tr>
<tr>
<td>8.5</td>
<td>25.3</td>
<td>93</td>
</tr>
<tr>
<td>32.0</td>
<td>54.2</td>
<td>94</td>
</tr>
<tr>
<td>86.0</td>
<td>84.3</td>
<td>91</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Isotonic</th>
<th>Anti-p120</th>
<th>Anti-C23</th>
<th>Anti-p120</th>
<th>Anti-C23</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>ND</td>
<td>93</td>
<td>ND</td>
<td>93</td>
</tr>
<tr>
<td>2</td>
<td>ND</td>
<td>17.5</td>
<td>ND</td>
<td>17.5</td>
</tr>
<tr>
<td>5</td>
<td>ND</td>
<td>91</td>
<td>ND</td>
<td>91</td>
</tr>
<tr>
<td>10</td>
<td>ND</td>
<td>93</td>
<td>ND</td>
<td>93</td>
</tr>
<tr>
<td>20</td>
<td>ND</td>
<td>93</td>
<td>ND</td>
<td>93</td>
</tr>
</tbody>
</table>

* ND, not determined.

RESULTS

Scrape Loading of Monoclonal Antibody Ascites Fluid into HeLa Cells. Initial studies were carried out to determine the optimal conditions for antibody loading following mechanical disruption of cells. Cells that were not scraped and incubated in the presence of antibody did not take up antibody (not shown). As shown in Table 1, an increase in the percentage of antibody-loaded cells was observed with increasing incubation times up to 20 min for both isotonic and hypotonic conditions, the cell viability was maintained at greater than 90%. With hypertonic conditions and for hypotonic and isotonic conditions where incubation times were greater than 20 min, decreased cell viability was observed (not shown). Approximately 4 times greater loading efficiency was observed by using isotonic conditions compared to hypotonic conditions with a loading time of 20 min. Similar amounts were loaded at 20 min for the monoclonal antibodies to both nucleolar antigen p120 and protein C23 (86% for p120 and 84% for C23). Similar results were obtained with purified antibodies as shown below.

Specific Localization of Monoclonal Antibodies to the Nucleolus. Indirect immunofluorescence of HeLa cells by using monoclonal antibodies to nucleolar antigens p120 and protein C23 showed that both antigens are predominantly localized to the nucleolus (Fig. 1).

Fig. 1. Indirect immunofluorescence of HeLa cells with monoclonal antibodies to nucleolar antigen p120 (A) and to nucleolar protein C23 (B). The primary monoclonal antibody was added to fixed and permeabilized HeLa cells cytocoentrifuged to slides. The primary antibody was detected with fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG.

In this study, we have used a modification of the scrape loading method of McNeil et al. (13) to inject cells with antibody-loaded cells was determined by immunofluorescence at 24 h after loading. A maximum of 81% of the cells were labeled (75-84% in three separate experiments) with an antibody concentration of 2.5 mg/ml (Fig. 3). Increasing the concentration of antibodies above 2.5 mg/ml did not result in an increase in the percentage of cells loaded (Fig. 3). A linear decrease in the percentage of antibody-loaded cells (Fig. 3) as well as a decrease in nucleolar staining intensity was noted at concentrations below 2.5 mg/ml of antibody (Fig. 4, A-F).

Purified p120 monoclonal antibody loaded at a concentration of 5 mg/ml (Fig. 4A) resulted in a significant increase in cytoplasmic staining compared to 2.5 mg/ml (Fig. 4B), but there was no increase in the number of loaded cells (Fig. 3). This result suggests that the increased cytoplasmic staining at antibody concentrations greater than 2.5 mg/ml represents excess antibody.

DISCUSSION

In this study, we have used a modification of the scrape loading method of McNeil et al. (13) to inject cells with antibody.
bodies to nucleolar antigens. In contrast to McNeil et al. (12), who incubated scraped cells for 10 s with macromolecules, we found that antibody penetration required a 20-min incubation in antibody containing medium for optimal loading.

It is probable that the number of micro tears created by scraping relates to the degree of cell attachment to substratum. Accordingly, HeLa cells may require a longer loading time than well-spread fibroblasts for penetration of equal numbers of molecules. The size of the macromolecules loaded may be important. Smaller molecules such as ovalbumin ($M_r, 30,000$) may be less likely to be excluded than whole immunoglobulins ($M_r, 150,000$). This study suggests that a loading time of 20 min does not significantly decrease cell viability and yields a maximum percentage of HeLa cells loaded with antibody. This longer incubation time may be critical for larger molecules such as antibodies.

Another factor relating to nucleolar binding sites is that a greater concentration of antibody may be necessary for uptake into the nucleolus than would be required for cytoplasmic antigens since these antibodies must also penetrate the nuclear envelope. It seemed possible that antibodies might bind to nucleolar proteins during mitosis when the nuclear envelope is disrupted. However, mitosis does not seem to be related to their antibody binding since most cells (>80%) showed nucleolar localization by 12 h (Fig. 2B). It is unlikely that this high percentage of cells would have gone through mitosis in nonsynchronized cultures. Moreover in unscraped controls, no antibody uptake was observed. We have shown earlier that antinucleolar antibodies penetrate the nuclear envelope when microinjected into the cytoplasm (16). It is probable that this process reflects passive diffusion throughout the nuclear envelope since treatment of cells with cycloheximide did not influence nuclear penetration (17).

In agreement with Bennett et al. (17), the antibodies used had some nucleolar localization at 5 h and more at 24 h. Microinjection of ascites fluid exhibited similar characteristics as purified antibodies (5 mg/ml); the same type of localization and intensity of nucleolar staining were noted (Figs. 2 and 4).
SCRAPE LOADING OF NUCLEOLAR ANTIBODIES

Fig. 4. Immunofluorescence detection of purified p120 monoclonal antibody 24 h after loading, showing the effect of antibody concentration on efficiency of loading. Concentration of purified antibody was (A) 5.0 mg/ml; (B) 2.5 mg/ml; (C) 1.25 mg/ml; (D) 0.63 mg/ml; (E) 0.31 mg/ml; and (F) 0.16 mg/ml. Immunofluorescence detection was performed as described in “Materials and Methods.”

Based on purification studies, the ascites fluids for proteins p120 and C23 contained between 5 and 7 mg of antibody/ml of ascites. The total protein concentration of the ascites fluid was approximately 100 mg/ml. Accordingly, ascites fluids can be used for localization studies.

Schlegel and Rechsteiner (9) indicated that loading of RBC with macromolecules was optimal in hypotonic buffers. We compared hypotonic, isotonic, and hypertonic buffers for the antibody loading of scraped HeLa cells. Hypotonic buffers resulted in only low percentages of cells loaded (approximately 20%) compared to 80% for loading with isotonic buffer at 20-min loading times; good viability was maintained (>90%) (Table 1). Hypertonic buffer resulted in poor viability of loaded cells (<50% viable) at loading times of 20 min (not shown). Hypotonic buffers may result in diffusion of essential cytoplasmic elements of HeLa cells into the surrounding hypotonic media; this phenomenon may not occur with red cell ghosts or broken RBCs that are relatively free of cytoplasmic elements and are not nucleated. Loading of red cell ghosts essentially traps antibody molecules and hypotonic buffers may aid this process by altering spatial relations within the RBC ghost membrane.

The percentage of cells loaded is dependent upon the antibody concentration and loading time. Under these conditions, not all of the cells were loaded; a maximum of approximately 85% loaded cells was found with 2.5 mg/ml of purified antibody. Cells which do not absorb antibody may have failed to tear during the scraping process, possibly by virtue of not being tightly attached to the substratum.

The methods and conditions presented in this study reflect optimal parameters for loading the maximum percentage of cells with antibodies. This study provides further evidence that antibodies microinjected into the cytoplasm of cells penetrate the nucleus and attach specifically to intranuclear antigens. Accordingly, this method offers a means for assessing the function and localization of nuclear or nucleolar proteins. In this regard, the p120 nucleolar antigen was shown to localize to or around the chromosomes during mitosis; the antigen appeared to be distributed between daughter cells. This method should offer a means of injecting other macromolecules into the nucleus of cells including proteins shown to possess sequences that result in nuclear localization.

REFERENCES


5249
SCRAPE LOADING OF NUCLEOLAR ANTIBODIES


Optimal Loading of Scraped HeLa Cells with Monoclonal Antibodies to the Proliferation-associated $M_r$ 120,000 Nucleolar Antigen

James W. Freeman, Jeffrey E. Hazlewood, Patricia Auerbach, et al.