Histochemistry (1989) 91:81–88

Histochemistry

A simple fixation procedure for immunofluorescent detection of different cytoskeletal components within the same cell

U. Vielkind* and S.H. Swierenga
Drug Toxicology Division, Health and Welfare Canada, Ottawa, Ontario, Canada K1A OL2

Received December 14, 1987 / Accepted July 14, 1988

Summary. In recent studies on the cytoskeletal organization of T51B rat liver cells by indirect immunofluorescence microscopy, we have been unable to achieve double-staining of microtubules and intermediate filaments within the same cell. In acetone-fixed cells, microtubules were poorly preserved, and two out of three monoclonal antibodies tested did not stain them properly. In formaldehyde-fixed cells, the monoclonal anti-cytokeratin produced an incomplete staining pattern against a diffuse background. We have now developed a fixation protocol which includes simultaneous fixation and extraction with formaldehyde and nonionic detergent in the present of microtubule stabilization buffer. Although developed for a specific purpose, it is of general application as it yields excellent preservation of all cytoskeletal components tested so far, without masking antigenic determinants. The procedure is both simple and fast and will, therefore, be valuable for efficient processing of samples from large-scale experiments, such as the screening for cytoskeletal changes during longterm treatment of cells with drugs or carcinogens.

Introduction

Normal cells in culture are characterized by a highly organized cytoskeleton (Brinkley 1982), the structure and composition of which are specific for the cell type. Some cytoskeletal components undergo specific alterations during the cell cycle, while others remain largely unchanged. Neoplastic transformation of cells is often associated with a disorganization in one or more of the cytoskeletal elements. As the individual structures seem to be highly interconnected (Schliwa and van Blerkom 1981; Singer et al. 1982), an alteration in the organization of one component may induce an organization change in one or more of the other components as well as in cell morphology. For example, some cell types rearrange their intermediate filaments while going through mitosis and restore the original pattern as soon as the microtubules have assumed their interphase configuration (Lane et al. 1982; Franke et al. 1982).

When investigating the effects of specific environmental factors or drugs on the organization of the cytoskeleton in cultured cells, it is therefore important to examine more than one of its components within the same cell and to relate any changes to cell morphology and to specific stages of the cell during mitosis. This can be done by double-staining for two cytoskeletal elements with different fluorescent labels (Hynes and Destree 1978; Geiger and Singer 1980), subsequent counterstaining of nuclei and chromosomes with a fluorochrome (Hilwig and Gropp 1972), and correlating the fluorescent patterns with the phase contrast image of the cell (Berlin et al. 1979).

We have recently become interested in looking for changes in the cytoskeleton of T51B rat liver cells that have been treated with drugs (in particular griseofulvin) or certain heavy metals (such as nickel) which are carcinogenic. In earlier investigations of the normal cytoskeletal organization of T51B cells by indirect immunofluorescence microscopy (Marceau and Swierenga 1985), microtubules were studied in cells fixed with phosphate-buffered formaldehyde (fixation by cross-linking of proteins), while intermediate filaments of the cytokeratin and vimentin types were studied in cells fixed with either cold acetone, methanol, or ethanol (fixation by precipitation of proteins). Until recently, we have been unable to demonstrate microtubules and cytokeratin filaments within the same cell when applying the conventional fixation protocols and monoclonal antibodies. Microtubules were hardly visible in precipitation-fixed cells, whereas cytokeratin filaments produced an incomplete and faint staining pattern in formaldehyde-fixed cells. Although staining of both these structures were successful when cells were detergent-extracted under conditions described in the literature (Schliwa and van Blerkom 1981; Schliwa et al. 1981), the preservation of the original microtubule pattern was very poor. In contrast, vimentin filaments as well as actin microfilaments could be demonstrated after both types of fixation, although with different staining intensities.

For studying microtubules and other cytoskeletal components within the same cell, we have developed a protocol which yields excellent preservation of structure and antigenicity of all cytoskeletal components tested so far, while maintaining details of cell morphology in phase contrast. The procedure is fast and simple and includes the simultaneous fixation and extraction of cells with formaldehyde and a nonionic detergent in the presence of a microtubule stabilization buffer. Although developed for a specific purpose, the protocol should be of general application to any detergent-resistent component within cells or in the extracellular matrix. It has already been used successfully for the detection of neurofilaments and microtubule-associated

* Present address: Department of Neurobiology and Anatomy, University of Rochester, School of Medicine, Rochester, NY 14642, USA

proteins in early neurons developing from retinoic acid-stimulated embryonal carcinoma cells (Falconer et al. 1987; Vielkind, Falconer, and Brown, manuscript submitted).

Materials and methods

Cell cultures. T51B rat liver cells were dissociated by incubation with 0.125% trypsin (1:250, Difco) in Puck's calcium and magnesium-free saline G (Puck et al. 1958) for 20 min at 4° C and subsequent gentle pipetting in William's medium E (GIBCO) containing 10% fetal bovine serum (Dextran Products). Cells were plated in a small volume of medium (0.2–0.5 ml) on glass coverslips in 35-mm dishes at low densities ($1–2 \times 10^4$ cells per 22×22 mm coverslip), fed with fresh medium (2 ml) after 1–2 h, and incubated at 37° C in humidified atmosphere containing 5% CO_2.

Fixation of cells. After 2–3 days, colony cultures with a high incidence of mitotic figures were fixed in different ways (see Table 1) and processed for indirect immunofluorescence microscopy. All steps were carried out in the tissue culture dishes. Except for the first washing step, all washes and antibody dilutions were done with calcium- and magnesium-free phosphate-buffered saline (PBS, pH 7.0). Before adding fixative or antibody solution, excess fluid was carefully aspirated with a pasteur pipette. Fixatives were prepared prior to use by mixing 1 part of formalin (37% formaldehyde) and 9 parts of buffer with or without 0.2% Triton X-100. The buffer used was either calcium- and magnesium-free PBS or microtubule stabilization buffer (PEM buffer; 100 m*M* PIPES, 5 m*M* EGTA, 2 m*M* $MgCl_2$, pH 6.8; Soltys and Borisy 1985). The best results for routine investigations were obtained with formaldehyde in PEM buffer containing Triton X-100 (Form/PEM/TX).

In detail, the following protocol was used: Medium and serum were removed by washing the cultures twice with PBS containing calcium and magnesium. Immediately before fixation, each culture was briefly rinsed twice with PBS and drained well. It was then fixed and extracted for 5 min at room temperature, either simultaneously in the same solution or in two separate steps (for details, see Table 1) and postfixed with ice-cold methanol, without any washing steps in between. (It was important to use methanol rather than ethanol or acetone to avoid precipitation of EGTA from the stabilization buffer. For visualization of actin filaments with phalloidin, where methanol had to be avoided, the fixed and extracted cells were washed with PBS and postfixed with ice-cold ethanol).) Other cultures were fixed with ice-cold methanol or ethanol only. All fixed cells were washed once with PBS and rehydrated in PBS for at least 30 min before being processed for immunofluorescence microscopy. Samples prepared in this way could be stored for several weeks in PBS containing 0.02% sodium azide, without significant changes in morphological details or loss of antigenicity.

Immunofluorescence microscopy. The following primary antibodies were used: mouse monoclonal antibodies (IgG) against yeast tubulin (purchased from Viklický et al., Prague, Czechoslovakia; Viklický et al. 1982), against flagellar tubulin of the green alga *Polytomella* (a gift from D.L. Brown, University of Ottawa; Aitchison and Brown 1986), against rat liver 55 kDa cytokeratin and against rat liver vimentin (gifts from N. Marceau, Laval University; Marceau et al. 1986), and a rat monoclonal antibody (IgG) against yeast tubulin (Sera Laboratories, clone YOL1/34; Kilmartin et al. 1982). The following secondary antibodies were used: fluorescein-conjugated goat anti-mouse IgG serum (Rego Enterprises), rhodamine-conjugated rabbit anti-mouse IgG serum (cross-absorbed with rat serum) and fluorescein-conjugated rabbit anti-rat IgG serum (cross-absorbed with mouse serum; both from Zymed Laboratories). Rhodamine-conjugated phalloidin (Molecular Probes) was used to demonstrate actin microfilaments (Wulf et al. 1979). Each coverslip was drained while still in the culture dish, overlaid with 50 µl of a 1:100 to 1:500 dilution (in PBS) of the primary antibody, and incubated at room temperature for 30 min. After 3 washes with PBS, the coverslips were incubated with 50 µl of a 1:100 dilution (in PBS) of the secondary antibody. Controls were run by omitting the primary antibody. For double-staining, the samples were incubated with a mixture of one rat and one mouse monoclonal antibody, followed by a mixture of fluorescein-conjugated anti-rat IgG and rhodamine-conjugated anti-mouse IgG (Osborn and Weber 1982). Alternatively, samples were first stained with rat or mouse primary antibody and the corresponding fluorescein-labeled secondary antibody, and then incubated with a 1:20 dilution of rhodamine-labeled phalloidin. After 3 washes with PBS, the samples were counterstained with Hoechst 33258 fluorochrome (Aldrich) in PBS (0.15 µm/ml) for 2 min (Hilwig and Gropp 1972). After 3 washes with PBS, they were briefly dipped into distilled water, drained, and mounted in a semipermanent mounting medium (Elvanol/Tris/glycerol, pH 8.6; Heimer and Taylor 1974) to which either 0.1% *p*-phenylene diamine (Aldrich) or 2.5% DABCO (1,4-diazobicyclo-(2,2,2)-octane, Aldrich) had been added to prevent fading of the fluorescein label during microscopic observation (Johnson et al. 1982). The slides could be stored at −20° C or +4° C for several months without changes in the intensity or distribution of the fluorescent antibody label. Slides stained for actin showed diffusion of the rhodamine phalloidin label after a few weeks.

The cells were examined with a Zeiss photomicroscope equipped with epifluorescence and phase contrast optics, using immersion oil objectives (Plan-Neofluar/Ph3 40× and Planapo 40×) and the appropriate filter combinations for detecting fluorescein, rhodamine, and Hoechst fluorochrome. Photographs were taken with Kodak Ektachrome 400 and Kodak Tri-X Pan films, using automatic exposure times at 400 ASA setting for phase contrast and Hoechst stain, or manual exposure times of 5 to 60 s, depending on the intensity of the fluorescence. Tri-X Pan films were developed with Rodinal (1:100, Agfa) for 30 min at room temperature.

Results

Experimental design

The aim of the present study was to find conditions which would allow fixation and staining of microtubules and cytokeratin filaments within the same T51B cell. There are several reasons why previous efforts might have failed to produce satisfactory results: (*i*) Precipitation fixation with cold solvents may induce tubulin depolymerization and, hence, fragmentation or loss of microtubules, especially when extracellular calcium ions have not been removed prior to fixation. (*ii*) The fixative may have altered the antigenic determinant (epitope) required to bind the monoclonal antibody to tubulin or cytokeratin, respectively. (*iii*) The antigenic determinant may be masked by proteins associated with cytoskeletal structures. (*iv*) Detergent extraction may induce microtubule disassembly due to the release of calcium ions from intracellular stores.

To eliminate these possibilities, we tested the following modifications of the fixation procedure: (*i*) Extracellular calcium ions were removed by briefly washing the cells with calcium-free PBS prior to fixation or extraction. (*ii*) Different monoclonal antibodies against tubulin were used to demonstrate microtubules in precipitation-fixed cells. (*iii*) The cells were extracted with detergent before, during, or after formaldehyde fixation, respectively. (*iv*) Extraction was carried out in a microtubule stabilization buffer (PEM buffer; Soltys and Borisy 1985) containing relatively high concentrations of PIPES, EGTA, and magnesium.

Table 1. Effect of different procedures for fixation and/or extraction on the immunofluorescent detection of cytoskeletal components

Procedure	Morphological details in phase contrast	Microtubules (tubulin)						Intermediate filaments		Microfilaments (actin)
		A		B		C		(cytokeratin)	(vimentin)	
		cyt	sp	cyt	sp	cyt	sp			
Ice-cold ethanol	+	(±)	(++)	(±)	(+)	+++	++ *	+++	++	+
Ice-cold methanol	+	(±)	(++)	(±)	(+)	+++	++ *	+++	++	−
Form/PBS[a]	++	+++ *	+++ **	N.D.		+++ *	++ ***	(±) ***	++ **	+++
Form/PEM[a]	++	+++ *	++++ **	+++ *	++++ **	+++ *	+++ ***	(+) **	N.D.	N.D.
Form/PEM + PEM/TX[b]	++	+++	++++ *	+++	++++ *	+++	+++ *	(++) *	+++ *	N.D.
Form/PEM/TX[c]	+++	+++	++++	+++	++++	++++	+++	+++	+++	++++
PEM/TX[d] 10 min	(±)	(+)	(+)	N.D.		(+)	(+)	++++	++++	N.D.
4 min	(+)	(++)	(+++)	(±)	(+)	(++)	(++)	++++	++++	N.D.
1 min	(++)	+++	++++	(±)	(++)	++++	+++	++++	++++	++++

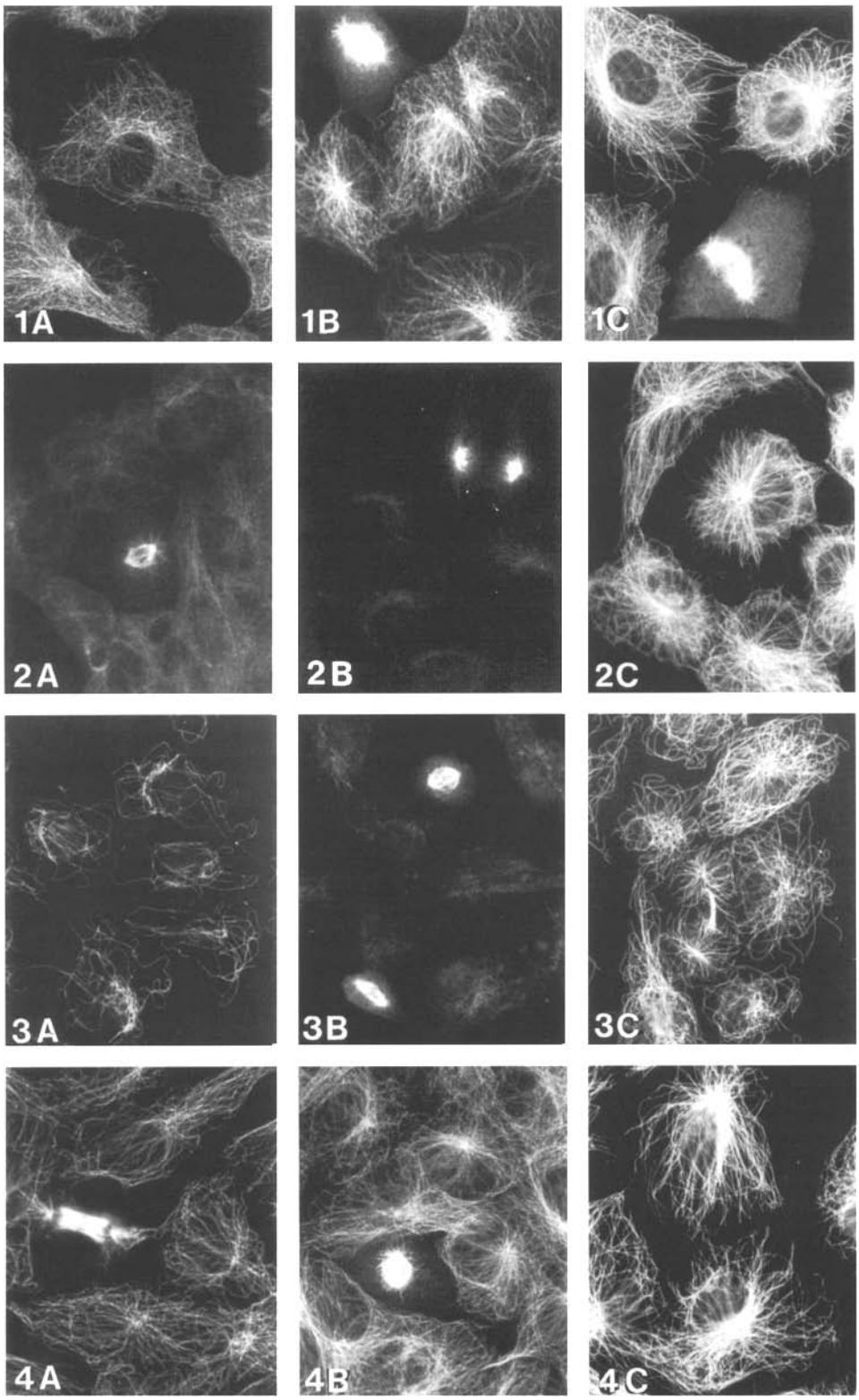
[a] Fixation in 3.7% formaldehyde in buffer (PBS or PEM)
[b] Fixation as in [a] but followed by extraction with 0.2% Triton X-100 in PEM buffer
[c] Simultaneous fixation and extraction in 3.7% formaldehyde, 0.2% Triton X-100 in PEM
[d] Extraction only, with 0.2% Triton X-100 in PEM
Procedures [a] to [d] were carried out at room temperature and were followed by postfixation in ice-cold methanol. For visualization of actin filaments, postfixation was carried out with ethanol rather than methanol.

Cytoplasmic (*cyt*) and spindle (*sp*) microtubules were demonstrated with mouse anti-yeast tubulin (*A*), rat anti-yeast tubulin (*B*), and mouse anti-*Polytomella* tubulin (*C*), respectively, and intermediate filaments were demonstrated with mouse anti-cytokeratin and mouse anti-vimentin, followed by fluorescein-labeled anti-mouse IgG or anti-rat IgG, respectively. Microfilament bundles were demonstrated with the actin-binding toxin phalloidin labeled with rhodamine. The symbols + and − stand for positive and negative staining, respectively, while the symbol * stands for diffuse background staining. Increasing numbers of symbols indicate an increase in staining intensity and/or structural details, while symbols in brackets () indicate an incomplete staining pattern and/or an abnormal arrangement of the structure in question. N.D., not done

Demonstration of microtubules following different fixation protocols

By using different monoclonal antibodies to tubulin, we were able to show that the structural preservation of microtubules in T51B cells by precipitation fixation was not a major problem, provided extracellular calcium ions had been removed prior to fixation. Microtubules were equally well preserved with the conventional fixatives, i.e. formaldehyde in PBS and cold ethanol or methanol (see column C in Table 1). Formaldehyde fixation produced a bright and typical microtubule pattern with all three anti-tubulins tested (Fig. 1A–C), although there were differences in the relative intensities of cytoplasmic versus spindle microtubules and in the intensity of a diffuse cytoplasmic background staining (Table 1). This background was particularly strong in cells at early mitotic stages (Fig. 1B and C) and was reduced when fixation was followed by detergent extraction. The replacement of PBS by PEM buffer during fixation improved the preservation of spindle details. When the cells were fixed with ethanol or methanol, however, only one out of three monoclonal anti-tubulins, namely the mouse antibody to *Polytomella* tubulin (C in Table 1), produced a bright staining pattern (Fig. 2C) similar to that observed in formaldehyde-fixed cells (Fig. 1C). The mouse and rat antibodies to yeast tubulin (A and B in Table 1) both resulted in a very faint (if any) staining pattern, in which the cytoplasmic microtubules appeared as faint dotted lines rather than continuous structures, while the mitotic spindles were more brightly stained but did not show many details (Fig. 2A and B). A similar pattern was obtained when formaldehyde-fixed cells were stained with a very low antibody concentration. The faint staining indicated that antigenic determinants were altered or masked during protein precipitation. As we intended to use the rat anti-tubulin (B in Table 1) in combination with a mouse anti-cytokeratin, precipitation fixation was not suitable for our purposes.

Brief detergent extraction of unfixed cells, followed by fixation with ethanol or methanol yielded a bright staining of cytoplasmic and spindle microtubules with both mouse anti-tubulins (Fig. 3A, C and A and C in Table 1) but, again, almost no staining of cytoplasmic microtubules with the rat anti-tubulin (Fig. 3B and B in Table 1). Obviously, the extraction procedure removed some cytoplasmic components which were masking the epitope required for binding one of the mouse anti-tubulins, while postfixation with ethanol or methanol altered the epitope for binding the rat anti-tubulin. However, the extraction of unfixed cells frequently resulted in a reduction or collapse of mitotic spindles and in a partial rearrangement or even loss of cytoplasmic microtubules (Fig. 3A and C). These features were more pronounced when the extraction period was pro-



Figs. 1–4. Preservation of microtubules in T51B rat liver cells fixed in different ways. The cells were stained with (**A**) mouse anti-yeast tubulin, (**B**) rat anti-yeast tubulin, or (**C**) mouse anti-Polytomella tubulin, respectively

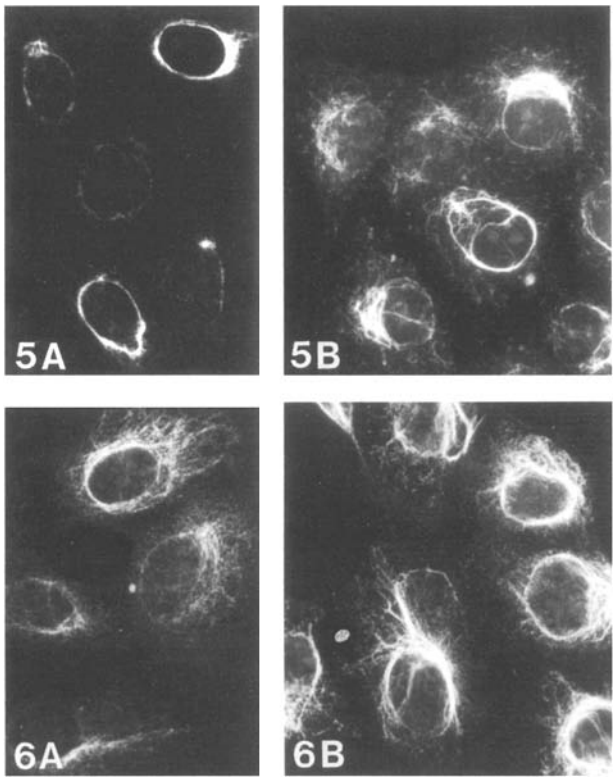
Fig. 1. Formaldehyde fixation (Form/PBS). Microtubules are well preserved and brightly stained with all three anti-tubulins. Note that cytoplasmic microtubules in **A and B** are less densely stained than in **C**, whereas staining of the mitotic spindle is more intense in **B** than in **C**. Note also the diffuse, cytoplasmic background staining in cells at prometaphase (**B and C**)

Fig. 2. Precipitation fixation with ice-cold ethanol or methanol. Microtubules are brightly stained only with one anti-tubulin (**C**). Cytoplasmic microtubules are hardly visible and mitotic spindles are incompletely stained in **A and B**. Note that the structural preservation of microtubules (**C**) is as good as after formaldehyde fixation.

Fig. 3. Detergent extraction (PEM/TX). Cytoplasmic microtubules are brightly stained with the two mouse anti-tubulins (**A and C**) but not with the rat anti-tubulin (**B**). Note that microtubules at the cell periphery lose their original organization after 1 min (**C**) and are partially lost after 4 min (**A**) of extraction

Fig. 4. Simultaneous formaldehyde fixation and detergent extraction (Form/PEM/TX). Microtubules are well preserved and brightly stained with all three anti-tubulins (**A–C**). Note details of the mitotic spindle and absence of cytoplasmic background staining in the cell at prometaphase (**B**). ×490

longed (Table 1). If, however, formaldehyde was included in the extraction buffer so that the cells were simultaneously fixed and extracted, microtubules stained brightly with all three monoclonal antitubulins (A, B and C in Table 1), and the structural preservation of both cytoplasmic and spindle microtubules was excellent (Fig. 4 A–C). There were some minor differences in the staining intensities of cytoplasmic versus spindle microtubules which were identical to those in unextracted, formaldehyde-fixed cells, but there was no diffuse cytoplasmic staining in early mitotic cells.



Figs. 5 and 6. Demonstration of cytokeratin and vimentin filaments. **Fig. 5. A** Incomplete staining pattern after formaldehyde fixation (Form/PEM) followed by detergent extraction (PEM/TX). **B** Typical cytokeratin pattern after simultaneous fixation and extraction (Form/PEM/TX), revealing perinuclear filament bundles and short fibres in the peripheral cytoplasm. **Fig. 6. A** Faint vimentin staining pattern and diffuse background staining after formaldehyde fixation. **B** Typical vimentin pattern after simultaneous fixation and extraction, revealing a dense network of filaments in the perinuclear cytoplasm. × 490

Demonstration of intermediate filaments and actin bundles

Staining of precipitation-fixed T51B cells with a monoclonal antibody to cytokeratin resulted in a bright pattern of thick bundles in the perinuclear area and some thin, short fibres in the peripheral cytoplasm, as has been described before (Marceau and Swierenga 1985). In contrast, fixation with formaldehyde caused a diffuse staining of the cells, with a faint perinuclear cytokeratin pattern superimposed in some but not all of the cells. Extraction of the cells after fixation resulted in some reduction of the background staining and a slight increase in the staining intensity of the perinuclear ring, but it did not result in visualization of fibres in the peripheral cytoplasm (Fig. 5A). Simultaneous formaldehyde fixation and extraction, however, produced a bright and typical cytokeratin pattern (Fig. 5B) which revealed more fibres in the peripheral cytoplasm and more details in the perinuclear filament bundles than that obtained after precipitation fixation. An equally distinct but brighter staining pattern was obtained after extraction of cells prior to fixation (Table 1).

Staining of T51B cells with a monoclonal antibody to vimentin resulted in a characteristic pattern of filament bundles concentrated in the perinuclear area and radiating into the cytoplasm, forming a dense network. This pattern has been described before (Marceau and Swierenga 1985) and was identical with all types of fixation tested. However, formaldehyde fixation without extraction produced some diffuse background staining which sometimes obscured details of the peripheral filament network (Fig. 6A). The vimentin pattern was especially bright and without any background when cells were extracted before or during fixation with formaldehyde (Fig. 6B and Table 1).

T51B cells counterstained with rhodamine-labeled phalloidin to demonstrate actin-containing structures revealed a distinct staining pattern of microfilament bundles (stress fibres; see Fig. 9A) which is characteristic of spread cells in culture (Wulf et al. 1979) including T51B cells (Marceau and Swierenga 1985). This pattern was equally well preserved with all types of fixation, but the staining was more intense after formaldehyde fixation and/or extraction than after ethanol fixation. No staining was obtained when methanol was used for fixation or postfixation (Table 1).

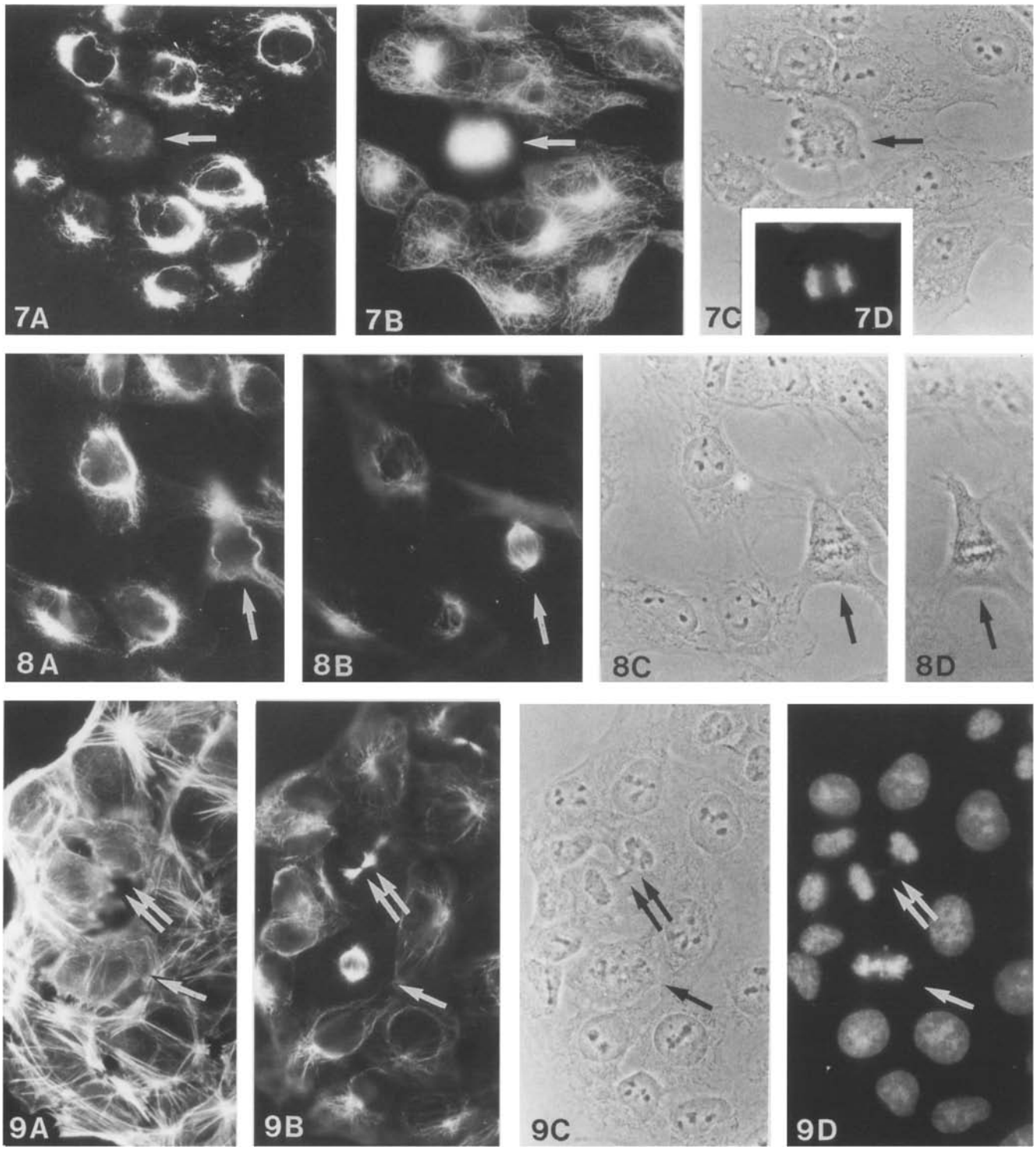
Preservation of cell morphology

A comparison of the phase contrast images of cells fixed in different ways (Table 1) showed that the stimultaneous fixation/extraction procedure yielded the best preservation of morphological details. In particular, the arrangement of chromosomes during all stages of mitosis (Figs. 7C, 8C and D, 9C) was much better visualized than with any other fixation tested. The contrast of the image, however, was significantly lower than after fixation with formaldehyde only. Extraction of cells prior to formaldehyde fixation resulted in the loss of structural details of the cytoplasm, while increasing the contrast of nuclei and chromosomes. Fixation with ethanol or methanol, on the other hand, caused numerous artefacts due to shrinking of the cells. Thus, the conditions that were optimal for the simultaneous demonstrating of microtubules and intermediate filaments also produced the best image with phase contast.

Double-staining for various cytoskeletal elements

Using the simultaneous fixation/extraction procedure, T51B cells were double-stained for various combinations of cytoskeletal components, counterstained with Hoechst fluorochrome, and examined for the following four aspects: (*i*) cell morphology and mitotic figures in phase contrast, (*ii*) morphology of nuclei and arrangement of mitotic chromosomes in the UV light channel, (*iii*) organization of microtubules or intermediate filaments in the channel for fluorescein fluorescence, and (*iv*) arrangement of intermediate filaments or actin bundles in the channel for rhodamine fluorescence. In all cases, the staining patterns of cytoskeletal elements were bright and distinct (Figs. 7–9), without diffuse background staining in interphase or mitotic cells. In addition, cross-reactivity between the secondary antibodies was negligible, each channel showing specific staining for only one cytoskeletal component.

Changes in cell morphology and cytoskeletal organization during the transition from interphase to metaphase, and vice versa, could easily be allocated to precise stages of the cells during mitosis by comparing the four different images of individual cells with each other (Figs. 7–9). Documentation of the staining patterns was greatly facilitated by the introduction of a semipermanent mounting medium that prevented fading of the fluorescence during observa-



Figs. 7–9. Correlation of double-immunofluorescent staining patterns with cell morphology of interphase and mitotic cells. **Figs. 7. A** Intermediate filaments stained with mouse anti-cytokeratin and rhodamine-conjugated anti-mouse IgG. **B** Microtubules stained with rat anti-tubulin and fluorescein-conjugated anti-rat IgG. **C** Cell morphology in phase contrast. **D** Anaphase chromosomes stained with Hoechst fluorochrome. Note that the cell in late anaphase (*arrow*), which is in a slightly different focal plane than the interphase cells, shows very little cytokeratin and a brightly stained mitotic spindle. **Fig. 8. A** Intermediate filaments stained with mouse anti-vimentin and rhodamine-conjugated anti-mouse IgG. **B** Microtubules stained with rat anti-tubulin and fluorescein-conjugated anti-rat IgG. **C and D** Cell morphology in phase contrast (**C**, same focal plane as in **A**; **D**, same focal plane as in **B**). Note that vimentin filaments form a cage around the mitotic apparatus of the cell in early anaphase (*arrow*). **Fig. 9. A** Microfilament (actin) bundles stained with rhodamin-conjugated phalloidin. **B** Microtubules stained with rat anti-tubulin and fluorescein-conjugated anti-rat IgG. **C** Cell morphology in phase contrast. **D** Interphase nuclei and mitotic chromosomes stained with Hoechst fluorochrome. Note the diffuse actin staining of the cells in metaphase (*arrow*) and late telophase (*double arrow*). × 490

tion. This made it possible to take a series of photographs from the same field of observation and to repeat photographing even after prolonged storage of slides.

Discussion

A failure to detect microtubules in cultured cells of various origins has been reported repeatedly in the literature and has been attributed to the fragmentation or complete disassembly of these structures due to the release of calcium ions from intracellular stores during methanol fixation or detergent extraction (for discussion, see Osborn and Weber 1982). Likewise, it has been reported that intermediate filaments did not react with specific antisera after aldehyde fixation (Osborn and Weber 1982), suggesting that antigenic determinants (epitopes) had been altered by the fixative. In recent investigations in T51B rat liver cells, we have encountered both these problems, and staining of microtubules and cytokeratin filaments within the same cell has been hampered.

Our search for a suitable fixative which would retain the structural integrity and antigenicity of both microtubules and cytokeratin filaments has been guided by two objectives: (*i*) to use a minimum of chemical treatment to produce a minimum of modifications, and (*ii*) to keep the preparation of fixative and the fixation procedure itself as simple as possible to facilitate large-scale processing of samples.

In the past, attempts to demonstrate intact microtubules in unfixed, detergent-extracted T51B cells have failed. Unlike other cytoskeletal elements, microtubules are highly unstable structures (for reviews, see Olmsted and Borisy 1973; Kirschner and Mitchison 1986) and may be lost during extraction or in the subsequent staining procedure (Osborne and Weber 1977b). In order to stabilize microtubules, a variety of buffers has been developed (Osborn and Weber 1977a; Bershadsky et al. 1978; Schliwa and van Blerkom 1981; Schliwa et al. 1981; Soltys and Borisy 1985). These buffers either contain components which promote microtubule assembly, or EGTA to chelate calcium ions, or any combination of these components to prevent disassembly of microtubules. Using such stabilization buffers, excellent preservation of microtubules has been achieved by other authors with brief detergent extraction of cells and subsequent mild fixation with glutaraldehyde (Schliwa and van Blerkom 1981; Osborn and Weber 1977a). However, the procedure is time-consuming as it involves several steps, including the reduction of free aldehyde groups with sodium borohydride to prevent non-specific binding of antibodies (Weber et al. 1978).

As confirmed in the present study, detergent extraction of unfixed T51B cells in the presence of an appropriate microtubule stabilizing buffer of simple composition (Soltys and Borisy 1985) does not result in a faithful preservation of the original microtubule organization. Rearrangement and loss of microtubules occurs already after very brief extraction periods. The rapid breakdown of microtubular organization during further extraction may be related to the fact that liver cells, in particular, store high levels of calcium in their mitochondria (Joseph et al. 1983) which is released during membrane permeabilization. Thus, in the case of T51B rat liver cells, detergent extraction prior to fixation is not a suitable routine procedure for the investigation of microtubules. In contrast, the simple addition of formaldehyde to the extraction buffer results in a good preservation of both interphase and mitotic microtubules. The use of commercial formalin rather than freshly prepared paraformaldehyde solution yields satisfactory results and saves time when preparing the fixative.

The present study shows that microtubules and cytokeratin filaments can be demonstrated within the same T51B cells, when formaldehyde fixation is carried out in the presence of detergent and microtubule stabilization buffer. It further shows that microtubules in these cells can be well preserved by precipitation with ethanol or methanol, but can then be detected with only one out of three monoclonal anti-tubulins tested. Detergent extraction prior to precipitation fixation significantly improves the stainability with one of the other two antibodies, indicating that the corresponding epitope becomes unmasked during extraction. Similarly, the bright staining patterns for cytokeratin and vimentin filaments in cells extracted prior to precipitation fixation or during formaldehyde fixation demonstrate that the antigenicity of these structures is not directly affected by either fixative. Rather, the extraction seems to remove cytoplasmic components that otherwise would mask some epitopes.

Double-staining of cells fixed and extracted simultaneously produces staining patterns of microtubules and intermediate filaments that are identical to those already described for acetone-fixed T51B cells (Marceau and Swierenga 1985). In addition, the cells reveal more details of the mitotic spindle and the perinuclear intermediate filament ring as well as a better phase contrast image than those fixed with conventional fixatives. The patterns are bright and clear, without any background staining. Particularly noteworthy is the absence of an intense cytoplasmic background staining usually observed in early mitotic stages of unextracted, formaldehyde-fixed cells after antitubulin staining. This can be attributed to the removal of depolymerized tubulin (Osborn and Weber 1977a) which is accumulating during the transition from interphase to metaphase

Conclusion

The present study shows that microtubules in T51B cells can be well preserved either by precipitation fixation, or by simultaneous formaldehyde fixation and detergent extraction. In addition, excellent staining of cytokeratin and vimentin filaments can be achieved when formaldehyde fixation is carried out in the presence of detergent. In principle, therefore, double-staining for microtubules and other cytoskeletal structures can be carried out using either method, provided an appropriate pair of antibodies is available (e.g. mouse versus rat IgG, or mouse IgG versus mouse IgM). For our purposes, precipitation fixation was not suitable, as the only monoclonal anti-tubulin that reacted with ethanol- or methanol-fixed cells was of the same type (mouse IgG) as the anti-cytokeratin and anti-vimentin. Apart from this, the fixation/extraction procedure is far superior to precipitation fixation. It does not result in artifacts, such as cell shrinkage induced by rapid dehydration, and it yields more details in both the staining patterns and the phase contrast image, particularly of mitotic cells.

In summary, the simultaneous fixation and extraction of cells with formaldehyde in the presence of microtubule stabilization buffer and nonionic detergent yields an excellent preservation of cytoskeletal elements in terms of both

structural integrity and antigenicity, without the need to follow a lengthy and complicated protocol. In combination with a semipermanent mounting medium that prevents fading of the fluorescence during observation, the procedure will be valuable for efficient processing of samples from large-scale experiments.

Acknowledgements. We thank Drs. D.L. Brown and K.R. Reuhl for helpful discussions. U.V. is a postdoctoral fellow of the Medical Research Council of Canada.

References

Aitchison WA, Brown DL (1986) Dublication of the flagellar apparatus and cytoskeletal microtubule system in the alga Polytomella. Cell Motil Cytoskeleton 6:122–127

Berlin RD, Oliver JM, Walter RJ (1979) Surface functions during mitosis I: phagocytosis, pinocytosis and mobility of surface-bound ConA. Cell 15:327–341

Bershadsky AD, Gelfand VI, Svitkina TM, Tint IS (1978) Microtubules in mouse embryo fibroblasts extracted with Triton X-100. Cell Biol Int Rep 2:425–432

Brinkley BR (1982) The cytoskeleton: a perspective. Methods Cell Biol 24:1–8

Falconer M, Vielkind U, Brown DL (1987) Alterations in microtubule stability and composition following commitment of embryonal carcinoma cells into neurons. J Cell Biol 105:203a

Franke WW, Schmid E, Grund C, Geiger B (1982) Intermediate filament proteins in nonfilamentous structures: transient disintegration and inclusion of subunit proteins in granular aggregates. Cell 30:103–113

Geiger B, Singer SJ (1980) Association of microtubules and intermediate filaments in chicken gizzard cells as detected by double immunofluorescence. Proc Natl Acad Sci USA 77:4769–4773

Heimer GV, Taylor CED (1974) Improved mountant for immunofluorescence preparations. J Clin Pathol 27:254–256

Hilwig I, Gropp A (1972) Staining of constitutive heterochromatin in mammalian chromosomes with a new fluorochrome. Exp Cell Res 75:122–126

Hynes RO, Destree AT (1978) Relationships between fibronectin (LETS protein) and actin. Cell 15:875–886

Johnson GD, Davidson RS, McNamee KC, Russel G, Goodwin D, Holborow EJ (1982) Fading of immunofluorescence during microscopy: a study of the phenomenon and its remedy. J Immunol Methods 55:231–242

Joseph SK, Coll KE, Cooper RH, Marks JS, Williamson JR (1983) Mechanisms underlying calcium homeostasis in isolated hepatocytes. J Biol Chem 258:731–741

Kilmartin JV, Wright B, Milstein C (1982) Rat monoclonal antitubulin antibodies derived by using a new nonsecreting rat cell line. J Cell Biol 93:576–582

Kirschner M, Mitchison T (1986) Beyond self-assembly: from microtubules to morphogenesis. Cell 45:329–342

Lane EB, Goodman SL, Trejdosiewicz LK (1982) Disruption of the keratin filament network during epithelial cell division. EMBO J 1:1365–1372

Marceau N, Swierenga SHH (1985) Cytoskeletal events during calcium- or EGF-induced initiation of DNA synthesis in cultured cells. Role of protein phosphorylation and clues in the transformation process. In: Shaw JW (ed) Cell and muscle motility, vol 6. Plenum Press, New York London, pp 97–140

Marceau N, Germain L, Goyette R, Noël M, Gourdeau H (1986) Cell of origin of distinct cultured rat liver epithelial cells, as typed by cytokeratin and surface component selective expression. Biochem Cell Biol 64:788–802

Olmstedt JB, Borisy GG (1973) Microtubules. Annu Rev Biochem 42:507–540

Osborn M, Weber K (1977a) The display of microtubules in transformed cells. Cell 12:561–571

Osborn M, Weber K (1977b) The detergent-resistent cytoskeleton of tissue culture cells includes the nucleus and the microfilament bundles. Exp Cell Res 106:339–349

Osborn M, Weber K (1982) Immunofluorescence and immunocytochemical procedures with affinity purified antibodies: tubulin-containing structures. Methods Cell Biol 24:97–132

Puck TT, Cieciura SJ, Robinson A (1958) Genetics of somatic mammalian cells. III. Long-term cultivation of euploid cells from human and animal subjects. J Exp Med 108:945–956

Schliwa M, van Blerkom J (1981) Structural interaction of cytoskeletal components. J Cell Biol 90:222–235

Schliwa M, Euteneuer U, Bulinski JC, Izant JG (1981) Calcium lability of cytoplasmic microtubules and its modulation by microtubule-associated proteins. Proc Natl Acad Sci USA 78:1037–1041

Singer SJ, Ball EH, Geiger B, Chen WT (1982) Immunolabeling studies of cytoskeletal associations in cultured cells. Cold Spring Harbor Symp Quant Biol 46:303–316

Soltys BJ, Borisy GG (1985) Polymerization of tubulin in vivo: direct evidence for assembly onto microtubule ends and from centrosomes. J Cell Biol 100:1682–1689

Viklický V, Oráber P, Hašek J, Bártek J (1982) Production and characterization of a monoclonal antitubulin antibody. Cell Biol Int Rep 6:725–731

Weber K, Rathke PC, Osborn M (1978) Cytoplasmic microtubular images in glutaraldehyde-fixed tissue culture cells by electron microscopy and by immunofluorescence microscopy. Proc Natl Acad Sci USA 75:1820–1824

Wulf E, Deboben A, Bautz FA, Faulstich H, Wieland T (1979) Fluorescent phallotoxin, a tool for the visualization of cellular actin. Proc Natl Acad Sci USA 76:4498–4502