FLUORESCENT ANTIBODY LOCALIZATION OF MYOSIN

IN THE CYTOPLASM, CLEAVAGE

FURROW, AND MITOTIC SPINDLE OF HUMAN CELLS

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ABSTRACT

We have studied the distribution of myosin molecules in human cells using myosin-specific antibody coupled with fluorescent dyes. Rabbits were immunized with platelet myosin or myosin rod. They produced antisera which precipitated only myosin among all the components in crude platelet extracts. From these antisera we isolated immunoglobulin-G (IgG)[1] and conjugated it with tetramethylrhodamine or fluorescein. We separated IgG with 2-5 fluorochromes per molecule from both under- and over-conjugated IgG by ion exchange chromatography and used it to stain acetone-treated cells. The following controls established the specificity of the staining patterns: (a) staining with labeled preimmune IgG; (b) staining with labeled immune IgG adsorbed with purified myosin; (c) staining with labeled immune IgG mixed with either unlabeled preimmune or immune serum; and (d) staining with labeled antibody purified by affinity chromatography. In blood smears, only the cytoplasm of platelets and leukocytes stained. In spread Enson and HeLa cells, stress fibers stained strongly in closely spaced 0.5 µm spots. The cytoplasm stained uniformly in those cells presumed to be motile before acetone treatment. In dividing HeLa cells there was a high concentration of myosin-specific staining in the vicinity of the contractile ring and in the mitotic spindle, especially the region between the chromosomes and the poles. We detected no staining of erythrocytes, or nuclei of leukocytes and cultured cells, or the surface of platelets and cultured cells.

To understand how a machine works, one must find the motor and determine how it is attached to the various cables, gears, and brakes. The same reasoning led us to study the distribution of myosin molecules inside cells as one way of learning how they move. We are particularly interested in myosin, because it is the energy transducing enzyme in those cellular contractile systems using actin filaments and it should be located at the sites of motile force production.

Previous efforts to localize myosin in vertebrate cells utilized electron microscopy of thin sections or staining with antibodies coupled to fluorescent dyes or electron-dense markers. Neither method has been completely successful.

The usefulness of conventional electron microscopy has been limited by difficulty in identifying the small aggregates characteristically formed by

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[1] *Abbreviations used in this paper:* IgG, immunoglobulin-G; Na-pp, sodium pyrophosphate; PBS, phosphate buffered saline.

vertebrate cytoplasmic myosins (1, 11, 47, 64). Four groups have described 2-10 nm-wide filaments in glycerinated cells (2, 6, 44, 62). These filaments could be myosin, since they are associated with actin and do not bind heavy meromyosin. Schroeder's (62) observations are particularly intriguing, because he found these presumptive myosin filaments along with actin in the contractile ring. In none of these studies were the filaments positively identified as myosin.

Antibody techniques have the potential advantages of specificity and high sensitivity. There have been a number of localization studies using antibodies against myosins (5, 7, 29, 45, 49, 72, 73, 74). We felt that additional antimyosin work was necessary, because antigen preparations were grossly impure in some cases, because antigens and antibodies were incompletely characterized in some cases, because there was evidence for nonspecific staining in some studies, and because there were conflicting results.

The objectives of the present work were to establish the immunological specificity of antibodies against platelet myosin, to couple these antibodies with fluorochromes in a way that avoids nonspecific staining, and to use the fluorescent antimyosin to localize myosin at the light microscope level in platelets, leukocytes, HeLa cells, and Enson cells. We have paid particular attention to the myosin distribution in the cytoplasm of stationary and motile cultured cells and in the cleavage furrow and mitotic spindle of dividing cells. In other reports, we used the antibodies to study platelet activation (53), serological relationships among cytoplasmic and muscle myosins (54), and the relationship between tubulin and myosin during cell division (22). Preliminary accounts of some of the present work were presented at the September 1975 Cold Spring Harbor Meeting on Cell Motility (54) and the November 1975 Meeting of the American Society for Cell Biology (21).

MATERIALS AND METHODS

Preparation of Immunizing Agents

We purified myosin and the rod portion of the myosin molecule from human platelets by the KI-gel filtration method of Pollard et al. (52). The purity of the preparations was assessed by electrophoresis in 7.5% polyacrylamide gels with 0.1% sodium dodecyl sulfate and a tris-glycine buffer (63). The gels were stained with Coomassie brilliant blue (18) and scanned at 550 nm with a Gilford spectrophotometer (Gilford Instrument Labora-

tories, Inc., Oberlin, Ohio). Protein concentrations were estimated by the method of Hartree (30) with the use of bovine serum albumin as the standard, or by absorbance at 280 nm, with 14.0 as the extinction coefficient for 1% immunoglobulin-G (75) and 5.9 as the extinction coefficient for a 1% platelet myosin solution (52).

Immunization

We used eight male white New Zealand rabbits (5-7 kg) to obtain antisera against human platelet myosin. We collected about 30 ml of preimmune serum from each rabbit to serve as controls for our experiments. Four rabbits were immunized with purified platelet myosin containing less than 5% of the myosin rod fragment (Fig. 2a, Table I). Another two were immunized with purified platelet myosin partially degraded into rod and head fragments (Fig. 2b, Table I). The remaining two rabbits were immunized with homogeneous platelet myosin rod (Fig. 2c, Table I). In all three cases, about 250 μg of protein emulsified in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) was injected subcutaneously at each of about 25 sites including a panel along the back, the foot pads, and the toe pads. A single boost consisting of the same amount of the original antigen in incomplete Freund's adjuvant (Difco Laboratories) was given subcutaneously at multiple sites during the 5th or 6th week after the initial injection (Table I). We bled the rabbits from ear veins three times during the 2nd wk after the boost, obtaining about 50 ml of blood each time. The blood was allowed to clot at 37°C for 1 h and further incubated at 4°C overnight. The serum was collected and freed of erythrocytes by centrifugation at 10,000 rpm for 30 min in a Beckman JA-20 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 4°C. The antisera were divided into 2 ml vials and stored at −20°C.

Evaluation of Antisera

We screened all eight antisera using double immunodiffusion and immunoelectrophoresis to estimate the strength and specificity of their reaction with platelet myosin. The test antigens were crude platelet extract and purified platelet myosin. From these we selected three antisera for more detailed evaluation and for staining.

TEST ANTIGENS

PURIFIED ANTIGENS FROM HUMAN PLATELETS: We obtained the following purified antigens directly from the KI-gel filtration column: myosin, myosin rod, myosin head mixed with some actin and tropomyosin, and actin.

CRUDE EXTRACTS: In addition to the purified antigens, we used crude extracts of platelets and HeLa cells to test the antisera. We prepared the cell extracts in 20 mM Na-pyrophosphate (Na-pp) buffer at pH 8.6, because (a) myosin is soluble in it at relatively low ionic strength, (b) it dissociates actin and myosin, and (c) it

appears to have no effect on immunological reactions. The platelet extract was made by hand-homogenizing 500 mg of frozen human platelets with a Teflon glass homogenizer in 0.5 ml 40 mM Na-pp buffer at pH 8.6. The homogenate was cleared by centrifuging for 30 min at 4°C in a Beckman JA-20 rotor at 14,000 rpm (23,700 g maximum force). This yielded an extract of soluble proteins in 20 mM Na-pp. We divided the crude platelet extract into small test tubes and stored them at −20°C until use. Whenever a platelet extract was needed, one tube was thawed, used once, and discarded. We prepared a crude extract of HeLa cells in a similar manner. The cells were harvested by treating the culture with Ca^{++}-Mg^{++}-free Hanks' solution (Grand Island Biological Co., Grand Island, N. Y.) containing 1 mM EDTA at 37°C for 10 min. The cells were collected by low speed centrifugation and washed once in Hanks' solution (Grand Island Biological Company). The volume of the packed cells was estimated and an equal volume of 40 mM Na-pp buffer added. After suspending the cells in the buffer, we froze them at −20°C for 10 min. The suspension was thawed and left on ice for 1 h to extract soluble components. The extract was clarified by centrifugation at the maximum speed of a clinical centrifuge for 5 min.

DOUBLE IMMUNODIFFUSION ANALYSIS

For the two dimensional double immunodiffusion (48), we used 1% agarose (Induboise A 45, L'Industrie Biologique Francaise 5. A. Gennevilliers, France) containing 0.01% Merthiolate (Eli Lilly and Co., Indianapolis, Ind.) and 20 mM Na-pp buffer at pH 8.6. The immunodiffusion plates were incubated in a moist chamber at either room temperature or 4°C for 2-4 days. With dark-field illumination most precipitin lines could be seen and recorded photographically, but we routinely stained the plates with Coomassie brilliant blue to detect faint precipitin bands. Before staining, the plates were washed first in 20 mM Na-pp buffer overnight with one change of buffer and then in distilled water for 2 h with one change. They were air-dried with filter paper on top. The dried plates were stained for 30 min with 0.25% Coomassie brilliant blue in a 5:1:4 mixture of methanol, acetic acid, and water. Destaining was done in the same solution without the dye.

IMMUNOELECTROPHORESIS

For immunoelectrophoresis (48) we prepared agarose plates using 2 inch × 3 inch glass slide and 11 ml of 1% agarose in 20 mM Na-pp buffer at pH 9.0. 10-20 µl of antigen solution were subjected to electrophoresis for 1 h at 15 mA per plate using the Na-pp buffer as the tank buffer. After electrophoresis, the antiserum was applied to troughs and the plate was incubated for 2-5 days in a moist chamber at room temperature. The precipitin bands were recorded photographically with or without staining by Coomassie brilliant blue.

CROSSED IMMUNOELECTROPHORESIS

We modified the crossed immunoelectrophoresis technique, described in the manual edited by Axelsen et al. (3, 37), to make it applicable for myosin. The condition for the first electrophoresis was identical with the immunoelectrophoresis, except that the pH of the Na-pp buffer was adjusted to 8.6. A strip of agarose containing electrophoretically distributed components of the antigen solution was cut out and placed at one edge of a second 2 inch × 3 inch microscope slide. We mixed 0.01 vol of antiserum with 1% agarose in Na-pp buffer at 50°C and poured it onto the remaining part of the second microscope slide. After this agarose layer had solidified, the second electrophoresis was performed perpendicular to the first at 1.5 mA per plate for 24 h at room temperature. The agarose plate was washed first in the Na-pp buffer overnight, then in distilled water for several hours before air-drying and staining with Coomassie brilliant blue. To identify unknown precipitin bands, we used tandem crossed immunoelectrophoresis (37). Two antigen wells were made on the first electrophoresis plate in such a way that one well was slightly closer to the anode. This anodic well was also off the electrophoretic passage of the cathodic sample. Purified platelet myosin was applied to the anodic well and the platelet extract to the cathodic well. All the other conditions for the tandem crossed immunoelectrophoresis were identical with the conventional crossed immunoelectrophoresis described above.

Preparation of Fluorescent Reagents for Antibody Staining

FLUORESCENT PREIMMUNE AND IMMUNE IgG

Immunoglobulin G (IgG) from one preimmune serum and three different batches of antisera were labeled with either fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate (Table I). Each of the three antisera was against different antigen preparations of platelet myosin (Table I and Fig. 2). Fig. 1 illustrates the protocol for preparing fluorescent antibodies, which is a minor modification of the procedure of Cebra and Goldstein (13).

Frozen serum was thawed and its volume measured. From this point on, all steps were carried out at 0-4°C. IgG was fractionated from the serum with 37% saturated ammonium sulfate. After 1 h with stirring, precipitated IgG was collected by centrifugation in a JA-20 rotor at 12,000 rpm (maximum force 17,600 g) for 15 min. The white pellet was resuspended into the original volume of phosphate-buffered saline (PBS) containing 0.85% NaCl in 15 mM phosphate buffer at pH 7.4, and the ammonium sulfate fractionation step was repeated once more. The pellet was redissolved in one-half volume of PBS, and dialyzed against several changes of 500 ml 0.01 M phosphate buffer at pH 7.5 for 24 h. At the end of the dialysis the small amount of white precipitate which
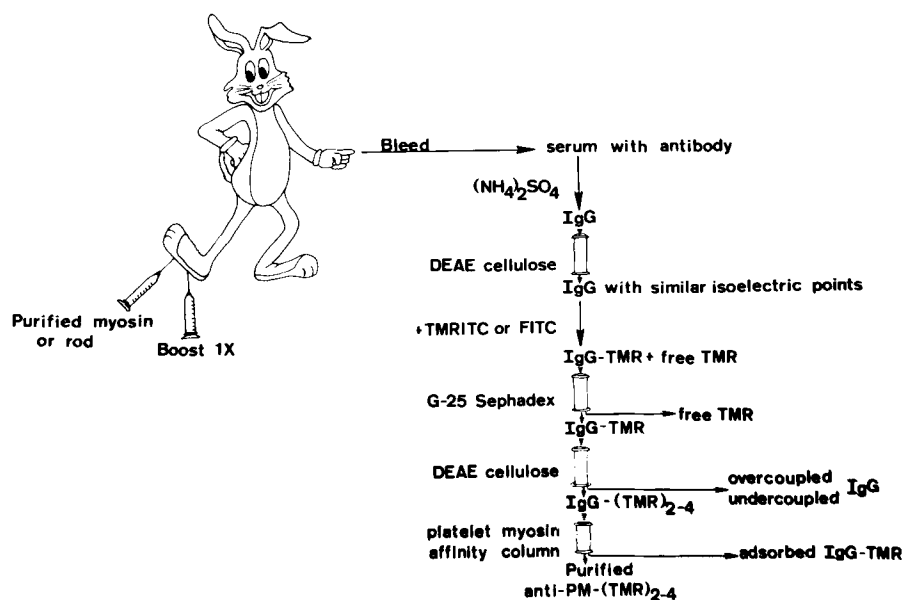
Bleed ————————► serum with antibody

$(NH_4)_2SO_4$

IgG

DEAE cellulose

IgG with similar isoelectric points

+TMRITC or FITC

IgG-TMR + free TMR

G-25 Sephadex ———————► free TMR

IgG-TMR

DEAE cellulose

IgG-(TMR)$_{2-4}$ overcoupled IgG
                     undercoupled

platelet myosin
affinity column ——————————► adsorbed IgG-TMR

Purified myosin
or rod   Boost 1X

Purified
anti-PM-(TMR)$_{2-4}$

FIGURE 1   The protocol for preparing the reagents used in fluorescent antibody staining. This method of Cebra and Goldstein (3) was adopted with a slight modification. The following abbreviations are used: IgG (immunoglobulin G), TMRITC (tetramethylrhodmine isothiocyanate), FITC (fluorescein isothiocyanate), TMR (tetramethylrhodamine), and PM (platelet myosin).

formed was removed by centrifugation at 17,600 g for 15 min. This IgG in phosphate buffer was fractionated on DEAE cellulose (DE 52, Whatman) to obtain two pools of IgG, each having a limited distribution of charges. The two pools were IgG which passed directly through the anion exchanger column in 0.01 M phosphate buffer (Pool I) and IgG which was eluted with 0.05 M NaCl in the phosphate buffer (Pool II). From this point on, the two pools were handled separately, although they were treated in exactly the same way. Each pool of IgG was precipitated with 40 or 50% ammonium sulfate, and the pellet dissolved in a small amount of PBS to make the IgG concentration roughly 10 mg/ml. To remove ammonium sulfate, the IgG solutions were dialyzed against PBS for 1 day with several changes. At the end of dialysis, the protein concentration in each solution was determined by absorption at 280 nm.

For conjugation with the fluorochromes, dry fluorescein isothiocyanate (FITC, BBL, Cockeysville, Md., lot K4EAGM11-76) or tetramethylrhodamine isothiocyanate (TMRITC, BBL, lot K3EAGL) was measured into a small conjugation vial. The amounts of FITC and TMRITC per milligram of IgG were 20 μg and 40 μg respectively. The IgG solution, whose pH was adjusted to 9.5 with 0.1 N NaOH at 0°C, was then added slowly with constant stirring to the reaction vial on ice. During the 1st h of the conjugation reaction, the pH was kept between 9.3 and 9.5 with 0.1 N NaOH. Then the reaction vial was covered with aluminum foil and left at 4°C

with stirring. After 12-18 h of conjugation, the reaction mixture was passed through a Sephadex G-25 column (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) equilibrated with 0.01 M phosphate buffer at pH 7.5 to separate most of the unconjugated fluorochrome from the conjugated IgG, to change the pH of the solution, and to remove the salt. Then the IgG in 0.01 M phosphate buffer was chromatographed on a DEAE cellulose column equilibrated with the same buffer to remove under- and over-conjugated IgG. For pool I the conjugated IgG was eluted sequentially with 0.01 M phosphate buffer containing 0 M, 0.04 M, and 0.1 M NaCl. For pool II, the steps were 0 M, 0.04 M, 0.06 M, 0.1 M, and 1 M NaCl in the buffer. The ratio of dye to protein in each fraction was determined by measuring the absorbance of rhodamine at 515 nm or fluorescein at 495 and IgG at 280 nm. We used 0.13 (1 μg/ml) as the extinction coefficient for rhodamine (43) and 0.20 (1 μg/ml) for fluorescein (32), and the correction factors for fluorochrome absorbance at 280 nm (0.56, 0.35) (8). As recommended, those fractions having dye to protein ratios of 2 to 5 were used to stain cells (13). All the labeled IgG was stored in phosphate buffer with 0.02% Na-azide at 4°C.

LABELED PURIFIED ANTIBODY AND
ADSORBED IgG

Part of the labeled IgG was further purified for specific antibodies against platelet myosin by affinity chro-

matography. Purified human platelet myosin was covalently bound to polyacrylamide beads by the method of Ternynck and Avrameas (71), except that we substituted Na-pp for phosphate buffer to keep the myosin in solution. The labeled IgG in PBS was run into the myosin-polyacrylamide column and the fraction which passed directly through the column was used as adsorbed IgG. The column was washed with PBS until no more protein eluted from it. The antibody that bound to the beads was then eluted with 0.2 M glycine-HCl buffer at pH 2.8. As each 20 drop fraction containing protein was collected, the pH was raised at once to 6.9 with 3 drops of 1 M sodium phosphate at pH 7.5. The purified antibody was dialyzed against PBS with 0.02% Na-azide and stored in solution at 4°C. We also prepared adsorbed immune IgG by mixing 50 μl of labeled IgG solution containing 16 μg of protein with either the myosin bound to beads or with purified platelet myosin in PBS containing 6–45 μg protein. The mixture was incubated at room temperature for 30 min and 4°C for 10 h. Then it was centrifuged for 45 min in Sorvall SS-34 rotor (Ivan Sorvall, Inc., Norwalk, Conn.) at 10,000 rpm and the supernatant was used as adsorbed IgG.

Staining Cells with Fluorescent Antimyosin

HUMAN BLOOD SMEAR

We tested the immunocytochemical specificity of our fluorescent reagents on human blood smears. A small drop of human blood was smeared between two 18 × 18 mm coverslips, which were then submerged into acetone at −20°C for 5 min with or without brief air-drying beforehand. We found that air-drying the smear briefly before the acetone treatment enhances the adhesion of erythrocytes to the coverslip. Acetone-treated smears were air-dried at room temperature. To examine surface staining, we fixed blood smears in 3% formalin (Mallinckrodt, St. Louis, Mo.) in PBS at room temperature for 30 min and washed in PBS for 10 min.

Blood smears were then stained by inverting the coverslips onto 50–100 μl of labeled antibody solution (2.5–320 μg/ml IgG) for 30 to 45 min at room temperature in a moist chamber. The stained coverslips were washed by submersion in several changes of 30 ml PBS over a period of 1–2 h and finally mounted in 90% glycerol in PBS. The coverslips were sealed to the slide with nail polish.

HUMAN TISSUE CULTURE CELLS

Human cell lines, HeLa and Enson (CRL 1139; skin fibroblast), were grown on 18 × 18 mm microscope coverslips using Eagle's basic medium with 10% fetal calf serum. The cells on the cover glass were briefly washed in PBS at 37°C, then treated in acetone at −20°C for 5 min, and air dried at room temperature. For surface staining, the cells were fixed in 3% formalin as described above for blood smears. Fixed tissue culture cells were stained with the labeled antibody, washed in PBS, and mounted exactly as the blood smears.

CONTROLS FOR IMMUNOFLUORESCENCE

To examine the specificity of the staining patterns, we carried out the following controls: (a) staining with labeled IgG from preimmune serum, (b) staining with labeled IgG adsorbed with purified myosin, (c) staining with labeled antibody in the presence of an excess amount of unlabeled specific antiserum, (d) staining in the presence of preimmune serum, and (e) staining with labeled purified antibody. For controls (c) and (d), cells were stained with a mixture of 20 μl fluorescent IgG containing antimyosin antibodies and 80 μl either immune or preimmune serum.

Fluorescence Microscopy and Photomicroscopy

We observed cells stained with fluorescent antibody with a Leitz Orthoplan microscope stand equipped with a Ploem vertical illuminator using a Xenon lamp (Xenon Corp., Watertown, Mass.) (XBO-150) as the light source. The entire filter set-up is described in the order through which the illuminating light passes. For fluorescein the set-up was 3 mm BG-38 (red suppression filter), KP-500 (excitation interference filter), TK-510/515 (built-in dichroic mirror and barrier filter), and K-530 (barrier filter); and for tetramethylrhodamine, 3 mm BG-38, KP-560 (excitation interference filter), 2 mm BG-36 (exciter filter), TK-580/K-580 (built-in dichroic mirror and barrier filter), and K-580 (barrier filter).

Objective lenses with high NA were used to obtain both an efficient illumination and high resolution. They included × 100 (Leitz, NA 1.3, oil), × 63 (Zeiss, Planapo phase NA 1.4, oil), × 63 (Zeiss, Planapo, NA 1.4, oil). For phase contrast observation, both × 63 (Zeiss, Planapo phase NA 1.4, oil) and × 40 (Leitz, Planapo phase NA 1.0, oil) were used with Leitz phase contrast condenser modified to match the phase ring of the Zeiss lens.

Kodak 35 mm Tri-X negative film was used to record the fluorescent image. The exposure time varied depending upon the preparation as well as the fluorochrome used. Average exposure times were 1–2 min for fluorescein and 15–30 s for tetramethylrhodamine. The film was developed in Acufine (Acufine Inc., Chicago, Ill.) which gave an effective ASA of 1200. The photographic prints are an accurate representation of the fluorescence contrast in the original specimens, but different prints cannot be compared with respect to their intensity because we had to adjust the intensity to bring out the contrast.

RESULTS

Purity of Immunizing Agents

We used electrophoresis in sodium dodecyl sulfate on polyacrylamide gels to assess the purity of
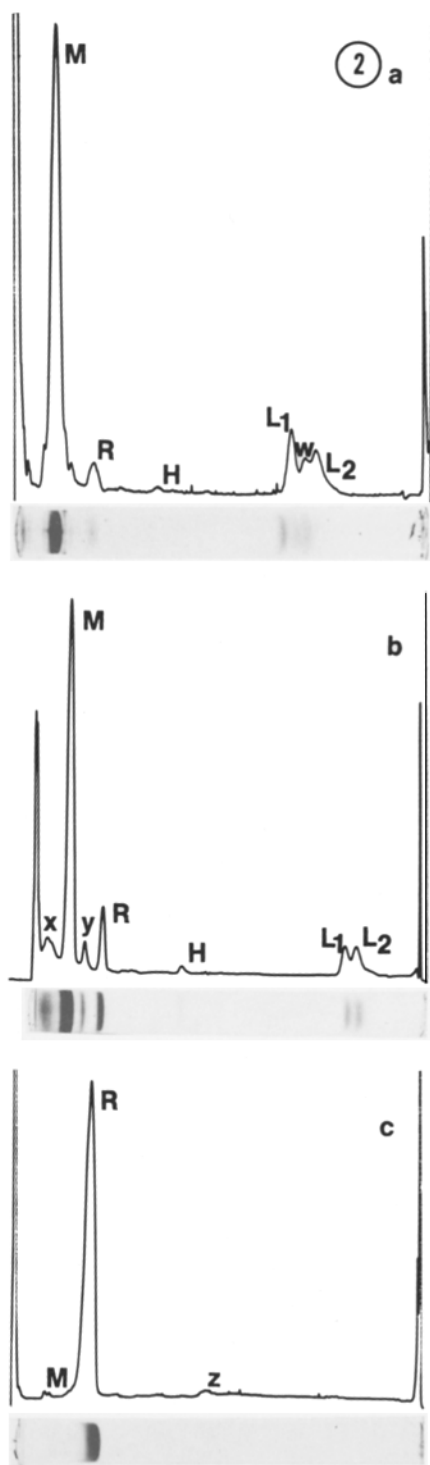
the three immunizing agents: purified platelet myosin, purified platelet myosin partially degraded into rod and head fragments, and purified platelet myosin rod (Fig. 2). Densitometer scans of stained gels indicated that the purified myosin (Fig. 2a) and purified myosin rod (Fig. 2c) were contaminated with 1-2% unidentified polypeptides. Thus we injected about 2-5 μg of unidentified polypeptide along with each dose of antigen. The partially degraded myosin (Fig. 2b) contains 16% unidentified polypeptides, but we believe that they are mostly aggregated myosin and proteolytic fragments of myosin.

Specificity of the Antisera

We screened immune sera from eight rabbits for antibodies against human platelet myosin using double immunodiffusion and immunoelectrophoresis. In both tests, all of the immune sera formed a single precipitin line when reacted with either purified platelet myosin or a crude platelet extract. For fluorescent antibody studies, we selected three antisera which reacted strongly with myosin and subjected these three sera to more rigorous immunological tests. These tests showed that each of these antisera reacts with the rod portion of the platelet myosin molecule. Reaction with other platelet components was not detected. These antisera also reacted with a component in HeLa cells which is immunologically identical with platelet myosin and is presumably HeLa myosin. Examples of these tests are presented in the following sections and all of the data are summarized in Table I. These methods detect only precipitating antibodies. Nonprecipitating antibodies against

Figure 2 Electrophoretic analysis of immunizing agents. The polyacrylamide gels are stained with Coomassie blue and scanned at 550 nm. The following sym-

bols are used to identify the polypeptides: myosin heavy chain (M), myosin light chains (L-1 and L-2), myosin rod fragment (R), myosin head frgment (H), and various unidentified polypeptides (w, x, y, and z). (a): Purified platelet myosin. This preparation consists of 94% myosin (M + L-1 + L-2), 4% myosin rod, a trace of myosin head, and 1.6% unidentified material (w). (b): Partially degraded platelet myosin. This material consists of 73% myosin (M + L-1 + L-2), 10% myosin rod, 1% myosin head, and 16% unidentified polypeptides (x and y). Since the amounts of the heavy chain (M) and the light chains (L-1 + L-2) are 60% and 13%, respectively, there is not enough heavy chain to account for the excess amount of the light chains. Therefore we suspect that bands x and y must be aggregates and fragments of intact myosin. (c): Purified myosin rod. This preparation contains 1% myosin (M), 98% myosin rod, and 1% unidentified polypeptide (z).

TABLE I

Evaluation of Antisera

Rabbit sera		Preimmune	1	2	3	3	4	5	6	8	9
Immunizing agents isolated from human platelets			Myosin				Partially degraded myosin		Myosin	Myosin rod	
No. of injections		0	2	2	2	5	2	2	2	2	2
Double immuno-diffusion	Platelet extract	−	+	+	+	+(+)	+	+	+	+	+
	" myosin	−	+	+	+	+	+	+	+	+	+
	" myosin rod	+	+	+	+	+	+		+	+	+
	" myosin head				−	−	−		−	−	
	" actin						−		−	−	
	HeLa extract						+		+	+	
Immunoelectrophoresis	Platelet extract				+	++	+	+	+	+	+
	" myosin				+	+	+	+	+	+	+
	" myosin rod				+	+	+		+	+	+
	" myosin head						−		−	−	
	" actin						−		−	−	
Crossed immunoelec-trophoresis	Platelet extract						+		+	+	
	" myosin						+		+	+	
Fluorochromes		F					R		F	R	

+, one precipitin line; ++, two precipitin lines; +(+), two precipitin lines, but sometimes one; −, no precipitin line, F, fluorescein isothiocyanate; R, tetramethylrhodamine isothiocyanate.

myosin or contaminating antigens would escape detection.

DOUBLE IMMUNODIFFUSION

The antiserum against purified platelet myosin reacted with purified myosin, myosin rod, and the crude extracts of platelets and HeLa cells to form a single precipitin line (Fig. 3). The fusion of the precipitin lines shows that the four reactions are immunologically "identical." The antiserum did not react with the head region of platelet myosin or with platelet actin (Fig. 3). The other two antisera used for staining work gave identical results (Table I). Elsewhere we report that none of the antisera against platelet myosin reacted with myosins from human skeletal and cardiac muscles, but that some of them reacted weakly with extracts of human uterus (54).

IMMUNOELECTROPHORESIS

Immunoelectrophoresis is a useful adjunct to double immunodiffusion for assessing the specificity of antisera, because the antigens are separated according to their electrophoretic mobility before reaction with the antiserum. This test confirmed the results of immunodiffusion: the three antisera chosen for staining reacted with platelet myosin, myosin rod, and a single component in crude platelet extracts (Fig. 4; Table I). The antigen in the crude platelet extract had the same electro-
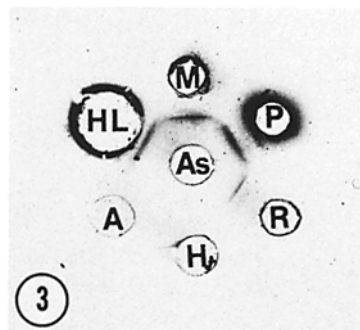

FIGURE 3 Double immunodiffusion analysis of antiserum against human platelet myosin. The antiserum (rabbit 4, single boost) is in the center well and test antigens are placed in the six outer wells. The antiserum forms a single precipitin line with purified human platelet myosin (M), purified human platelet myosin rod (R), a crude extract of human platelets (P) and a crude extract of HeLa cells (HL). It does not react with human platelet myosin head (H) or human platelet actin (A). Stained.

phoretic mobility as myosin. The antisera did not react with platelet myosin head or actin.

The experiment shown in Fig. 4 also illustrates the danger of repeated immunization and the necessity of using crude antigen mixtures when evaluating antisera. An antiserum obtained after four boosts with purified myosin formed precipitin lines with *two* components in the crude platelet extract
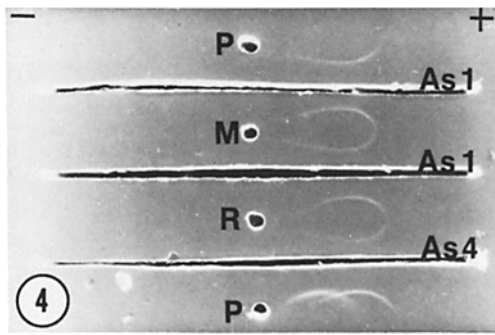
FIGURE 4 Immunoelectrophoretic analysis of the antisera obtained after one boost (*As1*) and four boosts (*As4*) of rabbit 3. Purified antigens, human platelet myosin (*M*), and human platelet myosin rod (*R*), form a single precipitin line with both antisera. A crude extract of human platelets (*P*) forms a single precipitin line with *As1* and two precipitin lines with *As4*. Unstained.

and only *one* precipitin line against purified platelet myosin rod. The lines formed by reaction with the crude extract did not fuse, indicating that they are serologically different. From their electrophoretic mobility, it is clear that the anodic precipitin line represents myosin. The identity of the contaminating antibody is unknown; however it must be against a minor component contaminating the myosin preparation used to immunize the rabbit. The concentration of this contaminating component in the purified antigen was so low that the second precipitin line was undetectable when the antiserum from the hyperimmunized rabbit reacted with the purified test antigens. However, the concentration of the contaminant was high enough in the crude extract to form a precipitin line and reveal the presence of the second antibody. This experiment also shows that a high resolution test such as immunoelectrophoresis is essential in evaluating antisera because this hyperimmune serum sometimes forms a *single* precipitin line on double immunodiffusion against the crude extract.

CROSSED IMMUNOELECTROPHORESIS

By conventional crossed immunoelectrophoresis we showed that the antisera against platelet myosin reacted with a single component in the crude platelet extract (54). By tandem crossed immunoelectrophoresis we identified this component as myosin (Fig. 5). This technique is very sensitive, being capable of detecting less than 0.1 μg of myosin. In addition, it can be used for quantitative analysis. For example, from the ratio of the crude extract and myosin peak heights in

Fig. 5, we estimated that the myosin content of the extract was about 1.5%. This figure is close to estimates of the myosin content in human platelets made by other methods (52).

Preparation of Fluorescent Antibody

We used the Cebra and Goldstein (13) procedure to obtain fluorescent IgG giving bright specific staining and minimal nonspecific staining. During the fractionation more than 80% of the IgG was lost, but this sacrifice was necessary to obtain reliable staining reagents.

Fixation and Staining Conditions

To stain the cytoplasm with fluorescent antibody, it was necessary to fix cellular components in place and to give the large immunoglobulin-fluorochrome conjugate access to the cell interior. For localizing myosin, we used cold acetone to immobilize the myosin and lyse the cell membrane. This treatment preserved general cell morphology and gave the brightest anti-myosin staining. We usually used acetone at $-20°C$, but temperatures between $-70°C$ and room temperature gave similar results. Acetone treatment followed by fixation with 1–1.5% formalin in PBS (61) preserved the life-like morphology of cells and gave staining patterns identical with acetone treatment alone. With cells fixed in 10% formalin in PBS and then treated with acetone, we obtained similar antimyosin staining patterns but the intensity was less than with acetone alone.
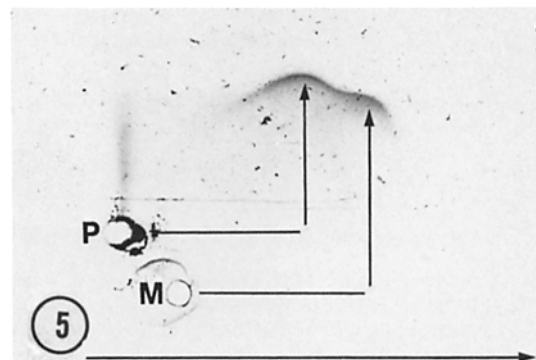

FIGURE 5 Analysis of an antiserum (rabbit 6) against human platelet myosin by tandem crossed immunoelectrophoresis. The two precipitin peaks formed by a crude human platelet extract (*P*) and purified human platelet myosin (*M*) fuse completely. The two long arrows indicate the directions of the two electrophoretic fields. Stained.

Inverting small coverslips onto drops of fluorescent antibody allowed us to use as little as 50 μl of reagent per coverslip. We used 2.5-320 μg/ml of fluorescent immune IgG and 1-32 μg/ml of fluorescent purified antimyosin for staining. The fluorescence intensity of platelets, measured with a microscope photometer, was roughly proportional to the concentration of fluorescent antibody used for staining. All concentrations gave similar staining patterns.

We have confirmed the observations of Cebra and Goldstein (13) on the staining properties of IgG coupled with different amounts of fluorochrome. Specific staining was achieved with conjugates having 2-4 fluorochrome molecules per IgG. The staining intensity was low with conjugates having less than 1 fluorochrome per IgG. IgG coupled with more than 4 fluorochromes per IgG stained cells nonspecifically in locations like the nucleus.

Staining of the Human Blood Smear

Of the formed elements in the blood, only the platelets and the cytoplasm of leukocytes stained with fluorescent antimyosin IgG (Fig. 6b). Occasionally there was a very faint staining of erythrocytes, but it was so weak that it was difficult to record photographically. There was no staining of platelets or leukocytes with fluorescent preimmune IgG (Fig. 6d) or with fluorescent immune IgG adsorbed with platelet myosin (Fig. 6f). The specific fluorescence was greatly reduced or completely blocked when the smear was stained with fluorescent immune IgG mixed with an excess of unlabeled antiserum (Fig. 6h), but a bright specific fluorescence was seen when the smear was stained with labeled immune IgG mixed with the same concentration of preimmune serum (Fig. 6j). There was no detectable staining of erythrocytes in the presence of preimmune IgG.

Staining of Human Tissue Culture Cells during Interphase

We used fluorescent antibodies against human platelet myosin to localize myosin in two human tissue culture cell lines, Enson and HeLa. This was reasonable because the antisera reacted with a single component in HeLa cells which was serologically identical with platelet myosin.

ENSON CELLS

Enson cells are very large, flat epithelial cells whose morphology suggests that they are not ac-

tively motile. Their cytoplasm is laced with phase-dense straight fibers, known as stress fibers. These stress fibers survived cold acetone treatment (Figs. 7a and 7c) and were the only structure in these cells which stained strongly with fluorescent immune IgG or purified fluorescent antibody against platelet myosin (Figs. 7b and 7d). Individual stress fibers were about 0.5-2 μm wide and up to several hundred micrometers long. The thicker fibers appeared to be formed by the lateral fusion of thin fibers. The long stress fibers tended to be arranged parallel to each other, but short fibers often ran obliquely across the cell. The ends of some of these fibers converged at focal points forming pine needle-like patterns. The edge of some fibers was close to the margin of the cell, but the antimyosin staining at the ends of the stress fibers usually terminated several micrometers away from the edge of the cell. As established by electron microscopy (10, 27, 41) most of the stress fibers were parallel to the substrate and close to the plasma membrane at the base of the cell. In some parts of these cells, dense parallel arrays of very fine fibers formed a sheet across the base of the cell. A few fibers arched over the nucleus. We have confirmed the earlier report (73), that the antimyosin staining of stress fibers is discontinuous.

HeLa Cells

The pattern of antimyosin staining in HeLa cells depended on the stage of the cell cycle and the culture conditions. During interphase three patterns were found: concentration in phase-dense stress fibers, concentration in fibers invisible by phase contrast optics, and general distribution throughout the cytoplasm.

Some HeLa cells had phase-dense fibers, especially if the culture medium was not changed for a week or longer (Figs. 8a and 8c). In these cells, the staining with antimyosin was confined to these stress fibers (Figs. 8b and 8d). Morphologically, the fluorescent staining pattern was similar to the staining pattern of Enson cells, although the HeLa cells were much smaller. At high magnification the discontinuous nature of the staining was clear (Fig. 9). Each unit of staining appeared as a spot about 0.5 μm in diameter. Detection of asymmetry in these spots was beyond the resolution of the light microscope. The fluorescent spots were arranged in lines along the stress fibers like beads on a string. They were separated from each other by unstained spaces of about 0.5 μm. The spots on
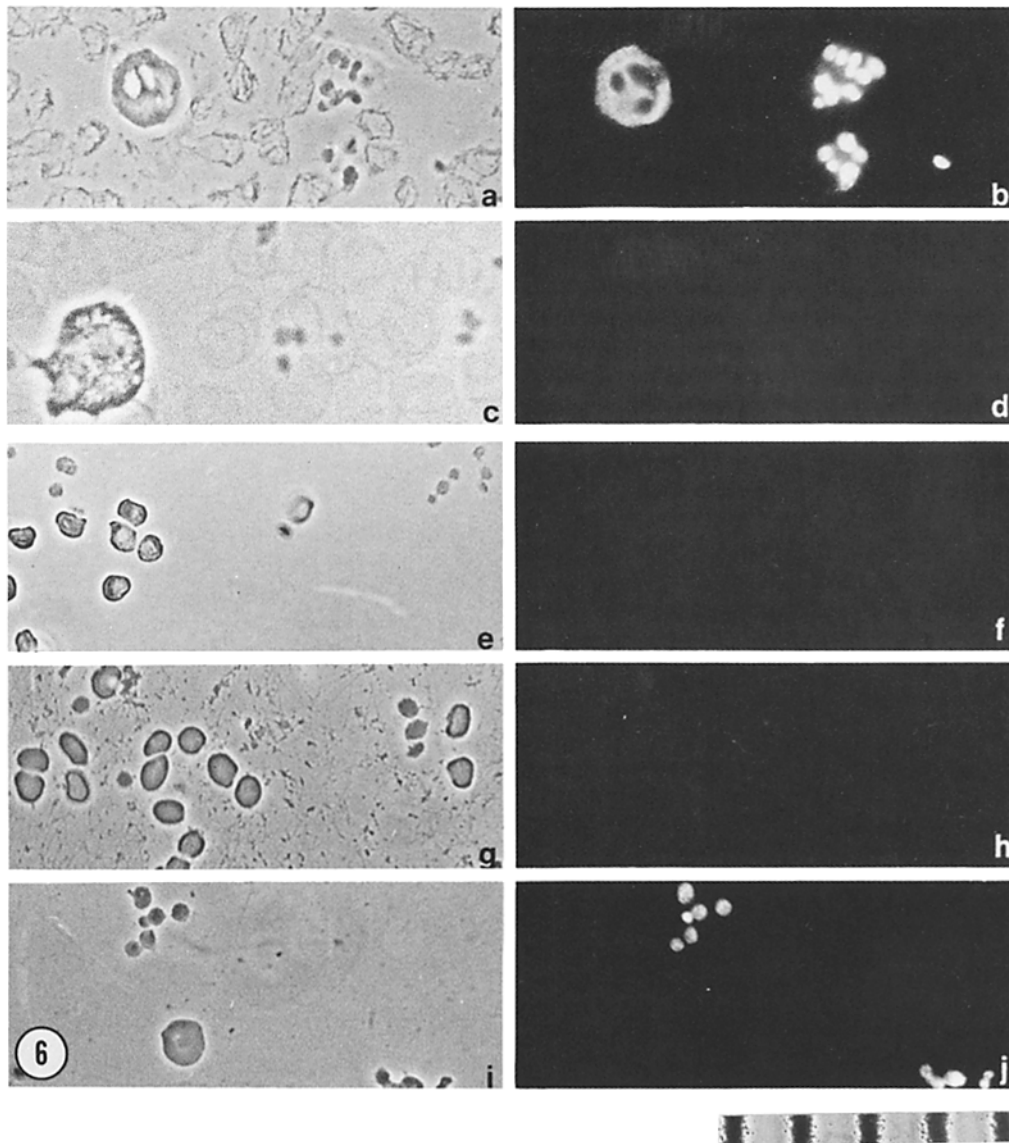
FIGURE 6   Human blood smears treated with acetone and then stained with various fluorescent reagents. These experiments represent the minimum controls necessary to interpret the significance of the staining. Each fluorescence micrograph is accompanied on its left by a phase-contrast image of the same field. (a) and (b) 60 μg/ml of immune IgG from rabbit 8 labeled with 2 rhodamines per molecule. The platelets and the cytoplasm of the leukocyte are brightly stained. (c) and (d) 90 μg/ml of preimmune IgG from rabbit 6 labeled with 3.5 fluoresceins per molecule. None of the platelets or leukocytes stain. (e) and (f) 56 μg/ml of immune IgG from rabbit 8 labeled with 1.8 rhodamines per molecule and adsorbed with purified human platelet myosin coupled to polacryalmide beads. No staining is observed. (g) and (h) 60 μg/ml of immune IgG from rabbit 8 labeled with 2 rhodamines per molecule mixed with 80% immune serum from rabbit 8. No staining is observed. (i) and (j) 60 μg/ml of immune IgG from rabbit 8 labeled with 2 rhodamines per molecule mixed with 80% preimmune serum from rabbit 8. The platelets and leukocytes (not shown) stain brightly, but not the erythrocytes. Scale: 1 division = 10 μm.
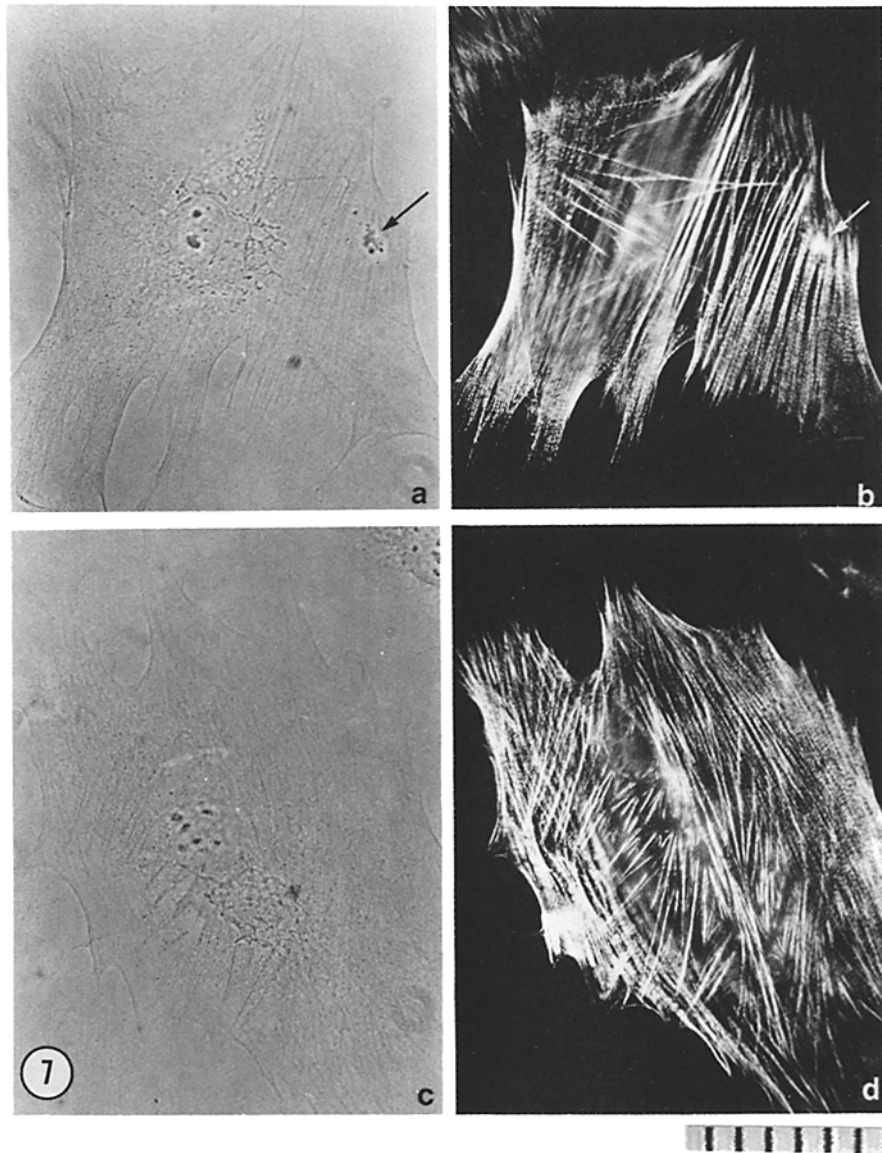
FIGURE 7 Enson cells stained with labeled immune IgG (rabbit 8, 72 µg/ml IgG with 2 rhodamines per molecule). Stress fibers which are detectable with phase-contrast microscopy (a and c) stain strongly with the labeled immune IgG (b and d). In short stress fibers branching is easily recognized. There is no nuclear staining. The arrow indicates some debris which stains nonspecifically (a and b). Scale: 1 division = 10 µm.

adjacent fibers were usually not in register. When several of these fine fibers merged to form a thicker fiber, the staining appeared continuous. This is probably due to the periodicity of the spots on adjacent subfibers being out of register with their neighbors and obscuring the dark intervals between the spots. We speculate that the spots stained with fluorescent antimyosin may represent individual myosin filaments, because the spots are about the same size as the cytoplasmic myosin aggregates formed in vitro (1, 10, 47, 64).

Most HeLa cells did not have phase-dense stress fibers either before or after fixation (Figs. 10a, 11a and 11c). In some of these cells the anti-
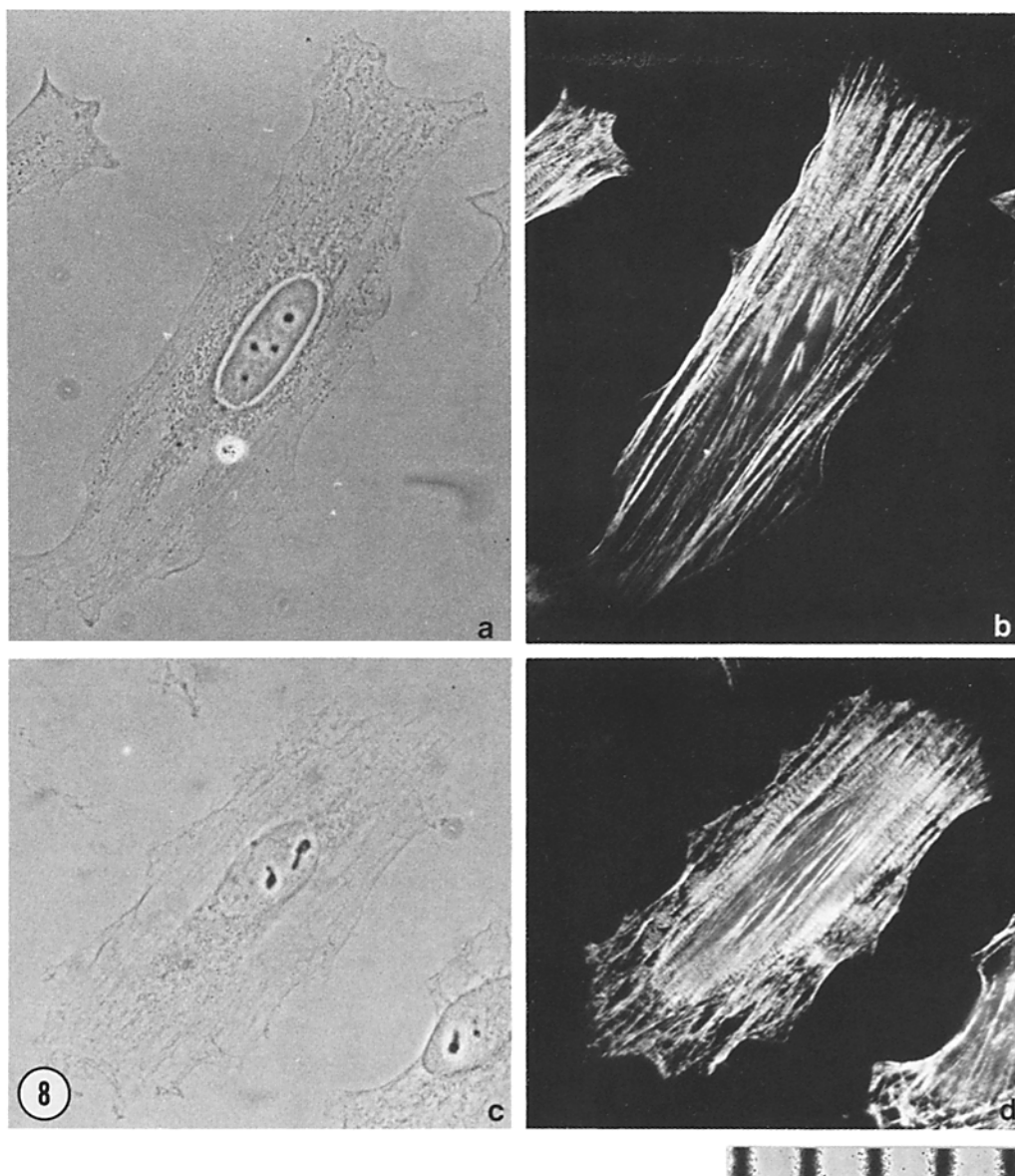
FIGURE 8   HeLa cells stained with labeled immune IgG (rabbit 8, 72 μg/ml IgG with 2 rhodamines per molecule). The phase-contrast micrographs (a and c) show stress fibers, which stain brightly (b and d). There is no nuclear staining. Scale: 1 division = 10 μm.

myosin staining was concentrated in fibers that closely resemble stress fibers (Fig. 10b), but which were undetectable by phase contrast microscopy. These cells were usually found in confluent parts of the cultures. Given their spread morphology, we suspect that they are not actively motile.

In other cells, without phase-dense stress fibers, the antimyosin staining was spread throughout the cytoplasm (Figs. 11b and 11d). Typically, these cells were found in nonconfluent cultures or at the edge of cell colonies. Judging from their morphology, it is our impression that they are actively motile.

Most nuclei did not stain with fluorescent immune IgG, although there was some nuclear and nucleolar staining in isolated regions of the prepa-
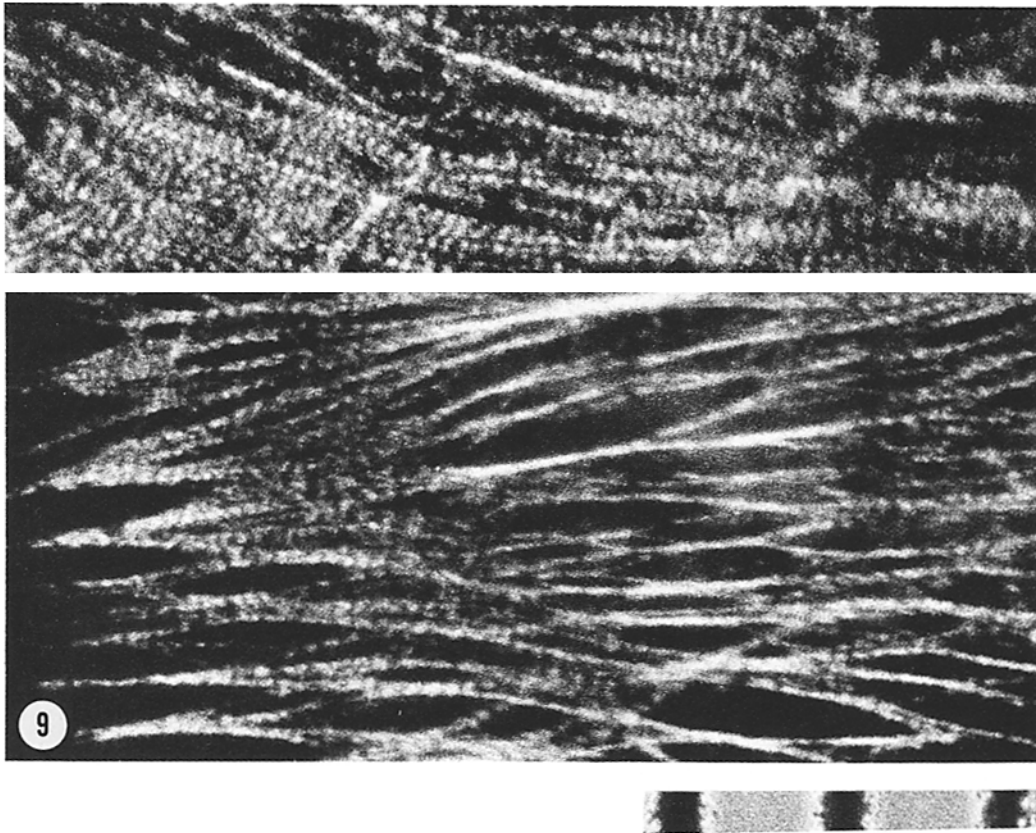
FIGURE 9   HeLa stress fibers stained with labeled immune IgG (rabbit 8, 60 μg/ml IgG with 2 rhodamines per molecule). These high magnification fluorescence micrographs show the staining pattern within stress fibers in detail. Many stress fibers are made up of linearly arranged discrete fluorescent spots. These spots have a diameter of about 0.5 μm. Branching is seen in many fibers. Scale: 1 division = 10 μm.
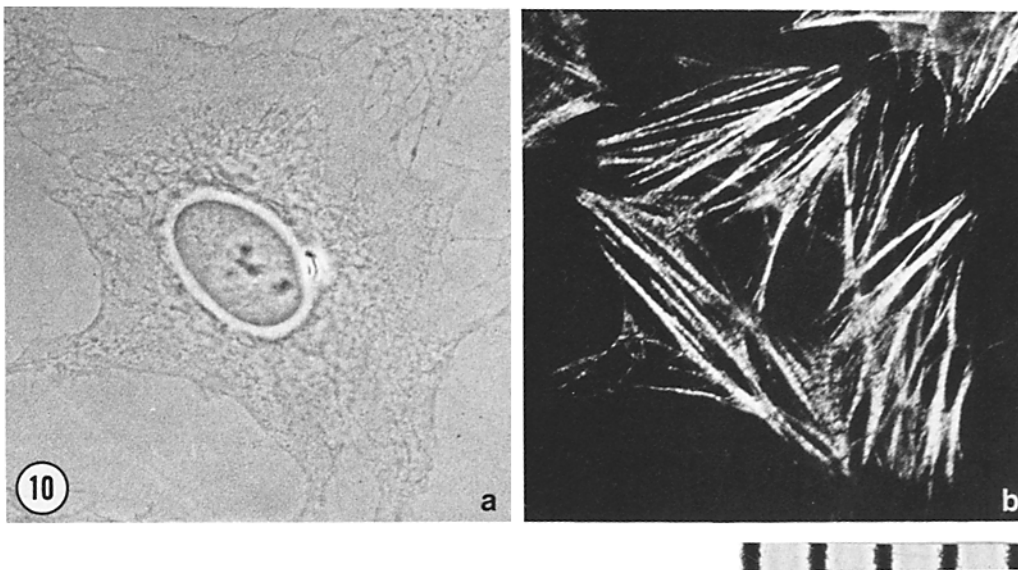

FIGURE 10   HeLa cells stained with labeled purified antibody (rabbit 8, 22 μg/ml IgG with 2 rhodamines per molecule). The phase-contrast image (a) reveals no stress fibers, but the fluorescent image (b) shows many cytoplasmic fibers which are morphologically identical to the stress fibers shown in Figs. 8 and 9. There is no nuclear staining. Scale: 1 division = 10 μm.
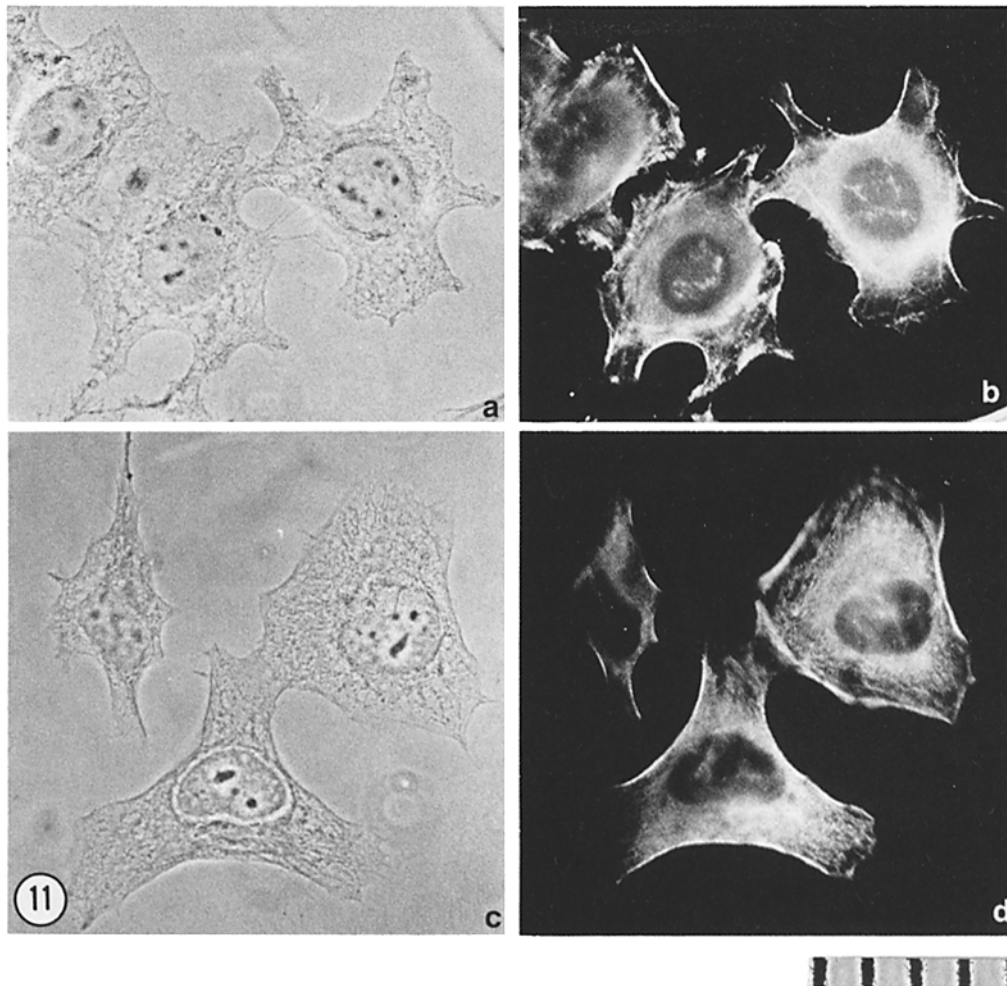
FIGURE 11 HeLa cells stained with labeled purified antibody (rabbit 8, 22 μg/ml IgG with 2 rhodamines per molecule). These cells are less spread and show no stress fibers in their cytoplasm (a and c). The antimyosin staining is found throughout the cytoplasm (b and d). Judging from their morphology, we believe that these cells are actively motile. The staining seen in the nuclear region is outside the nuclei. Scale: 1 division = 10 μm.

rations. Because this staining was rarely found with purified fluorescent antimyosin, we believe that it is artifactual. This conclusion is supported by the control experiments described in the following paragraphs.

CONTROLS

Various control experiments showed that all of the staining patterns described above were specific for myosin. When cells were stained with fluorescent IgG from preimmune serum (Fig. 12b) or with fluorescent IgG adsorbed with purified myosin (Fig. 12d), there was no detectable stain-

ing of stress fibers, myosin-containing cytoplasmic fibers, and general cytoplasm; however occasional nuclear staining could be seen (Figs. 13b and 13c). The myosin-specific fluorescence was greatly reduced and usually completely blocked by staining cells with a myosin-specific fluorescent reagent mixed with an excess amount of unlabeled antiserum against myosin (Fig. 14b). However, the presence of preimmune serum did not block the myosin-specific staining (Fig. 15). In these preparations, the cytoplasmic fibers were somewhat thinner and their staining discontinuity was clearer. There was no nuclear fluorescence in cells
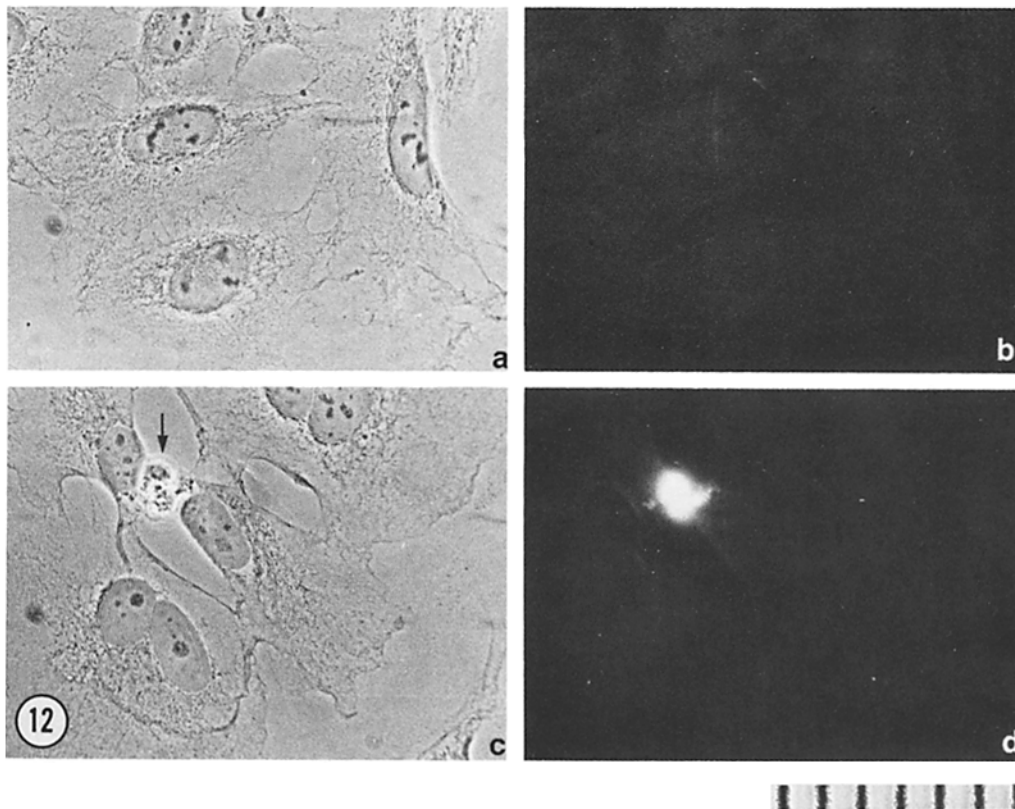
Figure 12 HeLa cells stained with labeled preimmune IgG (rabbit 6, 90 µg/ml IgG with 3.5 fluoresceins per molecule) and labeled adsorbed immune IgG (rabbit 6, 40 µg/ml IgG with 4 fluoresceins per molecule). (b) cells stained with preimmune IgG. (d) cells stained with adsorbed immune IgG. There is very weak or no fluorescence detectable in these preparations. The phase-contrast micrographs of the same field (a and c) are shown on the left of the fluorescent micrographs. The bright fluorescent spot in (d) is cell debris (c; arrow) which stains nonspecifically. Scale: 1 division = 10 µm.

stained in the presence of preimmune serum, which blocked nonspecific interactions of IgG with the cell. Labeled purified antibody gave staining patterns identical with labeled immune IgG (Figs. 10 and 11).

There was strong, nonspecific staining of cell debris and decomposing cells with labeled preimmune IgG, labeled immune IgG, purified antibody, and adsorbed immune IgG (Figs. 12c and 12d). This intense staining was not blocked by unlabeled immune or preimmune serum. Its cause is not known.

Surface Staining

We were unable to detect any myosin on the outside of platelets or HeLa cells by staining intact cells with fluorescent antimyosin (Figs. 16b and 16d). Elsewhere, we have reported physiological

evidence that none of our eight antiplatelet myosin sera bind to the surface of human platelets (53).

Staining of HeLa Cells during Division

STAINING WITH FLUORESCENT IMMUNE IgG

Antimyosin staining patterns during mitosis and cytokinesis are illustrated in Figs. 17 and 18. During these important events most of the antimyosin staining was spread evenly through the cytoplasm but staining was concentrated in the spindle during mitosis and in the cleavage furrow during cytokinesis.

When flat interphase cells rounded up in prophase, the entire cytoplasm stained uniformly with fluorescent immune IgG. The nucleus containing the condensing chromosomes was unstained. After the nuclear membrane broke down, the
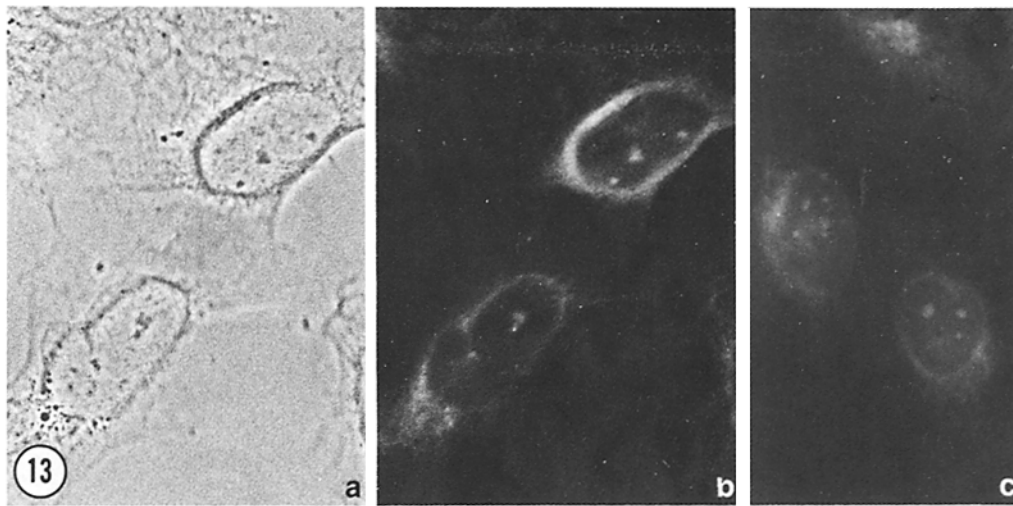
FIGURE 13 Staining with labeled immune IgG adsorbed with purified myosin (*b*) and labeled preimmune IgG (*c*). The staining reagents are described in the legends for Fig. 12. This illustrates an occasional nuclear, especially nucleolar, staining with these fluorescent reagents. (*a*) phase contrast, and (*b*) and (*c*) fluorescence. Scale: 1 division = 10 μm.
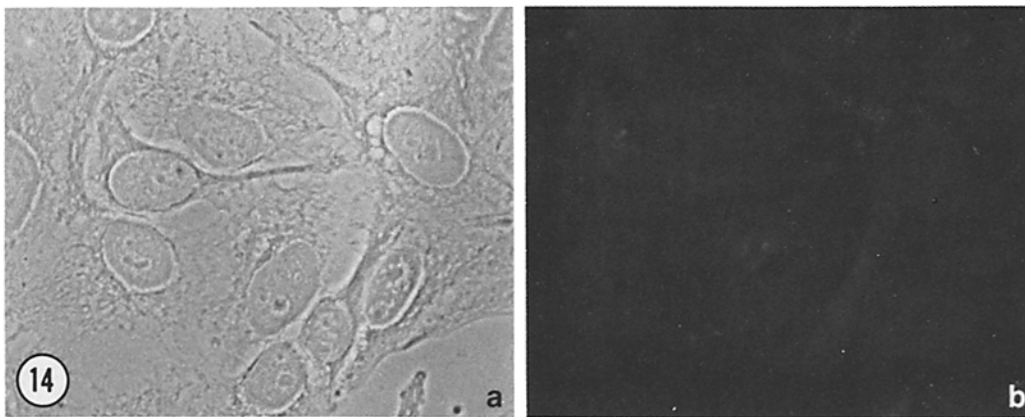

FIGURE 14 HeLa cells stained with labeled purified antibody mixed with unlabeled immune serum (rabbit 8, 22 μg/ml IgG with 2 rhodamines per molecule mixed with 80% immune serum from rabbit 8). The myosin-specific staining is completely blocked. (*a*) phase-contrast image of the field shown in (*b*). Scale: 1 division = 10 μm.

unstained chromosomes were outlined by bright antimyosin fluorescence (Figs. 17*b* and 17*g*). In metaphase and early anaphase, staining was found throughout the cytoplasm, but was more intense in the mitotic spindle than in other regions (Figs. 17*d* and 17*h*). The low fluorescence contrast of the mitotic spindle, relative to the brightly stained surrounding cytoplasm usually made it difficult to photograph the spindle fluorescence. Nevertheless, the fluorescence of a spindle was easily perceived at the microscope by focusing through the spindle region. In metaphase and anaphase, staining was most intense from the chromosomes to the poles, but we were unable to resolve clearly
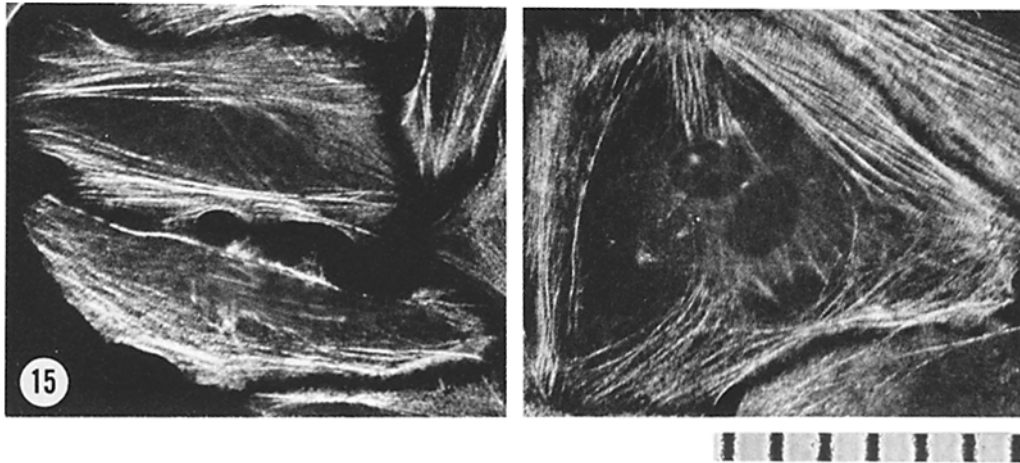
FIGURE 15 HeLa cells stained with labeled immune IgG mixed with unlabeled preimmune serum (rabbit 8, 60 μg/ml IgG with 2 rhodamines per molecule mixed with 80% preimmune serum from rabbit 8). The specific staining of the stress fibers is not blocked by the preimmune serum. There is no nuclear staining. Scale: 1 division = 10 μm.
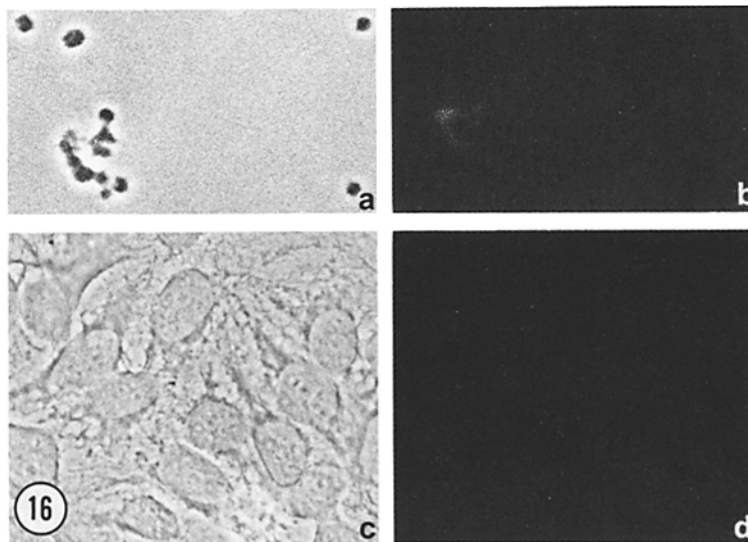

FIGURE 16 Absence of surface staining in human platelets (b) and HeLa cells (d) with labeled immune IgG (rabbit 4, 52 μg/ml IgG with 2.5 rhodamines per molecule). (a) and (c): phase-contrast images of the field shown in (b) and (d).

any fluorescent fibrous structure comparable to a spindle fiber. The fiber-like staining patterns seen in some metaphase spindles (Figs. 17 d and 17 h) represent spaces between chromosomes and should not be mistaken for spindle fibers. In late anaphase the staining was most intense in the polar regions (Fig. 17 f). The region between the separated chromosomes was also stained, but

much of this intensity is attributable to the cleavage furrow surrounding this part of the cell.

Throughout cytokinesis, antimyosin staining was strongest in the cleavage furrow (Fig. 18). In late anaphase, before the cleavage furrow had formed, a bright fluorescent ring was found around the equator, midway between the separated chromosomes (Fig. 18 b). By focusing
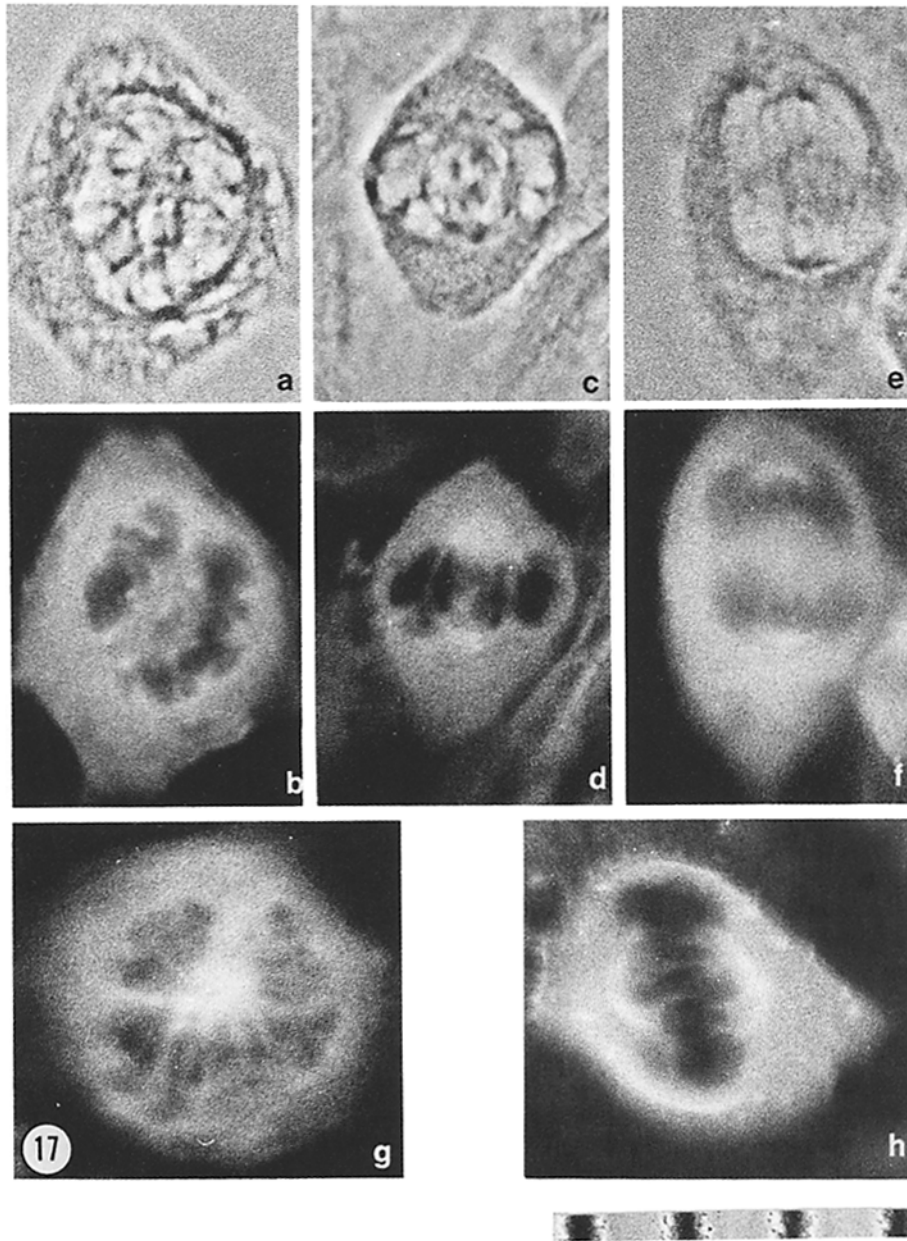
FIGURE 17   Staining of HeLa cells during mitosis with labeled immune IgG (72 μg/ml IgG with 2 rhodamines per molecule). (a, c, and e) phase-contrast images of the cells in (b, d, and f). Myosin-specific staining in the mitotic HeLa cells occurs throughout the cytoplasm except the region occupied by the chromosomes. In prophase cells, the fluorescence is found throughout the cell except the chromosomes (b and g). During metaphase stronger staining is detectable in the mitotic spindle (d and h) especially from chromosomes toward the poles. In late anaphase the two polar regions stain intensely (f). The fluorescence at the cell equator is due to the staining of the cleavage furrow. Scale: 1 division = 10 μm.
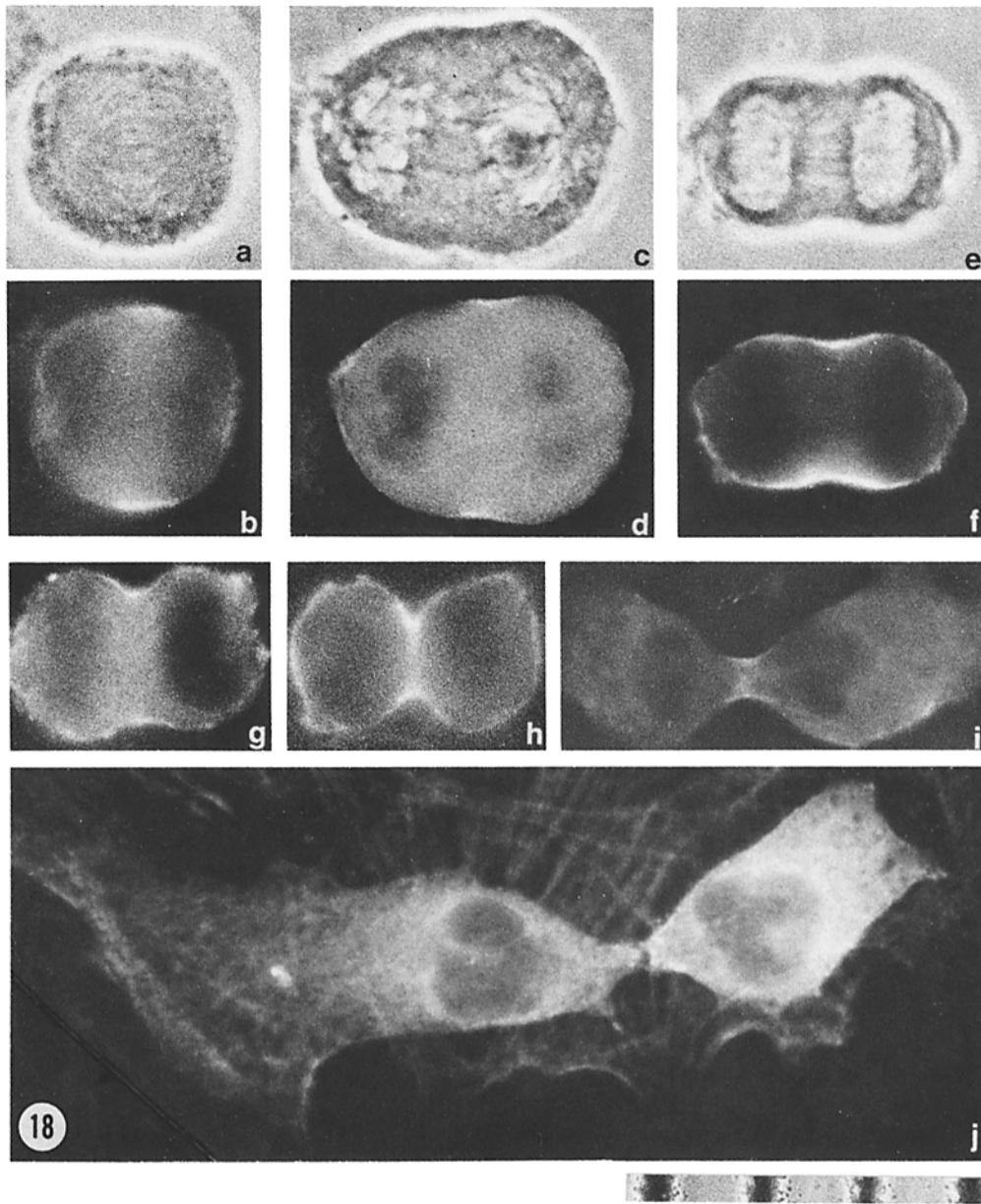
FIGURE 18 Staining of HeLa cells during cytokinesis with myosin-specific fluorescent reagents. (b, g, and h) stained with 72 μg/ml IgG (rabbit 8) with 2 rhodamines per molecule. (d) stained with 60 μg/ml IgG (rabbit 8) with 2 rhodamines per molecule. (f) stained with 22 μg/ml purified antibody (rabbit 8) with 1.8 rhodamine per molecule. (i and j) stained with 70 μg/ml IgG (rabbit 6) with 3.5 fluoresceins per molecule. (a, c, and e) phase-contrast images of (b, d, and f). High concentration of myosin-specific staining is associated with the cleavage furrow, presumably the contractile ring, throughout cytokinesis (d, f, g, h, and i). The formation of the cleavage furrow (b) is detected in late anaphase (a). After the completion of the cytokinesis process, myosin staining is generalized (j). Note that the stem body does not stain (i and j). Scale: 1 division = 10 μm.

through the cell, it was possible to follow the band of intense staining all the way around the equator. When the center of a cell was in focus, the ring appeared as two bright spots on either side at the equator due to the tangential view (Figs. 18b and 18d). This fluorescent ring marked the site where furrowing begins (Fig. 18d). At later stages of cytokinesis this fluorescent ring was found in the cleavage furrow (Figs. 18f, 18g, 18h and 18i). At the completion of cytokinesis, the fluorescent ring disappeared and the staining pattern was identical with that of a motile interphase cell (Fig. 18j). The stembody was completely free of any staining.

STAINING WITH PURIFIED FLUORESCENT ANTIMYOSIN

Purified fluorescent antimyosin stained the cleavage furrow exactly like fluorescent immune IgG (Fig. 18); however, we found that purified antibody stained the spindle with the same intensity as the rest of the cytoplasm (Figs. 19b and 19d). In some cases the intensity of staining in the spindle region was slightly greater than in other regions, but the contrast was not nearly as striking as in cells stained with immune IgG.

CONTROLS

Neither the mitotic spindle nor the cleavage furrow stained with fluorescent preimmune IgG or with fluorescent immune IgG adsorbed with purified myosin (Figs. 20b and 20d). The staining of the mitotic spindle and the cleavage furrow with fluorescent immune IgG was blocked by unlabeled immune serum but not by unlabeled preimmune serum.

An additional control was provided by our unsuccessful attempts to stain the mitotic spindle and cleavage furrow of cells from some nonhuman species. There was no staining of these structures or other regions of the cytoplasm in dividing rat kangaroo PtK-2 cells or salamander lung cells. Thus, staining of the mitotic spindle and cleavage furrow in human cells with antimyosin is the result of a specific immunological reaction and is not due to nonspecific binding.

DISCUSSION

In the past decade, a number of investigators have used Coons' powerful fluorescent antibody technique (14, 15) to localize motility-related proteins in nonmuscle cells (4, 5, 7, 9, 12, 16, 17, 24, 25, 35, 38, 39, 40, 45, 46, 49, 72, 73, 74). The
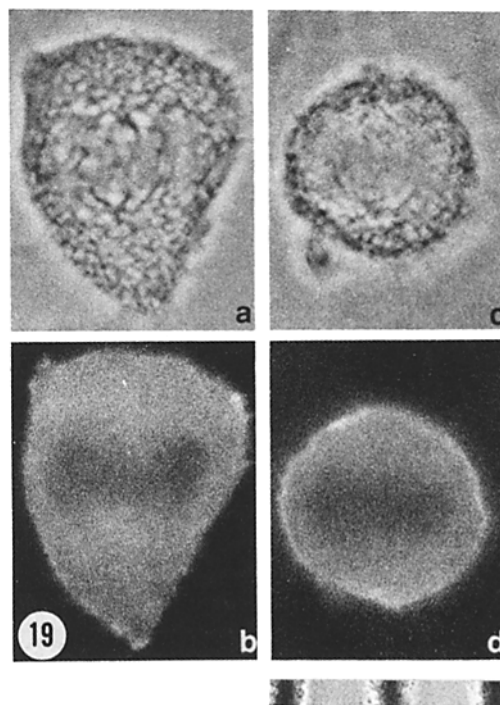

FIGURE 19 Mitotic HeLa cells stained with labeled purified antibody (rabbit 8, 22 μg/ml IgG with 1.8 rhodamine per molecule). The metaphase spindle is clearly visible in the phase-contrast micrographs (a and c), but the corresponding fluorescent image reveals only slight fluorescent contrast between the spindle and the cytoplasm (b and d). Scale: 1 division = 10 μm.

aesthetic beauty of the resulting micrographs makes the work so appealing that one may be inclined to accept uncritically the staining patterns as the actual distribution of a specific protein in the cell. Interpretation of the fluorescent staining patterns is straightforward if the localization of the stained antigen can be established by an independent technique like electron microscopy. This is the case with microtubules in the mitotic spindle (9, 16, 46, 61) and actin filaments in stress fibers (40). On the other hand, if fluorescent antibodies provide the only information on the localization of a protein, the reliability of the data depends heavily on control experiments. Myosin, tropomyosin, and α-actinin are cytoplasmic motility proteins whose localization is unknown, uncertain, and/or difficult to demonstrate by other means. In these cases, it is essential to establish that the staining obtained is specific for a single protein and that the antigen remains in place during fixation, staining,
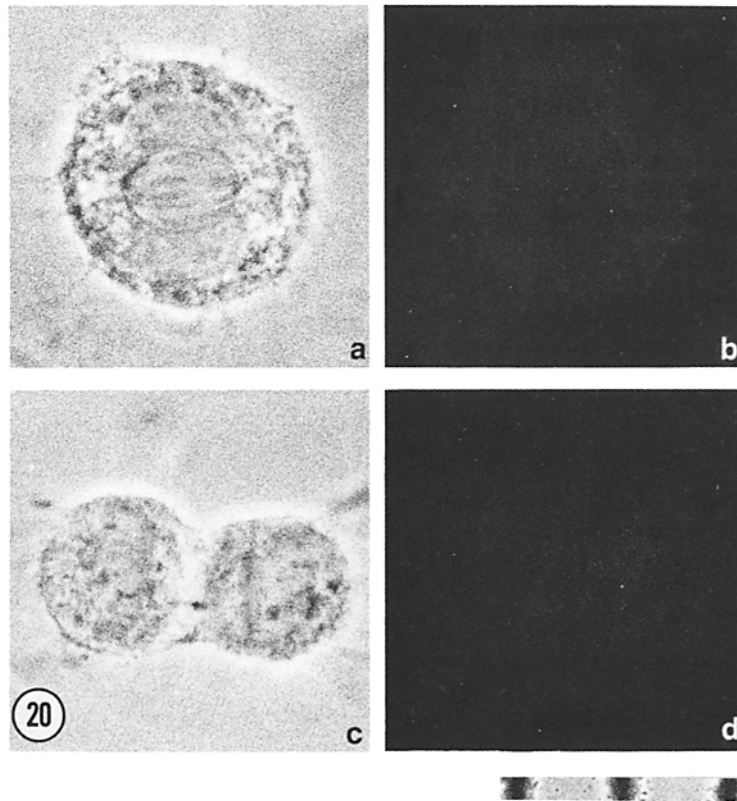
FIGURE 20 HeLa cells in metaphase (a) and in cytokinesis (c) stained with adsorbed labeled immune IgG (rabbit 8, 90 µg/ml IgG with 2 rhodamines per molecule). There is no staining of the spindle (b) and the contractile ring (d). Scale: 1 division = 10 µm.

and washing. In the first four sections of the discussion, we consider the extent to which the methods we employed in the present study meet these criteria. In the two concluding sections of the discussion, we present an interpretation of our results and some thoughts on their biological significance.

Antibody Production and Evaluation

To capitalize on the potential sensitivity and specificity of the fluorescent antibody method, the antibodies must be directed against a single cellular component. To make such antibodies, it is desirable to immunize animals with a homogeneous antigen, but, realistically, most protein preparations isolated from cells contain at least low levels of contaminants. Depending on the concentration and the antigenicity of these contaminants, they may give rise to unwanted antibodies.

To minimize the possibility of unwanted anti-

bodies in our antimyosin sera, we purified the myosin antigens as carefully as possible, measured the extent of contamination, immunized rabbits with care, and tested the sera for antibodies against myosin and other platelet components. The extent of unidentified polypeptide contamination was about 1 to 2% in our best myosin and myosin rod preparations. Faced with the unavoidable presence of these contaminants, we chose to elicit antibodies with a minimal immunization procedure in which we injected small amounts of antigen in adjuvant at multiple sites. With each dose of 250 µg of myosin or myosin rod, we injected 2 to 5 µg of unidentified polypeptide. After a single boost, we detected antibody against myosin alone. Multiple boosts gave rise to additional precipitating antibodies against contaminants. To detect the presence of these unwanted antibodies in the hyperimmune sera, we had to use immunoelectrophoresis of crude cellular extracts

containing high concentrations of both the specific antigen and all possible contaminants. Using the high resolution and sensitivity of crossed immunoelectrophoresis together with crude antigen preparations, we detected only myosin antibodies in the sera used for the fluorescent antibody staining work.

Differences in antigen composition may explain, in part, why our results differ in some ways from previous antimyosin work. Some early work (5, 29, 45) used as antigen crude actomyosin preparations, now known to contain many components besides myosin (52). Some myosin preparations were shown to contain other polypeptides (5, 7). In other cases (72, 74), polyacrylamide gels of purified myosin were underloaded to the extent that the light chains were not visible. Since each light chain comprises more than 6% of the mass of the myosin, fairly high concentrations of contaminants could have gone undetected. Contamination by antigens which are not detected by polyacrylamide gel electrophoresis and staining with protein dyes are a more serious problem. For example, there are small membrane vesicles which are difficult to remove from platelet actomyosin by cycles of low ionic strength precipitation. These vesicles are cleanly separated from myosin by gel filtration on 4% agarose (52). The vesicles elute in the void volume on these columns where they can be detected by their turbidity, by phase contrast microscopy or by electron microscopy, but their presence is not obvious from protein determination or by gel electrophoresis (see Fig. 2 in reference 52). The presence of these vesicles in platelet myosin prepared by other methods may elicit antibodies against membrane components.

Fluorescent Reagents

We stained cells directly with antibody coupled to tetramethylrhodamine and fractionated by the method of Cebra and Goldstein (13). An alternative is to use "indirect" staining methods employing unlabeled specific antibody followed by an anti-immunoglobulin coupled to a fluorescent dye. This "indirect" approach is more convenient for screening many batches of antiserum since it requires only a single fluorescent reagent which can be purchased from commercial sources. It may also amplify the fluorescent image. Nonetheless, we believe that direct staining is superior because (a) there is but one antigen-antibody reaction to be controlled, (b) the immunological nature of

the staining can be demonstrated by blocking the staining with unlabeled antibody, and (c) one can stain simultaneously with two antibodies labeled with contrasting fluorochromes, as we have done with antimyosin and antitubulin (22).

As a fluorochrome, rhodamine is superior to fluorescein, because it resists bleaching in the exciting beam of the microscope. Its negligible bleaching makes it easier to observe and photograph the stained cells and is a prerequisite for quantitative studies by fluorometry.

It is essential to separate over-conjugated and under-conjugated fluorochrome-IgG from the optimal conjugates, because IgG with either insufficient or excess fluorochrome causes staining artifacts. As reported by Cebra and Goldstein (13) and confirmed here, under-conjugated IgG gives very dull staining, while over-conjugated IgG stains cells nonspecifically.

Controls for Staining

While the specificity of fluorescent antibody staining ultimately depends on the quality of the antibody, control experiments can eliminate likely causes of staining artifacts. The occurrence of non-immunological binding of fluorescent-IgG to the cell by the attached fluorochrome, or by regions of the antibody molecule outside the antigen combining site, is eliminated by the absence of staining with adsorbed fluorescent immune IgG or fluorescent preimmune IgG and the presence of staining with fluorescent immune IgG mixed with preimmune serum. The possibility that some of the staining is due to the presence of naturally occurring antibodies is eliminated by the absence of staining with fluorescent preimmune IgG and adsorbed fluorescent immune IgG, and the presence of staining with fluorescent purified antibody. That some of the staining is due to a *low* titer of nonprecipitating antibody against a minor contaminant of the myosin cannot be ruled out, because the control reactions could follow the myosin specific pattern exactly. This hypothetical contaminating antibody would be adsorbed out along with the antimyosin by the small amount of contaminating antigen in the myosin preparation. In the same way, such a contaminating antibody might be purified along with the antimyosin by affinity chromatography. On the other hand, these same controls do eliminate the possibility that the staining is due to a *high* titer of antibody against one or more contaminants. There would not be enough

of any contaminant present at the 1% level in the myosin preparation to adsorb out all of a high concentration of contaminating antibody. Thus, those structures listed in Table I which stain with fluorescent immune IgG, fluorescent purified antibody and fluorescent immune IgG mixed with preimmune serum, and which do not stain with fluorescent preimmune IgG, adsorbed fluorescent immune IgG and fluorescent immune IgG mixed with immune serum most likely contain myosin.

Preservation of Natural
Antibody Distribution

Even the best fluorescent antibody reagents would be useless if applied to cells whose antigen is extracted, whose antigen molecules have moved from their natural sites, or whose structure has been seriously distorted. Unfortunately there is presently no assurance that any fixation method avoids all of these problems while simultaneously opening the plasma membrane to provide access to the fluorescent antibody to the cytoplasm.

We prepared the cells for fluorescent antimyosin staining by treatment with cold acetone because the natural cellular morphology was generally preserved and there was minimal evidence for nonspecific staining. With formalin fixation followed by acetone treatment, the distribution of specific antimyosin staining was identical but less intense. In addition, there was definite nonspecific staining. It is reasonable to use acetone treatment as a "fixation" of myosin, because acetone is known to render myosin insoluble in dilute aqueous buffers. In fact, work in progress indicates that little or no myosin is extracted from acetone-treated HeLa cells by our washing procedures. On the other hand, some other proteins remain soluble after acetone dehydration and can be extracted during staining and washing.

Whether or not the myosin retains its natural distribution in the cytoplasm during preparation for staining is not known, although it is our unproven impression that the acetone treatment is a freeze substitution process which might immobilize the myosin in situ. When coverslips are plunged into the −20°C acetone, the thin film of buffer over the cells appears to freeze. Subsequently the ice appears to dissolve in the cold acetone, dehydrating and "fixing" the frozen cells. Two additional observations are consistent with the natural distribution of myosin being maintained inside the freeze-dried cells: (a) the specific

staining patterns are the same with and without prior formalin fixation and (b) double staining the cells with a second fluorescent antibody shows that myosin and tubulin are located in different parts of the same cell (22), showing that there is morphological specificity to the distribution of myosin in the fixed cell.

Interpretation of Observations

Using all of the criteria for specificity discussed in the previous sections, we conclude that myosin is located in the general cytoplasm of platelets, leukocytes, and motile cultured cells and that myosin is concentrated in the cleavage furrow during cytokinesis (Table II). Although unproven by light microscopic observations, it seems likely that the fluorescent antibody in the region of the cleavage furrow is bound to myosin in the contractile ring. We confirm an earlier report (73) that myosin is concentrated at intervals along the stress fibers of cultured cells.

Using these same criteria for specificity, we conclude that there is no detectable myosin on the outer surface of platelets, erythrocytes or cultured cells, inside erythrocytes, in cell organelles, in the nucleus, or in chromosomes. Our observations fail to confirm earlier reports of myosin staining on the outer surface of platelets (45, 55) and cultured cells (25, 74).

The question of myosin in the mitotic spindle presents a special problem. By all criteria, myosin is present throughout the cytoplasm of dividing cells, including the spindle. By most criteria, myosin is concentrated in the mitotic spindle during metaphase and anaphase, especially between the chromosomes and the poles. The uncertainty regarding the concentration of myosin in the spindle arises because labeled purified antimyosin does not stain the spindle much more intensely than the rest of the cytoplasm. One interpretation may be that high affinity antibodies, which are lost during antibody purification, are necessary to stain part of the myosin in the spindle. This fraction of myosin could be aggregated differently or associated with different molecules than the bulk of the cell's myosin which, in contrast, stains the same with labeled immune IgG and labeled purified antibody. It is clear that the antibody which stains the spindle intensely is actually lost or destroyed during antibody purification, because it is present in the sample applied to the affinity column and is absent from both the adsorbed material which passes directly through the column and the puri-

TABLE II

Summary of Fluorescent Antibody Staining

Fluorescent reagents	Immune IgG	Purified anti-body against myosin	Preimmune IgG	Adsorbed im-mune IgG	Immune IgG or antibody mixed with unlabeled immune serum	Immune IgG or antibody mixed with unlabeled preimmune se-rum
Cytoplasm						
Human blood smear						
Leukocyte	+	+	−	−	−	+
Platelet	+	+	−	−	−	+
Erythrocyte	−(+)	−(+)	−(+)	−(+)	−	−
Enson stress fiber	+	+	−	−	−	+
HeLa						
Stress fiber	+	+	−	−	−	+
Cytoplasmic fiber	+	+	−	−	−	+
Cytoplasm	+	+	−	−	−	+
Nucleus	−(+)	−(+)	−(+)	−(+)	−	−
Mitotic spindle (HeLa)	+	+?	−	−	−	+
Cleavage furrow (HeLa)	+	+	−	−	−	+
Cell surface						
Blood smear	−	−				
HeLa	−	−				
Cell debris and dead cells*	+	+	+	+	+	+

+, strong staining; −, no staining; −(+), generally no staining, but occasional staining; +(?), the spindle and the surrounding cytoplasm stain with equal intensity; *, cell debris and dead cells stain non-specifically with all the fluorescent reagents.

fied antibody eluted with acid. Thus we believe that myosin is concentrated in the mitotic spindle, but feel that this point needs further investigation.

Biological Significance of the Results

The broadest conclusion of the work is that myosin molecules change their cytoplasmic distribution in a predictable way as the cell passes through the cell cycle. The patterns of myosin distribution are similar to those previously established for actin by electron microscopy (20, 26, 27, 28, 31, 33, 34, 41, 44, 50, 62), fluorescent heavy meromyosin staining (58, 59, 60), and fluorescent antiactin staining (38, 40). Thus, there is a mechanism to control the spatial and temporal distribution of both actin and myosin to meet particular needs of the cell at each stage in its life cycle. The myosin may simply be following the actin around the cell, although its localization could be controlled independently.

Myosin is a gregarious contractile protein. Its constant association with actin inside cells means

that myosin localization studies can be used to predict sites of potential force generating capacity. We emphasize that this is only potential force generating capacity, because it is likely that the actual utilization of this potential is controlled by local variations in calcium ion concentration (67, 68, 69) or other factors.

There are now two good examples of the coexistence of actin and myosin within cells: the contractile ring and stress fibers. It was previously established that the cleavage furrow develops the force for cytokinesis (56) and that the contractile ring which is located in the cleavage furrow contains actin filaments (50, 62). Our demonstration that myosin is concentrated in the cleavage furrow provides further evidence that actomyosin may be responsible for generating the force for cytokinesis. It is also well established that stress fibers contain both actin (27, 28, 40) and myosin (72, 73, present study). Although there is little evidence relating stress fibers to cell motility, Isenberg et al. (33) showed that isolated stress fibers

can contract. Now ultrastructural studies are necessary to determine how the myosin and actin relate to each other in these organized actomyosin bundles.

These two examples encourage us to think that other sites where actin and myosin are found together are also sites of motile force generation. In the following paragraphs we consider mitosis and cell locomotion. In neither case is there any direct proof for the involvement of actomyosin, though in both cases our studies on myosin localization provide some clues about mechanisms.

The localization of both actin (12, 20, 26, 31, 42, 59) and myosin in the mitotic spindle forces one to consider seriously a contractile mechanism for chromosome movements. If actin were present in the spindle without myosin, it might be attributed an exclusively structural role. However, the coexistence of actin and myosin between the chromosomes and the poles means that these contractile proteins could develop the force to move the chromosomes. Theoretical calculations show that very few myosin filaments would be needed to develop the force to pull a chromosome to the pole (54). As discussed by others (19, 57, 59), contractile proteins might interact with spindle microtubules in two ways: (a) the microtubules may be the attachment sites for the actin or myosin filaments, linking them indirectly to the chromosomes and the poles; and (b) the kinetochore microtubules are likely to resist the action of the contractile proteins so that their depolymerization rate could determine the rate of chromosome movement (23). Further physiological and morphological data will be needed to confirm the role of actomyosin in mitosis and to refine the present sketchy ideas about mitotic mechanisms.

It is our impression that the most motile cells such as leukocytes, have myosin and probably actin (see Fig. 2a in ref. 38) distributed fairly uniformly throughout their cytoplasm. This suggests that all parts of the cytoplasmic matrix are potentially contractile. Taylor has reached the same conclusion from microinjection studies on giant amoebae (67). This suggests that locomotion could be generated by the concerted contractile activity of various regions of the cytoplasmic matrix. There is now evidence that the contractile proteins form a gel in the cytoplasmic matrix (36, 51, 65, 66, 70), so that contractile force generated at any site could be transmitted through the matrix to the cell surface and the substrate. Although this

sort of mechanism is not well understood, it seems to differ from the situation in the contractile ring where force is generated locally and constricts a limited region of the cell surface.

REFERENCES

1. ADELSTEIN, R. S., M. A. CONTI, G. S. JOHNSON, I. PASTAN, and T. D. POLLARD. 1972. Isolation and characterization of myosin from cloned mouse fibroblasts. *Proc. Natl. Acad. Sci. U. S. A.* **69:**3693-3697.
2. ALLERA, A., R. BECK, and K. E. WOHLFARTH-BOTTERMAN. 1971. Extensive fibrillar protoplasmic differentiations and their significance for protoplasmic streaming. VII. Identification of plasma filaments in *Physarum polycephalum* as F-actin by *in situ* binding of heavy meromyosin. *Cytobiologie.* **3:**437.
3. AXELSEN, N. H., J. KRØLL, and B. WEEKE, editors. 1973. A manual of quantitative immunoelectrophoresis. Methods and Applications. *Scand. J. Immunol.* **2**(suppl. 1).
4. BECKER, C. G., and G. E. MURPHY. 1969. Demonstration of contractile proteins in endothelium and cells of the heart valves, endocardium, intima, arteriosclerotic plaques and Aschoff bodies of rheumatic heart disease. *Am. J. Pathol.* **55:**1.
5. BECKER, C. G., and R. L. NACHMAN. 1973. Contractile proteins of endothelial cells, platelets and smooth muscle. *Am. J. Pathol.* **71:**1-22.
6. BEHNKE, O., B. I. KRISTENSEN, and L. E. NIELSEN. 1971. Electron miroscopical observations on actinoid and myosinoid filaments in blood platelets. *J. Ultrastruct. Res.* **37:**351.
7. BOOYSE, F. M., L. A. STERNBERGER, D. ZSCHOCKE, and M. E. RAFELSON. 1971. Ultrastructural localization of contractile protein (thrombos-

thenin) in human platelets using an unlabeled antibody peroxidase staining technique. *J. Histochem. Cytochem.* **19**:540-550.

8. BRANDTZAEG, P. 1975. Rhodamine conjugates: specific and nonspecific binding properties in immunohistochemistry. *Ann. N. Y. Acad. Sci.* **254**:35-54.

9. BRINKLEY, B. R., G. M. FULLER, and D. P. HIGHFIELD. 1976. Tubulin antibodies as probes for microtubules in dividing and non-dividing mammalian cells. *In* Cell Motility, R. D. Goldman, T. Pollard, and J. Rosenbaum, editors. Cold Spring Harbor, N.Y. In press.

10. BUCKLEY, I. K., and K. R. PORTER. 1967. Cytoplasmic fibrils in living cultured cells. A light and electron microscope study. *Protoplasma.* **64**:349-380.

11. BURRIDGE, K., and D. BRAY. 1975. Purification and structural analysis of myosins from brain and other non-muscle tissues. *J. Mol. Biol.* **99**:1-14.

12. CANDE, W. Z., E. LAZARIDES, and J. R. McINTOSH. 1975. Visualization of actin in functional lysed mitotic cell preparations. *J. Cell Biol.* **67**:54a.

13. CEBRA, J. J., and G. GOLDSTEIN. 1965. Chromatographic purification of tetramethylrhodamine-immune globulin conjugates and their use in the cellular localization of rabbit γ-globulin polypeptide chains. *J. Immunol.* **95**:230-245.

14. COONS, A. H., H. J. CREECH, and R. N. JONES. 1941. Immunological properties of an antibody containing a fluorescent group. *Proc. Soc. Exp. Biol. Med.* **47**:200.

15. COONS, A. H., and M. H. KAPLAN. 1950. Localization of antigen in tissue cells. II. Improvements in a method for the detection of antigen by means of fluorescent antibody. *J. Exp. Med.* **91**:1.

16. DALES, S. 1972. Concerning the universality of a microtubule antigen in animal cells. *J. Cell Biol.* **52**:748-754.

17. FAGRAEUS, A., K. LIDMAN, and R. NORBERG. 1975. Indirect immunofluorescence staining of contractile proteins in smeared cells by smooth muscle antibodies. The influence of chelating agents. *Clin. Exp. Immunol.* **20**:469-477.

18. FAIRBANKS, G. T., L. STECK, and D. F. H. WALLACH. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocytes membrane. *Biochemistry.* **10**:2606-2617.

19. FORER, A. 1974. Possible roles of microtubules and actin-like filaments during cell division. *In* Cell Cycle Controls. G. M. Padilla. I. L. Cameron, and A. M. Zimmerman, editors. Academic Press, New York, 319-336.

20. FORER, A., and O. BEHNKE. 1972. An actin-like component in spermatocytes of a crane fly (Nephrotoma suturalis Loew). I. The spindle. *Chromosoma* (*Berl.*). **39**:145-173.

21. FUJIWARA, K., and T. D. POLLARD. 1975. Staining of cells with fluorescent antibody against myosin. *J. Cell Biol.* **67**:125a.

22. FUJIWARA, K., and T. D. POLLARD. 1976. Simultaneous intracellular localization of myosin and tubulin during cell cycle by direct double immunofluorescence. *J. Cell Biol.* **70**(Pt. 2):181a. (Abstr.)

23. FUSELER, J. W. 1975. Temperature dependence of anaphase chromosome velocity and microtubule depolymerization. *J. Cell Biol.* **67**:789-800.

24. GABBIANI, G., G. B. RYAN, J. LAMELIN, P. VASSALI, G. MAJNO, C. A. BOUVIER, A. CRUCHAUD, and E. F. LUSCHER. 1973. Human smooth muscle autoantibody. Its identification as antiactin and a study of its binding to "nonmuscular" cells. *Am. J. Pathol.* **72**:473-487.

25. GARNETT, H., U. GRÖSCHEL-STEWART, B. M. JONES, and R. B. KEMP. 1973. Immunofluorescent detection of a myosin-type protein at the surface of trypsin-dissociated embryonic chick cells. *Cytobios.* **7**:163-169.

26. GAWADI, N. 1974. Characterization and distribution of microfilaments in dividing locust testis cells. *Cytobios.* **10**:17-35.

27. GOLDMAN, R. D. 1975. The use of heavy meromyosin binding as an ultrastructural cytochemical method for localizing and determining the possible functions of actin-like microfilaments in nonmuscle cells. *J. Histochem. Cytochem.* **23**:529-542.

28. GOLDMAN, R. D., J. SCHLOSS, and J. STARGER. 1976. Structure and possible physiological functions of actin-like microfilaments. *In* Cell Motility. R. Goldman, T. Pollard and J. Rosenbaum, editors. Cold Spring Harbor, N.Y. In press.

29. GRÖSCHEL-STEWART, U., B. JONES, and R. KEMP. 1970. Detection of actomyosin-type protein at the surface of dissociated embryonic chick cells. *Nature* (*Lond.*). **227**:280.

30. HARTREE, E. F. 1972. Determination of protein. A modification of the Lowry method that gives a linear photometric response. *Anal. Biochem.* **48**:422-427.

31. HINKLEY, R., and A. TELSER. 1974. Heavy meromyosin binding filaments in the mitotic apparatus of mammalian cells. *Exp. Cell Res.* **86**:161.

32. HOLBOROW, E. J., and G. D. JOHNSON. 1967. Immunofluorescence. *In* Handbook of Experimental Immunology. D. M. Weir, editor. Blackwell Scientific Publications, Ltd. Oxford. 571-596.

33. ISENBERG, G., P. C. RATHKE, N. HULSMANN, W. W. FRANKE, and K. E. WOHLFARTH-BOTTERMAN. 1976. Cytoplasmic actomyosin fibrils in tissue culture cells-direct proof of contractility by visualization of ATP-induced contraction in fibrils isolated by laser microbeam dissection. *Cell Tissue Res.* **166**:427-443.

34. ISHIKAWA, H., R. BISCHOFF, and H. HOLTZER. 1969. Formation of arrowhead complexes with

heavy meromyosin in a variety of cell types. *J. Cell Biol.* **43**:312.

35. Jorgensen, A. O., L. Subrahmanyan, and V. I. Kalnins. 1975. Localization of tropomyosin in mouse embryo fibroblasts. *Am. J. Anat.* **142**:519-525.

36. Kane, R. E. 1975. Preparation and purification of polymerized actin from sea-urchin egg extracts. *J. Cell Biol.* **66**:305-315.

37. Krøll, J. 1973. Tandem-crossed immunoelectrophoresis. *In* A Manual of Quantitative Immunoelectrophoresis. N. H. Axelsen, J. Krøll, and B. Weeke, editors. Universitetsforlaget, Oslo. 57-59.

38. Lazarides, E. 1975. Tropomyosin antibody-specific localization of tropomyosin in nonmuscle cells. *J. Cell Biol.* **65**:549-561.

39. Lazarides, E. 1976. Actin, alpha-actinin and tropomyosin interaction in structural organization of actin-filaments in non-muscle cells. *J. Cell Biol.* **68**:202-219.

40. Lazarides, E., and K. Weber. 1974. Actin antibody: the specific visualization of actin filaments in non-muscle cells. *Proc. Natl. Acad. Sci. U. S. A.* **71**:2268-2272.

41. Luduena, M. A., and N. K. Wessels. 1973. Cell locomotion, nerve elongation and microfilaments. *Dev. Biol.* **30**:427-440.

42. McIntosh, J. R., W. Z. Cande, J. A. Snyder, E. Lazarides, and K. McDonald. 1976. Studies on the formation and function of the mammalian mitotic spindle. *In* Cell Motility. R. Goldman, T. Pollard, and J. Rosenbaum, editors. Cold Spring Harbor, N.Y. In press.

43. McKinney, R. M., and J. T. Spillane. 1975. An approach to quantitation in rhodamine isothiocyanate labeling. *Ann. N.Y. Acad. Sci.* **254**:55-64.

44. Mooseker, M. S., and L. G. Tilney. 1975. Organization of an actin filament-membrane complex. Filament polarity and membrane attachment in microvilli of intestinal epithelial cells. *J. Cell Biol.* **67**:725-743.

45. Nachman, R. L., A. J. Marcus, and L. B. Safier. 1967. Platelet thrombosthenin: subcellular localization and function. *J. Clin. Invest.* **46**:1380-1389.

46. Nagayama, A., and S. Dales. 1970. Rapid purification and the immunological specificity of mammalian microtubular paracrystals possessing an ATPase activity. *Proc. Natl. Acad. Sci. U. S. A.* **66**:464-471.

47. Niederman, R., and T. D. Pollard. 1975. Human platelet myosin. II. In vitro assembly and structure of myosin filaments. *J. Cell Biol.* **67**:72-92.

48. Ouchterlony, Ö. 1968. Handbook of Immunodiffusion and Immunoelectrophoresis. Ann Arbor Publ., Ann Arbor, Michigan. 215 pp.

49. Painter, R. G., M. Sheetz, and S. J. Siner. 1975.

Detection and ultrastructural localization of human smooth muscle myosin-like molecules in human nonmuscle cell by specific antibodies. *Proc. Natl. Acad. Sci. U. S. A.* **72**:1359-1363.

50. Perry, M. M., H. A. John, and N. S. T. Thomas. 1971. Actin-like filaments in the cleavage furrow of newt egg. *Exp. Cell Res.* **65**:249-252.

51. Pollard, T. D. 1976. The role of actin in the temperature dependent gelation and contraction of extracts of Acanthamoeba. *J. Cell Biol.* **68**:579-601.

52. Pollard, T. D., S. M. Thomas, and R. Niederman. 1974. Human platelet myosin. I. Purification by a rapid method applicable to other nonmuscle cells. *Anal. Biochem.* **60**:258-266.

53. Pollard, T. D., K. Fujiwara, R. Handin, and G. Weiss. 1976. Contractile proteins in platelet activation and contraction. *Ann. N.Y. Acad. Sci.* In press.

54. Pollard, T. D., K. Fujiwara, R. Niederman, and P. Maupin-Szamier. 1976. The importance of cytoplasmic actin and myosin for cellular structure and motility. *In* Cell Motility. R. Goldman, T. Pollard, and J. Rosenbaum, editors. Cold Spring Harbor, N.Y. In press.

55. Puszkin, E. G., M. B. Zucher, and T. H. Spaet. 1975. Effect of antibody to platelet rod myosin on platelet function. *Blood.* **46**:1031a.

56. Rappaport, R. 1967. Cell division: direct measurement of maximum tension exerted by furrow of echinoderm eggs. *Science (Wash. D. C.).* **156**:1241-1243.

57. Rickards, G. K. 1975. Prophase chromosome movements in living house cricket spermatocytes and their relationship to prometaphase, anaphase, and granule movements. *Chromosoma (Berl.).* **49**:407-455.

58. Sanger, J. W. 1975. Changing patterns of actin localization during cell division. *Proc. Natl. Acad. Sci. U. S. A.* **72**:1913-1916.

59. Sanger, J. W. 1975. Presence of actin during chromosomal movement. *Proc. Natl. Acad. Sci. U. S. A.* **72**:2451-2455.

60. Sanger, J. W. 1975. Intracellular localization of actin with fluorescently labeled heavy meromyosin. *Cell Tissue Res.* **161**:431-444.

61. Sato, H., Y. Ohnuki, and K. Fujiwara. 1976. Immunofluorescent anti-tubulin staining of spindle microtubules and critique for the technique. *In* Cell Motility. R. Goldman, T. Pollard, and J. Rosenbaum, editors. Cold Spring Harbor, N.Y. In press.

62. Schroeder, T. 1973. Actin in dividing cells. Contractile ring filaments bind heavy meromyosin. *Proc. Natl. Acad. Sci. U. S. A.* **70**:1688.

63. Stephens, R. E. 1975. High resolution preparative SDS-polyacrylamide gel electrophoresis: fluorescent visualization and electrophoretic elution-concentration of protein bands. *Anal. Biochem.* **65**:369-379.

64. Stossel, T. P., and T. D. Pollard. 1973. Myosin in polymorphonuclear leukocytes. *J. Biol. Chem.* **248**:8288-8294.

65. Stossel, T. P., and J. H. Hartwig. 1975. Interactions between actin, myosin and an actin-binding protein from rabbit alveolar macrophages. Alveolar macrophage myosin Mg^{++} ATPase requires a cofactor for activation by actin. *J. Biol. Chem.* **250**:5706-5712.

66. Stossel, T. P., and J. H. Hartwig. 1976. Interactions of actin, myosin and a new actin-binding protein of rabbit pulmonary macrophages. II. Role in cytoplasmic movement and phagocytosis. *J. Cell Biol.* **68**:602-619.

67. Taylor, D. L. 1974. In vivo contractility of amoeba cytoplasm. *Biol. Bull.* (*Woods Hole*). **147**:501.

68. Taylor, D. L. 1976. Motile models of amoeboid movement. *In* Cell Motility. R. Goldman, T. Pollard, and J. Rosenbaum, editors. Cold Spring Harbor, N.Y. In press.

69. Taylor, D. L., J. S. Condeelis, P. L. Moore, and R. D. Allen. 1973. The contractile basis of amoeboid movement. I. The chemical control of motility in isolated cytoplasm. *J. Cell Biol.* **59**:378-394.

70. Taylor, D. L., J. A. Rhodes and S. A. Hammond. 1976. The contractile bais of amoeboid movement. II. Structure and contractility of motile extracts and plasmalemma-ectoplasm ghosts. *J. Cell Biol.* **70**:123-143.

71. Ternynck, T., and S. Avrameas. 1972. Polyacrylamide-protein immunoadsorbents prepared with glutaraldehyde. *FEBS* (*Fed. Eur. Biochem. Soc.*) *Lett.* **23**(1):24-28.

72. Wang, K., J. F. Ash, and S. J. Singer. 1975. Filamin, a new high-molecular weight protein found in smooth muscle and non-muscle cells. *Proc. Natl. Acad. Sci. U. S. A.* **72**:4483-4486.

73. Weber, K., and U. Gröeschel-Stewart. 1974. Antibody to myosin: the specific visualization of myosin-containing filaments in nonmuscle cells. *Proc. Natl. Acad. Sci. U. S. A.* **71**:4561-4564.

74. Willingham, M. C., R. E. Ostlund, and I. Pastan. 1974. Myosin is a component of the cell surface of cultured cells. *Proc. Natl. Acad. Sci. U. S. A.* **71**:4144-4148.

75. Wood, B. T., S. H. Thompson, and G. Goldstein. 1965. Fluorescent antibody staining. III. Preparation of fluorescein-isothiocyanate-labeled antibodies. *J. Immunol.* **95**:225-229.