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Monoclonal antibodies against trophectoderm-specific markers during mouse blastocyst formation

(intermediate filaments/embryo/cellular differentiation)

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ABSTRACT **Two-dimensional gel electrophoresis has allowed the detection of proteins characteristic of inner cell mass and trophectoderm in mouse blastocyst. Certain of the proteins characterizing trophectoderm copurify with intermediate filaments from trophectoderm and a trophoblastoma cell line. A monoclonal antibody prepared against proteins of these intermediate filaments labels a filament network in trophectoderm but not in inner cell mass cells.**

The first morphologically observable divergence of mouse embryonic cells occurs during blastocyst formation. At the eight-cell stage, the embryo undergoes a process of compaction, with the formation of junctions between the blastomeres (1). After one or two more cleavages, cavitation begins. By day 3.5, the fully expanded blastocyst, composed of 32–64 cells, comprises two tissues: an external layer of differentiated cells, the trophectoderm, and an inner cell mass (ICM) composed of a few multipotential cells. The currently favored hypothesis assumes that, at the eight-cell stage, all cells still retain full potentialities, their fate resulting subsequently from their relative position, inside or outside the morula (see ref. 2). Analysis of this first differentiation has been heretofore hampered by a lack of markers characteristic of each cell type. In this paper, we describe such a marker of trophectoderm cells.

MATERIALS AND METHODS

Cells. The following teratocarcinoma cell lines were cultured under standard culture conditions: PCC3/A/1 (ref. 3) and PSA-1-NG2 (ref. 4) are embryonal carcinoma lines (the latter was a gift from Gail Martin, San Francisco); T.D.M.1 is a trophoblastoma (3), PYS-2 a parietal yolk-sac (5), and 3/A/1-D-3 an embryonic fibroblast-like (3) cell line. 3T3 and HeLa cells were cultured in Eagle's medium (Dulbecco's modification) supplemented with 15% (vol/vol) fetal calf serum (GIBCO).

Preimplantation Embryos. Embryos were recovered from virgin superovulated C57BL/6 × CBA female mice, 3–5 weeks old, that had been mated with C57BL/6 × CBA males, as described (6). Zona-free blastocysts were cultured on coverslips in modified Eagle's medium supplemented with 10% fetal calf serum. After 60–70 hr in culture, blastocysts had attached on the coverslip as a layer of well-spread trophoblastic cells with a bulging mass of ICM cells (blastocyst outgrowth).

ICMs were prepared by immunosurgery (7). Trophoblastic vesicles were prepared by a microsurgical method (V. Papaioannou, personal communication; see ref. 8); artificial hatching was promoted by splitting zona pellucida mechanically at the level of ICM. After a few hours, ICM with the polar trophectoderm herniated through the zona. The herniated part of the blastocyst was then cut with two needles acting as scissors. The mural trophectoderm left inside the zona soon collapsed but reexpanded as a trophoblastic vesicle after a few hours at 37°C in Whitten's medium (9). These vesicles were then labeled with [^{35}S]methionine.

Labeling of Embryos and Two-Dimensional Gel Electrophoresis. Ten to twenty embryos were labeled for 90 min with [^{35}S]methionine according to Levinson *et al.* (10), and the pattern of proteins synthesized was analyzed by the O'Farrell technique as modified by Garrels (11). The gels were then processed by the dimethyl sulfoxide/2,5-diphenyloxazole technique (12) and fluorographed at −70°C for 5 days to 2–3 weeks with Kodak X-Omat films.

Biochemical Extraction of Intermediate Filaments from Embryos. The procedure was adapted from that of Franke *et al.* (13). All buffers contained 0.25 mM phenylmethylsulfonyl fluoride (Boehringer Mannheim) and *p*-tosyl-L-arginine (Aldrich) at 0.5 mg ml^{-1} as protease inhibitors. Fifty to one hundred labeled embryos or ICM were washed with phosphate-buffered saline containing 0.25% bovine serum albumin and lysed on ice in 10 μl of 10 mM Tris·HCl, pH 7.5/140 mM NaCl/1% Triton X-100 containing 2 μg of unlabeled intermediate filament proteins isolated from trophoblastoma cells. This mixture was mixed gently on a Vortex, 25 μl of the above buffer was added, and the tube was centrifuged for 5 min in an Eppendorf centrifuge. The pellet was washed once with the same buffer and resuspended into 25 μl of 10 mM Tris·HCl (pH 7.5). This was adjusted to 5 mM $MgCl_2$/100 μg ml^{-1} micrococcal nuclease/100 μg ml^{-1} DNase I/50 μg ml^{-1} RNase A. The digestion was allowed to proceed for 10 min on ice. The 1.5-ml siliconized Eppendorf tube was filled with 10 mM Tris·HCl, pH 7.5/1.5 M KCl/140 mM NaCl/0.5% Triton X-100 and left on ice for 20 min after being mixed on a Vortex. This extraction was repeated once. The pellet was then washed twice with 100 μl of 10 mM Tris·HCl (pH 7.5), resuspended into 25 μl of sample buffer containing 9 M urea, 1% (wt/vol) Nonidet P-40, 2% (wt/vol) $NaDodSO_4$, and 5% (wt/vol) mercaptoethanol, and stored at −80°C before analysis by gel electrophoresis.

Monoclonal Antibodies Against Intermediate Filaments Isolated from Trophoblastoma Cells. Rats were immunized with a preparation of intermediate filaments that had been dialyzed against phosphate-buffered saline. A first injection was done intraperitoneally and subcutaneously with incomplete Freund's adjuvant, 250 μg of protein per animal. After 3 weeks, one rat received 250 μg of protein in phosphate-buffered saline intravenously; 4 days later its spleen was used for fusion.

Splenocytes were fused with myeloma cells, SP-2-O (a gift from G. Köhler, Basel Institute of Immunology, Basel, Switzerland), according to ref. 14. Sp-2-O is a hypoxanthine ribosyltransferase-negative (HPRT$^-$), immunoglobulin nonproducing cell line. Hybrid growth was selected in hypoxan-

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Abbreviations: ICM, inner cell mass; DBM, diazobenzyloxymethyl.

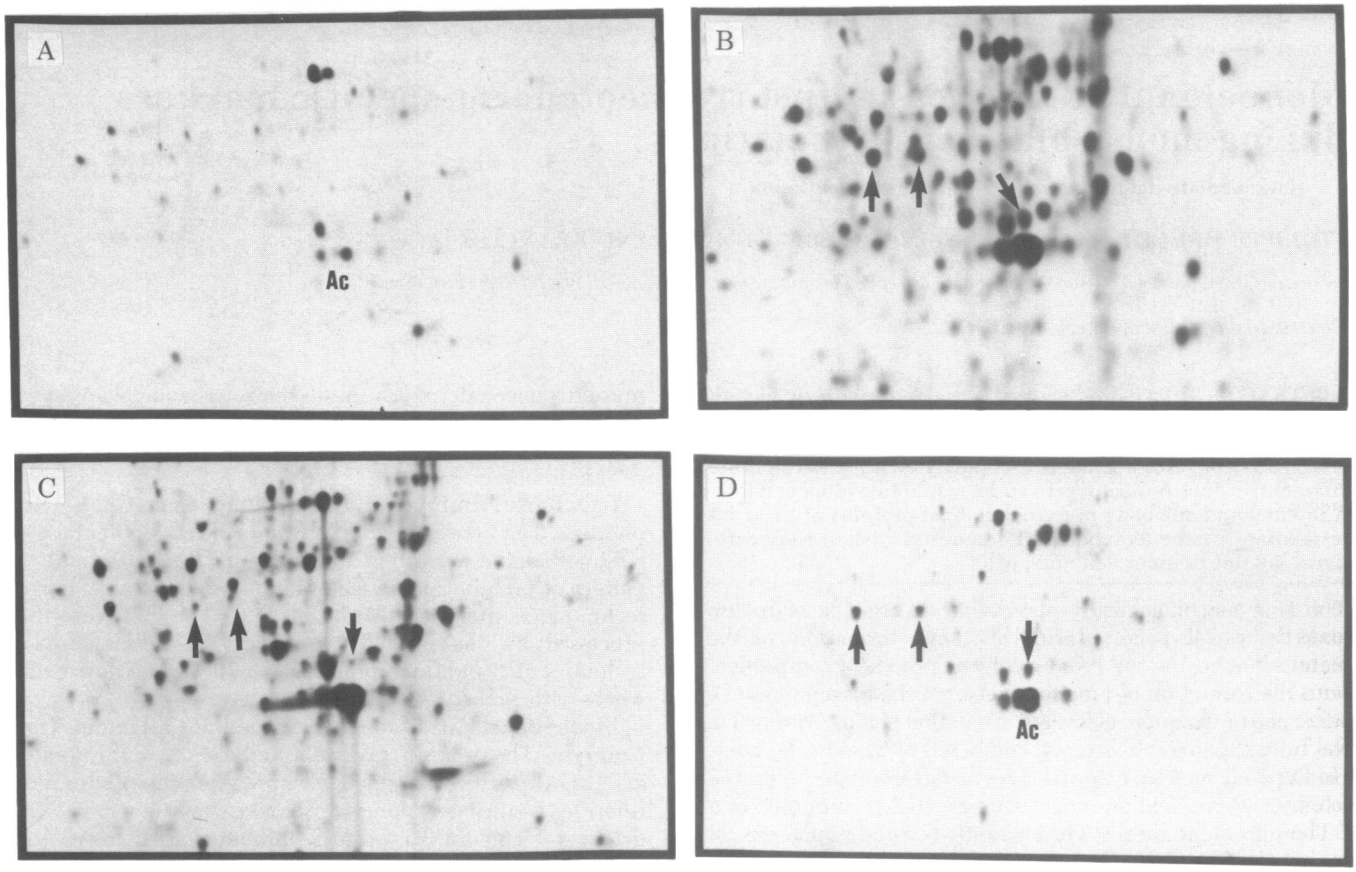

FIG. 1. Corresponding regions of fluorograms from two-dimensional gels showing the pattern of protein synthesized after a 90-min labeling. For the isoelectric focusing, 100 μl of Ampholine pH 3–10 was mixed with 150 μl of Ampholine pH 5–7. The pH range shown was estimated to be between 4.75 and 6.75. For the second dimension a 10% acrylamide/NaDodSO$_4$ gel was used; the origin is at the top; the ratio of acrylamide to bisacrylamide is 30:0.8. Ac indicates the position of actin (gels *A* and *D*). (*A*) Eight-cell embryos before compaction; (*B*) old morulae; (*C*) ICM; (*D*) trophoblastic vesicles. Ten to twenty embryos were used for *A–C*; five were used for *D*. Arrows indicate proteins characteristic of trophectoderm.

thine/aminopterin/thymidine. Details about fusion and selection of positive hybrid cell lines will be described elsewhere. Hybrid cell lines were subcloned twice in 1% methylcellulose (Hercules, New York). For analysis of Ig produced, hybridoma cells were grown in the presence of [^{14}C]leucine; supernatant was immunoprecipitated with rabbit anti-rat Ig and analyzed by gel electrophoresis.

Transfer of Proteins from Polyacrylamide Gels to Diazobenzyloxymethyl (DBM)-Paper. The protein mixture was fractionated by two-dimensional gel electrophoresis. The gel (17 × 18 cm) was then washed twice in 10 mM Na acetate (pH 5.2) at room temperature for 30 min. DBM-paper was prepared according to Alwine *et al.* (15) and washed in 10 mM Na acetate (pH 5.2). The transfer was made in a similar fashion as Towbin *et al.* (16) except that the electrode buffer used was 10 mM Na acetate (pH 5.2) at 4°C. A voltage gradient of 10 V cm^{-1} was applied for 45 min–1 hr. After transfer, the sheet of DBM was left overnight in 10% ethanolamine/0.1 M Tris·HCl, pH 8.0/0.25% bovine serum albumin and washed in phosphate-buffered saline.

Immunological Detection of Proteins on DBM-Paper. The sheet was incubated at 37°C or at room temperature with shaking for 1 hr with 40 ml of hybridoma supernatant in sealed plastic bags, washed with phosphate-buffered saline for 1 hr, and incubated with 35 ml of phosphate-buffered saline containing 0.25% bovine serum albumin, 5 mM EDTA, and rabbit F(ab)$_2$ anti-rat Ig (10^7 cpm) purified by affinity column chromatography and iodinated by the procedure of Bolton and Hunter (17) to a specific activity of 7.5 Ci/g (1 Ci = 3.7 × 10^{10} becquerels). The sheet was then exhaustively washed in phosphate-buffered saline, dried in air, and exposed at −70°C with an Ilford intensifying screen between 4 and 12 hr.

Immunolabeling of Cells and Embryos. Cells grown on coverslips were washed twice in cold phosphate-buffered saline, dipped into cold methanol for 15 min, and rinsed in phosphate-buffered saline. For indirect immunofluorescence, cells were incubated for 1 hr at 37°C with 100 μl of hybridoma culture supernatant, washed twice in phosphate-buffered saline for 15–30 min, incubated with 50 μl of rabbit anti-rat Ig labeled with fluorescein isothiocyanate (Miles), 1:25 dilution, and rinsed again in phosphate-buffered saline for 1–2 hr. The slides were examined under oil with a Leitz objective Phaco 63/1.3.

Preimplantation embryos were air-dried on albumin-coated slides and fixed in cold methanol. Indirect immunoreaction was done as described above, by using purified sheep anti-rat IgG antibodies conjugated with horseradish peroxidase (a gift of J. C. Antoine, Institut Pasteur). Peroxidase activity was revealed with 3-amino-9-ethylcarbazole as described (18).

RESULTS

Two-Dimensional Gel Electrophoresis of Proteins Synthesized by Preimplantation Embryos. Fig. 1 reproduces parts of the two-dimensional gel electrophoretic chromatograms of the proteins synthesized by eight-cell embryos before compaction, of old morulae, of ICM, and of trophectoderm. As reported by several groups (19–22), differences appeared in the pattern of proteins synthesized by the different cell types. For instance, one actin subunit is not synthesized before compaction.

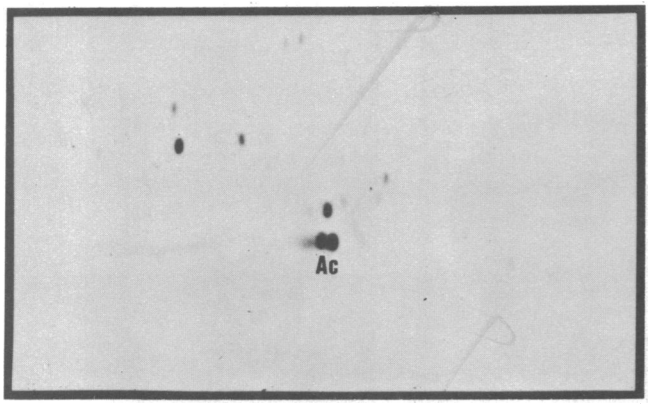

FIG. 2. Part of a fluorogram from a two-dimensional gel showing the proteins extracted as intermediate filaments from total blastocysts. The same regions are shown and same conditions of labeling were used as for Fig. 1.

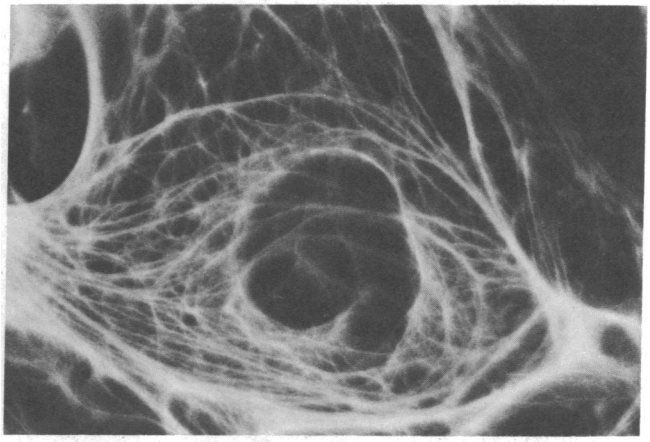

FIG. 4. Immunofluorescence on trophoblastoma cells after incubation with monoclonal antibody. The cells were cultured and treated as explained in the text. (×1100.)

Several proteins are characteristic of the ICM; several others are characteristic of the trophectoderm. Characteristic here means that the protein is synthesized by one cell type in much greater amount than by the other. Careful analysis of fluorograms that were exposed for a longer time indicates that such characteristic proteins are often also synthesized at very low level by the latter.

In this paper, we shall concentrate exclusively on the three proteins indicated by arrows in the trophectoderm pattern (Fig. 1). These three proteins were also observed in the trophoblastoma cell line. These proteins, with molecular weight in the range 45,000–60,000, are abundant in this tissue. The mapping of these proteins in two-dimensional gels is reminiscent of that described for components of intermediate filaments. These structures were therefore investigated in more detail.

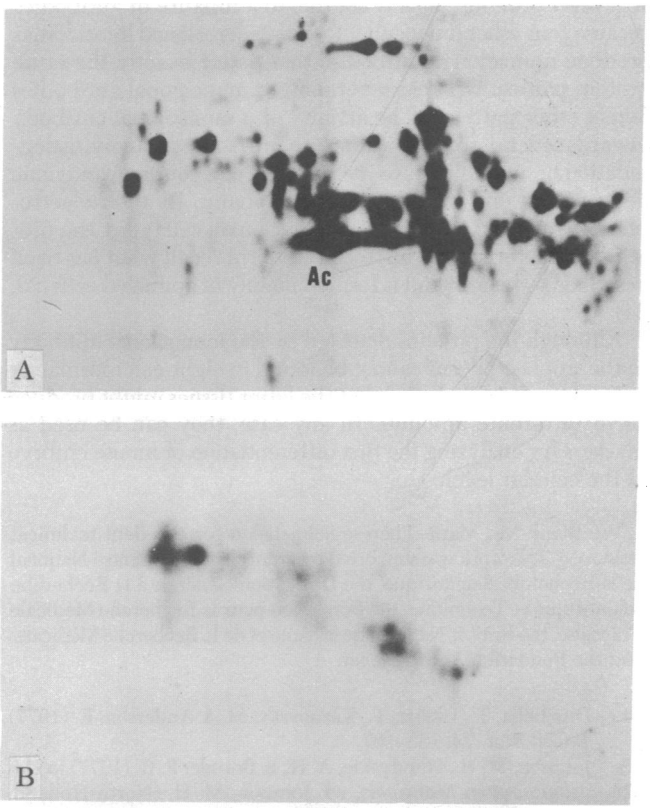

FIG. 3. (*A*) Fluorogram from a two-dimensional gel of a preparation of intermediate filaments extracted from trophoblastoma cells (same region as shown in Figs. 1 and 2). The cells were labeled for 15 hr with [^{35}S]methionine. (*B*) Immunological detection of proteins after transfer to DBM-paper. A preparation of unlabeled intermediate filament proteins from trophoblastoma cells was fractionated and treated with monoclonal antibodies.

Extraction and Analysis of Intermediate Filaments. The intermediate filaments were extracted from various cell types and from preimplantation mouse embryos. The purification is based upon the insolubility of the structures in 1% Triton X-100. To the radioactive material from embryos, 2 μg of unlabeled proteins from intermediate filaments isolated from trophoblastoma cells was added as carrier. Although some proteins may bind nonspecifically onto the carrier, this appeared the only way to minimize a loss of proteins. Fig. 2 shows the fluorogram of such a preparation made from total labeled blastocysts. It is clear that the three marker proteins are present in this preparation. More than 50% of them appear to be recovered at the end of the fractionation. Overexposure of the fluorograms reveals a few more protein spots in the gel. In cell lines, these proteins are not observed in embryonal carcinoma cells (PCC3 and NG2) or in fibroblasts (3T3). They are formed in a trophoblastoma line (Fig. 3). The complexity of the trophoblastoma spectrum as compared with that of the blastocyst trophectoderm (Figs. 2 and 3*A*) reflects a higher specific activity and a longer exposure. In similar preparations, the three marker proteins have been found in the embryo only in the trophectoderm and not in the ICM or the eight-cell morula.

Monoclonal Antibodies Against Proteins of Intermediate Filaments of Trophoblastoma Cells. Monoclonal antibodies were then prepared against the crude preparation of intermediate filaments extracted from trophoblastoma cells (Fig. 3*A*). This preparation was renatured by dialysis before injection into rats. Some 100 independent hybridomas producing immunoglobulins against intermediate filaments were obtained and will be described elsewhere. Several of these hybridomas were subcloned twice, and the culture supernatants were assayed by indirect immunofluorescence or by peroxidase on various cell lines and on preimplantation embryos. Results obtained with one of these monoclonal antibodies are reported here.

On cells from the trophoblastoma line, attached to the dish and fixed with methanol, this monoclonal antibody labels a network of intracellular filaments very similar to what has been described on various cell types as intermediate filaments (Fig. 4). Under similar conditions, the same result was obtained with PYS-2 cells, a parietal yolk sac derivative of teratocarcinoma. In contrast, this monoclonal antibody does not react with two

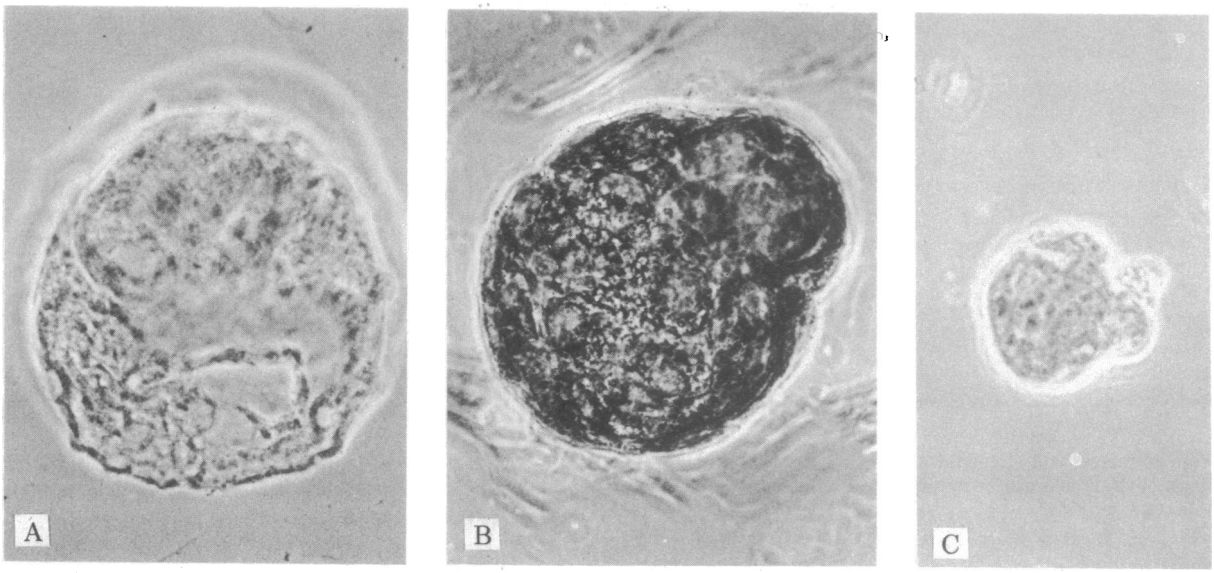

FIG. 5. Indirect immunoperoxidase reaction on the embryo after incubation with the monoclonal antibody. (*A*) Blastocyst with a control monoclonal antibody; (*B*) blastocyst; (*C*) ICM labeled with the specific monoclonal antibody against intermediate filaments. (×1000.)

embryonal carcinoma cells (PCC3 and NG2) or with fibroblasts (3T3). The latter result indicates that the monoclonal antibody is not directed against actin, tubulin, or vimentin. Furthermore, treatment of trophoblastoma cells with cytochalasin B (10 μg/ml for 30 min) or with vinblastine sulfate (50 μM for 15 hr) did not alter the labeling of the filaments by monoclonal antibody (see ref. 23).

In order to identify the protein detected by the monoclonal antibody, preparations of intermediate filaments from various cell types were chromatographed in one- or two-dimensional gels and transferred electrophoretically onto DBM-paper. The proteins detected by the monoclonal antibody were revealed by autoradiography. In one-dimensional gels, extracts of intermediate filaments from trophoblastoma, but not from PCC3 embryonal carcinoma and 3/A/1-D-3 cells, reacted with the monoclonal antibody. When extracts from trophoblastoma cells were chromatographed in two-dimensional gels, the monoclonal antibody was found to react with several proteins (Fig. 3*B*). By comparing Figs. 3 *A* and *B* and 2, the two more basic proteins indicated by arrows in Fig. 2 were found among the pattern of spots labeled by the monoclonal antibody.

The reaction of the monoclonal antibody with embryos was first investigated on blastocysts that had attached to culture dishes. Under these conditions, the antibodies label a network of filaments similar to that observed in trophoblastoma cells.

Total blastocysts, ICM prepared by immunosurgery, and eight-cell morulae in suspension in culture medium have also been analyzed for their reactivity towards the monoclonal antibody. Under these conditions, no network can be detected immunohistochemically. Only strong positive diffuse reactions can unambiguously be ascertained. Such a reaction was observed only in total blastocysts with the trophectoderm cells. ICM and eight-cell morulae exhibited only a very weak reaction similar to the control tests (Fig. 5).

DISCUSSION

The primary aim of this work was to define specific markers for analyzing the first differentiation of the mouse embryo at the cellular level. Three proteins characterizing the trophectoderm copurified with preparations of intermediate filaments from trophectoderm and a trophoblastoma cell line. Among a series of monoclonal antibodies against intermediate filaments from trophoblastoma cells, one was selected for detailed analysis. This monoclonal antibody decorates a network of intermediate filaments in the trophectoderm of the blastocyst, in trophoblastoma parietal yolk sac, and HeLa cells but does not react with ICM, embryonal carcinoma cells, or fibroblasts.

Among the protein spectrum of intermediate filaments from trophoblastoma cells, the monoclonal antibody detects a pattern of several proteins. This is not due to a mixture of antibodies because four other independently isolated, recloned hybridomas produce monoclonal antibodies that detect exactly the same protein pattern whereas several other monoclonal antibodies detect other patterns. The affinity of a monoclonal antibody towards several proteins is due either to some proteolytic degradation or, more likely, to the presence of a common antigenic determinant on several polypeptide chains. By their electrophoretic mobility and their distribution on cell types, the proteins characteristic of the trophectoderm recall what has been described as keratin and is formed mainly in epithelial cells (23, 24).

Although the proteins detected by the monoclonal antibody in the trophectoderm cannot be found in eight-cell morulae or in ICM, it is not excluded that the latter tissues might produce them in minute amounts. In any case, they can be used as markers for analyzing the first differentiation of mouse embryo at the cellular level.

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