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MB1, a quail leukocyte–endothelium antigen: Partial characterization of the cell surface and secreted forms in cultured endothelial cells

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ABSTRACT **We describe here conditions allowing the selective growth in culture of embryonic capillary endothelial cells from quail yolk sac. Such cultures were set up to characterize an antigen present on the endothelial cell surface and to study whether it was secreted in the culture medium. This antigen, MB1, was previously evidenced by a monoclonal antibody raised to quail IgM heavy chain. It is present at the surface of all endothelial and hemopoietic cells (except mature erythrocytes) starting from the hemangioblast, the early mesodermal precursor of blood and vascular endothelial cells. The MB1 epitope is also found on quail plasma molecules of 80 and 125–200 kDa. By immunoprecipitation of either surface or metabolically labeled endothelial cellular material, we have chemically characterized MB1-bearing components as glycoproteins of apparent molecular mass ranging from 80 to 200 kDa and provided evidence for their release into the culture medium. This is consistent with the hypothesis that, in the quail, vascular endothelium participates in the secretion of the α-MB1-positive plasmatic components.**

An antigenic determinant common to all vascular endothelial and hemopoietic cells, with the exception of erythrocytes, has been evidenced in an avian species, the Japanese quail (1). After immunizing a mouse with quail plasma immunoglobulin μ chain, we obtained a hybridoma antibody that recognizes a surface determinant (referred to as MB1) on endothelial and blood cells and also on the hemangioblast—the early mesodermal precursor of the embryonic yolk sac blood islands. The MB1 antigen is species specific. No cells of the chicken bind the antibody. We showed, in addition, that the MB1 epitope is included in several blood proteins of apparent molecular masses 125–200 kDa in reducing conditions (1), as well as the 80-kDa monomer used to raise the antibody. However, the immunoreactivity observed on endothelial cells is not merely the result of binding to the cell surface of MB1 molecules present in the plasma. This was clearly demonstrated by embryonic heterospecific grafting experiments in which quail endothelial cells developing in chicken or mouse organs are labeled by the anti-MB1 monoclonal antibody (α-MB1-mAb) (2–5).

Surface antigen(s) distributed like MB1 have not been reported in other species. However, this is evocative of the murine T200 antigens, first evidenced at the surface of T cells (6) and later found on B lymphocytes (7, 8). T200 antigens are glycoproteins that, depending on their localization, are slightly heterogeneous in molecular mass (170–220 kDa) and isoelectric point, as a result of variations in glycosylation (8, 9). An equivalent of T200 was described in rat species. It is a 150-kDa glycoprotein, named leukocyte-common antigen because of its cellular distribution (10). However, the presence of these molecules on endothelial cells has not been reported so far.

The present work was aimed at further characterizing the MB1 antigen carried by quail endothelial cells and determining whether the vascular endothelial system contributes to the molecules carrying the MB1 determinant in the plasma. To achieve such a goal, we first set up appropriate culture conditions for quail endothelial cells. Biosynthetic and surface labeling was then carried out on these cultures, so that the synthesis of MB1-bearing molecules by the endothelium and their shedding into the culture medium could be demonstrated.

MATERIALS AND METHODS

Culture of Endothelial Cells. The culture medium used was minimal essential medium (MEM) containing D-valine (GIBCO; Hoofddorp, The Netherlands) supplemented with 10% heat-inactivated newborn calf serum (GIBCO), 10% 11-day chicken embryo extract, 200 μg of heparin per ml (Sigma), and 10% of a 1:1 mixture of two endothelial cell growth factors: endothelial mitogen (10 mg/ml) (Biomedical Technologies, Norwood, MA) and endothelial cell growth supplement (3 mg/ml) (Collaborative Research, Waltham, MA).

Quail endothelial cell cultures were prepared from yolk sac capillaries according to a modification of the method described by Folkman *et al.* (11). Ten yolk sacs removed from 7-day-old quail embryos were extensively rinsed in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (P_i/NaCl) and soaked in P_i/NaCl supplemented with 0.5% (wt/vol) collagenase (Worthington) and 0.5% bovine serum albumin (Sigma). The tissue was cut into small pieces and vigorously pipetted and incubated for 40 min at room temperature. The resulting suspension was passed through a nylon-covered funnel (Nitex Monoscreen Cloth HC 3-110, Tetko, Elmsford, NY) and centrifuged at 50 × *g* for 7 min at 4°C. After three washes with MEM containing D-valine supplemented with 10% newborn calf serum, the cell pellet was resuspended in 1.2 ml of complete medium, seeded in a 35-mm tissue culture dish (Nunc), and incubated at 37°C in 5% CO_2/95% air. At 2-hr intervals, the supernatant was replated in a second and then in a third dish. Each dish was filled with fresh complete medium and incubated at 37°C without disturbance for 48 hr. Then the nonadherent cells were washed out and one-half of the culture medium was replaced. The cells were fed every 2 or 3 days thereafter by changing 50% of the medium until confluency was reached.

Characterization of the Endothelial Cells in Culture. *Antibodies.* The α-MB1 and α-MD2 (which recognizes the quail

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Abbreviations: mAb, monoclonal antibody; DiI-Ac-LDL, fluorescent acetylated low density lipoprotein.

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μ chain) monoclonal antibodies were obtained as described (1). A rabbit antiserum to MB1 was prepared by three injections, at 2-week intervals, of 200 μg of the affinity-purified plasma 160-kDa MB1 antigen in Freund's complete adjuvant.

Immunostaining with anti-MB1 antibody. α-MB1 mAb directly conjugated to fluorescein isothiocyanate was diluted 1:100 in P_i/NaCl and incubated for 20 min with the living cells at 37°C.

Labeling of endothelial cells with fluorescent acetylated low density lipoprotein cell marker. Acetylated low density lipoprotein combined with the fluorescent probe 1,1'-dioctadecyl-1-(3,3,3',3'-tetramethyl)indocarbocyanine perchlorate (DiI-Ac-LDL) selectively labels endothelial cells (12), which possess lysosomal enzymes that can degrade it, resulting in the accumulation of fluorescence in the intracellular membranes. Living cells were first incubated for 4 hr at 37°C with DiI-Ac-LDL (Biomedical Technologies) diluted at 10 μg/ml in the culture dish. After extensive washing, the cells were treated with α-MB1 conjugated to fluorescein isothiocyanate and washed. DiI-Ac-LDL fluorescence was visualized by using standard rhodamine excitation/emission filters.

Biochemical Characterization of the MB1 Antigen Produced by Endothelial Cells. *Biosynthetic labeling of cultured endothelial cells.* Semiconfluent cultures were washed twice for 45 min in methionine-free MEM (GIBCO) supplemented with 5% dialyzed newborn calf serum. [^{35}S]Methionine (>800 Ci/mmol; 1 Ci = 37 GBq; Amersham, U.K.) was added at a final concentration of 200 μCi/700 μl of this medium and synthetic labeling was performed during 3 hr at 37°C. Then the cells were washed three times for 10 min with unlabeled P_i/NaCl/10 mM L-methionine. In case of antibiotic treatment, the cells were preincubated for 3 hr in methionine-free MEM supplemented with newborn calf serum and tunicamycin (Sigma) or swainsonine (Boehringer Mannheim) at a concentration range of 25–50 μg/ml. Thereafter, incorporation of [^{35}S]methionine was carried out in the continuous presence of antibiotic.

Cell surface iodination. Semiconfluent cell cultures were washed and labeled for 10 min at room temperature with 1 mCi of Na^{125}I (100 mCi/ml; Amersham) in P_i/NaCl, in the presence of lactoperoxidase (Sigma) and H_2O_2. The cells were washed at least six times with unlabeled P_i/NaCl/5 mM KI/0.5 mM phenylmethylsulfonyl fluoride before lysis or preparation of conditioned medium.

Preparation of conditioned medium. Endothelial cell culture conditioned medium was prepared as described by Goridis *et al.* (13). Cells labeled either for 3 hr with [^{35}S]methionine or for 10 min with ^{125}I were washed and cultured for 24 hr in serum-free medium. The conditioned medium was then centrifuged (100 × *g* for 10 min, then 15,000 × *g* for 20 min), dialyzed against 3 mM NaCl/1 mM Tris·HCl, pH 7.4/0.25 mM phenylmethylsulfonyl fluoride for 36–48 hr (one change), and concentrated to 200 μl, to which bovine serum albumin was added to a final concentration of 0.2 mg/ml.

Cell lysis and immunoprecipitation. Cells were lysed at 0°C for 15 min with 250 μl of P_i/NaCl/0.5% Nonidet P-40/0.5 M NaCl/1 mM phenylmethylsulfonyl fluoride/1% aprotinin (Sigma). Immunoprecipitation on cultured cell lysates or supernatants was performed as described by Barbu *et al.* (14).

Electrophoresis. Samples containing 2% 2-mercaptoethanol were boiled for 5 min and loaded on 10% acrylamide gels according to the technique of Laemmli (15). After fixation and fluorography with Enlightning (New England Nuclear) in the case of ^{35}S incorporation, labeled components were revealed by autoradiography of dried gels on Kodak x-ray films.

Endoglycosidase H digestion. Immunoprecipitates from swainsonine-treated cells were eluted in 20 μl of 0.15 M citrate buffer, pH 5.5/0.2% $NaDodSO_4$/1% 2-mercaptoethanol at room temperature for 30 min and then boiled for 2 min. Endoglycosidase H (Miles) dissolved in the same buffer without $NaDodSO_4$ (50 milliunits/20 μl) was added. Digestion was carried out for 12 hr at 37°C and was stopped by addition of concentrated electrophoresis sample buffer.

Two-dimensional polyacrylamide gel electrophoresis. Immunoprecipitates were eluted in nonequilibrium pH gel electrophoresis sample buffer at room temperature for 15 min. Two-dimensional polyacrylamide gel electrophoresis (2-D gel) was performed as described by Charron and McDevitt (16).

Immunoblotting. Quail plasma proteins were transferred from polyacrylamide gels to nitrocellulose sheets. Indirect staining with α-MB1 or α-MD2 mAbs was carried out by the peroxidase-antiperoxidase/diaminobenzidine method as described (1).

RESULTS

Culture of Quail Capillary Endothelial Cells. Collagenase treatment of 7-day-old yolk sac provided a mixture of various cells—i.e., endodermal, mesenchymal, endothelial, and blood cells. The culture conditions described above were determined after numerous preliminary experiments in which, as judged by morphology and α-MB1 staining, we found that more than one-half the cells growing in the cultures were fibroblasts. The main requirements for successful selection of endothelial cells appeared to be the following: (*i*) the combined presence in the culture medium of D-valine, which favors the growth of endothelial cells while inhibiting that of fibroblasts (17), and of endothelial cell growth factors, whose effect was significantly enhanced by heparin, as already observed (18); (*ii*) seeding in a single culture dish 10^9 cells, which after sequential plating provided a suitable final cell density of ≈3 × 10^8 cells per dish; (*iii*) incubation of the freshly seeded cultures for at least 48 hr before washing, followed by elimination of all nonadherent cells. Under these conditions, the cultures contained fibroblasts (≈10%), a few widely spread macrophages, and other adherent cells of various shapes (round, oval, or hexagonal), which had a dark cytoplasm and were filled with round strongly refractile lipidic vesicles. These cells were distributed throughout the culture dish, either individually or grouped as small colonies; they reacted positively with α-MB1 mAb and were thus considered to be endothelial cells. During further incubation, the number of fibroblasts did not increase because of the mitosis-inhibiting effect of D-valine on this cell type. In contrast, endothelial cells selectively proliferated and yielded cultures whose aspect was somewhat variable. When endothelial cells were particularly abundant after the first 48-hr incubation period, they continued to proliferate quickly, giving rise to a typical cobblestone-like monolayer that covered 50–75% of the dish within 7 days (Fig. 1*a*). All the cells forming those layers were positive for both MB1 and low density lipoprotein uptake markers, which confirmed their endothelial nature (Fig. 2). In other cases, when fewer endothelial cells had attached to the dish during the first incubation period, they were generally larger and fusiform and became arranged in parallel lines all oriented in the same direction, evocative of the tube-like structure already described for mammalian capillary endothelial cells (refs. 19–21; Fig. 1*b*). Such cultures also failed to achieve confluency, again occupying 50–75% of the dish, but never formed the continuous pavement-like monolayer described above. However, in that type of culture too, all cells were α-MB1 positive and degraded acetylated low density lipoprotein. In both types of cultures, degeneration began in a few cell groups, which overlapped and aggregated as colonies of round cells with a thick dark plasma membrane prior to

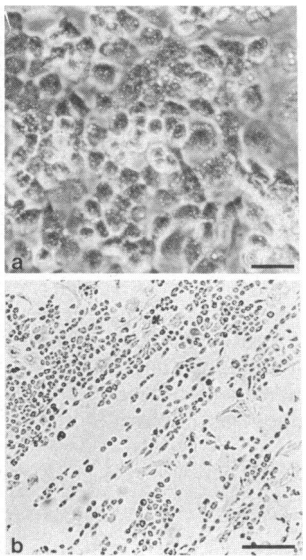

FIG. 1. Quail capillary endothelial cells viewed by phase-contrast microscopy after 7 days in culture. (*a*) Cobblestone-like monolayer of cells exhibiting typical endothelial morphology. (Bar = 30 μm.) (*b*) Tube-like organization of aligned cells growing in parallel lines. (Bar = 100 μm.)

bursting. This phenomenon propagated rapidly in zones of confluency, and the culturres could not be maintained beyond 10 days.

Partial Characterization of the Endothelial Cell MB1 Antigen. *Biosynthetic labeling.* After incorporation of [^{35}S]methionine, the MB1-bearing molecules were immunoprecipitated from endothelial cell lysates by both monoclonal and polyclonal α-MB1 antibodies. They appeared as three or four bands distributed over a zone ranging from 80 to 200 kDa, showing smears typical of membrane glycoprotein migration patterns. Major bands were observed at 200, 160, 100, and 80 kDa (Fig. 3, lanes c and d). In an immunoblot of plasma proteins (lane a), the 160 to 200-kDa bands were also found. The 80-kDa product in the plasma corresponds to (or comigrates with) the μ chain, as demonstrated by its reaction with an anti-μ mAb (lane b) (1). In contrast, the 80-kDa band obtained from biosynthetically labeled product of the endothelial cell lysate does not react at all with the anti-μ reagent (lane e).

When the cultures were pretreated with tunicamycin, which totally inhibits the formation of N-glycosidic bonds in glycoproteins (22), no reactivity with either monoclonal (lane h) or polyclonal α-MB1 (not shown) was found. In contrast, swainsonine treatment, which blocks the final processing of N-linked mannosidic groups into complex glycans by inhibiting the Golgi mannosidase II (23), did not abolish the immunoreactivity with both monoclonal (not shown) and polyclonal α-MB1. In this case, the major component immunoprecipitated had a reduced apparent molecular mass of $\approx$90 kDa, which was further lowered to 60 kDa by endoglycosidase H digestion (lanes f and g). It thus appears that the MB1 antigen is a glycoprotein containing complex N-linked oligosaccharides and that its α-MB1 mAb-defined epitope is not carried by the terminal sugar residues of the glycan groups. In contrast, the presence of N-glycosidic bonds seems to be necessary for antigen–antibody complex formation.

Surface labeling. The radioiodinated endothelial cell surface molecules immunoprecipitated by α-MB1 migrated in PAGE essentially as a 160- to 200-kDa doublet included in a smear extending down to $\approx$80 kDa (Fig. 4, lanes a and b). The same material, further analyzed by two-dimensional gel

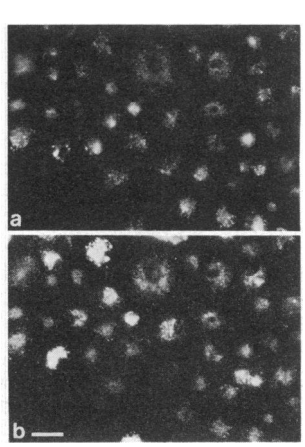

FIG. 2. Double labeling of quail capillary endothelial cells after 7 days in culture. (*a*) Surface immunostaining with α-MB1 mAb conjugated to fluorescein isothiocyanate. (*b*) The same field: Metabolic labeling with DiI-Ac-LDL. All the cells are MB1-positive and have incorporated acetylated low density lipoprotein. (Bar = 20 μm.)

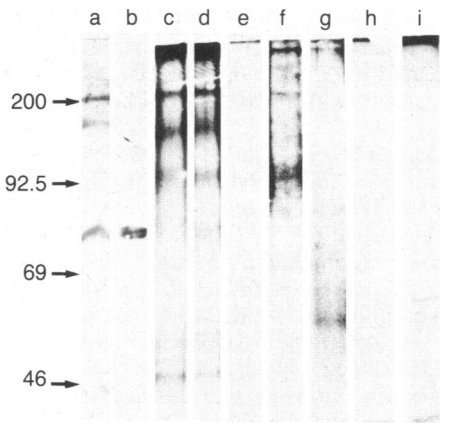

FIG. 3. Immunoprecipitation of endothelial cell lysate after incorporation of [^{35}S]methionine for 3 hr. Analysis by 10% NaDodSO$_4$/PAGE under reducing conditions and autoradiography. Comparison with quail plasma material run at the same time and blotted onto nitrocellulose. Lanes: a, plasma from 3-day quail stained with α-MB1 mAb; b, the same plasma stained with α-MD2 mAb; c, d, and e, endothelial cell lysate immunoprecipitated with α-MB1 mAb (lane c), polyclonal α-MB1 (lane d), and α-MD2 mAb (lane e); f and g, lysate of endothelial cells labeled with [^{35}S]methionine after treatment with swainsonine (30 μg/ml); immunoprecipitation with polyclonal α-MB1. Electrophoresis before (lane f) and after (lane g) digestion by endoglycosidase H; h, α-MB1 mAb-precipitated material from lysate of tunicamycin-treated endothelial cells; i, as in lane h, but using an irrelevant mouse ascitic fluid. Arrows indicate standard molecular mass markers (kDa).

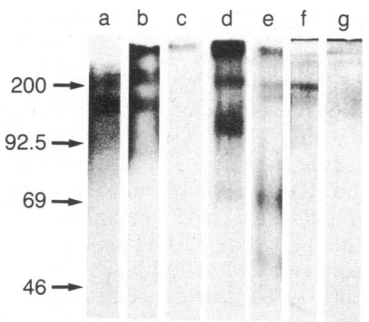

FIG. 4. Immunoprecipitation of lysate and conditioned medium from iodinated endothelial cells; comparison with conditioned medium obtained after [^{35}S]methionine metabolic labeling of endothelial cells. Analysis by 10% NaDodSO$_4$/PAGE under reducing conditions and autoradiography. Iodinated endothelial cell lysate immunoprecipitated with rabbit polyclonal α-MB1 (lane a), α-MB1 mAb (lane b), or α-MD2 mAb (lane c). Lane d, α-MB1 mAb-immunoprecipitated material from endothelial cell lysate after iodination and preparation of conditioned medium. Lane e, α-MB1 mAb immunoprecipitate of the resulting conditioned medium. Lane f, α-MB1 mAb immunoprecipitate of endothelial cell conditioned medium after [^{35}S]methionine incorporation. Lane g, α-MB1 mAb immunoprecipitated material from endothelial cell lysate after preparation of ^{35}S conditioned medium. Arrows indicate standard molecular mass markers (kDa).

electrophoresis, was resolved into four acidic spots of apparent molecular mass between 200 and 80 kDa (Fig. 5). The various entities identified, varying both in charge and size, may represent different degrees of glycosylation and particularly of sialylation. Alternatively, they may result from partial degradation of the final high molecular mass product.

Release of MB1 Molecules into the Culture Medium. After labeling either with [^{35}S]methionine or ^{125}I as described above, the conditioned medium was removed and the cells, >90% of which were still attached and healthy after 24 hr in serum-free medium, were lysed. Immunoprecipitation was performed both on cell extracts and culture medium, and the resulting material was subjected to one-dimensional PAGE. As shown in Fig. 4, the endothelial cell conditioned medium contained MB1-labeled material appearing as a doublet of 180–200 kDa after iodination (Fig. 4, lane e) of membrane molecules. In the case of metabolic labeling, the material immunoprecipitated by α-MB1 showed up as a diffuse band extending from 160 to 200 kDa (lane f). Immunoprecipitable material was still present in the cell extract from 24-hr cultures in nonradioactive medium, as shown in Fig. 4 (lanes d and g).

DISCUSSION

The recently developed *in vitro* culture of endothelial cells has already been used in a number of studies concerning the structure (24–26), physiology (27–29), and immunological function (30–33) of these components of the vessel wall. We report here conditions allowing the growth in culture of capillary endothelial cells from quail yolk sac that exhibit *in vitro* the typical properties of this cell type. The cells growing in culture were first identified by the use of a mAb defining a hemangioblastic cell lineage marker (1). Further evidence of the endothelial nature of cultured cells was found in their ability to degrade acetylated low density lipoprotein, a metabolic property of endothelial cells (12), and by the morphology of the cultures. Cultured quail capillary endothelial cells either formed a pavement-like pattern by close apposition of polygonal cells, as described for mammalian endothelial cells from large vessels (34–36), or formed tubular structures. The latter is a less common phenotype, which was first described in cultures of bovine and human capillary endothelia (19). Since it resulted from a rearrangement of an original cell monolayer, it may be considered to reflect some kind of *in vitro* angiogenesis. Several authors have confirmed this observation of tube-like organization for human venous (37) and rat capillary endothelial cells (20). It has been demonstrated that the phenotypic modulation between the monolayer and the tube-like structure depends on culture conditions, and especially on the nature of the extracellular matrix components provided (38). For example, rat capillary endothelial cells formed monolayers in cultures grown on interstitial collagens, while more differentiated tube-like structures appeared on basement membrane collagens (20). In our cultures, endothelial cells were initially seeded along with variable amounts of other yolk sac cell types, which may have conditioned the substrate differently in the various dishes.

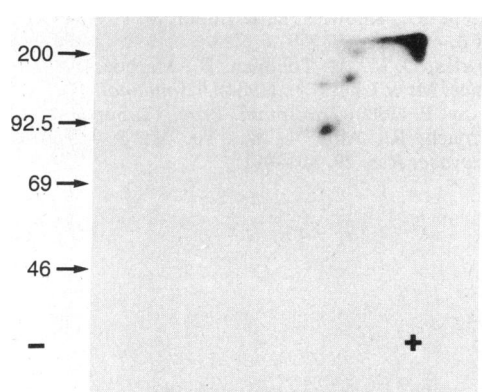

FIG. 5. Two-dimensional gel analysis of an iodinated endothelial cell lysate immunoprecipitated by α-MB1 mAb. Nonequilibrium pH gel electrophoresis was used in the first dimension (basic side at left, acidic side at right), and 10% NaDodSO$_4$/PAGE in the second dimension. Arrows indicate standard molecular mass markers (kDa).

Although healthy for about a week in culture, quail capillary endothelial cells unlike those of bovine, human, or rat (11, 20), but similar to those of chicken (11), could not be subcultured. However, the relative purity of the primary cultures (>90% cells double positive for MB1 expression and low density lipoprotein uptake and exhibiting typical endothelial shape), allowed us to pursue the main goal of this investigation—i.e., the characterization of the MB1 antigen expressed on endothelial cells and the contribution of the latter to the high molecular mass glycoproteins carrying the MB1 determinant in the quail plasma.

Quail endothelial cells in culture retain their *in vivo* property of expressing the MB1 surface antigen that we identified here as glycoproteins of 80–200 kDa. Although immunoreactivity with α-MB1 was significantly altered when the cells were incubated in the presence of tunicamycin, an inhibitor of N-glycosylation, the exclusively oligosaccharide nature of the epitope recognized by the antibody cannot be deduced with certainty. The participation in the epitope of the polypeptide moiety of the molecule remains a possibility. The antigenic determinant may even be exclusively peptidic, if located in a region of the protein that is protease-susceptible when cleared of carbohydrates. The molecular mass heterogeneity from 80 to 200 kDa, which was observed in the different experiments, could reflect different degrees of glycosylation of the reacting molecules. They may also represent either sequential steps of synthesis or degradation of a single 200-kDa final product.

The hypothesis of the endothelial origin of the MB1 molecules present in the plasma is supported by the production and release in the culture medium of material with a similar molecular mass by pure endothelial cell cultures. Since cell death in the course of radioactive precursor incorporation was very low, it is unlikely that material shed passively from dead cells contributed significantly to the proteins immunoprecipitated by α-MB1 from the culture supernatant. Cultured endothelial cells of various origins have already been described to secrete compounds spontaneously into the medium; these include glycosaminoglycans (39), various cell-associated glycoproteins (40–43), and serum components such as fibronectin (44) and factors implicated in hemostasis and vascular integrity (45–48). However, secreted molecules or cell-surface antigens analogous to MB1 have not so far been described in any other species. Endothelial cell-surface antigens have been identified as a 66-kDa glycoprotein in the rat (40), as a glycoprotein composed of 190-kDa subunits in the cow (43), and as a probable glycolipid in the mouse (49). In humans, a surface antigen shared by leukemic cells and both endothelial and epidermal basal cells was also identified as a glycolipid (50), while another, proteinaceous in nature with a mass of 92 kDa, was evidenced at the surface of both endothelial cells and fibroblasts (51). There is also a surface antigenic determinant shared by human vascular endothelium and a particular population of peripheral blood macrophages (52). Conversely, endothelial monocyte antigens have been evidenced with alloantibodies obtained from human kidney allografts undergoing rejection (53). In the case of capillary endothelial cells, expression of organ-specific antigens has been shown, common to the various cell types within a given organ (brain,

ovary, lung) and conserved among different animal species (54).

However, the particular distribution of MB1 antigen throughout the endothelial and hemopoietic lineages (excluding erythrocytes), and its additional presence as soluble material in the quail plasma, remains so far unique.

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