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Surface marker for hemopoietic and endothelial cell lineages in quail that is defined by a monoclonal antibody

(immunoglobulin μ chain/species-restricted phenotype)

BRUNO M. PEAULT, JEAN-PAUL THIERY, AND NICOLE M. LE DOUARIN

Institut d'Embryologie du Centre National de la Recherche Scientifique et du Collège de France, 49bis, Avenue de la Belle Gabrielle, 94130 Nogent-sur-Marne, France

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ABSTRACT **A mouse monoclonal antibody raised in response to quail immunoglobulin μ chain was found to exhibit a broad reactivity towards hemopoietic and endothelial cells in the quail (*Coturnix coturnix japonica*). Indirect immunofluorescence assays were performed at several stages of embryonic development and until 3 weeks after hatching, on either isolated cells or tissue sections. They revealed that the defined surface marker, referred to as MB1, (*i*) is expressed early on both intra- and extraembryonic hemopoietic stem cells and is transmitted to the whole progeny of these precursors, with the exception of mature erythrocytes, and (*ii*) is a constant feature of the endothelial cell surface throughout ontogenesis and adult life. In addition, this epitope is included in several soluble plasma components. MB1 expression was not detected in chicken tissues, and this characteristic was used to confirm its lineage restriction in quail–chicken chimeras. We stress the value of this species- and lineage-specific marker in study of the development of the hemopoietic and endothelial cell families, with special reference to their possible early common embryonic origin.**

The hemopoietic system is entirely derived from the mesodermal germ layer. Its differentiation fits especially well with a model in which broadly committed precursor cells become progressively more restricted in their developmental potential. According to this model, the initial determination event in ontogenetic processes gives rise to a multipotential stem cell, the differentiation capacities of which are not restricted to a single phenotype but include a series of related lineages. The first visible step of hemopoietic cell differentiation in amniote vertebrates consists in the appearance of angioblastic foci within the yolk sac mesoderm; these foci give rise to both endothelial and blood cells (1). However, before this histogenetic process begins, there are indications that hemopoietic precursors are already determined, because at the primitive streak stage some cells in the mesodermal germ layer of the chicken embryo are selectively susceptible to infection by avian erythroblastosis virus (2). If putative common precursors of the whole hemopoietic cell lineage, including endothelial cells, could be identified, this would be of great interest, especially in attempts to understand how the various hemopoietic cell lines become segregated during development.

We now report that all endothelial and hemopoietic cells of the quail (*Coturnix coturnix japonica*), with the exception of mature erythrocytes, have a common antigenic surface determinant, recognized by a monoclonal antibody raised against the quail immunoglobulin μ chain. The usefulness of such a reagent to study ontogeny of the hemopoietic system is discussed.

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MATERIALS AND METHODS

Materials. Embryos and adult birds of Japanese quail and White Leghorn chicken (*Gallus gallus*) were used throughout this work. Embryonic stages were determined by the duration of incubation and, for quail embryos, precisely recorded according to Zacchei's table (3). Monoclonal antibodies were raised in BALB/c mice bred in our own facilities.

Cells. Hemopoietic organs were minced and teased in phosphate-buffered saline (P_i/NaCl) supplemented with 2% heat-inactivated newborn calf serum (GIBCO Europe, Hoofddorp, The Netherlands). Debris was discarded after sedimentation and cells were washed twice in the same buffer. Leukocytes were isolated by centrifugation on a Ficoll density gradient (Sigma).

Tissue Sections. Fresh organs from hatched birds were quickly frozen by immersion into liquid nitrogen and cut in 5- or 7-μm sections with a cryostat (Bright Instrument, Huntingdon, England). Sections were attached on gelatin-coated glass slides and fixed for 1 sec in acetone.

Embryonic tissues were processed as described by Drews (4). Briefly, organs were fixed for 4 hr in 2% (wt/vol) paraformaldehyde in 0.05 M sodium phosphate (pH 7.4), dehydrated in alcohol, and embedded in polyethylene glycol 1,000 (Serva, Heidelberg). Serial 10-μm sections were stuck on gelatin-coated glass slides.

Electrophoresis. Analytical electrophoresis in polyacrylamide gels containing $NaDodSO_4$ was carried out according to Laemmli (5) in 6.5% or 10% acrylamide slab gels 0.75 mm thick; preparative $NaDodSO_4$ electrophoresis was performed in a 3-mm-thick 15% acrylamide gel. After the run, gels were processed either for protein staining with Coomassie blue or for protein transfer to nitrocellulose (see below).

Isolation of the Quail μ Chain. Polymeric IgM was purified from adult quail plasma (a generous gift of A. Perramon, Institut National de la Recherche Agronomique, Jouy-en-Josas). Fifty milliliters was defatted by $MnCl_2$ precipitation (6). Proteins were isolated from the clarified plasma by successive precipitations with 18%, 14%, 9%, and 5% sodium sulfate (7). The 5% fraction was dissolved in 1 ml of 0.1 M Tris·HCl buffer, pH 8.0, containing 0.5 M NaCl and submitted to gel filtration on Sephacryl S-300 (Pharmacia, Uppsala). Fractions corresponding to the first elution peak, determined by absorbance at 280 nm, were pooled, dialyzed against water, lyophilized, and dissolved in 0.5 ml of a 0.2% solution of $NaDodSO_4$ containing 4 μl of a 10% solution of fluorescamine (Fluram, Roche) in acetone. Plasma macroglobulins were then reduced and submitted to preparative $NaDodSO_4$ electrophoresis. Fluorescent bands were detected under UV light and cut out. Proteins were then

Abbreviations: P_i/NaCl, phosphate-buffered saline; kDa, kilodalton(s).

extracted from polyacrylamide by electroelution. Finally, samples of total reduced macroglobulins and of each migration band were run on an analytical 10% acrylamide gel.

Monoclonal Antibodies. Two- to 3-month-old female BALB/c mice were immunized against purified quail μ chain by intraperitoneal injection of a 50% (vol/vol) emulsion of complete Freund's adjuvant in water containing 30 μg of μ chain, followed, 19 days later, by a booster intravenous injection of 75 μg of μ chain in P_i/NaCl. Fusion, selection, cloning, and injection into mice of positive hybrids were performed as described (8).

Immunofluorescence. Cells in suspension were indirectly stained and examined as described (9). Alternatively, they were attached by centrifugation to polylysine-coated glass slides laid in 24-well plates, before staining. The glass slides were taken out and mounted for observation. Hybridoma supernatants were used undiluted during the screening step. Ascitic fluid was routinely used at 1:1,000 dilution in P_i/NaCl. Rabbit Ig anti-mouse Ig labeled with fluorescein isothiocyanate (Nordic, Tilburg, The Netherlands) was diluted 1:50. Tissue sections and fibroblasts grown on coverslips were stained by successive incubations in a drop of each antibody, for 30 min at room temperature. Controls were performed by replacing the specific antibody, in the first step of the method, with either P_i/NaCl or ascitic fluid from a mouse injected intraperitoneally with unhybridized Sp2/0 cells.

Plasma Protein Blotting and Indirect Staining by Monoclonal Antibody. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose was carried out by using the procedure of Towbin *et al.* (10), slightly modified. Briefly, the gel was laid onto a nitrocellulose sheet (Millipore) and submitted to constant current (90 V, 350 mA) for 2 hr, in 25 mM Tris/192 mM glycine/20% (vol/vol) methanol electrode buffer at pH 8.5. The sheet was then stained for 10 min in a 0.2% solution of Ponceau S dye (Serva) in 2% trichloroacetic acid and washed in several changes of distilled water (11). Pink stained bands were photographed, then individual tracks were cut out and incubated successively in the following reagent solutions: 3% bovine serum albumin (Serva) in P_i/NaCl, ascitic fluids 1:200 (one is directed against the heavy chain of IgM and called anti-MD2; the other, with broader specificity to be described below, is called anti-MB1), goat Ig anti-mouse Ig (Nordic) 1:50, and peroxidase–antiperoxidase complex of mouse origin (Sternberger–Meyer, Jarrettsville, MD) 1:80. Alternatively, nitrocellulose was incubated with a goat antiserum to chicken IgM Fc fragment (Nordic) 1:50 as first antibody and then processed for peroxidase–antiperoxidase staining. Each incubation step was carried out for 2 hr at room temperature, with rocking, and followed by extensive washing of nitrocellulose in P_i/NaCl containing 0.5% bovine serum albumin and 0.2% Tween 80 (Prolabo, Paris). The same buffer was used to dilute all antibodies. Reactions were visualized with diaminobenzidine (Serva) in the presence of H_2O_2.

Grafting Techniques. As described previously by Le Douarin and Jotereau (12), chimeric thymuses were obtained by grafting interspecifically the third and fourth branchial pouches before the thymic primordium has been seeded by hemopoietic precursor cells (i.e., at 4 days and 5 days, respectively, for quail and chicken).

Primary Culture and Embryonic Fibroblasts. Eleven-day quail embryos were used as a source of fibroblasts. These were grown on glass coverslips in BT 88 medium (GIBCO), containing 10% tryptose phosphate, 8% fetal calf serum (GIBCO), and 2% chicken serum.

RESULTS

Isolation and Characterization of Quail μ Chain. The gel filtration of precipitated plasma proteins from quail on Sephacryl S-300 gave rise to a main peak of elution of macroglobulins, containing essentially polymeric IgM (Fig. 1). Previous studies have demonstrated that the major IgM from chicken serum is pentameric with a molecular mass of 860 kilodaltons (kDa) and that a minor tetrameric IgM (680 kDa) can also be detected (13). Reduced quail macroglobulins were resolved, by $NaDodSO_4$ electrophoresis, into three main bands (Fig. 1 *Inset*, lane a). The μ chain was the most abundant, and exhibited an apparent molecular mass calculated at about 70–72 kDa (Fig. 1 *Inset*, lane d). Light chains were isolated as a band at about 23 kDa (Fig. 1 *Inset*, lane b), whereas accessory bands at 55–62 kDa likely represent aggregates. The identity of the third band (Fig. 1 *Inset*, lane c, apparent molecular mass close to 45 kDa) is unknown. However, the presence of such an accessory band (apparent molecular mass = 45 kDa) among reduced chicken IgM bands has been previously described (13).

Monoclonal Antibodies Against Quail μ Chain. After the hybridization of myeloma and spleen cells, the fused cells were distributed into 48 wells of multiwell plates. When screened by indirect immunofluorescence with B and T lymphocytes, one hybridoma supernatant (anti-MD2) reacted only with B cells, of both quail and chicken. Moreover, the ontogenic emergence of the antigen recognized by this antibody confirmed its μ chain nature (to be published elsewhere). Another supernatant, which strongly stained quail B cells, exhibited the same reactivity on quail thymocytes and leukocytes. The corresponding hybridoma population was cloned twice. All clones exhibited the same specificity and one was chosen for further propagation and ascites production. This monoclonal antibody binds to a determinant referred to as MB1.

Tissue Distribution of MB1: Expression on Hemopoietic Cells. In the adult quail, anti-MB1 was found to strongly label thymic and bursal lymphocytes, and very few cells in each sus-

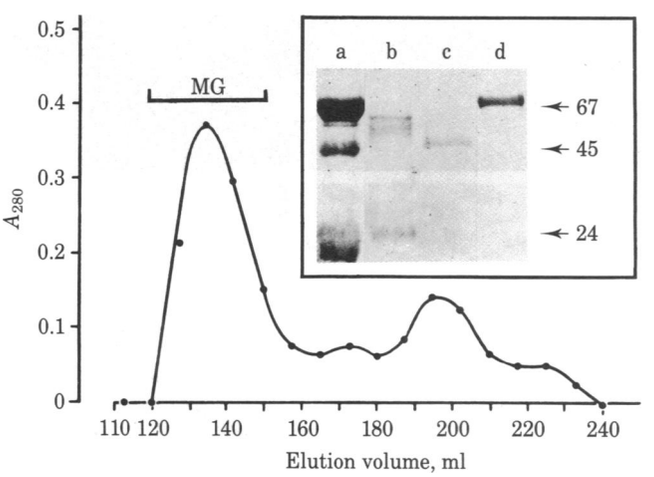

FIG. 1. Purification of quail μ chain. Sodium sulfate-precipitated plasma globulins were filtrated on a Sephacryl S-300 gel. Fractions forming the first main peak of elution, corresponding to macroglobulins (MG) were pooled for further purification of IgM subunits. Preparative $NaDodSO_4$ electrophoresis, in a 15% acrylamide gel, of macroglobulins reduced in the presence of 2-mercaptoethanol gave rise to three main migration bands (not shown); each of the three units was electroeluted from the polyacrylamide. (*Inset*) Analytical $NaDodSO_4$ electrophoresis, in a 10% acrylamide gel, of reduced macroglobulins. The three units are visible in lane a. Each one was run separately, from the light to the heavy, in, respectively, lanes b, c, and d. Lane d contains μ chain and lane b probably corresponds to light chains. Standard molecular mass (kDa) markers were run on the same gel (arrows).

pension or section were unstained. All leukocytes, including granulocytes, isolated from spleen or blood bound the antibody as strongly as did thymocytes and bursal lymphocytes (Fig. 2). MB1 reactivity appeared as a continuous ring of bright fluorescence. Under appropriate conditions, capping was observed on lymphocytes. Erythrocytes were MB1-negative, whereas immature erythroid cells exhibited dim spots of fluorescence (not shown), suggesting that the MB1 phenotype is progressively lost, or hidden, in the course of erythroid differentiation. In the embryo, lymphocytes from bursa and thymus taken at various stages were labeled with the same intensity as in hatched animals. To investigate a possible MB1 expression at early stages of lymphoid differentiation, we screened the lymphoid precursors present in the quail thymic primordium at $6^1/_2$ days of incubation; at this stage, the first colonization of the thymic anlage by extrinsic precursor cells is just completed (12, 14, 15). These precursor cells were found to be strongly MB1-positive, whereas we have shown previously that they do not yet express specific T-cell markers (8).

To examine the expression of the MB1 antigen on yolk sac hemopoietic precursor cells, the antibody was tested on cell suspensions from the quail extraembryonic area, at 2 days of incubation. Such suspensions are very heterogeneous and contain both hemopoietic cells and cells from the germ layers. About 20% of this cell population expressed MB1. Cell lineage specificity was further demonstrated on primary cultures of 11-day quail fibroblasts, which were not reactive with anti-MB1 except for some cells with the morphology of macrophages, and on tissue sections of the whole embryo at various ages [from 10-somite stage (36 hr) to 6 days of incubation]. This revealed that MB1 was exclusively expressed on hemopoietic and endothelial cells. The endocardium of the early developing cardiac tube showed both surface and cytoplasmic staining (Fig. 3A). So did the endothelia in young embryos. Later on in development, binding of the antibody to endothelial cells was somewhat polarized, with a much stronger affinity for the lateroapical than for the basal cell membrane (Fig. 3 *B* and *C*). Cytoplasmic staining was less bright than at earlier stages. Blood cells inside blood vessels were stained, with the exception of mature erythrocytes. In the trunk of the 6-day quail embryo, all tissues were unstained except for the endothelia of all blood and lymphatic vessels. In the vicinity of the duct of Cuvier, the intraembryonic hemopoietic foci described by Dieterlen-Lièvre and Martin (16) were conspicuously stained (Fig. 4).

No chicken tissue, at any stage of development, was found to bind anti-MB1. This was particularly striking in chimeric tissue combinations resulting from the construction of interspecific thymuses in which the endodermal epithelium and the thymic mesenchyme belong to one species (quail or chicken) and the hemopoietic cells to the other (chicken or quail). On sections of chimeric thymuses derived from the colonization of chicken anlagen by quail precursor cells, anti-MB1 stained exclusively the hemopoietic cell population, including lymphocytes and accessory cells. Reverse combinations gave rise to thymuses in which only some endothelial cells lining intrathymic blood vessels, derived from the third, fourth, and sixth aortic arches included in the graft, were found to be MB1-positive. The quail origin of these endothelial cells was confirmed by Feulgen–Rossenbeck staining (17) (data not shown).

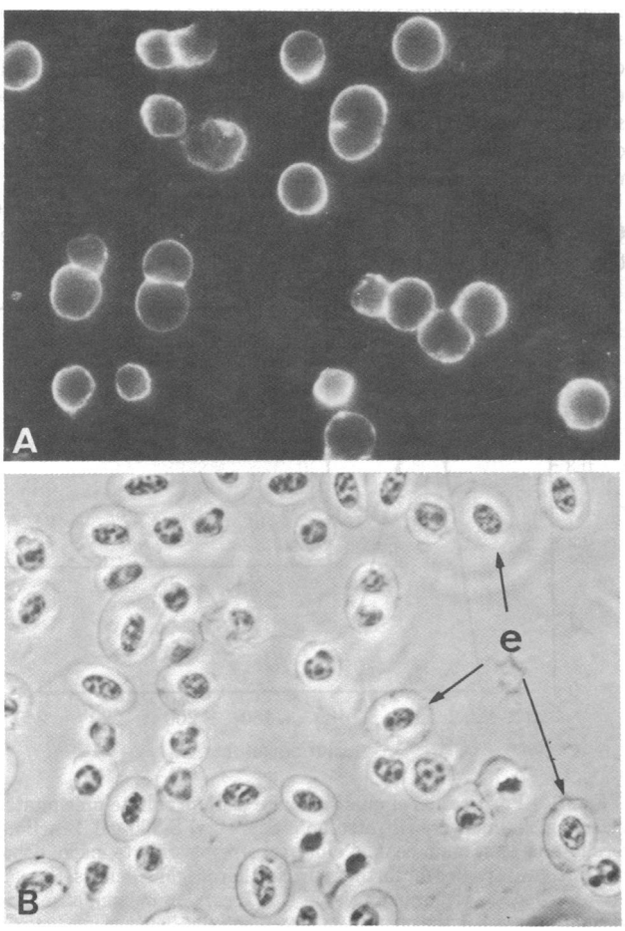

FIG. 2. Staining of leukocytes by anti-MB1. Citrated blood from a 3-week-old quail was centrifugated upon a Ficoll density gradient to obtain a cell fraction enriched in leukocytes. Erythrocytes (e), exhibiting typical ellipsoidal shape on the phase-contrast photograph (*B*), are unstained, whereas all leukocytes present in the field are brightly labeled (*A*). (×600.)

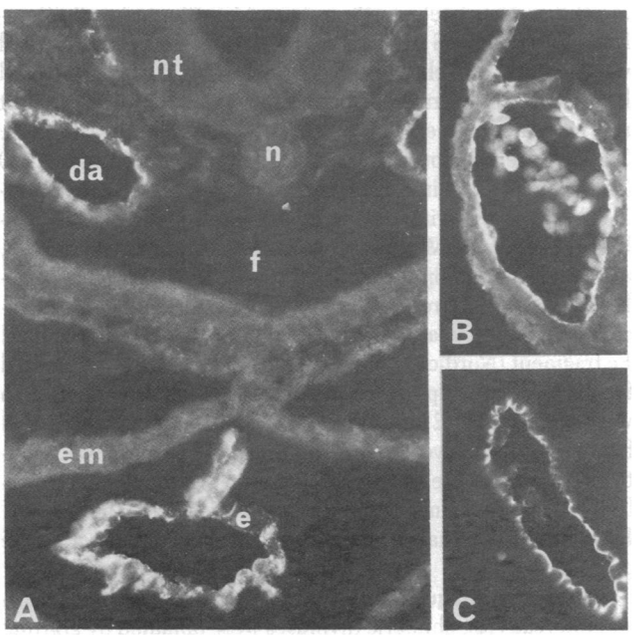

FIG. 3. Staining of embryonic endothelial cells by anti-MB1. (*A*) Transverse section in the anterior region of a quail embryo at stage 9 of development (33 hr of incubation). Dorsal aorta primordia (da) and the endocardium (e) are stained. (×150.) nt, neural tube; n, notochord; f, foregut; em, epimyocardium. (*B*) Transverse section of an extraembryonic vessel at stage 15 of development (65 hr of incubation). Endothelial and some blood cells are labeled. (×240.) (*C*) Intraembryonic vessel in the truncal region, at 6 days of incubation. MB1 antigen is expressed essentially on the lateroapical aspect of endothelial cells. (×240.)

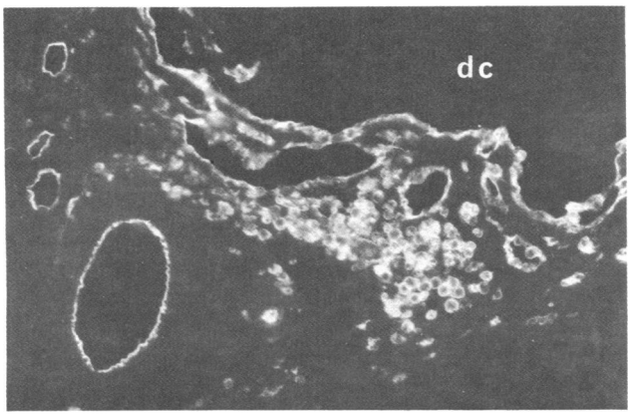

FIG. 4. MB1 expression at the surface of intraembryonic hemopoietic precursor cells. Transverse section in the truncal region of a 6-day quail embryo was indirectly labeled by anti-MB1. Hemopoietic cells proliferating in close vicinity of the duct of Cuvier (dc) are labeled. (×450.)

Anti-MB1 and Anti-MD2 Binding to Plasma Components. On 3-day quail plasma, anti-MB1 reacted predominantly with two proteins of apparent molecular masses about 160 and 80 kDa (Fig. 5). The 78- to 80-kDa component probably corresponds to the μ chain, because this band was also recognized by anti-MD2 (Fig. 5, lane c) and by a commercial antiserum to chicken IgM. These three antibodies also bound to the μ chain prepared as the original immunogen and to the 45-kDa component present in the same preparation (not shown). The strongest reactivity of anti-MB1 was with the 160-kDa band (Fig. 5, lanes a and b). This component did not react with either anti-MD2 or the commercial anti-IgM and therefore must not be considered as μ chain dimers.

Anti-MB1 also recognizes some minor bands. Those at 170–180 kDa and around 125 kDa did not react with the anti-μ antibodies (Fig. 5, lanes b and c). In contrast, 64- and 55-kDa materials were detected with anti-MB1, anti-MD2, and the commercial anti-IgM; they might be degradation products of the μ chain (13).

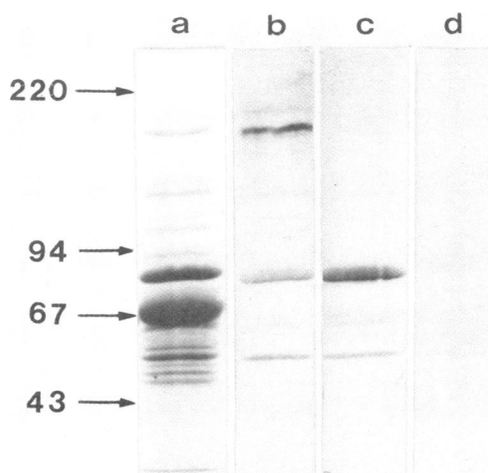

FIG. 5. Anti-MB1 and anti-MD2 binding to quail plasma components. Plasma from 3-day-old quail was submitted to $NaDodSO_4$ electrophoresis in a 6.5% acrylamide gel, in reducing conditions, and material was blotted to nitrocellulose. Lane a, Ponceau S staining of transferred proteins. After destaining, individual lanes were treated with anti-MB1 (lane b) or anti-MD2 (lane c), and reactions were visualized by the peroxidase–antiperoxidase/diaminobenzidine technique. In lane d, as a control, the first antibody was omitted. Arrows indicate standard molecular mass markers (kDa) run in the same gel.

DISCUSSION

The monoclonal antibody described in the present report specifies a determinant displayed on embryonic and adult quail hemopoietic and endothelial cells, whereas it does not bind to chicken cells of any kind. It has been raised in mice immunized with the isolated plasma μ chain of immunoglobulin. From protein blotting experiments, we tentatively conclude that MB1 epitope is included in μ chains. It is interesting to see that this epitope is also associated with plasma soluble material of higher molecular weight, whose cellular origin (endothelial or blood cells) will be interesting to determine. The relationship between the high molecular weight material detected in plasma and the antigens present on hemopoietic and endothelial cells is at present time a question.

Although MB1 material is abundant in the plasma, the reactivity located at the apical pole of endothelial cells is not due to deposition of the circulating material on the internal aspect of the blood vessel wall, because the MB1 phenotype is maintained on the progeny of quail vessel walls grafted into chicken embryos (not shown). The chemical structure of the MB1 epitope is not yet known. Treatment of tissues with a chloroform/methanol solution did not modify the staining pattern, indicating that the antigen is not associated with glycolipid or ganglioside moieties. Whether it could be a common region of the peptidic or of the glycosydic components of the various antigens identified is unknown at the present time. Capping of MB1 observed on lymphocytes indicates that the antigen is part of a transmembrane component. Similar crossreactivities between Ig chains and hemopoietic cell surface antigens have been previously reported in amphibians (18) and in mammalian species (19, 20), including humans (21). It was strongly suggested that they were due to homologous carbohydrate moieties on distinct components. In amphibians (18), similar to our own results, such a ubiquitous determinant was also expressed by non-Ig plasma proteins. However, reactivity with endothelia or with embryonic cells of the hemopoietic lineage has not been reported. Moreover, the interspecies distribution of these "jumping specificities" (22) was emphasized by some of these authors (19).

The antigenic determinant recognized by anti-MB1 antibody is a stable feature of the hemopoietic cell lineage, because it is maintained at all stages, from the early cells of both the intra- (16) and extraembryonic hemopoietic foci (1) to terminal steps of differentiation of endothelia and blood cells, the only exception being that it disappears during maturation of the erythrocytes. Such a disappearance of surface antigens has been described for erythrocytes of both avian (23) and mammalian species (24). Coexpression of MB1 determinant on blood and endothelial cells and its absence from all other cell types so far tested is consistent with the hypothesis of a common embryonic origin of these cell lines from precursors derived from mesoderm and termed hemangioblasts (1). This antibody specificity is of unique value for probing the very early emergence of the hemopoietic system in embryos. Moreover, this reagent will be useful in studies involving quail–chicken chimeras to examine the lineage derivation of various elements whose hemopoietic affiliation has been proposed but not clearly demonstrated.

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